

Rickets in Denmark

Prevalence of nutritional and hereditary rickets among children living in Denmark and characteristics of patients with hypophosphatemic rickets

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This review has been accepted as a thesis together with three previously published papers by University of Southern Denmark the 7th of September 2009 and defended on the 6th of November 2009.

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Dan Med J 2012;59(2):B4384

THIS THESIS IS BASED ON THE FOLLOWING THREE PAPERS:

- I: Beck-Nielsen SS, Brock-Jacobsen B, Gram J, Brixen K, Jensen TK. Incidence and prevalence of nutritional and hereditary rickets in Southern Denmark. *European Journal of Endocrinology* 2009; 160(3):491-497
- II: Beck-Nielsen SS, Jensen TK, Gram J, Brixen K, Brock-Jacobsen B. Nutritional rickets in Denmark: a retrospective review of children's medical records from 1985 to 2005. *European Journal of Pediatrics* 2009; 168(8):941-949
- III: Beck-Nielsen SS, Brusgaard K, Brixen K, Rasmussen LM, Brock-Jacobsen B, Poulsen MR, Vestergaard P, Ralston SH, Albagha OME, Poulsen S, Haubek D, Gjørup H, Hintze H, Andersen MG, Gram J. Phenotype presentation of hypophosphatemic rickets in adults. *Calcified Tissue International* 2010; 87(2):108-119

1. INTRODUCTION

Rickets is a disease of the past, but during the last decades, cases of nutritional rickets have reappeared in the industrialized countries. As nutritional rickets has become a rarity, it is now the general perception that hereditary rickets is the most prevalent cause of rickets in the industrialized countries. Data on the incidence of nutritional rickets in Scandinavia, however, are not available. Similarly, the incidence and prevalence of hereditary rickets are unknown. The clinical presentation of nutritional rickets and the risk factors in Scandinavian children has not previously been described. Therefore, the aims of this study were to establish the incidence and prevalence of rickets in Denmark, to characterize the clinical presentation of nutritional rickets, and to identify risk factors.

Especially in adults, the most common type of hereditary rickets, HR, is not well-characterized and diverging reports of possible gender differences have been published. This thesis, therefore, also aimed to characterize the genotype and phenotype in a large group of patients with HR, to evaluate the effects of medical treatment, and to determine differences in disease severity in X-linked HR according to gender.

2. AIMS

The aims of the Ph.D.-study were to:

- Estimate the incidence of nutritional rickets and the incidence and prevalence of hereditary rickets in Southern Denmark.
- Describe symptoms, clinical and biochemical characteristics at diagnosis of nutritional rickets in children living in Southern Denmark
- Identify current risk factors for nutritional rickets
- Determine the geno- and phenotype in a large group of patients with HR
- Evaluate possible effects of medical treatment in patients with HR
- Assess possible gender differences in disease severity in patients with genetically verified X-linked HR

3. BACKGROUND

NUTRITIONAL RICKETS

Historical review

The term rickets comes from the Greek word of spine, and rickets is derived from the old English word for "twist", or "wrick". A common term for rickets is the English Disease, in Danish "Engelsk syge", which most people in Denmark will associate to the disease.

The present thesis is not the first to describe rickets. In 1645, the 26 year old medical student Daniel Whistler presented his doctoral thesis; 'De morbo perili Anglorum' - The Children's disease of the English. Dr. Whistler was the first to describe the clinical characteristics of rickets. He also proposed a new term for the disease, which apparently was not to be applied; "Paedoplanchnosteocaces is a children's disease which attacks all the viscera and the bony skeleton on account of the unequal combination of the elements of the blood" (2). Dr. Whistler proposed a wide range of suggestions for treatment from crow's or frog's livers, application of leeches, purgation, poultices of snails and salt placed on the belly, and grease from mainly pork fat, goose-grease and butter to be smeared on the swollen epiphyses (2). The day after submitting his dissertation covering 8 pages, Dr. Whistler was examined and received the degree (3). Only five

years later, Francis Glisson published his thesis on rickets and received all the credits for being the first to describe rickets (4).

Rickets has presumably been present even earlier, but a large epidemic was seen during the industrial revolution, especially in England by the beginning of the 17th century. Families moved from the country side into the narrow streets of the cities where exposure to sunlight was limited and blocked by air pollution. In the late 17th century, signs of rickets were reported in 80% of infants in Boston (5). In 1822, Sniadecki assumed that lack of sunlight in children of Warsaw caused development of rickets and showed that exposure to sunlight cured the disease (6). That cod liver oil could also heal rickets, was first described by Schutte in 1824 (1), but a century had to pass before cod liver oil was implemented as a preventive treatment of rickets. The efficacy of cod liver oil in preventing rickets was assessed by Hess and Unger in 1917, based on a clinical trial including 65 primarily black children (7). In 1922, Hess proposed that rickets could be eradicated if cod liver was given to all children in New York City and this was the first step to overcome the rickets epidemic. Again, exposure to sunlight was observed to heal rickets in infants and a marked seasonal incidence of rickets was also noted (8). In 1919, Huld-schinsky discovered the healing effects when children with rickets were exposed to light from a Mercury lamp (9). By 1922, McCollum determined the anti-rachitic substance in cod liver oil to be vitamin D (10,11). The addition of vitamin D precursors to milk, subsequently irradiated by a mercury lamp (12,13), and the advice of one teaspoon of cod liver oil a day defeated this first epidemic of rickets (6). In Denmark, the prevalence of rickets among young children admitted to hospital was reduced from 41% during 1924-35 to 4% during 1946-51, following the implementation of health visitors in 1937 who encouraged the mothers to give their children the advised cod liver oil (14).

In the late 1970's, a new wave of rickets was reported in industrialized countries, especially in children of immigrants and in children on prolonged breastfeeding (5,15-18). During the last decades, several reports of rickets from industrialized countries (19-29) and in developing countries (30-38) have been published.

Pathophysiology

The mineralization of bone as well as teeth depends on the presence of adequate amounts of the major constituents, calcium and phosphate, as well as a balanced and undisturbed control of bone mineralization. Especially during periods of rapid growth, the demands for calcium and phosphate for bone mineralization are increased. Consequently, nutritional rickets is predominantly diagnosed in infants and young children and again during adolescence (39). The diagnosis of nutritional rickets might be suspected when clinical signs or symptoms of rickets are discovered, and laboratory findings and radiological signs of rickets confirms the diagnosis.

Nutritional rickets most frequently arise in the late stages of longstanding vitamin D deficiency, it might be inborn due to vitamin D deficiency in utero, and may also be caused by a low calcium intake (40). Nutritional rickets may be subdivided into acquired primary vitamin D deficiency rickets, acquired vitamin D deficiency rickets secondary to other diseases, and acquired calcium deficiency rickets (table 1). Vitamin D in its active form of 1,25-dihydroxyvitamin D (1,25(OH)₂D) stimulates intestinal calcium and phosphate absorption (41). In the state of vitamin D deficiency, dietary calcium absorption is reduced to 10-15% and phosphate to 50-60% (42). Vitamin D derives from photoconver-

sion of 7-dihydrocholesterol in the skin, from dietary sources, or from supplementation (figure 1). The cutaneous synthesis of vitamin D depends on exposure to ultraviolet-B (UVB) radiation and is diminished by skin pigmentation (43,44), may be completely prevented by clothing (45), or by the application of sunscreen (46). Overcast and extensive air pollution limits the amounts of UVB rays reaching the surface of the earth. In addition, the photo conversion by sun exposure cannot take place in Denmark (situated on latitude 55-58°N) from October till March due to a large solar zenith angle (SZA). A large SZA extends the travelling distance through the atmosphere of the rays, attenuating the UVB rays, and the rays reaching the surface of the Earth are spread over a larger area (44). In countries situated on latitudes 50°N and higher (44), the vitamin D source during winter months is dependent on intake from foods and/or supplementation, however, the dietary sources of vitamin D are limited and the average intake of vitamin D in Denmark is only 3.3 µg/day (47).

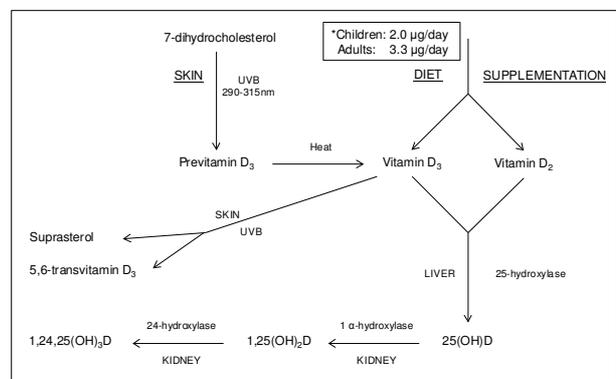


Figure 1. Sources, synthesis, and degradation of vitamin D.

The precursor, 7-dihydrocholesterol in the skin is transformed into previtamin D₃ by UVB radiation from the sun. Heat converts previtamin D₃ to vitamin D₃, which along with vitamin D₂, might also be provided by dietary sources or supplementation. 25-hydroxylation to 25-hydroxyvitamin D (25(OH)D) takes place in the liver, and the final transformation to the active metabolite 1,25(OH)₂D in the kidneys (48,49). Vitamin D₃ is degraded by UVB to suprasterol and 5,6-transvitamin D₃, and 1,25(OH)₂D is degraded by 24-hydroxylase in the kidneys and other organs to 1,24,25-trihydroxyvitamin D (1,24,25(OH)₃D) (50).

* Source: Dietary habits of the Danish population 2000-2002, [Danskernes kostvaner 2000-2002] (47).

Serum 25(OH)D has a half life of 25 days and reflects the vitamin D deposits in the body. The serum levels of the active metabolite 1,25(OH)₂D has a short half life of 7 hours, and is regulated by several factors as parathyroid hormone (PTH), fibroblast growth factor 23 (FGF23), hypocalcaemia, and hypophosphatemia. In vitamin D deficiency, serum values of 1,25(OH)₂D might be low, normal, or even elevated, reflecting the stage of vitamin deficiency and the availability of the precursor, 25(OH)D. This renders serum 1,25(OH)₂D unhelpful in the diagnosis of vitamin D deficiency (51).

Inadequate serum levels of calcium and phosphate decrease the mineralization of the newly formed osteoid on the surface of bone tissue (41). In the growth plate, the expansion of the late hypertrophic chondrocyte layer is characteristic of rickets (52). Moreover, mineralization of the cartilage is impaired (53). Presenting symptoms vary with age, where hypocalcemic seizures are most frequently seen in young children, whereas in adolescence, uncharacteristic symptoms as pains of legs, skeleton, and back, and muscle weakness are frequently reported (39). In young

children, the impaired mineralization leads to characteristic bowing of weight bearing extremities, and the widening of growth plates at the distal end of the long bones. In addition, craniotabes, rachitic rosary, and Harrison groove are seen (53,54). In adolescence, clinical signs are less prominent but may include enlargement of wrists, knees or ankles and bowing of weight bearing extremities (42). In the teeth, enamel hypoplasia may be seen depending on the timing and duration of the mineralization disturbance (55).

Treatment of vitamin D deficiency rickets is oral vitamin D supplementation and in case of hypocalcemia, in addition oral calcium supplements. Alphacalcidol is not recommended when treating vitamin D-deficiency rickets (56). Calcium deficiency rickets heals upon treatment with oral calcium supplementation (57).

HEREDITARY RICKETS

Hypophosphatemic rickets (HR)

Historical review

HR was first described in 1937 by Albright (58) and its X-linked inheritance was demonstrated in 1957 by Winters (59). Later, however, several different forms of HR have been described. A mutation identified in 1995 in the phosphate regulating gene with homologies to endopeptidases on the X-chromosome (PHEX) is responsible for the most prevalent form of HR, X-linked HR (XLH) (60). In 2000, the principal regulator of the phosphate homeostasis, FGF23, was isolated. The same year, a mutation in the FGF23 gene was associated with autosomal dominant HR (ADHR) (61). FGF23 was soon thereafter isolated from mesenchymal tumors causing tumor induced osteomalacia (TIO) and characterized as the causative factor of TIO (62). This was a breakthrough in elucidating the underlying biochemical disturbances, characteristic of the FGF23-associated forms of HR. In 2006, a mutation in the dentin matrix protein 1 (DMP1) was identified in patients with autosomal recessive HR (ARHR) (63) and the same year a mutation in the sodium-phosphate cotransporter gene, SLC34A3, was detected in hereditary HR with hypercalciuria (HHRH) (64,65). Most recently, a translocation causing increased α -Klotho levels, was described in a patient with HR and hyperparathyroidism (66). Klotho is a co-receptor binding to the fibroblast growth factor receptor and thereby increasing the receptor affinity for FGF23 activation (67).

Medical treatment of HR has evolved over time. Initially, very high doses of vitamin D were used (40-50,000 IU/day) (68), however, this treatment carried a considerable risk of vitamin D intoxication. A study describing adjuvant use of phosphate salts was published in 1964 (69). In 1978, the activated vitamin D alphacalcidol was launched in Denmark, and treatment with vitamin D was replaced by alphacalcidol. Addition of thiazide diuretics (70) and also treatment with growth hormone (71) have been suggested to raise the tubular threshold for phosphate. Finally, adjuvant therapy with calcimimetics has recently been proposed to oppose the hyperparathyroidism often induced by high dosages of oral phosphate (72).

Pathophysiology

Children with HR develop the characteristic clinical signs of rickets at approximately 6 months of age (54), and at that age growth retardation appear (73). The clinical signs are identical to those described in nutritional rickets, therefore, it is impossible clinically to distinguish nutritional rickets from the different forms of hereditary rickets. X-ray changes of rickets develop within the first 3-6 months of living (74). HR patients often experience numerous of spontaneous dental abscesses in both primary and permanent teeth not preceded by decay or trauma (75). The main biochemical characteristic of HR is the excessive renal phosphate wasting, as evaluated by a low renal threshold value for reabsorption of phosphate in the urine in relation to GFR (TPO_4/GFR). Serum levels of phosphate during the first 6 months may be normal (73) as well as decreased (54,74). Increased serum values of alkaline phosphatase (ALP) present within the first 2-3 months of living (76) and often remain elevated throughout childhood, however, values are often normal in adult patients (54).

HR may be subdivided according to the underlying pathophysiology into FGF23-associated and non-FGF23-associated HR (table 1). FGF23 is a phosphaturic peptide, secreted primarily by osteocytes (77). The main function of FGF23 is to maintain serum phosphate within its normal range and to serve as a counter regulatory hormone for $1,25(OH)_2D$ (78). In addition, FGF23 coordinates renal phosphate reabsorption with the demand for phosphate for bone mineralization (79). The phosphaturic effect of FGF23 is exerted by a decreased expression of the sodium-phosphate co-transporter in the kidneys (80), with concomitant reduction of the renal phosphate reabsorption and phosphaturia (figure 2). Moreover, FGF23 decreases the synthesis of $1,25(OH)_2D$ by inhibition of the 1α -hydroxylase and increases the degradation of $1,25(OH)_2D$ by induction of the 24-hydroxylase enzyme (80) (figure 1). This in turn results in decreased intestinal absorption of phosphate and calcium (81) (figure 2).

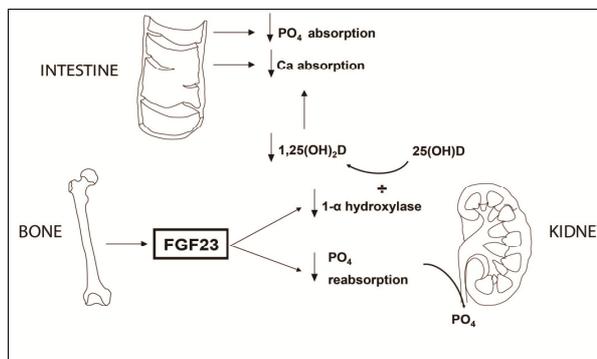


Figure 2. Outline of FGF23 and its regulation of the phosphate homeostasis.

FGF23 is primarily produced in bone. FGF23 inhibits the reabsorption of phosphate (PO_4) in the renal tubuli, and in addition inhibits the synthesis of $1,25(OH)_2D$. Hereby, the renal PO_4 excretion is increased and the absorption of intestinal PO_4 and calcium (Ca) is decreased.

Hereditary FGF23-associated HR (figure 3)

XLH is caused by an inactivating mutation in the PHEX-gene. Under normal circumstances, PHEX binds to matrix extracellular phosphoglycoprotein (MEPE), whereby proteolysis of the acidic, serine- and aspartic acid-rich motif (ASARM)-peptide attached to the C-terminal end of MEPE is regulated. MEPE belongs to a group of extracellular matrix proteins, small integrin-binding ligand, N-linked glycoproteins (SIBLINGS), all involved in the mineralization

of bone and teeth (82,83). Both MEPE and ASARM peptides demonstrate inhibition of bone mineralization (83,84). Free MEPE and free ASARM-peptides increase the FGF23 level by a

pathway presumably involving PHEX inhibition (84), which most likely is a regulatory effort to decrease the renal phosphate reabsorption in terms of the decreased demand for phosphate for bone mineralization (79).

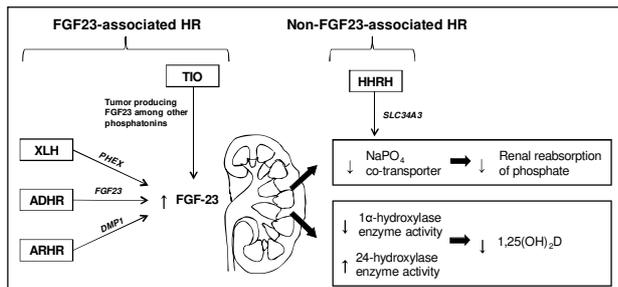


Figure 3. Pathways for the development of hypophosphatemia in FGF23-associated HR and non-FGF23-associated HR.

The FGF23-associated HR types are characterized by excessive renal phosphate wasting and an inappropriately low serum $1,25(\text{OH})_2\text{D}$ caused by the elevated levels of FGF23. By different pathways bone mineralization is decreased and FGF23 levels increased in XLH and ARHR. In ADHR, the increased FGF23 is caused by a cleavage resistant FGF23 gene product. Tumors in TIO produce FGF23 in excessive amounts along with other phosphatonins. In the non-FGF23-associated HR rickets type, HHRH, a mutation in the sodium-phosphate (NaPO_4) co-transporter causes decreased renal phosphate reabsorption. In contrary to the FGF23-associated HR types, an appropriate increase in $1,25(\text{OH})_2\text{D}$ induced by the hypophosphatemia is seen, causing hypercalciuria.

FGF23-associated HR:

- XLH: X-linked HR
- ADHR: Autosomal dominant HR
- ARHR: Autosomal recessive HR
- TIO: Tumor induced osteomalacia

Non-FGF23-associated HR:

- HHRH: Hereditary HR with hypercalciuria

ADHR is caused by a mutation in the FGF23 gene, leaving the FGF23 protein resistant to cleavage (85). The mutated FGF23 protein thereby has a longer half-life, and retain the phosphaturic effects characteristic of wild type FGF23 (86).

ARHR is caused by a loss of function mutation in the DMP1 gene (63). DMP1 is a matrix protein required for normal mineralization (83). Similar to MEPE, DMP1 also belongs to the SIBLING proteins and carry an ASARM motif as well. It has been proposed that full length DMP1 is a mineralization inhibitor, and that a subsequent cleavage initiates bone mineralization (87).

The complete pathways from the PHEX- and DMP1 mutations to the defect bone mineralization and elevation of FGF23 remain to be elucidated. The FGF23-induced hypophosphatemia also negatively affects the bone mineralization (88). In mice, however, normalization of the biochemical environment does not rescue the defect bone mineralization (89).

Acquired FGF23-associated HR (figure 3)

TIO is caused by a tumor, phosphaturic mesenchymal tumor, mixed connective tissue variant (PMT-MCT), producing FGF23 along with other phosphatonins as DMP1, and MEPE 90. TIO

patients and the hereditary forms of FGF23-associated HR share the same biochemical phenotype, but TIO patients display low

bone mineral density (BMD) (91-94), and the hereditary forms of FGF23-associated HR are characterized by a high BMD (95,96).

Hereditary non-FGF23-associated HR (figure 3)

HHRH is caused by a mutation of the SLC34A3-gene, resulting in loss of function of the sodium-phosphate co-transporter (65). The hypophosphatemia leads to an appropriate increase in $1,25(\text{OH})_2\text{D}$, causing hypercalciuria and subsequently renal calcifications and nephrolithiasis (97).

Biochemical implications of medical treatment of HR

Current recommendations on medical treatment of FGF23-associated HR are intermittent oral phosphate supplementation in combination with alphacalcidol (72,98,99). Phosphate loads transiently decrease serum ionized calcium with concomitant increase in serum PTH (72,100-103). Addition of alphacalcidol opposes this phosphate induced elevation of PTH (104,105). An adverse side effect of phosphate treatment is the development of secondary hyperparathyroidism that may even progress into tertiary hyperparathyroidism (106-111). Elevated PTH levels decrease TPO_4/GFR , accelerate bone turnover, and may be responsible for the development of hypertension in HR (106), all being undesired side effects of treatment. High dose phosphate treatment ($> 100 \text{ mg/kg/day}$) (108,112-114) and high doses of alphacalcidol ($>20 \text{ ng/kg/day}$) (115) may also increase the risk of nephrocalcinosis, a complication not seen in untreated patients (107,112). Adverse effects of alphacalcidol treatment are increased urinary calcium excretion and the risk of hypercalcaemia (115-117). In combination with the main feature of HR, which is an abnormally high renal phosphate excretion, it is likely that hypercalciuria adds to the risk of developing nephrocalcinosis. Finally, phosphate and alphacalcidol treatment increase circulating FGF23 (72,118,119), whereby treatment itself may establish a vicious circle.

Rickets due to disturbance in vitamin D synthesis

1α -hydroxylase deficiency (VDDR type I) (table 1)

In VDDR type I, the final activation of vitamin D in the kidneys by the enzyme 1α -hydroxylase (figure 1) is defective due to mutations in the coding gene, *CYP27B1*. Characteristically, very low serum values of $1,25(\text{OH})_2\text{D}$ are present despite normal levels of $25(\text{OH})\text{D}$ (120,121).

Hereditary vitamin D-resistant rickets (VDDR type II) (table 1)

VDDR type II is caused by mutations in the gene coding for the vitamin D-receptor, VDR. The mutation may decrease the receptor binding capacity to $1,25(\text{OH})_2\text{D}$ or impair binding of the ligand-receptor complex to DNA causing a post-receptor defect. High serum levels of $1,25(\text{OH})_2\text{D}$ and normal $25(\text{OH})\text{D}$ are the biochemical characteristics of the disease (122).

CLASSIFICATION OF RICKETS

Today's insight into the underlying pathophysiology of rickets constitutes the framework of the classification of rickets as listed in table 1.

Table 1. Suggested classification of rickets

Vitamin D deficiency rickets		Gene
Acquired, primary	Lack of sun exposure	
	Inadequate dietary vitamin D intake	
	Congenital vitamin D deficiency	
Acquired, secondary to	Malabsorption of vitamin D	
	Hepatic disease causing decreased hepatic 25-hydroxylation	
	Chronic kidney disease causing decreased renal 1,25-hydroxylation	
	Medically induced degradation of / decreased synthesis of vitamin D	
Rickets due to disturbance in vitamin D synthesis		
Hereditary	1 α -hydroxylase deficiency (VDDR type I)	CYB27B1
	Hereditary vitamin D-resistant rickets (VDDR type II)	VDR
Calcium deficiency rickets		
Acquired	Inadequate dietary calcium intake	
FGF23-associated HR		
Hereditary	X-linked dominant HR (XLH)	PHEX
	Autosomal dominant HR (ADHR)	FGF23
	Autosomal recessive HR (ARHR)	DMP1
Acquired/ sporadic	Tumor induced osteomalacia (TIO)	
	McCune Albright's syndrome	GNAS1
	Epidermal nevus syndrome	FGFR3
	HR and hyperparathyroidism De novo balanced translocation t(9;13) near the KLOTHO gene	
Non-FGF23-associated HR		
Hereditary	Hereditary HR with hypercalcaemia (HHRH)	SLC34A3
	X-linked recessive HR (XLRH)	CLCN5
	Lowe oculocerebrorenal syndrome	OCRL
Diseases causing rickets like bone changes		
Hereditary	Hypophosphatasia, Mucopolysaccharidosis II, Jansen's methaphyseal dysplasia	

4. MATERIALS AND METHODS

Identification of patients with rickets for the three studies (I, II, III) were based on a register survey in the Danish National Patient Registry (DNPR) performed from 1977 to 2005, identifying patients with diagnosis codes referring to rickets (table 2).

The diagnosis codes were assigned at referral as well as at discharge. The DNPR comprises data on all hospitalized patients from 1977 and onwards, and in addition all outpatient contacts from 1995 and onwards.

Table 2: Diagnosis codes used for register survey

DIAGNOSIS CODES 1994-2004 (ICD 10)	
Source: <i>Klassifikation af sygdomme, 10. revision, Munksgaard, 1992</i>	
Diagnosis code	Description
E 55	Vitamin D deficiency
E 55.0	Rickets, active (Osteomalacia: infantilis + juvenilis)
E 55.9	Vitamin D deficiency, unspecified
E 64.3	Sequelae of rickets
E 83.3	Disorders of phosphorus metabolism (Vitamin D resistant: Osteomalacia + rickets)
DIAGNOSIS CODES 1985-1993 (ICD 8)	
Source: <i>Klassifikation af sygdomme, 8. revision, Schultz Grafisk A/S, 1986</i>	
Diagnosis code	Description
265	Vitamin D deficiency
265.09	Rickets, active
265.19	Rickets, late effect
265.29	Osteomalacia
265.99	Vitamin D deficiency, unspecified
273.40	Rickets, vitamin D resistant (hypophosphatemia familiaris)

STUDY I: EPIDEMIOLOGICAL STUDY ESTIMATING THE INCIDENCE AND PREVALENCE OF RICKETS IN SOUTHERN DENMARK

Identification of patients

Using the same diagnosis codes as for the DNPR survey, an additional survey in the hospital registers of southern Denmark was performed from 1985 to 2005. No register data were available from 1985 to 1991 from Southern Jutland County (comprising 19% of the region of Southern Denmark), as electronic registration was only implemented thereafter. Medical records from patients retrieved in the different registers were identified by their unique personal identification number (CPR number), and reviewed to validate the diagnosis of rickets.

Patients living in the region of Southern Denmark and fulfilling both biochemical inclusion criteria and clinical signs/symptoms or radiological signs of rickets were included. The diagnostic criteria used are listed in table 3. Patients above the age of 15 years at time of first diagnosis were excluded to limit this study to patients with rickets and to exclude patients with osteomalacia. Moreover, patients with secondary rickets due to e.g. prematurity, anti-tuberculosis treatment, liver/bile duct disease, hypophosphatasia or malabsorption were excluded (figure 4). Also, patients with serum 25(OH)D \geq 50 nmol/l were excluded to limit the study to vitamin D deficiency rickets.

To identify patients treated exclusively in the primary health care sector, a questionnaire survey was undertaken among all general practitioners (GPs) and pediatricians working in primary care in the region of Southern Denmark. They were asked to assess whether they had treated and/or referred patients to hospital evaluation in suspicion of rickets in 2005, and if so, they were contacted by phone for verification.

Table 3. Diagnostic criteria of nutritional rickets, VDDR type 1, and HR (123).
Biochemical inclusion criteria and clinical signs/symptoms or radiological signs of rickets

	Biochemical criteria	Clinical signs / symptoms	Treatment effect	Radiologic signs of rickets
Nutritional rickets	[ab]25(OH)D available: 25(OH)D < 12.5 nmol/l or 25(OH)D: 12.5-25 nmol/l and at least one of the following; ↑ ALP[c] ↑ PTH[d] ↓ Ca[e] No 25(OH)D measures: At least one of the following; ↑ ALP ↑ PTH ↓ Ca	<i>Infants and young children:</i> At least one of the following; craniotabes rachitic rosary Harrison groove enlargement of the wrists, knees or ankles bowing of weight bearing extremities hypocalcemic seizures <i>Adolescents:</i> At least one of the following; enlargement of the wrists, knees or ankles bowing of legs muscle weakness pain of the lower limbs or in the back hypocalcemic seizures	Heals on vitamin D treatment	Widening of the growth plates with irregularity and cupping of their metaphyseal borders
Vitamin D resistant rickets	All of the following; ↑ ALP ↑ PTH ↓ ↔ Ca ↓ PO4 [f] 1,25(OH)2D [g] < 15 pmol/l 25(OH)D: 50-178 nmol/l	At least one of the following; rachitic rosary Harrison groove enlargement of the wrists, knees or ankles bowing of weight bearing extremities pain of the lower limbs	Refractory to vitamin D	
Hypophosphatemic Rickets	All of the following; ↑ ALP ↔PTH ↓ PO4 ↔ Ca	At least one of the following; rachitic rosary Harrison groove enlargement of the wrists, knees or ankles bowing of weight bearing extremities pain of the lower limbs		
[a]Definition of stages of vitamin D insufficiency (124): 25(OH)D < 50 nmol/l: vitamin D insufficiency 25(OH)D: 12.5-25 nmol/l: vitamin D deficiency 25(OH)D < 12.5 nmol/l: severe vitamin D deficiency		[b]25(OH)D: 25-hydroxyvitamin D [c]ALP: alkaline phosphatase [d]PTH: parathyroid hormone [e]Ca: calcium [f]PO ₄ : phosphate [g]1,25(OH) ₂ D: 1.25-dihydroxyvitamin D		

Epidemiological data analyses

The likelihood of patient recruitment changed during the study period, as the DNPR register did not comprised data on outpatient contacts before 1995. Therefore, the incidence of nutritional rickets was calculated in two study periods (1985-1994 and 1995-2005).

The average yearly incidence during the two study periods was calculated as the mean of the total number of children diagnosed with nutritional rickets for the first time during the study periods divided by the mean of the reported yearly total number of children in that age group living in Southern Denmark during the two study periods, assuming each child to contribute one year of risk time. Information about the total number of children living in the area on the 1st of January each year was obtained from Statistics Denmark (<http://www.statistikbanken.dk/>). Immigrant patients were defined as children of at least one non-Danish parent. The incidence of nutritional rickets among immigrants was only calculated among children born in Denmark. Immigrant families who might only be in Denmark for a short period of time while their application for asylum is considered are not recorded in the population registers. Consequently, data on these families were not included in the calculations. The incidence among immigrant children of different ethnic origin born in Denmark was calculated as for ethnic Danish children but dividing by the population of children of that ethnic group during the study period.

Data on the ethnicities of the parents and place of birth were collected by linkage to The Central Office of Civil Registration

(COCR). As only 10 immigrant children born in Denmark were diagnosed from 1985 to 1994, the incidence was not calculated during that period.

Patients with hereditary rickets appeared several times in the registers throughout the study period due to continued attendance at outpatient clinics for treatment control. This allowed a calculation of the incidence of hereditary rickets throughout the entire study period. As only one patient with hereditary rickets was non-Danish, the incidence was calculated among ethnic Danish children only. With the exception of one patient, all cases were diagnosed before the age of 3 years. The average yearly incidence was therefore calculated as the mean of the total number of ethnic Danish children diagnosed with hereditary rickets during the study period (1985-2005) and born from 1982 to 2002 divided by the mean of the reported yearly total number of ethnic Danish children aged 0-11 months living in Southern Denmark from 1982 to 2002. The prevalence of hereditary rickets among children 0-14.9 years of age on the 1st of January 2002 was calculated by dividing the total number of children with hereditary rickets on that date by the total number of children 0-14.9 years of age living in Southern Denmark at that date. Children with hereditary rickets were assumed to be diagnosed before the age of 4 years and therefore children born before 2002 would be diagnosed by the end of the study period in 2005.

STUDY II: DESCRIPTIVE STUDY OF NUTRITIONAL RICKETS; BASED ON A REVIEW OF MEDICAL RECORDS

Identification of patients and data obtained from medical records

Patients included in this study were the patients identified with nutritional rickets from study I. Age at diagnosis divided the case series into two groups, infants/young children (0-3.9 years) and older children/adolescents (4-14.9 years), and the patients were described according to these two age groups. Symptoms, clinical signs, height and head circumference at diagnosis were recorded. Similarly, biochemical measures analyzed by the local laboratories including plasma ALP, serum calcium (total and ionized), plasma phosphate, serum PTH, serum 25(OH)D, and serum 1,25(OH)₂D, were recorded. Potential risk factors as breastfeeding without concomitant vitamin D supplementation, omitted vitamin D supplementation, consumption of dairy products, and veiling were recorded if available in the medical records.

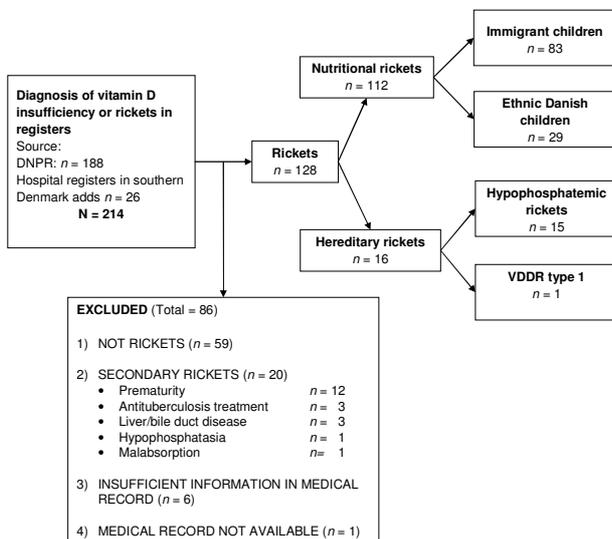


Figure 4. Flow diagram of patient inclusion, study I + II
 DNPR: Danish National Patient Registry
 VDDR type 1: Vitamin D-dependent rickets type 1
 Adapted from paper I (123)

Statistical analyses

Statistical analyses were performed using SPSS 15.0. One sample t-test was used when comparing means and Mann-Whitney's test was used when comparing skewed variables between groups. In ethnic Danish patients, z-scores of height and weight were calculated from growth charts of age and gender matched Danish children 125 and similarly head circumference by age and gender matched Swedish children 126 by use of the growth calculation program AUXOLOGY®. In immigrant patients aged 0-3.9 years, z-scores were calculated from WHO Child Growth Standards (<http://www.who.int/childgrowth/en/>, accessed 1st of oct 2008) by use of the growth calculation program ANTHRO®v2.0.2. In immigrant patients aged 4-14.9 years: z-scores were calculated from WHO Child Growth Standards according to the formula in "Computation of centiles and z-scores for height-for-age, weight-for-age and BMI-for-age", by WHO (<http://www.who.int/childgrowth/en/>, accessed 1st of Oct 2008).

Normal values for weight-for-height were only available for the age group 5-10 years.

Biochemical findings in patients presenting with generalized seizures were compared to patients without generalized seizures presenting within the same age interval, and biochemical findings in Danish patients were compared to immigrant patients presenting within the same age interval. P-values < 0.05 were considered significant.

STUDY III: CROSS SECTIONAL STUDY OF HR PATIENTS

Study design and recruitment of HR patients

This cross-sectional study of HR patients was performed at Odense University Hospital, Hospital of Southwest Denmark and in the School of Dentistry, Aarhus University, during 2006-2008. Participants were children (aged < 18 years) and adults recruited from 2006-2007. Participants were identified in three different ways (figure 5 and 6);

1) By register search based on the diagnosis codes 273.40 and E83.3 in the DNPR from 1977 to 2005. An identical survey was performed in hospital registers in the Region of Southern Denmark from 1985 to 2006 and in the former Aarhus County from 1989 to 2006. By inquiry to COCR availability, names and addresses of potential participants were obtained. Medical records were retrieved and reviewed to ensure that the diagnosis of hereditary rickets was plausible. Patients misclassified or with hypophosphatemia/rickets caused by other underlying diseases than hereditary rickets were excluded (figure 5).

2) Doctors known to treat patients with HR were contacted. Patients in their care were invited to participate. These patients were either diagnosed but not recorded in the registers by the diagnosis codes 273.40 or E83.3, or they were recorded in the registers but had assigned for protection from contact by scientists. After permission from the Ethics Committee of Southern Denmark their treating doctor handed out written information about the study to these patients, and if they were interested in participation they contacted me (figure 6).

3) First degree relatives to participants were offered screening for HR. In case of HR, they were invited to participate in the study and their first degree relatives were offered screening for HR. In addition, participants were asked if they had knowledge of second degree relatives experiencing symptoms of HR, as childhood bowing of legs, present bowing of legs or if they had experienced spontaneous dental abscesses. Symptomatic second degree relatives were then offered screening for HR, and were invited to participate in case of HR (figure 6).

Eligibility criteria

Patients with hereditary, FGF23-associated HR were included in study III. The diagnostic criteria were genetically verified HR or biochemically verified HR. Biochemical criteria of HR were at least one of the following; serum phosphate below normal range, low TPO₄/GFR, or elevated serum FGF23. In addition, a history of childhood rickets or spontaneous dental abscesses was required to exclude TIO. Two brothers denied blood samples, however, their sister, mother and grandmother had PHEX-positive HR. The boys were included in the study since they both displayed clinical signs of HR and radiological signs of rickets or spontaneous dental abscesses, respectively. At present, an attempt of collecting DNA

samples from saliva to test for the identified PHEX-mutation is performed. In addition, one male patient had no evidence of childhood rickets or dental abscesses. He had late onset of HR and no mutation in the HR genes was demonstrated, however, he had a low serum phosphate, his renal phosphate wasting was associated to an elevated serum level of FGF23, as well as marked osteosclerosis with coarse trabeculae on X-ray and markedly elevated BMD of the lumbar spine. Since the generalized osteosclerosis supported that his disease was not due to TIO, he was included in suspicion of inborn, hereditary FGF23-associated HR. Ten previously undiagnosed adult patients were included from a large kindred of HR exhibiting an X-linked dominant trait, but currently undetected genetic mutation. From this family, four females had no history of rickets or spontaneous dental

abscesses, but they all had children with verified HR.

Exclusion criteria

The exclusion criteria were non FGF23-associated HR, acquired HR due to TIO, and sporadic, non-hereditary HR e.g. McCune Albright's Syndrome. Exclusion of patients with these differential diagnoses was performed by review of their medical records, screening for mutations in the SLC34A3-gene (HHRH), the CLCN5-gene (XLRH), and by biochemical evaluation. Paper III describes adult phenotype presentation only, for which reason children (aged < 18 years) were excluded.

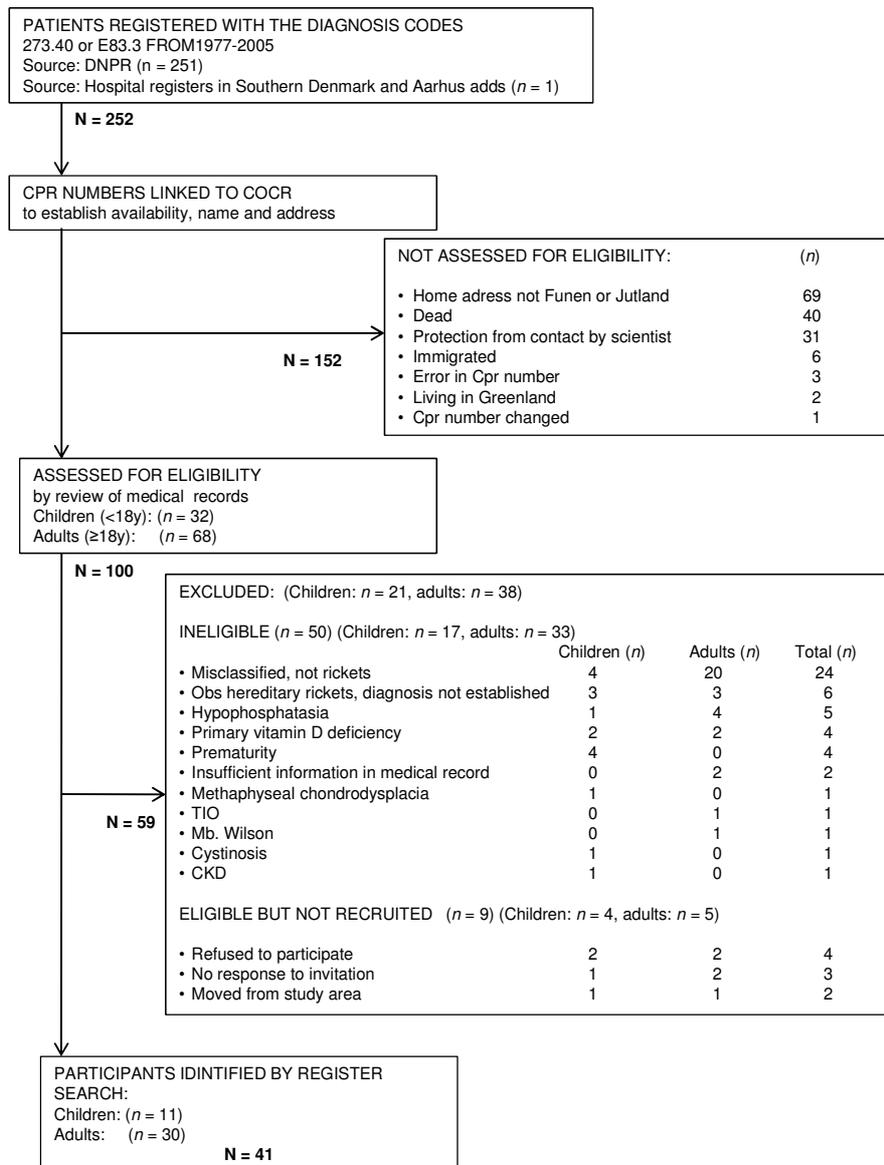


Figure 5. Flow chart of patients recruited from register search, study III

COCR: Central Office of Civil Registration
TIO: Tumor Induced Osteomalacia
CKD: Chronic Kidney Disease

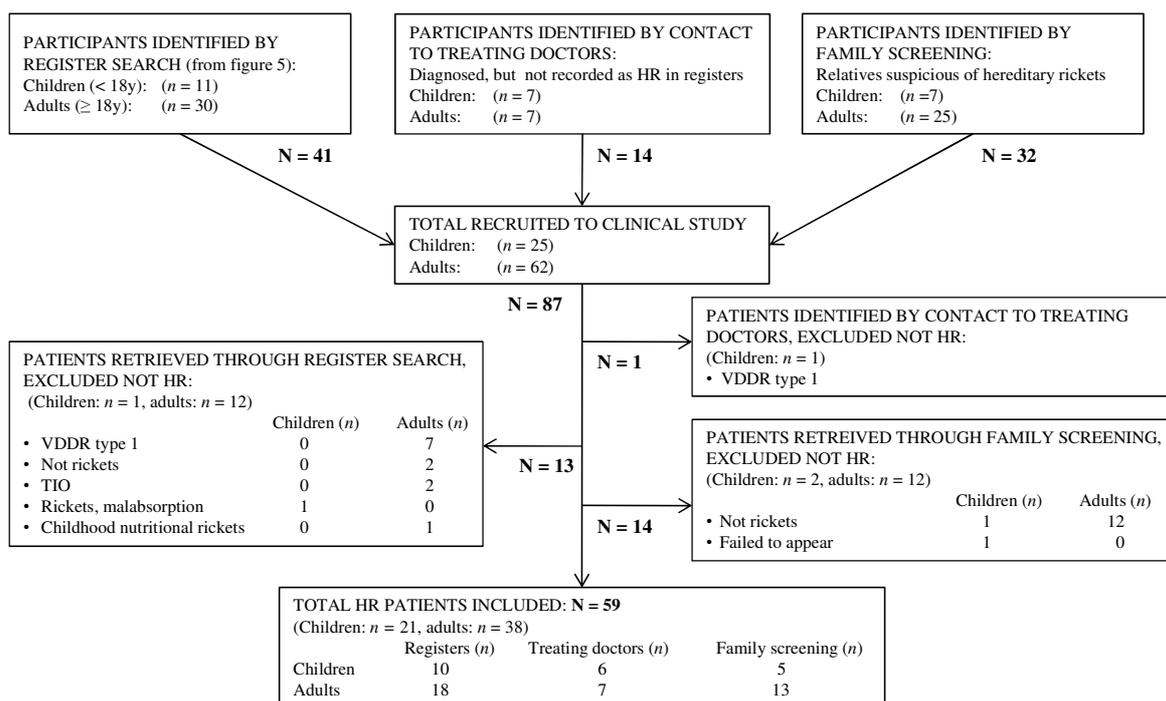


Figure 6. Flow chart of included patients; Identification by register search, by contact to treating doctors, and by family screening, study III. VDDR type 1: Vitamin D resistant Rickets type 1. Adapted from paper III.

Genetic analyses

Genomic DNA was extracted from full blood using a DNA purification robot (Maxwell® Promega, Ramcon Denmark) and analyzed for mutations in the PHEX-, FGF23-, DMP1-, SLC34A3-, and CLCN5-genes. The method used was polymerase chain reaction covering all introns and intron/exon-boundaries followed by denaturing high performance liquid chromatography (dHPLC) analysis (WAVE 3500HT High Sensitivity System; Transgenomic Inc, Elancourt, France) testing for small deletions, insertions or point mutations in all exons and exon-intron boundaries of all genes. Samples with deviating chromatographic profiles were sequenced in both directions using the BigDye® Terminator v3.1 Cycle Sequencing Kit and analysed on a 3730XL DNA Analyzer (Applied Biosystems, Foster City, USA). Sequence analysis was performed using SeqMan Software (DNA STAR, Madison, USA). Mutational analysis of the PHEX- and FGF23 gene was performed by use of the primers published by Goji et al.,¹²⁷ and mutational analysis of the CLCN5-gene was performed by use of the primers published by Lloyd et al.¹²⁸ Primers were designed using Primer Select Software (DNA STAR, Madison, USA) for mutational analysis of the DMP1, FGF23 and SLC34A3-genes. To detect larger deletions of the PHEX-, and FGF23 genes, a Multiplex Ligation-dependent Probe Amplification (MLPA) analysis was performed in patients with no mutations detected by dHPLC. The MLPA procedure was performed according to the manufacturers recommendations (MRC, Holland) and run on the 3730XL DNA analyzer using GeneMarker software (Softgenetics, USA) for the analysis.

Variables collected

A medical history was obtained including age at debut, previous and current treatment, dental complications, and surgical treatment. The following clinical observations were obtained: height, sitting height, leg length, arm span, head circumference, and leg deformities. Sitting height ratio was calculated as sitting height divided by height. Calculations of z-scores by, e.g. height, were done by the equation:

$$Z = \frac{\text{Patient's height} - \text{mean normal reference height}}{\text{SD of reference height}}$$

Z-scores of anthropometric data were calculated by comparing to reference data as follows; height: Denmark, Andersen, 1982 (125). Sitting height, sitting height ratio, leg length and arm span: Denmark, Hertel, 1995 (129). Head circumference in adults: UK, Bushby, 1992 (130), and in children: UK, Cole, 1998 (131). In children, the z-scores were calculated by use of the growth calculation program AUXOLOGY®. One patient had a 10 centimeter surgical lengthening of the legs and her data on height, sitting height ratio, and leg length, were omitted in the data analysis. Z-scores of leg deformities: (genu varus: distance between medial femoral condyles, genu valgus: distance between medial tibial malleolus, equal leg length) were calculated by comparing to reference data, France (132). As reference data for leg deformities were only available for the age group from 10-16 years, z-scores of leg deformities were not calculated in children aged less than 10 years. In adults, the reference data for 16 year olds were used for z-score calculations. The following definitions were used: genu varus: distance between medial femoral condyles, genu valgus: distance between medial tibial malleolus. In adults, ranges of movement (ROM) of the hip were assessed and when a differ-

ent ROM in right and left hip was recorded, the lowest ROM was used.

Fractures were defined as any clinical fracture and based on self-reports. The number of fractures in each HR patient was compared to the number of fractures experienced by three age and gender matched Danish normal controls (133). The relative risk (RR) of fractures in patients was compared to controls using incidence rate ratios. The number of childbirths and caesarean sections was recorded in adult females.

A skeletal severity score was defined based on the presence of surgical corrections of leg deformities, severity of leg deformities, and height reduction (figure 7). The skeletal severity score was not applied on children aged less than 10 years due to missing z-score calculation of leg deformities.

Surgical correction of leg deformities	Leg deformity / Height reduction	Severity	Score
YES		Severe	2
NO	Leg deformity: $z \geq 2$ SD or Height: $z \leq -2$ SD		
NO	Leg deformity: $z < 2$ SD and Height: $z > -2$ SD	Mild	1

Figure 7. Skeletal severity score

SD: Standard deviation
Adapted from paper III

Phosphate and alphacalcidol treatment was paused from the evening before the examination. After 2 hours of fasting, blood samples were drawn and urine samples were collected. In exception of serum PTH, serum creatinine, serum calcium ion and the urine analyses, the frozen samples were all analyzed in the same batch. Methods of biochemical analyses are listed in table 4.

According to the method described by Stark et al. (136), TPO_4/GFR based on the 2 hour fasting blood- and urine samples was calculated by use of the equation:

$$TPO_4/GFR = Sp - \frac{Up \times Scr}{Ucr}$$

Sp: serum PO_4 , Up: urine PO_4 , Scr: serum creatinine, Ucr: urine creatinine

In adults, x-rays of ankles, knees, pelvis including hips, and lumbar spine anterior and posterior projection were obtained. Osteoarthritis of ankles, knees and hips, and enthesopathies, defined as bone proliferation at sites of ligament attachments or calcification of ligaments, were recorded.

DEXA scans of BMD lumbar spine and hip were obtained by use of two DEXA scanners (Delphi W and Discovery A, Hologic, Waltham, MA). Hologic reference data were used for calculation of z-scores in adult patients. In children, spine data are presented as bone mineral apparent density (BMAD, g/cm³) as this measure is considered less dependent on bone size than areal bone mineral density (aBMD, g/cm²) (134).

Table 4. Method description of biochemical analyses

Analysis	Test principle	Manufacturer/ apparatus	Intra-assay coefficient of variation	Inter-assay coefficient of variation
Serum PTH [1]	Two-site chemiluminescent enzyme-labelled-immunometric	Immulite 2000	5.7% at 7.6 pmol/l	6.3% at 5.7pmol/l
Serum 25(OH) _{2,3} D [2]	Isotope dilution liquid chromatography-tandem mass spectrometry	LC-MS/MS	25(OH) ₂ D 8.5% at 23.4 nmol/l	-
			25(OH) ₃ D 9.6% at 24.8 nmol/l	-
Serum 1.25(OH) ₂ D [2]	Radioimmunoassay	IDS, Phoenix, Arizona, USA	6.8% at 90 pmol/l	-
Serum FGF23 [3]	Two-site enzyme-linked immunosorbent	Kainos Laboratories, Tokyo, Japan	2.0% at 33.6 pg/ml	-
Serum BSALP [2]	Enzyme immunoassay, Metra BAP EIA kit	Quidel, San Diego, CA, USA	5,7% at 41.9 U/l	-
Serum PO ₄ [4]	Phosphomolybdate Method	Modular P, Roche	0.9% at 1.38 mmol/l	-
Serum Creatinine [1,4]	Jaffé reaction	Modular P, Roche	0.9% at 148 μmol/l [4]	3.0% at 120 μmol/l [1]
Serum calcium ion [5]	Potentiometric	NOVA 8	2.0%	3.0%
Urine PO ₄ [4]	Phosphomolybdate Method	Modular P, Roche	0.7% at 28 mmol/l	1.3% at 27 mmol/l
Urine Creatinine [4]	Jaffé reaction	Modular P, Roche	1.1% at 5.39 mmol/l	1.2 % at 5.22 mmol/l

-: Samples analyzed by the same assay on the same day, rendering the inter-assay coefficient of variation obsolete.

Source:

[1]Method description, Department of Biochemistry, Pharmacology, and genetics, Odense University Hospital

[2]Method Description, Department of Biochemistry, NBG, Aarhus University Hospital

[3]Kainos Laboratories, Tokyo, Japan

[4]Roche

[5]Reference manual for NOVA 8

BMAD was calculated by the formula given by Ward et al. (135):

$$\text{BMAD} = \frac{(\text{BMC1} + \text{BMC2} + \text{BMC3} + \text{BMC4})}{(\text{v1} + \text{v2} + \text{v3} + \text{v4})}$$

BMC1: the bone mineral content of L1

v1: the estimated volume of lumbar vertebra L1, calculated by the formula $v1 = a1^{1.5}$

a1: the area of L1.

Z-scores of BMAD L1-L4 in children were calculated by the reference data from healthy 6-17 years old Caucasian children from United Kingdom, provided by Ward et al. (135).

Endodontic examination

The patients were all offered a clinical endodontic examination in the Department of Pediatric Dentistry, School of Dentistry, University of Aarhus. Furthermore, a digital panoramic radiograph, examined for endodontically treated teeth and teeth with periapical bone lesions (apical periodontitis) was performed. An endodontic severity score was calculated as the number of permanent teeth with periapical periodontitis or previous endodontic treatment divided by the total number of teeth. The endodontic severity score was only calculated in adults, as reference data were only available from 20+ years of age (137).

Endodontic severity score =

$$\frac{\text{Number of permanent teeth with present periapical periodontitis or endodontically treated teeth}}{\text{Total number of permanent teeth}}$$

Grouping of patients

In the analysis on possible gender differences, only patients with positive PHEX-mutation or established X-linked disease were included. Presence of enthesiopathies was limited to patients aged 40+ years. As this influenced the z-scores of BMD lumbar spine, these data were analyzed according to age above or below 40 years. Medical treatment with phosphate and calcitriol increases serum FGF23 (72,118,119), therefore, when biochemical values were compared between groups, only patients not treated medically for the past 6 months were included.

Statistical analyses

Statistical analyses were performed using SPSS 16.0. Normally distributed data were presented as mean (95% CI), and skewed data as median [range]. Student's t-test was used when comparing normally distributed data, and Mann-Whitney's test was used when comparing skewed variables between groups. Wilcoxon's paired t-test was used when comparing paired variables following a normal distribution, and Wilcoxon's signed rank test when comparing paired, skewed variables. Pearson's Chi2 was used for analysis of bivariate variables and Fisher's exact test when observed frequencies were less than 5. For correlation analysis of skewed variables, Spearman's correlation coefficient (Rho) was calculated.

Ethics

Study I, II, and III were approved by the Ethics Committee of Southern Denmark (M-2678-05) and by the Danish Data Protection Agency (2004-41-4699).

5. RESULTS

STUDY I: EPIDEMIOLOGICAL STUDY ESTIMATING THE INCIDENCE AND PREVALENCE OF RICKETS IN SOUTHERN DENMARK

Nutritional rickets

In the DNPR 188 medical records were retrieved from children aged 0-14.9 years of age with a diagnosis code referring to rickets from 1985 to 2005 in the region of Southern Denmark. The regional registers lead to inclusion of additional 26 cases. After review of these medical records, 86 cases were excluded as they did not fulfill the diagnostic criteria of nutritional rickets. A total of 112 children were verified with the diagnosis of nutritional rickets comprising 83 (74%) immigrant children and 29 (26%) ethnic Danish children (figure 4). Ethnic Danish children were only diagnosed at age 24 months or less. The majority (18 patients) was diagnosed from 12-24 month and 31% of these patients received vitamin D according to the guidelines as reported by their parents.

From 1995 to 2005, the average incidence of nutritional rickets in children aged 0-14.9 years was 2.9 per 100,000 per year. In children aged 0-2.9 years the average incidence was 5.8 per 100,000 per year. Among immigrant children aged 0-14.9 years and born in Denmark the average incidence was 60 per 100,000 per year. The incidence of nutritional rickets among ethnic Danish children declined from 5.0 to 2.0 per 100,000 per year from 1985-1994 to 1995-2005 (table 5).

Table 5. Average yearly incidence of nutritional rickets in Southern Denmark 1995-2005

Age group (years)	Ethnic group	Average incidence (per 100 000 per year)
0-14.9	All children	2.9
0-14.9	All immigrant children born in Denmark (Middle East, Africa, Asia, The Balkans)	60
0-14.9	Middle East countries	85
0-14.9	African countries	59
0-14.9	The Balkans	37
0-14.9	Asia	18
0-2.9	All children	5.8
0-2.9	Immigrant children born in Denmark	100
0-2.9	Ethnic Danish children	2.0

Adapted from paper I (123)

Hereditary rickets

During the study period 1985-2005, 16 children were diagnosed with hereditary rickets giving an average incidence of 4.3 per 100,000 (0-11months) per year (table 6). The prevalence of hereditary rickets was 5.2 per 100,000 children and the prevalence of HR was 4.8 per 100,000 children (table 7). With exception of one child diagnosed at age 9.4 years, all patients were diagnosed before the age of 3 years. All patients were ethnic Danish, apart from one child whose parents were from Lebanon.

Table 6. Average yearly incidence of hereditary rickets in ethnic Danish children in Southern Denmark 1985-2005

Age group (months)	Type of hereditary rickets	Average incidence (per 100 000 per year)
0-11	Hereditary rickets, overall	4.3
0-11	HR	3.9
0-11	VDDR type 1	0.3

HR: Hypophosphatemic rickets
 VDDR type 1: Vitamin D resistant Rickets type 1
 Adapted from paper I (123)

Table 7: Prevalence of hereditary rickets in children younger than 15 years of age, living in Southern Denmark on the 1st of January 2002

Type of hereditary rickets	Total number of children	Total number of cases	Prevalence [per 100,000]
Hereditary rickets, overall	251,234	13	5.2
HR	251,234	12	4.8
VDDR type 1	251,234	1	0.4

VDDR type 1: Vitamin D resistant Rickets type 1
 Adapted from paper I (123)

STUDY II: DESCRIPTIVE STUDY OF NUTRITIONAL RICKETS; BASED ON A REVIEW OF MEDICAL RECORDS

Characteristics at diagnosis

Among immigrant children, the age at diagnosis occurred in two incidence peaks, in age group 0.3-3.6 years and again in age group 5.1-14.8 years. Ethnic Danish children were only diagnosed between age 5 and 24 months. They accounted for 53% of all children diagnosed at age 0-24 months (figure 8).

Patients diagnosed before the age of 4 years displayed the classic clinical signs of rickets; enlargements of wrists, knees or ankles, bowing of legs, and rachitic rosary. Presenting symptoms in the young age group were predominantly refusal to support weight on legs and generalized seizures. Only immigrant patients were diagnosed after the age of 4 years and 69% were girls. They had few clinical signs of rickets and the presenting symptoms were predominantly pain in the legs, skeleton, and back.

Ethnic Danish children accounted for 73% of all cases presenting with hypocalcemic seizures and three of five cases presenting with fractures. There were no biochemical differences between ethnic Danish patients and age-matched immigrants at time of diagnosis (table 8).

The diagnosis of rickets was suspected by the referring physician in half of all patients but only in 24% of ethnic Danish patients. There was a seasonal variation with 75% of all cases presenting from January to June. In exception of one adolescent, generalized seizures at diagnosis were only seen in children aged 5-19 months and predominantly during winter and early spring (figure 9).

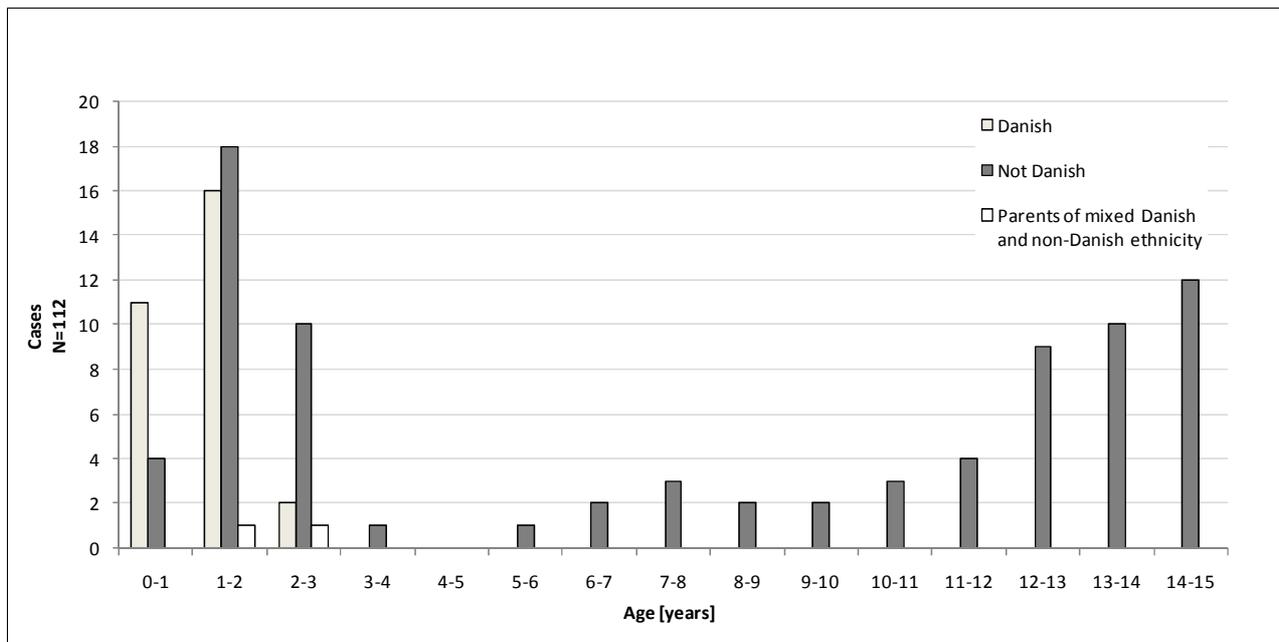


Figure 8. Age at diagnosis of nutritional rickets in ethnic Danish and non-Danish patients in Southern Denmark. Age at diagnosis occurred in two incidence peaks; among infants and young children aged 0.3-3.6 years and in older children and adolescents aged 5.1-14.8 years. Remarkably, ethnic Danish children were only diagnosed between age 5 and 24 months.

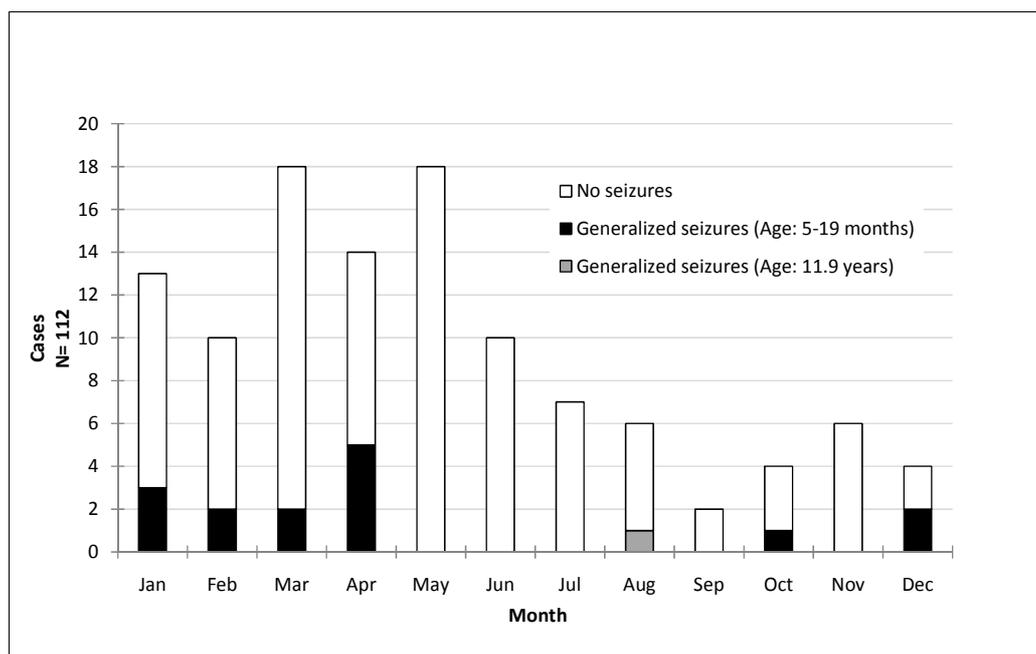


Figure 9. Seasonal variation in diagnosis and of generalized seizures as presenting symptom in patients with nutritional rickets. Diagnosis of nutritional rickets appeared more often during winter and early spring, and also generalized seizures were more often a presenting symptom during that time of the year.

Risk factors for nutritional rickets

Vitamin D was not given as recommended by The Danish National Board of Health in any of the immigrant children, however, in 24% of ethnic Danish children the parents reported that vitamin D had been given according to the guidelines. Among immigrant girls older than 4 years of age, 78% were veiled.

Table 8: Biochemical findings in ethnic Danish patients compared to age matched immigrant patients aged 0-24 months

	Ethnic Danish	n	Immigrants	n	p
Age [Months]	14 (5-24)	29	17 (4-24)	26	0.13
ALP [U/l] (100-400)	1961 [767-10080]	26	1748 [559-6570]	24	0.14
Ca ionized [mmol/l] (1.19-1.29)	1.04 [0.71-1.32]	18	1.17 [0.74-1.3]	13	0.42
Ca total [mmol/l] (2.15-2.70)	2.08 [1.13-2.64]	21	2.26 [1.23-2.49]	22	0.93
PTH [pmol/l] (1.1-6.9)	16.7 [1.1-112.0]	19	23 [4.9-59.7]	13	0.49
25(OH)D [nmol/l] (50-178[a])	7 [4-20]	4	12 [3-20]	8	0.86
1,25(OH) ₂ D [pmol/l] (51-177)	99 [16-553]	13	144 [29-473]	9	0.97

(reference value), mean (95CI), median [range]

[a] Definition of stages of vitamin D insufficiency (124):

25(OH)D < 50 nmol/l: vitamin D insufficiency,

25(OH)D: 12.5-25 nmol/l: vitamin D deficiency,

25(OH)D < 12.5 nmol/l: severe vitamin D deficiency

STUDY III: CROSS SECTIONAL STUDY OF PATIENTS WITH HR

Determination of genotype in patients with HR

The 59 included patients with hereditary FGF23-associated HR originated from 24 families and in 12 cases, HR was sporadic. Thus, 12 families accounted for 47 individual patients (table 9). In 20 of 24 probands (83%) a PHEX-gene mutation was detected. Seven of these PHEX-mutations have been reported previously in the literature or entered in the PHEXdb encountering 260 mutations (<http://www.phexdb.mcgill.ca/>, accessed 28th of May 2009), and to our knowledge 13 were new mutations.

Among the 12 patients with sporadic HR, a PHEX-gene mutation was detected in 11 patients (92%), and in one patient no mutations in the genes (PHEX-, FGF23-, DMP1-, SLC34A3 and CLCN5-genes) were detected. The parents of these probands were screened for the mutation found. In 7 probands, the parents did not carry the PHEX-mutation detected in their offspring, and the analysis of the parents of the remaining 3 PHEX-positive probands is currently being performed. Two probands' parents had passed away.

Among the 12 probands from families with HR, 9 (75%) were PHEX-positive. In 2 probands no genetic mutation was detected and in one proband a DMP1 mutation was found. This patient was the child of biochemically healthy, non-consanguineous Lebanese parents with no history of rickets. After termination of the inclusion period, the patient had a little brother, in whom the diagnosis of HR was recently established. Genetic analysis to confirm the DMP1-mutation in the parents and the diseased little brother is currently being performed. There was a male to female ratio of 1:2 in sporadic cases and of 1:1.9 in cases occurring in families (table 9).

In one family denoted 'family X', no mutation in the genes (PHEX-, FGF23-, DMP1-, SLC34A3 or CLCN5-genes) was detected. The trait was inherited in an X-linked dominant fashion. A genome wide linkage scan in this family revealed a strong evidence of linkage to the PHEX-locus (Lod score +4.8) and their disease was most likely due to a mutation in PHEX that was not detected by either the dHPLC or the MLPA analysis.

Table 9: Genetic mutations grouped by familial and sporadic cases

	Familial	Sporadic
Individuals (n)	47	12
Probands(n)	12	12
PHEX-positive (n Probands (%))	9 (75%)	11 (92%)
DMP1-positive (n Probands)	1	0
No mutation detected (n Individuals (n families))	16 (2)	1
Males	16	4
Females	31	8

Phenotype characteristics of patients with HR

Adult HR patients were characterized by a significantly lower final height with a z-score of -1.9 (95% CI = -2.4(-1.4); $p < 0.001$) compared to reference data. Compared to paired z-scores of final height, z-scores of leg length (-2.7 [-7.5(-0.8)]), were significantly more reduced, ($p < 0.001$) and paired z-scores of sitting height (-1.1 (-3.5-1.7)), were significantly higher, ($p < 0.001$). Accordingly, the patients appeared disproportioned, as indicated by the significantly elevated z-scores of sitting height ratio (2.6, 95% CI = 2.1-3.1; $p < 0.001$). The paired z-scores of height and arm span (-2.1 (-7.8-1.3)) indicated relatively short arms compared to height ($p = 0.06$). Their head circumference (1.4 [-0.4-5.5]) was significantly elevated compared to their final height, ($p < 0.001$), and in 30 (81%) patients this more than 2SD (table 10). To calculate the possible contribution of the deformities of the legs to the shortness of stature, Spearman's correlation coefficient of paired z-scores of leg deformities and z-scores of height was calculated in patients with no previous surgeries. No correlation was found in the total HR group or in the group comprising PHEX positives only, with $Rho = -0.31$, $p = 0.2$ and -0.11 , $p = 0.8$, respectively.

In HR children, the same anthropometric findings were present but though elevated, the z-scores of head circumference in children were significantly less enlarged compared to adult HR patients (table 11).

Assessing skeletal severity score and pain score

In adult HR patients the skeletal severity score was 1.6 (1-2). One or more surgical corrections of the leg deformities by osteotomy were performed in 18 (47%) of HR adults and in 5 (24%) of the children (table 11). Joint pain was commonly reported in adults and to a lesser degree in children. Predominantly, pain was reported in the knees (61% of adults and 29% of children), ankles (50% and 14%), and hips (40% and 10%). The average pain score was significantly higher in adults compared to children (1.4 (0-3) and 0.8 (0-3), $p=0.01$). Severe pain with serious limitation of activities (score 3) was only reported by 4 patients, all aged 40+ years. ROM of the hip was assessed in 37 of 38 adult patients, all having an internal rotation below the normal 40 degrees. In 14 (38%), no internal rotation of at least one hip was present. Limited flexion (below 140 degrees) was present in 36 (97%), extension below 15 degrees was seen in 18 (49%), of whom 8 (44%) had no extension in both hips. Patients aged 40+ years with de-

creased ROM, had significantly lower ROM of internal rotation ($p < 0.001$), flexion ($p = 0.03$), and extension ($p = 0.04$) compared to patients younger than 40 years of age.

Presence of arthrosis and enthesiopathies

Skeletal X-rays were obtained in 35 of 38 adult patients. Radiologic signs of arthrosis were present in at least one hip, knee, or ankle in 9 (26%), 9 (26%), and 4 (11%) of adult HR patients, respectively. Arthrosis of at least one of the above mentioned joints was present in 4 (25%) of the 16 patients aged less than 40 years and in 11 (58%) of the 19 patients aged 40+ years ($p = 0.05$). Enthesiopathies and calcification of the collateral ligaments between the vertebrae were seen only in patients aged 40 years or more and were present in 16 (84%) in that age group.

Bone mineral density in HR patients

BMD z-scores of lumbar spine and hip were elevated in adult patients (table 10). Lumbar BMD z-score was significantly higher in patients with one or more enthesiopathies of L2-L4 compared to patients without (4.3 [-1.0 - 7.2] and 0.5 [-1.5 - 5.3], respectively, $p=0.01$). BMD z-scores were also significantly higher in patients aged 40+ years of age compared to patients younger than 40 years of age (3.9 [-1.0 - 8.6] and 0.5 [-1.5 - 4.2], respectively, $p=0.001$), however, no difference was found in BMD of the hip in the two age groups (1.0 (-1.7-2.9) and 0.8 (-2.1-5.1), respectively). A high BMAD L1-L4 was found in HR children aged median 10.2 years [6.2-12.2]. Their z-score of BMAD L1-L4 was 2.3 [0.1-4.3].

Risk of fracture

One or more fractures had been experienced by 7 (18%) of HR adults and 1 (5%) of HR children. Comparing each HR patient with three age and gender matched Danish individuals gave an overall reduced RR of fracture among all HR patients of 0.34 (0.21-0.55), $p < 0.001$. Patients from Family X had not experienced any fractures, giving a reduced RR of fractures of 0.00 (0.00-0.00), $p < 0.001$. In PHEX positive patients the RR of fractures was 0.46 (0.27-0.78), $p < 0.01$.

Frequency of caesarean section

The 25 HR women had a total of 36 pregnancies and in 3 women (representing 5 of the 36 deliveries (14%)) a caesarean section was performed.

Dental findings

In HR adults aged 20+ years, the endodontic severity score was greater than two SD over the normal age matched reference 137 in 22 (67%) patients. Three children had radiologic signs of present or previous dental abscesses on x-ray, one in the primary teeth. Of the 21 HR children, 15 (71%) reported having had one or more dental abscesses in primary or permanent teeth, and 5 (33%) of these children had experienced more than five abscesses.

Table 10: Overview of the adult study population

	Total HR	PHEX positive	PHEX negative [a]	Family X
N (M/F) (ratio M:F)	38 (13/25) (1:1.9)	25	13	10 (3/7) (1: 2.3)
Age (years)	41.1 [18.6 - 74.0]	33.3 [20.8 - 74]	42.3 [18.7 - 73.4]	41.1 (18.7 - 66.6)
Anthropometrical data (z-score)				
Height	-1.9 (-6.7 - 0.8)	-2.1 (-4.4 - 0.8)	-1.5 (-6.7 - 0.5)	-0.9 (-1.9 - 0.5)
Sitting height	-1.1 [-3.5 - 1.7]	-0.9 [-2.6 - 1.7]	-0.9[-3.5 - 1.4]	-0.5 [-1.7 - 1.4]
Sitting height ratio	2.6 (-0.5 - 5.9)	2.9 (0.9 - 5.9)	2.0 (-0.5 - 5.9)	1.5 (-0.5 - 2.5)
Arm span	-2.1 [-7.8 - 1.3]	-2.2 [-7.8 - 1.3]	-1.9 [-4.2 - 0.4]	-1.3 [-3.7 - 0.4]
Leg length	-2.7 [-7.5 - (-0.8)]	-3.2 [-5.7 - (-0.9)]	-2.3 [-7.5 - (-0.8)]	-1.7 [-2.7 - (-0.8)]
Head circumference	1.4 [-0.4-5.5]	1.4 [-0.4-5.5]	1.4 [-0.2-3.2]	1.1 [-0.2-3.2]
Skeletal severity				
Skeletal severity score	1.6 (1 - 2)	1.8 (1 - 2)	1.3 (1 - 2)	1.2 (1 - 2)
Correcting surgery LL [b] (%)	47	60	15	0
Deformity LL (z-score)	1.7 [0.2 - 5.6]	1.7 [0.2 - 5.6]	1.5 [0.2 - 4.5]	1.4 [0.2 - 2.4]
Fracture %	18	24	8	0
Hip internal rotation ≤ 10 degrees (%)	43	52	58	50
Pain severity (score 0-3)	1.0	1.5	1.2	0.8
Arthrosis %	43	59	15	10
DEXA (z-scores)				
BMD lumbar spine	1.9 [-1.5 - 8.6]	1.9 [-1.0 - 8.6]	1.8 [-1.5 - 7.2]	1.5 [-1.5 - 6.5]
BMD hip	0.9 (-2.1 - 5.1)	1.1(-2.1 - 5.1)	0.4 (-1.7 - 2.9)	0.2 (-1.7 - 2.9)
Endodontic severity				
Endodontic severity score	0.26 (0 - 0.88)	0.29 (0 - 0.88)	0.18 (0 - 0.59)	0.18 (0 - 0.59)
Untreated for 6 months (N)	24	12	12	10
Laboratory values (z-score)				
Serum PTH	0.7 [-1.5 - 6.6]	0.2 [-1.5 - 6.6]	1.2 [-1.3 - 2.8]	0.7 [-1.3 - 2.8]
Serum PO4	-2.1 (-3.5 - (-0.7))	-2.3 (-3.1 - (-1.3))	-1.9 (-3.5 - (-0.7))	-1.7(-3.0 - (-0.7))
Serum FGF23	3.6 [0.9 - 18.9]	3.7 [1.6 - 8.7]	3.4 [0.9 - 18.9]	2.9 [0.9 - 7.3]
Serum 25(OH)D	-1.7 (-3.4 - 0.2)	-1.8 (-3.4 - (-0.2))	-1.6 (-2.9 - 0.2)	-1.6 (-2.9 - 0.2)
Serum 1.25(OH)2D	-0.7 (-2.9 - 1.8)	-0.6 (-2.4 - 1.5)	-0.8 (-2.9 - 1.8)	-0.7 (-2.9 - 1.8)
Serum BSALP	2.1 [-1.4 - 6.6]	1.8[-1.3 - 6.6]	2.3 [-1.4 - 5.8]	2.1 [-1.4 - 5.8]
TPO4/GFR	-2.7 [-4.0 - (-1.7)]	-3.1 [-4.0 - (-2.4)]	-2.5 [-4.0 - (-1.7)]	-2.4 [-3.2 - (-1.7)]

Mean (95CI), median [range]

[a]Family X family members are included in the group of PHEX-negative patients, [b]LL: lower limbs

Table 11: Comparison of anthropometric data in HR children (aged < 18 years) and adults

	HR children	HR adults	P-value
N (M/F) (ratio M:F)	21 (7/14) (1:2)	38 (13/25) (1:1.9)	0.9
PHEX-positive n (% of N)	16 (76%)	25 (66%)	0.4
Family X n (% of PHEX-negative)	4 (80%)	10 (77%)	1.0
Age (years)	10.1 [1.8-17.8]	41.1 [18.6 - 74.0]	< 0.001*
Correcting surgery LL [a] n (%)	5 (24%)	18 (47%)	
Anthropometrical data (z-score)			
Height	-1.6 (-5.4 - 1.5)	-1.9 (-6.7 - 0.8)	0.5
Sitting height	-0.4 [-2.2 - 1.4]	-1.1 [-3.5 - 1.7]	0.3
Sitting height ratio	2.3 (-0.1 - 4.6)	2.6 (-0.5 - 5.9)	0.4
Arm span	-1.3 [-4.3 - 1.4]	-2.1 [-7.8 - 1.3]	0.1
Leg length	-2.4 [-5.8 - 0.9]	-2.7 [-7.5 - (-0.8)]	0.6
Head circumference [b]	0.9 [-1.4 - 4.7]	1.4 [-0.4-5.5]	0.01*

Mean (95CI), median [range]

[a]LL: lower limb, [b]Reference data: Adults: UK, Bushby, 1992 (130), children: UK, Cole, 1998 (131)

*p< 0.05

Effects of medical treatment throughout childhood in PHEX-positive HR patients

Adult PHEX-positive patients treated with alphacalcidol and phosphate throughout childhood were significantly younger compared to the group of patients never treated during childhood (table 12). There were no differences in z-scores of final height or other anthropometric measures. Among the treated patients 6 (67%) had one or more osteotomies performed compared to 7 (54%) of the never treated patients, in whom the deformities of the legs tended to be more severe ($p = 0.09$). Thus, the severity score was not different between the groups. Treated patients had a significantly lower lumbar BMD z-score compared to those never treated ($p = 0.02$). With the exception of three patients, all those not treated during childhood had one or more enthesiopathies of L2-L4. In comparison, no enthesiopathies were seen in the younger group of patients treated throughout childhood. There was no difference in hip BMD z-score. The endodontic severity score was significantly lower in treated patients compared to never treated ($p = 0.001$) (table 12). In PHEX-positive patients treated during childhood, the endodontic severity score was greater than two SD over the normal age matched reference in 4 (44%). Among patients never treated during childhood, 10 (83%) had an endodontic severity score greater than two SD over the normal reference.

Biochemical implications of medical treatment

PHEX-positive patients currently treated with phosphate and alphacalcidol had significantly higher serum values of ionized calcium and FGF23 ($p = 0.01$ and 0.008 , respectively), and significantly lower values of TPO_4 /GFR and serum phosphate ($p = 0.001$ and 0.008) compared to never treated. Hyperparathyroidism (HPT) was not seen among PHEX-positive patients never medically treated, but it was present in 24% of currently treated patients. In three patients HPT was accompanied by hypercalcaemia (table 13).

Two patients had surgeries due to tertiary HPT, one of them was referred for surgery due to the results of blood samples

taken in this study. The other patient had surgery at the age of 17 years. Nephrocalcinosis and HPT were demonstrated at the age of 10 years and at the age of 18 years he developed hypertension. He was PHEX-positive, had a z-score of FGF23 of 240 and a decreased creatinine clearance of 62 ml/min, all contributing to the marked elevation of serum FGF23. Since treatment start at age 1.8 years phosphate doses were within the range of 100-200 mg/kg/day.

Due to a misinterpretation of a medication information leaflet, one child was by mistake treated with a phosphate dose of 185 mg/kg/day, being three times as high as intended. This patient had developed HPT (z-score PTH: 4.2), a severely decreased TPO_4 /GFR z-score of -7.7, and a z-score of serum phosphate of -5.3. In addition, nephrocalcinosis had been demonstrated.

Gender differences in disease severity in patients with genetically verified X-linked HR

In patients with X-linked HR, there were no significant differences according to gender in anthropometric or osteodensitometric measures (table 14+15). The skeletal severity score tended to be higher in males, 1.8 [1-2] compared to females 1.5 [1-2], ($p = 0.14$). One or more osteotomies had been performed in 7 (64%) of the males and in 8 (33%) of the females ($p = 0.14$). No difference in leg deformity was seen between genders (2.0 [0.2-5.6] in males and 1.7 [0.2-4.5] in females). Comparison of biochemical findings between males ($n = 7$) and females ($n = 15$) not treated during the past 6 months revealed a tendency of male patients having a higher z-score of serum FGF23, 4.1 [1.6-7.3] compared to females 2.4 [0.9-8.7], ($p = 0.13$). Endodontic examination was performed in 9 males and 21 females with X-linked HR. The endodontic severity score was greater than two SD over the normal age matched reference in 8 (89%) males and in 15 (71%) females. The endodontic severity score in males of 0.35 [0.0-0.88] was not different from the severity score in females 0.22 [0.0-0.70] (table 15).

Table 12: Differences between PHEX-positive adults treated medically throughout childhood compared to never treated during childhood

	PHEX-positive adults		p-value
	Treated (3-18y)	Never treated during childhood	
N (M/F) (ratio M:F)	9 (3/6) (1:2.0)	13 (3/10) (1:3.3)	0.6
Age (years)	25.6 [20.8 - 32.8]	53.7 [22.9 - 74]	0.01*
Anthropometrical data (z-score)			
Height	-1.8 (-3.4 - (-0.4))	-2.1 (-4.4 - 0.8)	0.6
Sitting height	-1.2 [-2.0 - 1.5]	-1.0 [-2.0 - 1.7]	0.6
Sitting height ratio	2.5 (0.9 - 3.9)	3.2 (0.9 - 5.9)	0.2
Arm span	-1.7 [-3.7 - 1.3]	-2.1 [-5.2 - 1.2]	0.5
Leg length	-2.7 [-4.1 - (-1.9)]	-3.6 [-5.7 - (-0.9)]	0.5
Head circumference	2.1 [1.1-5.5]	1.4 [-0.4-3.6]	0.1
Skeletal severity			
Skeletal severity score	1.8 (1 - 2)	1.8 (1 - 2)	1.0
Correcting surgery LLa n (%)	6 (67%)	7 (54%)	0.7
Deformity LL (z-score)	0.8 [0.2 - 2.6]	2.0 [0.2 - 5.6]	0.09
DEXA (z-scores)			
BMD lumbar spine	0.2 [-0.3 - 4.2]	3.1 [-1.0 - 6.1]	0.02*
BMD hip	0.5 (-2.1 - 5.1)	1.4 (-0.7 - 2.0)	0.6
Endodontic severity			
Endodontic severity score	0.08 (0 - 0.46)	0.43 (0.12 - 0.88)	0.001*

Mean (95CI), median [range], [a]LL: lower limbs

Table 13. Comparison of biochemistry in treated and never treated PHEX positive patients (children and adults)

	Current treatment Phosphate + Alphacalcidol	Never treated	p-value
N [a]	21	7	
Serum ionized Ca [z-score]	1.6 [-2.0 - 8.4]	-0.8 [-3.2 - 1.2]	0.01*
Serum PTH [z-score]	0.1 [-2.0 - 7.9]	-0.4 [-1.5 - 1.4]	0.2
TPO4/GFR [z-score]	-4.5 [-7.7 - (-2.7)]	-2.7 [-3.3 - (-2.4)]	0.001*
Serum PO4 [z-score]	-3.5 [-5.7 - (-2.0)]	-2.2 [-3.0 - (-1.3)]	0.008*
Serum FGF23 [z-score]	8.4 [3.0 - 76.2]	3.7 [2.0 - 8.7]	0.008*
Uca/cr	0.33 [0.03 - 1.23]	0.21 [0.01 - 0.35]	0.2
Hyperparathyroidism			
HPT n/N (%)	5/21 (24%)	0/7 (0%)	0.3
HPT + hypercalcaemia n/N (%)	3/5 (60%)		
Hypercalciuria			
Non-HPT + hypercalciuria n/N (%)	8/16 (50%)	2/7 (29%)	0.4
Skeletal severity (patients aged 10+y)			
n	14	7	
Skeletal severity score (1-2)	1.8	1.6	0.4

[a]Patients with vitamin D deficiency (S-25(OH)D < 25 nmol/l) were excluded to eliminate the contribution of vitamin D deficiency to biochemical changes, Median [range], *p < 0.05

HPT: hyperparathyroidism; S-parathyroid hormone > 7.0 pmol/l,

Hypercalcaemia: S-Ca ion > 1.30 mmol/l, Hypercalciuria: Uca/cr > 0.30 (139)

Table 14. Gender differences in anthropometric data in adults and children with X-linked HR

	Males/boys	Females/girls	p-value
N (adults/children)	17 (11/6)	38 (24/14)	
Age (years)	23.0 [4.3 - 66.5]	26.5 [1.8 - 74]	0.9
Anthropometrical data (z-scores)			
Height	-1.8 (-3.7 - 1.5)	-1.7 (-5.4 - 0.8)	0.5
Sitting height	-1.2 [-2.6 - 1.5]	-0.8 [-2.0 - 1.7]	0.4
Sitting height ratio	2.4 (0.3 - 4.3)	2.4 (-0.5 - 5.9)	0.9
Arm span	-1.9 [-7.8 - 1.4]	-1.5 [-5.2 - 1.3]	0.8
Leg length	-2.4 [-4.5 - 0.9]	-2.5 [-5.8 - (-0.1)]	0.7
Head circumference [a]	1.6 [-0.4 - 5.5]	1.1 [-1.4 - 3.6]	0.2

Mean (95CI), median [range],

[a]Reference data: Adults: UK, Bushby, 1992 (130), children: UK, Cole, 1998 (131)

Variations within a PHEX-positive family

One family comprised seven family members, all carrying the same PHEX-gene mutation. Four female HR patients were diagnosed through the family screening performed during the study. In exception of the two young girls, all patients had experienced dental abscesses and three had severe skeletal affection. Only the two young girls were on medical treatment with phosphate and alphacalcidol, the remaining patients had never had medical treatment. The characteristics of the family are summarized in figure 10.

Figure 10. Variations within a PHEX-positive family

Skeletal sev.: skeletal severity score

Dental sev.: endodontic severity score

z: z-score

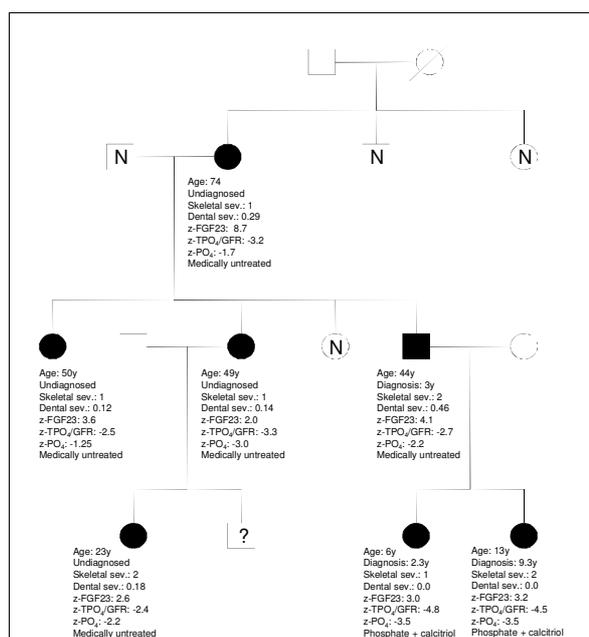


Table 15. Gender differences in adults with X-linked HR

	Males	Females	p-value
N	11	24	
Age (years)	43.5 [18.7 - 66.6]	39.8 [20.8 - 74]	0.9
Anthropometrical data (z-scores)			
Height	-1.6 (-3.7 - (-0.4))	-1.7 (-4.4 - 0.8)	0.8
Sitting height	-1.4 [-2.6 - 1.5]	-0.9 [-2.0 - 1.7]	0.4
Sitting height ratio	2.7 (0.7 - 4.3)	2.3 (-0.5 - 5.9)	0.5
Arm span	-1.5 [-7.8 - 1.2]	-1.9 [-5.2 - 1.3]	0.6
Leg length	-2.7 [-4.5 - (-1.3)]	-2.7 [-5.7 - (-0.8)]	0.9
Head circumference	1.6 [-0.2-5.5]	1.4 [-0.4-3.6]	0.9
Skeletal severity			
Skeletal severity score	1.8 (1 - 2)	1.5 (1 - 2)	0.14
Correcting surgery LL[a] n (%)	7 (64 %)	8 (33 %)	0.14
Deformity LL (z-score)	2.0 [0.2 - 5.6]	1.7 [0.2 - 4.5]	0.6
DEXA (z-score)			
BMD lumbar spine	1.8 [-1.0 - 8.6]	1.9 [-1.5 - 6.1]	0.8
BMD hip	1.6 (-0.7 - 5.1)	0.6 (-2.1 - 2.9)	0.19
Endodontic severity			
Endodontic severity score	0.35 (0.0 - 0.88)	0.22 (0.0 - 0.7)	0.2
Untreated for 6 months (N)	7	15	
Laboratory values (z-score)			
Serum PTH	0.7 [-0.4 - 2.6]	-0.3 [-1.5 - 6.6]	0.3
Serum PO4	-2.2 (-2.7 - (-1.5))	-2.0 (-3.1 - (-0.7))	0.5
Serum FGF23	4.1 [1.6 - 7.3]	2.4 [0.9 - 8.7]	0.13
TPO4/GFR	-3.0 [-3.3 - (-2.6)]	-2.5[-4.0 - (-1.7)]	0.17
Treatment			
Treatment throughout childhood	27%	25%	
Periodically treated	27%	4%	
Never treated during childhood	45%	71%	
Untreated for 6 months	64%	63%	

Mean (95CI), median [range], [a]LL: lower limbs

Patients diagnosed with HR but excluded from study III

In six adult patients previously diagnosed with and five of them currently on medical treatment for HR, the diagnosis of HR was not confirmed after participation in the study.

6. DISCUSSION

STUDY I AND II:

Incidence of nutritional rickets

Once considered largely eradicated, reappearance of nutritional rickets in the industrialized countries has given rise to several case reports. Our data demonstrated that nutritional rickets is still a rare disease in Denmark and primarily seen among immigrants. From the observed incidence among 0-14.9 year olds, approximately 29 new cases are expected per year in Denmark. Of these, an estimate of 11 cases will occur among the 0-2.9 year olds, and less than 4 of these cases will be among ethnic Danish children. The incidence of nutritional rickets in the Scandinavian countries has not been reported before. Recently, the incidence of nutritional rickets has been estimated in studies from Canada 28 and the United Kingdom (UK) 20 (table 16).

Table 16: Papers reporting incidence of nutritional rickets

	Callaghan et al., 2006 (20)	Ward et al., 2007 (28)
Country	United Kingdom	Canada
Study period	May 2000- April 2001	2002-2004
Number of patients	24	104
Age group[years]	0-5	0-18
Incidence [per 100,000 per year]	Overall: 7.5 Africans: 95 Asians: 38 White ethnics: 0.4	2.9

Thus, the incidence in Denmark (table 5) is similar to that reported from Canada (28) while the incidence in UK and among African and Asian immigrants in UK was higher compared to African and Asian immigrants living in Denmark. This may be attributable to the higher incidences among immigrants and a higher proportion of immigrants living in the West Midlands compared to Southern Denmark. The incidence among children of white ethnic origin in UK was lower compared to the incidence among ethnic Danish children, however, the UK series included only a single case.

One case report from Copenhagen, Denmark in 2003 described 40 cases of nutritional rickets, but only cases among immigrant children were identified (25). The incidence among ethnic Danish children in our study was therefore unexpectedly high, but tended to decrease throughout the study period. This

reduction coincided with launch in 1995 of recommendations on vitamin D issued by The Danish National Board of Health. Prior to 1995, vitamin D supplementation of 10µg/day was recommended to all children from 14 days until 1 year of age, irrespective of ethnicity. In 1995, the period of recommended vitamin D supplementation among immigrant children was extended to 2 years of age. Ethnic Danish children were advised to continue the existing recommendation of vitamin D supplementation. This announcement has most likely contributed to an increased awareness among general practitioners and health visitors to ensure vitamin D supplementation both among immigrants and in ethnic Danish children.

Characteristics at diagnosis of nutritional rickets

Our data confirmed that the age at diagnosis occurred in two incidence peaks i.e. among infants and young children aged 4 years or less and in older children and adolescents above the age of 4 years (figure 8) reflecting the greater demands of calcium and phosphate for mineralization of the skeleton at periods of high growth velocity. While infants and young children displayed the characteristic clinical signs of rickets, older children and adolescents had only few and unspecific symptoms and signs of rickets implying a risk of not being diagnosed. This view is supported by our observation that the diagnosis of nutritional rickets in ethnic Danish children was more than twice as often an incidental finding during hospitalization for other reasons compared to immigrant children. This diagnostic delay might have contributed to ethnic Danish children being clinically more severely affected compared to immigrant children diagnosed at the same age interval. Interestingly, however, ethnic Danish children were not more severely affected when evaluated by biochemical parameters suggesting a genetic difference in adaptation to vitamin D insufficiency (140).

We found a marked seasonal variation in the number of patients diagnosed with nutritional rickets as well as in frequency of hypocalcemic seizures as presenting symptom. Similar findings were described in New York by Hess, AF in 1922 (8), and in Copenhagen by Flensburg, EW et.al. in 1953 (14). In addition, a seasonal variation has been found in more sunny countries as Canada (28), Australia (26), and Iran (141). By the seasonal occurrence of nutritional rickets, our study reflects the inability of vitamin D synthesis by sun exposure from October to March in Denmark (44), which confines the vitamin D source during winter to food supply or supplementation. Since the average intake from the food supply of vitamin D is low, an adequate intake of vitamin D during winter months depends on vitamin D supplementation or possibly from foods enriched with vitamin D. At present, only a few margarine brands are fortified with vitamin D in Denmark (142), however, introduction of fortification of additional food items with vitamin D may prevent future rickets in Denmark.

Possible risk factors for nutritional rickets

Our data do not support breastfeeding as an independent risk factor in the development of rickets, but breast milk is a very poor source of vitamin D. A total of 95% of patients aged 4 years or less were breastfeed or weaned within 6 months before diagnosis and received no vitamin D supplement. It is well recognized that children exclusively breast feed by a vitamin D deficient mothers are at risk of developing rickets (143-147). Currently, vitamin D supplementation with 10 µg/day from 14 days after birth to 12

months in ethnic Danish children and until 24 months in immigrants are recommended in Denmark. Among the infants and young children, none of the immigrant patients and only 24% of the ethnic Danish patients had received vitamin D supplementation according to the guidelines. The majority of the ethnic Danish children were diagnosed from 12-24 months, and 31% of these patients received vitamin D according to the guidelines as reported by their parents.

Incidence and prevalence of hereditary rickets

Several authors have stated that hereditary rickets is now the most common cause of rickets in the western world (148-153). Our study indicates that hereditary rickets is the most common cause of rickets in ethnic Danish children, but overall, nutritional rickets among children younger than 3 years of age is still more common than hereditary rickets. The only previously published incidence of HR is a conservative estimate of 1:20-25,000 (154), corresponding to 4-5 per 100,000. However, this estimate is suspected to be a prevalence, since no information about age group, nationality or time period over which it was calculated was given. The incidence of HR in ethnic Danish children was calculated to 3.9 per 100,000 (0-11 months) and the prevalence to 4.8 per 100,000 (0-14.9 years). This corresponds to 2-3 new cases of HR per year and approximately 260 HR patients living in Denmark, assuming the prevalence in all age groups to be comparable to the prevalence among children aged 0-14.9 years. The family screening in study III performed among relatives of patients with HR revealed several undiagnosed adult patients with HR. No undiagnosed HR children were identified in the study region, thus, these calculations of incidence and prevalence are presumed to represent a true estimate of the actual number of new cases and of patients living in Denmark.

Evaluation of the method used in study I and II

Only patients diagnosed with rickets and assigned with one of the diagnosis codes referring to rickets were identified by this register based recruitment of patients. Our survey of GPs and pediatricians revealed that children with severe rickets were referred to hospital, but mild cases might not have been diagnosed and thereby escaped our attention. Nutritional rickets in adolescence often presents with unspecific symptoms and may therefore be misclassified or not be diagnosed. The calculated incidence of nutritional rickets therefore tends to be an underestimate. The prevalence of nutritional rickets was not estimated because the duration of the disease was unknown. The diagnosis of nutritional rickets is very often delayed several months and data on when complete healing of rickets occurred was not always available in the medical records. The identified cases of nutritional rickets were presumed to be predominantly vitamin D deficiency rickets, but in children with no measurement of serum 25(OH)D, calcium deficiency rickets cannot be excluded. Excluding patients suspicious of nutritional rickets and with serum 25(OH)D values > 50 nmol/l might potentially exclude patients with vitamin D deficiency rickets in stages of healing, as the treatment with vitamin D or sun exposure may have normalized their 25(OH)D serum levels. Re-evaluation of these cases revealed three excluded patients with no clinical or radiological signs of rickets described. In addition, only one child had elevated levels of ALP, otherwise no biochemical criteria of nutritional rickets were fulfilled. Thus, it

is not likely that they present cases of nutritional rickets in the early stages of healing.

When aiming to estimate the true incidence of nutritional rickets, a prospective study design might be superior to our retrospective, register based design. The prospective design is also capable of securing the observations needed for a correct diagnosis of nutritional rickets and in addition to distinguish between vitamin D deficiency- and calcium deficiency rickets. Results from a prospective study would still be limited to the cases where a diagnosis of rickets was suspected, but an increased awareness of the diagnosis is likely. Thus, a prospective study design was preferable but since nutritional rickets is a rare disease, a prospective study would have to proceed for decades to include the same number of patients as were included in our studies. A population based survey would be useful in estimating the prevalence of nutritional rickets. However, as nutritional rickets is a rare disease a very large population sample is needed. In addition, a large seasonal variation in diagnosis of nutritional rickets occurs in Denmark 39 whereby a large seasonal variation in prevalence might be expected.

The retrospective design based on data from medical records, limits the availability of data to those recorded. Well known clinical signs of nutritional rickets in infants and young children, as craniotabes and late dentition 56, were rarely mentioned in the medical records. It does not seem reasonable to assume that these clinical signs were not present, they were probably overlooked or not recorded.

The strengths of study I and II were the large number of patients included and the use of Danish registers providing data on diagnoses, ethnicity and place of birth.

STUDY III:

Determination of genotype in patients with HR

Mutations in the PHEX-gene were detected in 83% of all probands, and in 75% and 92% of familial and sporadic probands, respectively. Table 17 gives an overview of reports of the percentages of detected mutations in the PHEX-gene in HR patients. Along with Popowska's study 155 the percentage of detected PHEX-mutations in our study was the highest. In addition, a very high percentage of detected PHEX-mutations was found among sporadic probands. This high frequency of PHEX-mutations among sporadic probands probably reflects that our inclusion- and exclusion criteria did in fact exclude sporadic patients with acquired HR. A DMP1 mutation was detected in a Lebanese proband corresponding to the recessive mode of inheritance in that family. Indeed, PHEX-mutations were the most common cause of hereditary HR in our series.

Phenotype characteristics of patients with HR

Our study confirmed the well known short stature of HR adults as well as children. They were disproportioned with an average z-score of sitting height ratio significantly higher than normal reference. This was due to a relatively greater reduction of leg length compared to sitting height, which was also described in three other reports (167-169). Some of the reduction of leg length might be ascribed to deformities of the legs, but even in adult patients with a z-score of deformities of less than 2, the same significant association was seen. Their arm span was shorter than normal indicated by relatively short arms compared to height. Only one previous report on arm span in HR patients was re-

trieved reporting measures within the low-normal range, but the values were not correlated to height (167). The head circumference of HR patients was enlarged. This was most pronounced in adults where all patients had greater z-scores of head circumference compared to z-scores of height, and this more than 2SD in 81%. Significantly elevated head circumference in Polish HR patients was found in males but not in females (169). It is well known, that the skull undergoes mainly membranous ossification. Since the skull grows abnormally much and the rest of the skeleton grows significantly less than normal, we suggest that the influence of HR upon the growth of bones is partly determined by membranous or endochondral ossification, respectively. Thus, our data indicate that the long bones of legs as well as arms are more severely growth retarded than the axial skeleton, and that increased growth is seen in the skull.

Especially adult patients reported pain in the joints, most commonly in the knees, a finding also reported among HR patients of New Zealand (95). These joints are all affected by a changed anatomical strain due to the deformities of the legs, and we also found a high percentage of arthrosis in these joints in HR patients. Especially, ROM of the internal rotation of the hip was reduced in all HR adult patients also reported in 10 of 13 adult HR patients by Reid et al. (95), and ROM decreased further in patients aged 40+ years.

Table 17: Reports of verified mutations the PHEX-gene in HR probands

Reference	N probands (n familial / n sporadic)	Positive PHEX-mutation in probands		
		Total n (%) [a]	Familial n (%) [b]	Sporadic n (%) [c]
Francis 1997 (156)	43	33 (77%)	25 (86%)	8 (57%)
Rowe 1997 (157)	106 (99/7)	58 (55%)	56 (57%)	2 (29%)
Dixon 1998 (158)	68 (46/22)	31 (46%)	24 (52%)	7 (32%)
Popowska 2000 (155)	35	29 (83%)	NA	NA
Tynnismaa 2000 (159)	23 (8/15)	18 (78%)	4 (50%)	14 (93%)
Holm 1997 (160) and 2001 (161)	50	31 (62%)	NA	NA
Cho 2005 (162)	17 (5/12)	8 (47%)	3 (60%)	5 (42%)
Song 2007 (163)	15	9 (60%)	NA	NA
Ichikawa 2008 (164)	26	18 (69%)	NA	NA
Jehan 2008 (165)	91	70 (77%)	43 (NA)	27 (NA)
Gaucher 2009 (166)	116 (56/60)	93 (80%)	49 (88%)	44 (73%)
Beck-Nielsen Study III	24 (12/12)	20 (83%) [d]	9 (75%) [d]	11 (92%)
Mean percentage		68%	67%	60%

[a]percentage PHEX-positive probands of all probands

[b]percentage PHEX-positive familial probands of all familial probands

[c]percentage PHEX-positive sporadic probands of all sporadic probands

NA: Not available

[d]As a DMP1 mutation was found in one proband, a total of 21 probands (87%) and 10 (83%) of familial probands had a mutation causing HR identified

The z-scores of lumbar spine and hip in adults were elevated, also demonstrated in lumbar spine in two other studies (95,96). HR patients in our study with very high BMD L2-L4 were primarily aged 40+ years, and the majority had enthesiopathies of the spine visualized by X-ray. A BMD z-score of L2-L4 exceeding 2SD was also seen in four patients aged less than 40 years and with no enthesiopathies demonstrated on X-ray. These findings indicate that the high BMD of lumbar spine is not solely caused by extra-skeletal calcifications. A histologic study of adult patients with HR revealed an elevated trabecular calcified volume, elevated trabecular osteoid volume and mean osteoid seam thickness (170). A significant discrepancy was found between the in some cases, extremely elevated BMD L2-L4 and the more moderate elevation of BMD at the hip. Since the ratio of trabecular to cortical bone is higher in the lumbar spine as compared to the hip, the elevated BMD of L2-L4 might reflect an overabundance of partially mineralized osteoid or an increased trabecular bone mass. An elevated median z-score of BMAD in HR children was found as well. A study of BMD in HR children also described elevated BMD of lumbar spine (171). As enthesiopathies were not seen in children (172), the high BMAD cannot be ascribed to extra-skeletal calcifications, indicating a real elevated BMD at the lumbar spine.

In our study, 18% of the adult HR patients had experienced one or more fractures. The only previous report of risk of fracture is in German HR patients, where 39% suffered from one or more fractures (173). Their median age was 29 years, which was lower compared to patients in our study, thus, the higher percentage of the German patients experiencing a fracture could not be ascribed to a higher risk time. Patients from Family X did not experience any fractures, and among the PHEX positive adult patients the percentage was 24%, still considerably lower compared to the German HR patients. A significantly decreased RR of fracture in adult HR patients of 0.34 was calculated. Using the estimates by Marshall et al. (174) based on the observed spine BMD of +1.9 Z-scores, the expected RR of any fracture was $1.5 \cdot 1.9 = 0.46$, i.e. within the range observed. Much of the decrease in the risk of fractures may, thus, be ascribed to the increased BMD.

In nutritional rickets, the pelvis might become narrowed causing difficulty in vaginal delivery (1,175). A high frequency of caesarean section in 15 of 22 deliveries (68%) was described among HR women in New Zealand, however, only two of the caesarean sections were reported to be attributed to the presence of HR (95). Nine HR woman from USA had 31 children, all delivered vaginally (176). In our study population, the percentage of caesarean section in deliveries in HR women was 14%. These deliveries took place during the time period 1946-2006 (median year 1992). In comparison, the overall percentage of caesarean section in Denmark in 1991 was 12% (177). Our data support that HR women do not suffer from delivery complications due to contracted pelvis.

In 67% of HR patients, the endodontic severity score was greater than 2SD over the normal age matched reference, and increasing age was associated with a higher endodontic severity score as discussed in details in paper III. Although only three children had radiologic signs of present or previous dental abscesses, 71% reported having experienced abscesses and 33% of these children had had more than five abscesses. The low frequency of X-ray verified abscesses in children might be due to the abscesses being more prevalent in the primary teeth, or that abscessed primary teeth in children more often are extracted and thereby not visible on a subsequent x-ray.

Effects of medical treatment throughout childhood in PHEX-positive HR patients

Treatment throughout childhood did not reveal any differences in anthropometric measures or skeletal severity score compared to no childhood medical treatment. Other cross-sectional studies comparing final height between treated and not treated HR patients also report no treatment effect (162,173,176,178). Improvement of height z-scores during childhood growth upon treatment with alphacalcidol and phosphate has been demonstrated in prospective and retrospective studies (76,113,139,162,178-181). Some studies report a better treatment response in girls compared to boys (139,182), and two studies did not find improved height z-scores after treatment (183,184). Only one prospective trial of medical treatment of symptomatic adults is reported. Treatment improved bone or joint pain in 87% and improved, but did not normalize the osteoid thickness or volume (116).

In our study, patients in the treated group were significantly younger compared to never treated. They had a high frequency of osteotomies performed, but never treated patients tended to have more severe bowing of the legs, indicating that surgical correction might be indicated but not performed. The lumbar BMD z-score was significantly lower in the group of patients treated throughout childhood. However, with the exception of one patient, all never treated patients were aged 40+ years, and 73% had lumbar enthesiopathies, most likely contributing to the elevated BMD in the older untreated HR patients.

The endodontic severity was significantly lower in the group of treated patients compared to those not treated during childhood. In a large dental study of 48 children and adults with HR, a significant reduction of dental abscesses was seen in the group of patients treated with alphacalcidol during childhood compared to the group of never treated HR patients (75). This conclusion might be biased by the fact that patients in the never treated group were aged 28+ years compared to the younger group of patients treated with alphacalcidol, aged 3 to 25 years. Our findings are similar, but our data are also biased by the age difference between the groups. Thereby, ours as well as other author's data might primarily reflect the increasing age implying increased attrition as a potent risk factor for the development of dental abscesses.

Biochemical consequences of medical treatment

Patients on current medical treatment appeared more severely biochemically deranged compared to never treated patients. Patients on current medical treatment had significantly lower values of TPO_4/GFR , and serum phosphate, and higher values of FGF23. They had significantly higher serum ionized calcium, probably due to the alphacalcidol treatment. The skeletal severity score was not different among patients aged 10+y in the two groups, thus, it seems unlikely that patients on current medical treatment were so due to more severe skeletal disease. As HPT was not seen in never medically treated PHEX-positive patients with serum 25(OH)D values at or above 25 nmol/l, our data indicate that HPT is primarily caused by medical treatment. In three never treated adult patients from Family X, slightly elevated PTH levels of 7.0, 7.8, and 8.1 pmol/l, respectively, were present with concomitant serum levels of vitamin D of 46, 80, and 56 nmol/l, respectively. Elevated PTH levels have been reported in untreated HR patients, but with no genetic evaluation or concomitant data on vitamin D status in the individual patients (95,185,186). Therefore, HPT seems to be associated to medical treatment, at least in

Table 18. Reports of gender differences in HR patients

Study	Males /females (n)	Height	Head circ.	Lumbar BMD	Osteoid thickness	Skeletal severity	Endodontic severity	Serum PO4	TPO4/GFR
Winters et al. 1957 (59)	4/4	-	-	-	-	-	-	M<F	NS
McNair et al. 1969 (167)	9/16	NS	-	-	-	-	-	NS	-
Gloriux et al. 1972 (190)	-	-	-	-	-	-	-	-	M<F
Steendijk et al. 1984 (168)	5/11	M<F	-	-	-	-	-	-	-
Reid et al. 1989 (95) and 1991 (191)	5/14	NS	-	NS	M>F	-	-	NS	NS
Shields et al. 1990 (189)	6/11	-	-	-	-	-	M>F	-	-
Balsan et al. 1990 (139)	16/24	M<F	-	-	-	-	-	-	-
Petersen et al. 1992 (182)	7/13	NS	-	-	-	-	-	NS	NS
Rosenthal 1993 (96)	5/13	-	-	-	M>F	-	-	-	-
Whyte et al. 1996 (187)	7/23	NS	-	-	-	-	-	NS	NS
Miyamoto et al. 2000 (181)	5/17	NS	-	-	-	-	-	-	-
Holm et al. 2001 (161)	26/50	-	-	-	-	NS	NS	-	-
Cho et al. 2005 (162)	3/5*	-	-	-	-	NS	NS	NS	-
Ariceta et al. 2007 (183)	7/20	NS	-	-	-	-	-	-	-
Jehan et al. 2008 (188)	-	NS	-	-	-	NS	-	-	-
Beck-Nielsen et al paper III	11/24*	NS	NS	NS	-	NS	NS	NS	NS

- : No data available, M: Males, F: Females, NS: Not statistically significant, *: Patients with verified X-linked HR only

PHEX-positive patients. Hypercalcaemia was found in 43% of the patients with HPT, and two patients in the study had verified tertiary HPT.

Gender differences in patients with genetically verified X-linked HR

Since the PHEX-gene is located on the X-chromosome, a gene dosage effect with males being more severely diseased compared to females is expected. Several studies have investigated the possible gender differences, but in exception of one small study of HR children, no gene mutation analysis were performed or the comparison between genders were conducted on the whole group of studied patients with no regard to whether a PHEX-mutation was detected or not. Table 18 gives an overview of studies reporting gender differences in HR patients. The majority of the studies found no statistical significant gender differences in the variables reported (161,162,167,181-183,187,188). Males have been reported more severely affected compared to females regarding height, osteoid thickness, and endodontic severity (95,96,139,168,189). There are only reports of males being more severely affected than females. This study as well as other studies describes data where males tend to be more severely affected than females, but the differences did not reach statistical significance (table 15). This might be explained by small sample size and by the fact that asymptomatic females are rarely diagnosed and thereby not included in the comparison of gender differences.

Variations within a PHEX-positive family

Within a family of PHEX-positive family members all carrying the same genetic mutation, a great variation in severity was seen and the majority of the female patients were undiagnosed. The presence of undiagnosed XLH-patients has been reported by Econs et al. (192) and they also demonstrated the variability within family members carrying the same gene mutation (193).

Patients diagnosed with HR but excluded from study III

Six patients previously diagnosed with HR were excluded from the study as they did not meet the inclusion criteria. Two patients were suspicious of VDDR type 1, and two patients most likely had childhood nutritional rickets at diagnosis. They both had a relative diagnosed with HR which possibly contributed to an increased awareness of a hereditary form of rickets. The two remaining patients had no family history, adult onset of disease with multiple fractures, and were severely disabled at diagnosis. Investigations to locate a possible FGF23 producing tumor are currently being performed. These six patients represent possible differential diagnoses of HR. Reevaluating HR patients with no gene mutation detected is encouraged to ensure that the diagnosis of hereditary HR is verified.

Evaluation of the method used in study III

Our study revealed that a verified diagnosis of HR was only likely in 50% of the patients assigned with a diagnosis code of either 'Rickets, vitamin D resistant (hypophosphatemia familiaris)' or 'Disorders of phosphorus metabolism (Vitamin D resistant: Osteomalacia + rickets)' after review of the medical records. Register search without review of the medical journals is therefore not suitable in any evaluation of HR. First degree relatives to participants were offered screening for HR, but second degree relatives were only offered screening in case of symptoms. Thereby, asymptomatic second degree relatives with HR escaped our attention and a selection towards inclusion of more severely diseased HR patients was present.

Our methods of genetic evaluation and the inclusion criteria were satisfactory, as especially the percentages of PHEX-mutations detected in all probands and in sporadic probands were very high.

Medical treatment was only paused from the evening before the examination to reduce the influence on the biochemical parameters. Blood and urine samples were taken after 2 hours of fast at variable daytime hours. This method is appropriate for obtaining values for the calculation of TPO_4/GFR (136), but does not meet the diurnal changes of serum PTH. Ideally, all patients should have undergone a medication wash out period of 14 days prior to the study. In addition, the blood and urine samples could have been taken after an overnight fast. This was not considered realizable taken in consideration that some patients, especially children, were travelling for hours to reach the place of examination. Hospitalization during the study period was not considered due to the economical impact and the risk of fewer patients to be included in the study. The assessment of nephrocalcinosis would have been most interesting, but due to the already comprehensive examination program this examination was omitted to eliminate the risk of fewer patients to be included in the study. In characterizing patients with HR, Danish normal reference data were used to calculate z-scores for anthropometric data. Exceptions were calculations of z-scores of head circumference and leg deformities, where UK and French normal reference data, respectively, were used. An age and gender matched control group was desirable when assessing pain in the joints, ROM of the hips, BMD, and X-ray changes. Examination of a control group was declined as the economical and time expenses for this additional investigation could not be met.

Our study is the first to describe gender differences in a large group of adult HR patients with proven X-linked disease, whereby patients with i.e. ADHR, ARHR or acquired forms of HR were excluded. The detection rates of PHEX-mutation in the studies listed in table 17 were on average only 68%, whereby one third of the patients included in their calculations of gender differences might not have X-linked HR. Our data indicate males being more severely affected compared to females, but no statistical significant differences were obtained. This might be due to small sample size, but also by the fact that only symptomatic second degree relatives were offered screening. Until now, one asymptomatic PHEX-positive female not included in the study has been identified by the family screening. The remaining results from the family screening are awaited and patients identified with a PHEX-mutation will be offered participation in the study and subsequently included in the analysis of gender differences.

One aim was evaluation the possible effects of medical treatment throughout childhood. Only PHEX-positive adults were included to eliminate the possibility of a different treatment response in different groups of genotypes. Since a great variability

of disease severity was seen even within family members carrying the same PHEX-gene mutation, this strategy did not homogenize the group studied. A cross-sectional design does not take different starting points of i.e. skeletal severity into account. Therefore, when no differences between the groups were found, it does not necessarily mean that treatment did not change the outcome in the individuals. Patients medically treated throughout childhood might be more severely affected and if treatment improves their disease status, no difference is visible when comparing to never treated and milder affected patients. In addition, never treated patients were significantly older compared to treated patients providing a higher risk time for i.e. the development of dental abscesses. Today, all symptomatic patients are usually offered medical and/or surgical treatment when necessary, while this was probably not the case in all older patients despite their indications for treatment were fulfilled. Our finding that treatment did not affect the final height is in accordance with other cross-sectional studies while several retrospective and prospective studies find improvement in height z-scores upon treatment. In conclusion, the cross-sectional design is not suitable for evaluation of the effect of medical treatment. A prospective, randomized, controlled study design is clearly preferable.

7. CONCLUSIONS

STUDY I AND II - NUTRITIONAL AND HEREDITARY RICKETS:

Nutritional rickets is still observed in Denmark, predominantly among immigrant children, however, also among ethnic Danish children during early childhood. Overall, nutritional rickets is the most common type of rickets in Denmark, however, hereditary rickets was more common among ethnic Danish children. Since the majority of ethnic Danish children were diagnosed at age 12-24 months, we recommend an extension of their period of vitamin D supplementation until 2 years of age. Infants and young children displayed the characteristic clinical signs of rickets, but in older children and adolescents, clinical signs were few and symptoms unspecific implying a risk of not being diagnosed. Plausible risk factors in young children were omitted vitamin D supplementation and veiling in adolescent girls.

Nutritional rickets most frequently arise subsequent to long-standing vitamin D deficiency, and therefore diagnosed patients most likely only represent the tip of the iceberg considering the presence vitamin D deficiency in the general population of children. Since nutritional rickets were predominantly diagnosed during the winter months, our study indicates that the vitamin D intake during winter time was inadequate to prevent rickets in these children.

STUDY III - HR:

Mutations in the PHEX gene were found in 83% of all probands and were the most common cause of hereditary FGF23-associated HR in ethnic Danish patients. A DMP1 mutation in one Lebanese child was identified. Six patients previously diagnosed with HR were excluded from the study and they represent the possible differential diagnoses of HR.

The growth retardation and disproportioned body composition was associated to reduced growth of the long bones of the

legs. Reduced growth was also present in the arms, whereas the growth of the skull was increased. Elevated BMD of lumbar spine was characteristic. Especially in adults aged 40+ years, pain in the joints and decreased ranges of hip movement were frequent as were enthesiopathies of the spine and endodontic problems. HR patients had a reduced risk of fractures and the same percentage of caesarean sections compared with the background population. Among patients with proven X-linked disease, there was a tendency of males being more severely diseased compared to females.

The efficacy of medical treatment could not be assessed by the cross-sectional design. The severity of HR in PHEX-positive patients varied considerably even within family members carrying the same genetic mutation and considerable age differences were present. Hereby, discrimination between the causal effects of treatment, age contribution, and differences in disease severity was not possible. Current medical treatment in PHEX-positive patients was associated to HPT and hypercalcaemia. Two patients had developed tertiary HPT and one of them in addition hypertension and decreased renal function. These findings were not present in any untreated PHEX-positive patients. Treatment goals are healing of rickets, near normalization of serum phosphate and ALP with the caution of maintaining serum PTH and serum calcium within their normal ranges and preventing increased urinary calcium excretion.

8. FUTURE PERSPECTIVES AND FUTURE RESEARCH

Nutritional rickets imply a period of diminished mineralization of bone and dental tissues, but the long term effects of the disease are unknown. Studies to investigate whether these patients achieve the normal average of bone mineral density are pertinent. The dental implications of childhood nutritional rickets are not well characterized and future studies to describe these implications are needed.

Nutritional rickets only represents the most severe cases of long standing vitamin D deficiency, implying that less severe vitamin D deficiency in childhood might be widespread. As the prevalence of vitamin D deficiency in children is unknown, population-based studies should be directed to establish this. As several health benefits recently have been associated to adequate vitamin D levels (194), future considerations of how to improve the vitamin D status in children as well as adults living in Denmark are crucial. Food fortification with vitamin D, advice on sun exposure or diet may be considered.

As demonstrated in our study the advances in genetics allow DNA-analysis to be offered to all cases of suspected HR. Genetic evaluation in patients with HR has several advantages. Detection of a disease causing mutation in one of the HR genes ensures the correct diagnosis, enables early genetic evaluation of children allowing early initiation of treatment, enables prenatal diagnosis if relevant, and enables genetic evaluation of first degree relatives and symptomatic second degree relatives. If genetic analysis is negative, the diagnosis of HR should be re-evaluated and differential diagnoses considered.

Our study points to the difficulties in treating patients with HR. There is only little evidence of benefits from medical treatment in adult patients with HR and the risk of adverse effects has to be counterbalanced to the anticipated benefits of treatment. Current treatment options are not evidence-based and there is a need for a randomized, controlled treatment trial.

List of abbreviations

HR	hypophosphatemic rickets
1,25(OH) ₂ D	1,25-dihydroxyvitamin D
UVB	ultraviolet-B
SZA	solar zenith angle
25(OH)D	25-hydroxyvitamin D
PTH	parathyroid hormone
FGF23	fibroblast growth factor 23
PHEX	phosphate regulating gene with homologies to endopeptidases on the X-chromosome
XLH	X-linked hypophosphatemic rickets
ADHR	autosomal dominant hypophosphatemic rickets
TIO	tumor induced osteomalacia
DMP1	dentin matrix protein 1
ARHR	autosomal recessive hypophosphatemic rickets
HHRH	hereditary hypophosphatemic rickets with hypercalciuria
TPO ₄ /GFR	renal threshold value for reabsorption of phosphate in the urine
ALP	alkaline phosphatase
MEPE	matrix extracellular phosphoglycoprotein
ASARM	acidic, serine- and aspartic acid-rich motif
SIBLINGS	small integrin-binding ligand, N-linked glycoproteins
PMT-MCT	phosphatoretic mesenchymal tumor, mixed connective tissue variant
BMD	bone mineral density
VDDR type I	1 α -hydroxylase deficiency
VDDR type II	hereditary vitamin D-resistant rickets
XLRH	X-linked recessive hypophosphatemic rickets
DNPR	the Danish National Patient Registry
CPR number	personal identification number
GPs	general practitioners
COCR	Central Office of Civil Registration
dHPLC	denaturing high performance liquid chromatography
MLPA	Multiplex Ligation-dependent Probe Amplification
ROM	ranges of movement
RR	relative risk
BMAD	bone mineral apparent density
aBMD	areal bone mineral density
Rho	Spearman's correlation coefficient
HPT	hyperparathyroidism
SD	standard deviation

9. SUMMARY

Rickets is a heterogeneous group of diseases of the growing child caused by defect mineralization of bone. Nutritional rickets is caused by deficiency of vitamin D, calcium or both. Several hereditary forms of rickets exist where the disease proceeds into adulthood.

Nutritional rickets was common in the past, but by introduction of preventative administration of cod liver oil and vitamin D supplementation, nutritional rickets became a rarity. During the last decades, case reports of nutritional rickets reappear in the indus-

trialized countries. It is the general conception that in the industrialized countries, hereditary rickets is the most prevalent cause of rickets today. However, the incidence of nutritional rickets and the incidence and prevalence of hereditary rickets in Scandinavia are unknown.

The most common form of hereditary rickets is hypophosphatemic rickets (HR). The geno- and phenotype among Scandinavian patients have not been characterized. Especially, the disease in adult patients is not well described. Moreover, there are conflicting reports of the benefits of medical treatment throughout childhood, and in addition on gender differences in disease severity.

The aims of the Ph.D-study were to:

- Estimate the incidence of nutritional rickets and the incidence and prevalence of hereditary rickets in Southern Denmark
- Describe symptoms, clinical and biochemical characteristics at diagnosis of nutritional rickets in children living in Southern Denmark
- Identify current risk factors for nutritional rickets
- Determine the geno- and phenotype in a large group of patients with HR
- Evaluate possible effects of medical treatment in patients with HR
- Assess possible gender differences in disease severity in patients with genetically verified X-linked HR

Patients and methods:

By register search in the Danish National Patient Registry (DNPR) and in the hospital registers of Southern Denmark from 1985 to 2005, patients aged less than 15 years with rickets were identified. The search was based on diagnosis codes referring to rickets. After review of the medical records, patients fulfilling the diagnostic criteria of nutritional or hereditary rickets were included in the study. The ethnicity of the patients was determined by linkage to The Central Office of Civil Registration and the size of the background population in the age groups during the study period was obtained from Statistics Denmark. Based on these data, the incidence of nutritional rickets and the incidence and prevalence of hereditary rickets were calculated. Symptoms, clinical and biochemical characteristics of nutritional rickets were described upon data retrieved from the medical records.

Patients with HR were identified through register search in (DNPR) from 1977 to 2005 and in hospital registers from 1985 to 2006 based on the diagnosis codes of HR. In addition, doctors known to treat patients with HR were contacted and family screening performed.

Nutritional and hereditary rickets:

A total of 112 patients aged less than 15 years with nutritional rickets were identified. Of these, 83 were immigrant children and 29 were ethnic Danish. Hereditary rickets was diagnosed in 16 patients. Nutritional rickets was predominantly seen among immigrant children and was the most common type of rickets in all young children living in Denmark. However, hereditary rickets was more common than nutritional rickets among ethnic Danish children. Infants and young children displayed the characteristic clinical signs of rickets, but in older children and adolescents the clinical signs were few and symptoms unspecific indicating a risk

of under diagnosis. Possible risk factors in the young children were omitted vitamin D supplementation and veiling in adolescent girls. Since the majority of the ethnic Danish children were diagnosed at age 12-24 months, we recommend an extension of the period of vitamin D supplementation until 2 years of age in ethnic Danish children. In addition, a possible approach towards elimination of rickets in Denmark may be the introduction of food fortification with vitamin D.

Hypophosphatemic rickets:

A total of 59 patients with hereditary FGF23-associated HR were identified. Mutations in the PHEX gene were found in 83% of all probands and were the most common cause of hereditary FGF23-associated HR in ethnic Danish patients. A MDP1 mutation was identified in a single Lebanese child. Six patients previously diagnosed with HR were excluded from the study and they represent the possible differential diagnoses of HR.

Growth retardation and disproportioned body proportions were due to reduced growth of the long bones of the legs. Reduced growth was also seen in the arms, whereas the growth of the skull was increased. Elevated BMD of lumbar spine was characteristic. Especially in adult patients aged 40+ years, pain in the joints and decreased ranges of hip movement were frequent as were enthesiopathies of the spine and several spontaneous dental abscesses. HR patients had a reduced risk of fractures and the same percentage of caesarean sections compared with the background population. Among patients with proven X-linked disease, there was a tendency of males being more severely diseased compared to females. Current medical treatment in PHEX-positive patients was associated to hyperparathyroidism and hypercalcaemia, findings not seen in any untreated PHEX-positive patients.

In conclusion, nutritional rickets was predominantly seen among immigrant children but occasionally cases were seen among ethnic Danish children. We therefore recommend an extension of the period of vitamin D supplementation in ethnic Danish children until 2 years of age and/or introduction of food fortification with vitamin D. Genetic evaluation of patients with HR ensures a specific diagnosis, enables early diagnosis and treatment in children of HR parents. The medical treatment of HR is difficult. In adult patients, the adverse effects have to be counterbalanced to the benefits of treatment.

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