Vitamin K requirement in children

....beyond coagulation

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Vitamin K behoefte bij kinderen....meer dan alleen stolling
(met een samenvatting in het Nederlands)

Proefschrift ter verkrijging van de graad van doctor aan de Universiteit Utrecht op gezag van de rector magnificus, prof. dr. J.C. Stoof, ingevolge het besluit van het college voor promoties in het openbaar te verdedigen op woensdag 12 december 2007 des middags te 4.15 uur

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Slogan no 18:

an intuitive combination of opposing forces generates energy

(Start a New Art World, Ad de Jong)

Voor Marie van Summeren – van de Sande

(*16 april 1922 † 5 maart 2002)

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Abbrevations

ANA: antinuclear antigen

B: regression coefficient

BAP: bone alkaline phosphatase

BMI: body mass index

BMC: bone mineral content

BP: blood pressure

BUA: "broadband ultrasound attenuation"; decrease of the sound wave in bone

CC: compliance-coefficient, dynamic vascular parameter

CF: cystic fibrosis

CHQ: Child Health Questionnaire

CI: confidence interval

cMGP: carboxylated MGP

cOC: carboxylated osteocalcin

CRF: chronic renal failure

CVD: cardiovascular diseases

Δ: delta, difference

DC: distension-coefficient, dynamic vascular parameter

DEXA: dual X-ray absorptiometry, method to measure bone density

E: Young's modulus, dynamic vascular parameter which is used to express the elasticity independent of volume

ESR: erythrocyte sedimentation rate

ESRD: end stage renal disease

FN: femoral neck

Gla: γ-carboxyglutamate acid

Glu: glutamate acid

HDL: high density lipoprotein

hsCRP: high sensitivity C-reactive protein

IBM: inclusion-body myositis

ICTP: type I carboxy terminal telopeptides

IGF-1: insulin-like growth factor I

IMT: intima-media-thickness, morphologic vascular parameter

JDM: juvenile dermatomyositis

JIA: juvenile idiopathic arthritis

kDa: kiloDalton

Ln: natural logarithm

LS: lumbar spine

LDL: low density lipoprotein

MGP: matrix Gla protein

MK-n: menaquinones with a variable side chain length; together they form the group of vitamin K_2

MK-7: menaquinone-7, a form of vitamin K_2

n: number

NSAIDs: non-steroidal anti-inflammatory drugs

NTX: N-telopeptide cross-links of collagen breakdown

OC: osteocalcin

p: significance level

PBM: peak bone mass

PF: physical functioning score, derived from CHQ

PINP: procollagen type I amino terminal propeptides

PIVKA: protein induced in vitamin K absence

PIVKA II: prothrombin induced in vitamin K absence

pserMGP: phosphorylated MGP

PSS: psychosocial summary score, derived from CHQ

QUS: quantitative ultrasound, measure of bone

r: Pearson's correlation coefficient

RDA: recommended dietary allowance

RF: rheumatoid factor

RTX: renal transplantation

sd: standard deviation

sds: standard deviation score

serMGP: non-phosphorylated MGP

SOS: "speed of sound" in bone

TB: total body

UCR: ratio of undercarboxylated to carboxylated osteocalcin, measure of vitamin K status

ucOC: undercarboxylated osteocalcin

ucMGP: non-carboxylated MGP

VLDL: very low density lipoprotein

WTS: vessel wall tracking system which measures the distensibility of the vascular wall using ultrasound

y: years


Bente, 6 years

Chapter 1 General introduction

In past decades, novel treatment strategies of chronic diseases in childhood have lead to significant improvement in (functional) outcome and increased patient-survival. As a result, the treatment and prevention of long-term complications associated with chronic diseases have become of additional importance. These complications may give rise to considerable morbidity, extending into later (adult) life. For example, reduced bone mineral density and concurrent increased fracture risk are associated conditions in many pediatric chronic diseases (1;2). Furthermore, several chronic diseases in childhood may predispose patients to (subclinical) atherosclerosis resulting in early cardiovascular disease (3). The pathophysiological processes underlying osteoporosis and atherosclerosis are complex and multifactorial. In the adult population, the role of vitamin K and the vitamin K-dependent proteins osteocalcin and matrix Gla protein (MGP) in both conditions has been studied. However, limited research on these subjects has been performed in children. In this introduction, the role of osteocalcin in childhood bone health is outlined. In addition, the role of MGP in relation to ectopic calcification in pediatric disease is discussed.
For supplementary information on vitamin K, please refer to the appendix.

The vitamin K-dependent protein osteocalcin plays a role in bone health

Osteocalcin is a small matrix protein consisting of 49 amino-acids, three of which are γ-carboxyglutamate (Gla) residues (4;5). Osteocalcin is synthesized by osteoblasts and constitutes about 20% of the non-collagenous protein found in human bone (6-9). The Gla residues in osteocalcin are positioned in such a way that they point directly to the calcium ions in the crystal structure of hydroxyapatite, the mineral matrix in bone (10). The precise function of osteocalcin is not completely clear. Transgenic osteocalcin-deficient mice appeared to have larger bones than their wild type littermates, suggesting that osteocalcin is a negative regulator of bone formation (11). On the other hand, the protein has a role in the orderly deposition of hydroxyapatite crystals, both during bone growth and bone remodeling (12).

In order to adequately carboxylate osteocalcin, the osteoblast requires sufficient vitamin K (13). In case of vitamin K deficiency, undercarboxylated osteocalcin will be produced. In the healthy adult population, a variable degree of undercarboxylation of osteocalcin is naturally present (6).
Increase of vitamin K intake results in markedly higher carboxylation rate of

osteocalcin (6;14-16). Population studies in adults have shown that high concentrations of undercarboxylated osteocalcin are associated with lower bone mineral density and increased risk of hip fracture (17;18). Presumably, optimal carboxylation of osteocalcin contributes to improved bone quality, both by increased bone mineral density and improved bone morphology (19;20). This will result in diminished fracture risk.

The vitamin K-dependent protein matrix Gla protein (MGP) is an inhibitor of calcification

MGP is a secreted 84 amino acid Gla protein which is found most abundantly in bone and cartilage (21;22). MGP contains 5 Gla residues and is expressed by chondrocytes and vascular smooth muscle cells (23-25). Although the molecular mechanism of MGP action is not known, data from various studies demonstrate that MGP plays a major role in the inhibition of (soft) tissue calcification (26-28). The importance of MGP to prevent calcification in vivo is well illustrated in the MGP knockout mouse model, which exhibits intense arterial calcification leading to vessel wall rupture and premature death (26). In humans, a rare human recessive disorder named Keutel syndrome results in diffuse cartilage and arterial calcifications (29). Keutel syndrome is caused by nonsense mutations of the MGP gene (30).

Because of their calcium-binding properties, it is likely that the Gla residues within MGP are involved in the inhibition of calcification. Hence, it was demonstrated that the biological activity of MGP depends on the carboxylation of MGP and thus the presence of vitamin K (28;31). Population studies in adults have investigated the possible benefits of vitamin K in preventing vascular calcification and cardiovascular disease. It was shown that the presence of abdominal aortic calcifications is associated with a reduced vitamin K status in postmenopausal women (32). Furthermore, dietary intake of menaquinones is associated with a reduced risk of coronary heart disease (33). Braam et al. have described an improved elasticity of the vessel wall in vitamin K-treated postmenopausal women (34).

Vitamin K status in children

The role of vitamin K in bone health and prevention of ectopic calcification is fairly well described in the adult population (5). However, little is known about vitamin K and the vitamin K-dependent proteins osteocalcin and MGP in children. During recent years, the intake of vitamin K in children has

decreased gradually and is substantially lower than that of adults (35;36). It is uncertain whether this decreased intake may have consequences for the function of the vitamin K-dependent proteins in children.

During bone development and skeletal growth, both the metabolic activity and osteocalcin production are high (37), suggesting that also the vitamin K requirement of bone is high. The combination of high requirement and low intake may lead to subclinical vitamin K-deficiency in the rapidly growing bone. The importance of vitamin K status in bone health in children has only been studied by a small number of researchers (38). Kalkwarf et al performed a 4 year-study on vitamin K nutritive status and bone mass in a cohort of 245 healthy girls (3-16 yrs). It was concluded that better vitamin K status (high plasma phylloquinone and low percentage of non-carboxylated osteocalcin) was associated with reduction in bone turnover rate (39). In this study, biochemical measures of vitamin K status were inconsistently associated with current bone mineral content (BMC). The percentage of non-carboxylated osteocalcin (ucOC) was positively associated with 4-year changes in lumbar spine BMC. Another study in a large cohort of healthy girls aged 11-12 years showed that better vitamin K status was associated with increased bone mineral content (40). A cross-sectional study in 14 children showed that the ratio of serum carboxylated osteocalcin (cOC) to serum intact osteocalcin was positively related to bone mineral density of the right tibia, determined by ultrasound velocity (41). Furthermore, in children with long-standing vitamin K deficiency caused by the anti-coagulation drug warfarin, a reduced bone density was found (42). The latter study illustrates the potential consequences of vitamin K shortage in growing bone.

A limited number of studies on vitamin K and its relation to bone health have been performed in pediatric patient groups (e.g. cystic fibrosis, galactosemia). Patients with cystic fibrosis (CF) have an increased risk of subclinical vitamin K deficiency due to malabsorption (43-45). Nicolaidou et al have studied the effect of one year vitamin K supplementation in 20 children with CF (46). The intervention consisted of a weekly dose of 10 mg vitamin K_1. At baseline, lower levels of vitamin K and higher levels of ucOC were found in patients compared to healthy controls. After 1 year vitamin K supplementation, a decreased level of ucOC was seen in the patient group, together with improved bone formation markers. Galactosemic patients have an increased risk of low bone mineral density (47). In these children, a 2 year intervention study using a combination of 1 mg vitamin K_1, calcium and

vitamin D, demonstrated improved osteocalcin carboxylation and bone mineral content (BMC) of the lumbar spine in the treated group (48).

Until now, no papers have been published about the vitamin K-dependent protein MGP and its relationship to vitamin K in children. Results from several studies using the animal warfarin model in young rodents and lambs could support the hypothetical effect of (severe) vitamin K-deficiency in children. The warfarin model is based on complete blockade of the recycling enzyme in the vitamin K cycle, the KO-reductase, by excessive amounts of warfarin. In order to prevent bleeding complications, a moderate dose of vitamin K is administered which is used by the liver. In this way, the clotting system remains operational and a vitamin K deficiency is induced in the extra-hepatic tissues only. In young animals, warfarin treatment resulted in clinical features resembling the MGP-knockout mice or the fetal warfarin syndrome in humans (27;49;50). The animals showed medial elastocalcinosis, excessive tissue calcification in epiphyses and other cartilage. Furthermore, the level of MGP mRNA and MGP protein in the calcifying arteries was increased together with a decreased level of serum MGP (27). At the site of calcification, mainly undercarboxylated MGP was found, implying that the Gla-residues are probably required for the function of MGP as a calcification inhibitor (50). Spronk et al showed that the vascular calcification using the warfarin model can be prevented by administration of vitamin K_1 and menaquinone-4 (MK-4) which belongs to the group of vitamin K_2 (28).

Why are vitamin K and the vitamin K-dependent proteins osteocalcin and MGP of potential importance in childhood? If a (subclinical) deficiency of vitamin K, based on the extent of carboxylation of osteocalcin or MGP, is indeed present in children, this might affect bone health and contribute to the formation of ectopic calcifications in healthy children as well as in pediatric patients.

During childhood, the foundation for bone health is laid. A maximal peak bone mass (PBM) at skeletal maturity is considered to be the best protection against age-related bone loss and fracture risk (51;52). PBM is defined as the maximal amount of bone mineral accrued during life; eighty to ninety percent of the PBM is acquired between birth and adolescence (53;54). Supplementation of vitamin K in case of subclinically deficient but otherwise healthy children may be a strategy to optimize peak bone mass in childhood in order to prevent osteoporosis in later life.

Supplementation of vitamin K may also be of importance in pediatric

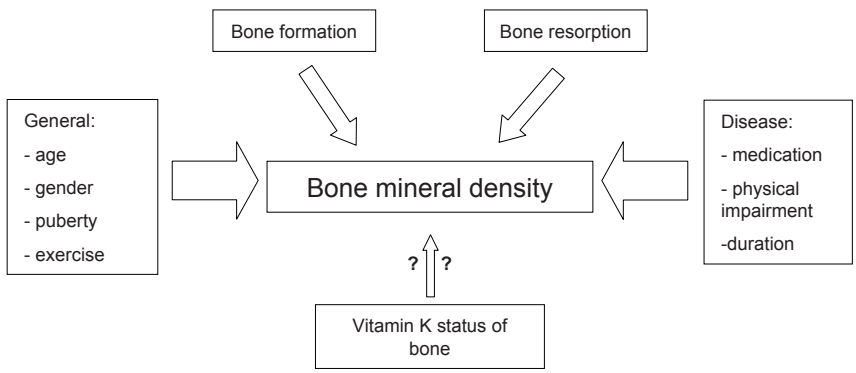
patients. Improved survival of chronically ill patients, due to improved treatment strategies, has resulted in an increased prevalence of late complications, such as osteopenia/osteoporosis. Reduced bone mineral density and an increased fracture risk have been documented in children with distinct conditions, e.g. juvenile idiopathic arthritis (JIA), juvenile dermatomyositis (JDM) and children with chronic renal failure (CRF) (55-57). Pathogenic factors of osteoporosis in these patients include chronic inflammation, use of glucocorticoid therapy or other medications, limited physical activity and malnutrition (figure 1) (58). In young adults with early-onset chronic disease, an increased fracture prevalence is also noted, probably due to an inadequate build up of peak bone mass in adolescence (59;60). Additionally, a relative shortage of vitamin K in bone may affect bone mineralization in a population already susceptible for osteopenia.

Another late complication of chronic pediatric disease may be the occurrence of cardiovascular diseases (CVD). Both atherosclerosis and arteriosclerosis contribute to the development of CVD (61). Atherosclerosis is an actively regulated, cell-mediated process in which local inflammation of the arterial wall plays a pivotal role (62). In end-stage atherosclerosis, vascular calcification may occur in the intima of the arterial wall (62). Risk factors for atherosclerosis are arterial hypertension, abnormal lipid profile, male sex, obesity, diabetes mellitus and systemic inflammation (3). Early signs of atherosclerosis have been described in children (63;64). Arteriosclerosis represents vascular calcification that is located in the media of the arterial wall and is related to factors like uremia and abnormalities in calcium-phosphate metabolism (61;65). Medial calcification leads to arterial stiffening resulting in increased pulse pressure and early reflection of the pulse wave (66). Ultimately, increased cardiac afterload results in left ventricular hypertrophy (66). Medial vascular calcification is also an actively regulated process that develops due to the imbalance between factors favouring calcium deposition and inhibitory mechanisms (67). One of these inhibitory factors is MGP (68;69). The Gla residues in MGP have a high affinity for calcium and are essential for its function (31). MGP was found to be present both in atherosclerotic plaques in the intima as well as in medial calcification (70;71). In a wide range of pediatric patient populations, the propensity to develop arterial calcifications is present; e.g. children with auto-inflammatory conditions and children with end stage renal disease (ESRD) (72). In these patients, a deficiency of vitamin K resulting in undercarboxylated

non-functional MGP might contribute to the atherogenic process.

Furthermore, subclinical vitamin K deficiency may also be of importance in JDM. JDM is the most common form of pediatric idiopathic inflammatory myopathies, often complicated by calcinosis (73). Calcinosis in JDM is usually noted between 4 months and 12 years after onset of the disease and often develops in a quiescent phase of the disease (74). Calcinosis is the massive deposition of insoluble calcium salts (hydroxyapatite crystals) in the skin and subcutaneous tissue (75). The presence of this complication is an important determinant of the functional outcome of JDM (76). Because of the calcium binding and calcification inhibitory properties of both osteocalcin and MGP, these vitamin K-dependent proteins may play an important role in the counteraction of calcinosis in JDM, but also in other rheumatologic disorders presenting with calcinosis (76;77). Recently, a higher expression of MGP was detected in skin biopsies from patients with systemic sclerosis with calcinosis, compared to patients without calcinosis (78). The question arises whether subclinical vitamin K deficiency may be an aggravating condition in the development of calcifications in rheumatologic conditions like JDM.

Figure 1: Bone mineral density and its influencing factors



This figure is a schematical representation of the interactions of different factors on bone mineral density in pediatric patients. Bone mineral density is influenced by general factors like age, gender, puberty and exercise, and by disease-specific factors. The model also includes vitamin K status of bone as possible factor.

Aims and outline of the thesis

The aim of this thesis was to investigate the role of osteocalcin in childhood bone health. In addition, the role of MGP in relation to ectopic calcification in pediatric disease is examined.

Questions to be solved in this thesis are:

- What is the level of the vitamin K status of bone, expressed as the ratio of undercarboxylated and carboxylated osteocalcin, in healthy children and how does it relate to that in adults?
- How does vitamin K status of bone relate to bone properties in healthy children and in JIA patients who are at risk of osteoporosis?
- What is the effect of vitamin K supplementation on the carboxylation of osteocalcin in healthy children?
- What is the level of MGP in healthy children compared to pediatric renal transplant patients who are at increased risk of early cardiovascular disease and how does it relate to vessel wall properties?
- Does MGP play a role in the pathophysiology of calcinosis in patients with JDM?

In **chapter 2**, the vitamin K status in healthy children is compared to that in adults. Furthermore, the relation between vitamin K status and bone turnover markers in children (high bone metabolic activity) and in adults (stable bone metabolic activity) is examined.

In **chapter 3**, vitamin K status in healthy children during puberty is studied together with its association with bone mass and changes in bone mass over 2 years. In addition, possible associations between vitamin K status and biochemical markers of bone metabolism, sex steroids and growth hormones were analyzed.

Chapter 4 describes the findings of a randomized controlled trial in which the effect of vitamin K_2 on osteocalcin carboxylation in healthy children, using an MK-7 containing food supplement, was studied.

In **chapter 5**, vitamin K status in children with JIA was compared to that in healthy children and the association between vitamin K status of bone, bone markers and quantitative ultrasound properties of calcaneal bone were studied in these children.

In **chapter 6**, the circulating levels of the vascular calcification inhibitors MGP and fetuin-A in children after renal transplantation are compared to healthy children. Possible associations between the levels of calcification inhibitors and vascular properties of the carotid artery in these children were studied as well.

In **chapter 7**, the localization of the different forms of MGP in muscle biopsies from JDM patients with and without calcification are studied. A summary and general discussion is presented in **chapter 8**.

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Fleur, 9 years

Chapter 2 Pronounced elevation of undercarboxylated osteocalcin in healthy children

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2.1 Abstract

The vitamin K-dependent protein osteocalcin is thought to play an important role in bone metabolism. Osteocalcin contains γ-carboxylglutamate acid (Gla) residues, which have a high affinity for calcium. Vitamin K acts as an indispensable cofactor for the formation of these residues. Inadequate dietary vitamin K intake results in the synthesis of undercarboxylated (i.e. inactive) osteocalcin (ucOC).

In adults, low vitamin K status of bone is associated with low bone density and increased risk of osteoporotic fractures. Little is known about vitamin K status and bone health in children. We used a cross-sectional study design to compare the vitamin K status of bone in healthy children (n=86) with that of adults (n = 30). In children, a marked elevation of the ratio of ucOC / carboxylated osteocalcin (cOC), indicative of a poor vitamin K status, was observed. This difference persisted after adjusting for age, gender, puberty, height and weight. Furthermore, a marked correlation between the markers for bone metabolism and ucOC and cOC was found in the children's group.

These findings suggest a pronounced low vitamin K status of bone during growth. The question remains, however, whether children would benefit from higher vitamin K intake, for instance, by improved bone health or stronger bones.

2.2 Introduction

Vitamin K and vitamin K-dependent proteins (also known as Gla-proteins) are best known for their role in blood coagulation. However, Gla-proteins are also involved in other physiologic processes (e.g. bone metabolism) (1;2). Osteocalcin is a bone Gla protein and synthesized by osteoblasts (3). It constitutes about 20% of the noncollagenous protein found in human bone (4).

Vitamin K acts as a cofactor in the posttranslational carboxylation of all Gla proteins. In this process, glutamate acid (Glu) residues are converted into y-carboxyglutamate acid (Gla) (5). Gla residues have a high affinity for calcium, and their complex formation with calcium ions was demonstrated to be essential for the function of all Gla-proteins presently known.

To adequately carboxylate osteocalcin, the osteoblast requires sufficient vitamin K (6). In case of vitamin K deficiency, undercarboxylated osteocalcin will be produced. Bioavailable vitamin K is mainly derived from nutritional sources. The gut flora also produces vitamin K, but at the site of synthesis (colon) its absorption is negligible (7;8). The two most important forms of vitamin K are phylloquinone (vitamin K_1) and the group of K_2 vitamins (menaquinones). Dietary sources of phylloquinone are green vegetables (spinach, broccoli) and some plant oils (9). Menaquinones are found in meat and fermented foods like cheese (10). Hence, inadequate intake of vitamin K will lead to the production of undercarboxylated (i.e. inactive) Gla-proteins.

Methods have been developed to distinguish between carboxylated (cOC) and undercarboxylated (ucOC) fractions of osteocalcin (11;12). The ratio between ucOC and cOC (UCR) as well as circulating ucOC levels are used as indicators for the vitamin K status of bone (2;13;14). The UCR is probably the most sensitive marker for bone vitamin K status (2). Serum vitamin K concentrations fluctuate with recent dietary vitamin K intake and are not reliable markers for tissue vitamin K status. In the healthy adult population, osteocalcin is carboxylated to a variable extent, suggesting that the dietary vitamin K intake is insufficient for full osteocalcin carboxylation (15). Markedly higher osteocalcin carboxylation is obtained by increased vitamin K intake (16).

The benefits of vitamin K on bone metabolism and the prevention of fractures are well described in the adult population (17-21). Little is known about the vitamin K status in growing bone such as in children. Both the metabolic activity and osteocalcin production (22) are high during bone development and skeletal growth, suggesting that also the vitamin K requirement of bone

is high. During recent years, the dietary intake of vitamin K in children has declined gradually (23). The combination of high requirement and low intake may lead to subclinical vitamin K deficiency in the rapidly growing bone. In a cohort of in 245 healthy girls (3-16 years), Kalkwarf et al. (24) recently reported a marked variation in the percentage of ucOC (% ucOC). In this study, better vitamin K status (expressed as %ucOC) was associated with decreased bone turnover. Furthermore, no evident correlations were found between %ucOC and dietary intake or serum levels of vitamin K_1. This is consistent with the view that serum vitamin K is a marker for recent dietary vitamin K intake but not for tissue vitamin K status.

The aim of this study was to compare the vitamin K status of bone in healthy children and adults in a cross-sectional study design. In addition, we studied possible correlations of vitamin K status with the level of bone turnover markers in children (high bone metabolic activity) and in adults (stable bone metabolic activity).

2.3 Subjects and methods

Study subjects

From October 2003 through January 2004, 86 healthy children (male and female) between 3 and 18 years of age were recruited from a population of children undergoing minor surgery (e.g. strabismus correction, phimosis correction), and friends or relatives of visiting patients in the Wilhelmina Children's Hospital. The department of Biochemistry, University of Maastricht recruited 30 healthy adult volunteers (25-35 years of age) via small ads. Subjects were included if they were within normal ranges for body weight and height according to reference growth charts. Exclusion criteria were current or previous medical diseases such as metabolic, gastrointestinal or endocrine disease; chronic degenerative diseases; current medication such as anticoagulants and corticosteroids; and vitamin concentrates or food supplements.

This study was approved by the Central Committee on Research Involving Human Subjects (CCMO, The Netherlands). Written informed consent was obtained from all adults, the parents of all children and also from children when 12 years of age or older.

General data elements

Body height and weight of all subjects were measured in a standardized manner

without shoes and heavy clothing, to the nearest centimeter and 100 g, respectively. From these values, the body mass index (BMI) was calculated. Information about medical history and current use of any medication was ascertained by a short interview. In children, the pubertal stage was determined by a short list of questions about the presence of menarche and breast development (in females), or genital development (in males), according to Tanner's sexual maturity scale (SMS). The groups were divided into three categories: prepuberty (prepubertal stage), puberty (pubertal stage II-IV) and end of puberty (pubertal stage V).

Assessment of vitamin K status and bone turnover markers

After blood sampling, all samples were frozen and kept at -80 °C until use. In serum, two biochemical markers of bone turnover were measured. Bone alkaline phosphatase (BAP) (Hybritech, Belgium) was measured as marker of bone formation by radio immunoassay. As a marker of bone resorption, N-telopeptide cross-links of collagen breakdown (NTX) (Ostex, Washington) were determined. Undercarboxylated and carboxylated fractions of osteocalcin (Takara, Japan) were used as indicators of vitamin K status and measured by enzyme-linked immunosorbent assay. The UCR is a sensitive indicator for the vitamin K status of bone (2;13). Elevated levels of UCR are indicative of an inferior vitamin K status of bone.

Statistical analysis

Normality of distribution for all subjects was checked by histograms for all study parameters. Independent t-tests and analysis of variance were used for comparison of parameters between children and adults. A chi-square test was performed for the differences in gender between adults and children. Pearson's correlation tests were performed to investigate possible correlations between demographic variables, bone markers and vitamin K parameters in all groups. Stepwise multivariate linear regression analysis for the difference between children and adults was performed, using ucOC, cOC and UCR as dependent variables with adjustments for the confounders age, gender, puberty, height, and weight.

The statistical tests were performed using a two-sided significance level of 5%. A p value <0.05 was considered to be statistically significant. SPSS Base 12.0.2 for Windows (SPSS Inc, Chicago, IL) was used for all analyses.

2.4 Results

Subjects

Eighty-six children (37 males, 49 females) versus 30 adults (14 males, 16 females) were included in the study. Baseline characteristics of the subjects are shown in table 1. The groups of adults and children differed in age, height and weight, as might be expected. In the children's group, boys and girls did not differ in age (mean: 10.06 versus 10.01 y; p = 0.958), weight (mean: 40.8 versus 40.2 kg; p = 0.889), height (mean: 155.6 versus 150.8 cm; p = 0.324) and pubertal stage (p = 0.103). Furthermore, no differences in these variables between male and female in adults were present.

Table 1: Demographic and anthropometric data, bone markers and vitamin K parameters of children and adults

	Children (n = 86)		Adults (n = 30)		p
Age, years	10.0	(4.0)	28.9	(3.5)	<0.001
Gender, male:female	37:49	(43:49)	14:16	(47:53)	0.729
Weight, kg	40.4	(17.3)	70.8	(12.0)	<0.001
Height, cm	152.8	(17.1)	176.9	(10.8)	<0.001
Bone markers					
BAP, ng/ml	170.0	(131.4)	20.2	(7.5)	<0.001
NTX, ng/ml	89.8	(38.9)	15.3	(2.5)	<0.001
Vitamin K parameters					
ucOC, ng/ml	31.3	(23.8)	3.6	(2.5)	<0.001
cOC, ng/ml	15.4	(7.3)	4.7	(1.5)	<0.001
UCR	2.3	(1.6)	0.8	(0.5)	<0.001

Results are presented as mean (sd), except for gender, male:female in n:n (%:%). Differences between the total group of children and adults were compared applying an independent t test.

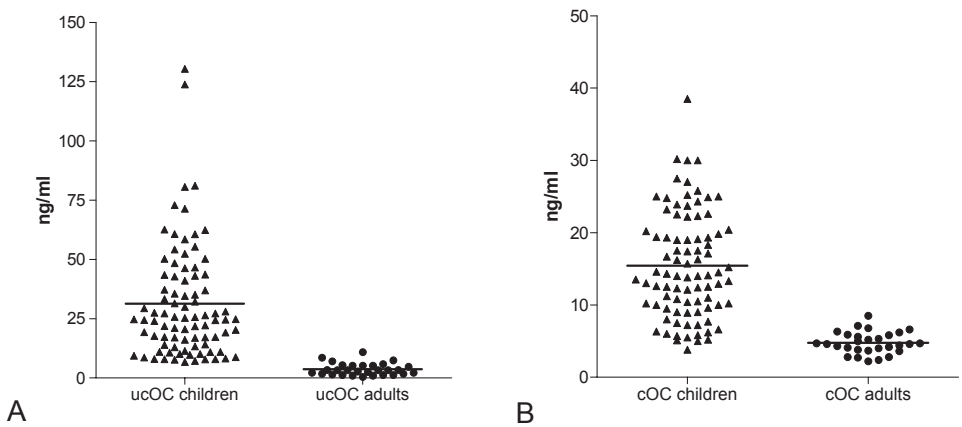
Bone turnover parameters

The levels of BAP and NTX were elevated in children compared to adults (table 1). This is consistent with the higher metabolic activity in growing bone in children. No differences in bone markers were found between boys and girls (data not shown). Both BAP and NTX significantly increased during puberty and declined by the end of puberty (data not shown).

Vitamin K status of bone

The levels of ucOC and cOC were elevated in children as well (table 1). In addition, the UCR in children was significantly higher than in adults, suggesting a poor vitamin K status of growing bone. Figure 1 shows the variation of the ucOC and cOC levels in children compared with the adult group. No differences in vitamin K status were found between boys and girls: ucOC (mean: 36.5 versus 27.4 ng/ml; p = 0.082), cOC (mean: 16.9 versus 14.3 ng/ml; p = 0.110) and the UCR (mean 2.46 versus 2.11; p = 0.445).

Figure 1: Scatterplot of ucOC (A) and cOC (B) in children (n = 86) and adults (n = 30)



The p value for the difference in the level of ucOC (t test) in children versus adults is <0.001 (A). The p value for the difference in the level of cOC (t test) in children versus adults is < 0.001 (B).

To investigate whether this apparent vitamin K-deficiency was associated with certain stages of development, we subdivided the children according to age and pubertal stage (table 2). In all age-groups, the UCR was above the adult level. A similar conclusion was obtained after subdivision according to pubertal stage. UcOC and cOC were highest during puberty and declined at the end of puberty. In postpubertal children, the cOC levels had almost reached adults values. Remarkably, the UCR remained high during all stages. Multiple linear regression analysis with adjustment for age, gender and pubertal stage showed that ucOC, cOC and the UCR in children were significantly higher than in adults (table 3). When also height and weight were introduced in the equation, only ucOC and the UCR remained significantly different from the adult levels (table 3).

Table 2: Vitamin K parameters in children of different age groups and pubertal stages

	n	ucOC (ng/ml)	p	UCR	p
Age groups					
3-8 years	28	18.9 (11.3)	0.021	1.8 (1.7)	<0.001
8-13 years	33	40.2 (25.2)	<0.0001	2.4 (1.4)	<0.001
13-18 years	25	33.4 (27.0)	<0.0001	2.6 (1.6)	<0.001
Pubertal stage					
Prepubertal (Tanner I)	55	26.8 (16.9)	<0.0001	2.0 (1.5)	0.01
Puberty (Tanner II-IV)	18	53.6 (33.7)	<0.0001	2.6 (1.7)	<0.001
End of puberty (Tanner V)	13	19.3 (11.3)	0.066	2.7 (1.4)	<0.001

Vitamin K parameters (ucOC and UCR) in different subgroups of age and pubertal stage. Data are presented as mean (sd). The p value is in regard to the differences between the different subgroups of children (age groups and pubertal stage) and adults. Differences were compared applying an independent t test.

Table 3: Differences in vitamin K status between adults and children (multivariate analysis)

	Δ Children - adults	95% CI	p
ucOC (ng/ml)	27.7	18.9 - 36.5	<0.001
	58.4	37.3 - 79.6	<0.001 *
	32.7	4.9 - 60.5	0.022 †
cOC (ng/ml)	10.7	8.0 - 13.4	<0.001
	14.4	8.0 - 20.9	<0.001 *
	5.6	-2.1 - 13.3	0.151 †
UCR	1.5	0.9 - 2.1	<0.001
	3.0	1.5 - 4.4	<0.001 *
	2.5	0.8 - 4.2	0.005 †

Differences in vitamin K parameters (ucOC, cOC and UCR) between the total group of children versus adults, adjusted for potential confounders. Data are presented as difference (Δ) between children and adults, 95% CI for the difference, and significance level of the difference (p)

Groups were compared applying a multivariate analysis adjusting for: * age, gender, and puberty and † age, gender, puberty, height, and weight.

Correlation of vitamin K status and bone turnover parameters

In the total group of children, a marked correlation was found between the markers for bone metabolism (BAP and NTX) and both ucOC and cOC. The bone markers did not correlate with the UCR (table 4). In adults, NTX, but not BAP, was correlated with the osteocalcin markers. In the total group (including adults and children), both BAP and NTX showed significant correlations with ucOC, cOC and the UCR.

Table 4: **Pearson correlation and significance level of relation between bone metabolic activity and the vitamin K status in different groups**

Group	Children, r	p	Adults, r	p	Total group, r	p
BAP - ucOC	0.435	<0.0001	0.268	0.160	0.577	<0.0001
BAP - cOC	0.535	<0.0001	0.136	0.481	0.666	<0.0001
BAP - UCR	0.051	0.641	0.303	0.111	0.251	0.007
NTX - ucOC	0.481	<0.0001	0.600	0.001	0.650	<0.0001
NTX - cOC	0.499	<0.0001	0.505	0.005	0.699	<0.0001
NTX - UCR	0.123	0.258	0.387	0.038	0.374	<0.0001

Correlation between bone markers (BAP, NTX) and vitamin K parameters (ucOC, cOC, UCR) in children, adults, and the total group (both adults and children). Data are presented as Pearson's correlation coefficient (r) and significance level (p).

2.5 Discussion

In this chapter, we report that high circulating levels of ucOC are found in the majority of children between 6 and 18 years of age. Moreover, the UCR was significantly higher in children than in adults. These findings suggest a poor vitamin K status of bone during growth. The extent of vitamin K deficiency shows a substantially interindividual difference when considering the large variations in the UCR. In the elderly, high serum levels of ucOC have repeatedly been mentioned to be associated with low bone mass and high fracture risk. Whether children would benefit from increased dietary vitamin intake remains to be seen.

Osteocalcin is a vitamin K-dependent protein almost exclusively synthesized by the osteoblasts in bone (4;25). On a molecular level, its function is still obscure, but the circulating antigen is frequently used as a marker for osteoblast activity. One of the characteristics of vitamin K-dependent proteins is that during episodes of low vitamin K-intake, they are synthesized as undercarboxylated species. Osteocalcin is the only Gla-protein known of which a relatively large fraction (20-30%) circulates in an undercarboxylated form in

healthy adults (15). The major part of newly synthesized osteocalcin is absorbed to the hydroxyapatite in bone, but a part of it leaks into the circulation where it can be detected (26).

The elevated levels of osteocalcin, BAP, and NTX in children, as found in the present study, are indicative of high metabolic activity in bone. Moreover, the high levels of ucOC suggest that the vitamin K-requirement of bone during growth is not met by the dietary vitamin K supply. It may be argued that high ucOC levels are representative for the high bone turnover state found in adolescence rather than the result of vitamin K deficiency. Indeed, the serum ucOC level is determined by both the total osteocalcin production and the fraction thereof that has been carboxylated by vitamin K action. Therefore, the UCR, which is independent of bone turnover, is a more reliable marker for vitamin K status of bone. In our study, we have found very high UCR values in children compared to adults, suggesting subclinical vitamin K deficiency during high bone turnover in children. This is consistent with observations in another population characterized by high bone metabolism, i.e. postmenopausal women (19;21;27;28). Several authors have demonstrated that, in the latter group, low dietary vitamin K intake, high circulating ucOC, and high UCR are associated with low bone mineral density and increased fracture risk. In these women, the serum ucOC levels are higher than in young adults. From our study, it is clear that both the absolute concentration of ucOC and the UCR in children are much higher than in healthy adults or even in postmenopausal women. Here we postulate that the metabolic activity of bone is associated with its vitamin K requirement. Children during growth have a much higher bone metabolism than adults. This may be a possible explanation for the apparent vitamin K-deficiency in children of all ages.

In children, the importance of vitamin K status in bone health has only been studied by a small number of research workers (29). Kalkwarf et al. (24) described that in healthy girls (3-16 y), greater vitamin K status was associated with decreased bone metabolic activity. Furthermore, in children with long-standing vitamin K deficiency caused by the anti-coagulation drug warfarin, a reduced bone density was found (30). This study illustrates the potential consequences of vitamin K deficiency in growing bone.

However, the question still remains whether improved bone health in children can be achieved by higher vitamin K intake. The value of vitamin K supplements has been shown in several studies in postmenopausal women.
In this group, vitamin K supplementation results in decreased bone loss (31)

or even gain of bone mass (32-34). Besides an effect on bone mineral density, recent data suggest that the most important benefit of vitamin K in bone is improved geometry. In an article that has only been published as an abstract thus far, Kaptoge et al. showed that in elderly women vitamin K supplementation resulted in increased femur neck width and increased bone strength at this site (Kaptoge S, Dalzell N, Welch A, Shearer MJ, Khaw KT, Reeve J; Vitamin K and fracture risk: an effect on bone not BMD? Bone and Tooth Society Anual Meeting, July 4-5, 2005, Birmingham, UK, Abstract no. 12). Recently, this was confirmed in galactosemic children (B. Panis et al., unpublished results), a group known to have low BMD.

Our study describes the finding of a possible vitamin K deficiency in bone. The major limitation of this study is that we were only able to link these results to markers of bone metabolism and not to assessment of bone mass or strength. The confirmation that this apparent deficiency may affect bone quality can only be established in prospective pediatric studies. Another limitation concerns the cross sectional design of our study. Prospective studies in which possible changes in UCR are monitored in a time-dependent manner may demonstrate whether vitamin K status is fluctuating during different episodes of growth or whether it is consistently associated with young age.

Our finding that, based on osteocalcin carboxylation, most of the children in a healthy population must be defined as vitamin K deficient is a point of interest and justifies follow-up studies in which bone quality is monitored as a function of vitamin K intake.

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Chapter 3 Vitamin K status is associated with childhood bone mineral content

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3.1 Abstract

In adult bone, vitamin K contributes to bone health, probably through its role as co-factor in the carboxylation of osteocalcin. In children, the significance of vitamin K in bone mass acquisition is less well known. The objective of this longitudinal study was to determine whether biochemical indicators of vitamin K status are related to (gains in) bone mineral content (BMC) and markers of bone metabolism in peripubertal children. In 307 healthy children (mean age 11.2 years), BMC of the total body, lumbar spine and femoral neck was determined at baseline and 2 years later. Vitamin K status [ratio of undercarboxylated (ucOC) and carboxylated (cOC) fractions of osteocalcin (UCR)] was also measured at both time points. Markers of bone metabolism, sex steroids, vitamin D status and growth hormones were measured at baseline only. Large variations in the levels of the UCR were found at both time-points, indicating a substantial interindividual difference in vitamin K status. Improvement of vitamin K status over 2 years was associated with a marked increase in total body BMC. The UCR was associated with pubertal stage, markers of bone metabolism, sex hormones and vitamin D status. A better vitamin K status was associated with more pronounced increase in bone mass in healthy peripubertal children. In order to determine the significance of these findings for childhood bone health, additional pediatric studies are needed.

3.2 Introduction

Several studies in adults suggest a beneficial role for vitamin K in bone mineral metabolism and bone fracture prevention, although precise mechanisms have not been entirely elucidated (1-3). A well recognized concept is the vital role of vitamin K as a co-factor in the posttranslational carboxylation of osteocalcin, a protein synthesized by osteoblasts (4;5). In this carboxylation process, glutamate acid (Glu) residues are converted into γ-carboxyglutamate acid (Gla) (6). The common property of all Gla-proteins is their high affinity for calcium. Chelation of calcium ions is essential for the function of these proteins. Osteocalcin, the most abundant non-collagenous protein found in human bone, consists of 49 amino-acids three of which are Gla (7-9). Here, we will designate the 3-Gla molecule as carboxylated osteocalcin (cOC). The Gla residues in osteocalcin are positioned in such a way that they point directly to the calcium ions in the crystal structure of hydroxyapatite, the mineral matrix in bone. In order to adequately carboxylate osteocalcin, the osteoblast requires sufficient vitamin K
(10). In case of vitamin K deficiency, undercarboxylated osteocalcin (ucOC) will be produced. In the healthy adult population, osteocalcin is carboxylated to a variable extent, suggesting that the dietary vitamin K intake is insufficient for full osteocalcin carboxylation (11). Markedly higher osteocalcin carboxylation is obtained by increased vitamin K intake (12;13). Previous studies in postmenopausal women found a clear association between elevated ucOC levels and increased fracture risk (14-16). Bioavailable vitamin K is mainly derived from nutritional sources such as green leafy vegetables and cheese (17;18).

Research in the elderly population has revealed that serum ucOC and the ratio between ucOC and cOC are reliable and stable markers for vitamin K status of bone, and that a high vitamin K intake may improve bone mineral content and strength and diminish fracture risk (2;13;15;19;20). Also in children, the amount of undercarboxylated osteocalcin relative to the total (or carboxylated) osteocalcin is used to study the relationship between vitamin K status and bone health (21-24). Using the UCR, we have recently shown that the majority of healthy children have a suboptimal vitamin K status of bone (24). Additionally, a marked correlation between the bone metabolism markers and the fractions of osteocalcin was found in these children (24). Recently, another study in a large cohort of healthy girls aged 11-12 years showed that better vitamin K status was associated with increased bone mineral content (21).

The objective of the present longitudinal study was to assess the vitamin K status in healthy children during puberty and to study its association with bone mass and changes in bone mass. In addition, the associations between vitamin K status and biochemical markers of bone metabolism, sex steroids and growth hormones were analyzed.

3.3 Subjects and methods

Originally, the present longitudinal study was designed to evaluate associations between bone markers, sex steroids and (changes in) bone mass throughout puberty in healthy children. The results of this study and a detailed description of the study design were previously published (25). A total of 307 children between 8 and 14 years, recruited from a number of primary and secondary schools in the villages around Amsterdam, participated in this study. The children were white, reported to be healthy and did not take any medication. At the first visit, height, weight, pubertal development and bone densitometry were measured and serum samples were collected. Height and weight were measured using a stadiometer and a calibrated scale, respectively, wearing underwear only. Pubertal development was assessed in boys on genital stages (G1-5) and in girls on breast stages (B1-5) according to Tanner. After 2.0 years (sd 0.10 y), bone densitometry was repeated in 281 children. At this study visit, height, weight and pubertal development were determined for the second time and collection of serum samples took place. The study protocol was approved by the Committee of Ethics on Human Research of the VU University Medical Centre.

Bone mineral content (BMC, g) and bone size (anterior-posterior projected bone area (BA), cm^2) of the L1-L4 region of the lumbar spine (LS), the femoral neck (FN) and the whole body (TB) were measured with dual-energy X-ray absorptiometry (DEXA) using the Hologic QDR-2000 (Hologic Inc., Waltham, MA, USA). All scans were carried out in the array mode and analyzed by the same investigator. The reproducibility of the different scans has been described previously (26). Delta-BMC (Δ-BMC) is defined as the difference in BMC at follow-up minus BMC at baseline.

Blood samples were drawn in the morning after overnight fasting. After blood sampling and serum preparation, samples were frozen and kept at -70° C until use.

Undercarboxylated (ucOC) and carboxylated (cOC) fractions of osteocalcin were measured by ELISA (Takara, Japan). The ratio of ucOC and cOC (UCR) was used as an indicator of vitamin K status. Elevated levels of the UCR are indicative of an inferior vitamin K status and are related to suboptimal nutritional vitamin K intake (11;27). Delta-UCR (Δ-UCR) is defined as the difference of the natural log-transformed UCR at follow up minus baseline. This means that a negative figure for Δ UCR indicates a decrease in UCR over time which means an improved vitamin K status. Vice versa, a positive figure for Δ UCR represents an increase in UCR over time, suggesting a deteriorated vitamin K status.

Furthermore, bone-specific alkaline phosphatase (BAP), marker of bone formation, was measured with an assay by wheat germ agglutinin (28). Procollagen type I amino terminal propeptides (PINP), marker of bone formation, and type I carboxy terminal telopeptides (ICTP), marker of bone resorption, were estimated by radioimmunoassay of Orion Diagnostica (Espoo, Finland). The level of $1,25(OH)_2$-vitamin D was measured using the IDS-assay (Tyne and Wear, UK). The level of 25(OH)-vitamin D_3 was measured using a competitive binding assay (Nichols) after alcohol extraction. Estradiol (in girls only) was determined by radioimmunoassay (Sorin Biomedica, Saluggia, Italy) as well as testosterone (in boys only; Coat-A-Count, DPC, Los Angeles, CA, USA). Insulin-like growth factor I (IGF-1) and IGF-BP_3 were determined by immunoradiometric assays (DSL, Webster, TX, USA).

UcOC and cOC were determined at baseline and after two years; all other biochemical markers of bone metabolism and vitamin D status, sex steroids, and IGF-1/IGF-BP_3 measurements were determined at baseline only.

Statistical methods

Normality of distributions was checked for all study parameters. The distributions of ucOC and UCR, testosterone and estradiol were skewed to the right, and their absolute values are given as median and range. The data of ucOC, UCR, testosterone and estradiol were converted to natural logarithms (ln), prior to use in regression- and correlation analyses. Paired samples t-tests were used for comparison of continuous parameters (age, height, weight, BMI, bone DEXA parameters) between baseline and follow-up. Comparisons of ucOC and UCR between baseline and follow-up were performed using Wilcoxon signed rank-tests. ANOVA was used to compare the distribution of pubertal stages at baseline and after 2 years. Possible associations of anthropometric data with

the UCR were investigated using bivariate correlation tests.

In order to explore the association between vitamin K status (UCR), bone markers and hormones, we used multivariate linear regression analysis. The UCR at baseline was used as dependent variable and bone markers and hormones at baseline as independent variables. Analyses were adjusted for gender, age, pubertal stage, weight and height, but only when these variables were associated with the UCR outcome at $p < 0.05$. In these analyses, pubertal stage was dichotomized into a prepubertal/early stage (Tanner stage 1-2) versus late/end of puberty-stages (Tanner stage 3-4-5). Because data on bone markers and hormones after 2 years were lacking, we could not perform their association with vitamin K status at 2 years.

Multiple linear regression analyses were also used to examine the association of (increase in) bone density and (changes in) vitamin K status. Independent variables were the TB-BMC, LS-BMC and FN-BMC at baseline and at follow up. In addition, differences (Δ) in TB-BMC, LS-BMC and FN-BMC, indicating gains in BMC over time, were also used as independent variables. In these analyses, we adjusted BMC for (site-specific) bone size in order to minimize size-related effects on (longitudinal) estimates of bone mass by DEXA, as is recommended for children (29). The dependent variables of interest in the regression analyses were the UCR at baseline and follow up, and the changes in UCR (Δ UCR) over time. Furthermore, besides adjustment for bone size, other potential confounders (gender, age, pubertal stage (early vs. late)) were included into the model, but only when these variables were associated with the BMC outcome at $P < 0.05$. Weight and height were also considered in the models but because of multicollinearity with bone area, these variables were omitted from the definitive models. In the regression analyses for Δ-BMC, besides gender, other potential confounders included in the model were the differences in bone size, weight, height and pubertal stage.

The statistical tests were executed using a two-sided significance level of 5%. A p value <0.05 was considered to be statistically significant. SPSS Base 12.0.2 for Windows (SPSS Inc., Chicago, Illinois, USA) was used for all analyses.

3.4 Results

In table 1, the anthropometric variables and DEXA-parameters at baseline and follow-up are shown. Over two years time, significant increases in weight,

height and BMI were noted.

Table 1: Characteristics of the study subjects at baseline and follow up (2 years)

	Baseline		Follow-up		p
Subjects (n)	307		281		
Anthropometry					
Male gender (n) [a]	156	(50.8 %)	139	(49.5 %)	
Age (years)	11.2	(1.3)	13.2	(1.3)	< 0.001
Height (cm)	150.6	(10.5)	162.5	(11.0)	< 0.001
Weight (kg)	39.9	(9.1)	50.7	(11.1)	< 0.001
BMI (kg/m^2)	17.4	(2.4)	19.0	(2.7)	< 0.001
Pubertal stage (n) [a b]					
1	112	(36.5 %)	28	(9.9 %)	< 0.001
2	96	(31.3 %)	46	(16.4 %)	
3	67	(21.8 %)	60	(21.4 %)	
4	23	(7.5 %)	48	(17.4 %)	
5	9	(2.9 %)	99	(35.2 %)	
Bone DEXA parameters					
BMC-LS (g)	30.9	(8.2)	42.9	(13.0)	< 0.001
BMC-FN (g)	3.3	(0.6)	3.9	(0.8)	< 0.001
BMC-TB (g)	1236.5	(310.1)	1619.9	(420.9)	< 0.001
Vitamin K status					
ucOC (ng/ml) [c]	35.1	(5.4 – 64.2)	43.1	(0.2 – 10.8)	0.004
cOC (ng/ml)	27.2	(8.0)	28.5	(9.2)	0.052
UCR [‡]	1.4	(0.2-8.3)	1.5	(0.2 - 10.8)	0.137

Values are presented as mean (sd); [a] male gender and pubertal stage are presented as number (percentage); [c] ucOC and UCR are presented as median (range). p values are presented for differences in values at baseline and follow-up. p values are based on paired t tests; except for [b], based on ANOVA; except for [c], based on Wilcoxon signed-rank test.

At baseline, most children were prepubertal or in early puberty. Expectedly, more children were found in later pubertal stages after 2 years. As expected, BMC increased significantly in the course of time in all children.

Table 1 also shows the levels of ucOC, cOC and UCR at the start of the study and after two years. Large ranges in the level of the UCR were found at both time-points, indicating a substantially inter-individual difference in vitamin K status. The median ucOC increased significantly from baseline to follow-up whereas the cOC showed a marginal, borderline-significant, increase. However, the median UCR did not change over time. The UCR at baseline was significantly associated with pubertal stage (r: 0,165; p: 0,004), baseline-weight (r: 0.183; p: 0.001) and baseline-BMI (r: 0.190; p: 0.001). The UCR at follow up was associated with gains in height (r: 0.342; $p < 0.01$) and weight (r: 0.204; p: 0.001) over 2 years.

In table 2, the associations of vitamin K status (UCR) with bone markers (resorption and formation), vitamin D status and hormones (growth, sex steroids) at baseline are shown. The UCR was found to have a positive correlation with markers of bone formation (BAP and PINP) and the marker for bone resorption (ICTP). The UCR was not related to the level of IGF-1 and IGF-BP_3. No significant association was found for 25(OH) -vitamin D_3 and UCR. However, an evident correlation between 1,25-$(OH)_2$ vitamin D and the UCR was found. In girls, estradiol was associated with the UCR, whereas in boys, testosterone was correlated to the UCR.

Table 3 shows the associations between bone mass and vitamin K status (UCR) at baseline and follow up. In addition, this table depicts the associations between the increase in bone mineral content and changes in UCR over time. The association of bone mass and vitamin K status was more evident for TB-BMC than for the other sites (FN and LS). At baseline, the UCR was inversely associated with TB-BMC, but this association merely showed a statistical trend. At follow-up, the UCR was inversely associated with TB-BMC after 2 years, even when adjusted for covariates. Considering changes over time, improvement of the UCR was inversely associated with more pronounced increase in whole body bone mass (table 3). The association of FN-BMC with vitamin K status was not consistent at the different time-points. At baseline, the UCR was related to FN-BMC whereas at follow-up, no significant association was found.

In addition, improvements in UCR were inversely associated with more pronounced increases in FN-BMC. No statistically significant associations were found between LS-BMC and vitamin K status.

Table 2: Bone markers and hormones at baseline and their associations with vitamin K status

	Mean	(sd)	B	95% CI	p
Bone formation					
BAP, U/L	194.6	(61.0)	0.001	0.000; 0.003	0.040
PINP, µg/L	754.0	(308.5)	0.001	0.000; 0.001	<0.001
Bone resorption					
ICTP, U/L	15.8	(6.9)	0.014	0.002; 0.026	0.018
Growth factor					
IGF-1, U/L	29.5	(14.1)	0.005	-0.012; 0.012	0.177
$IGF\text{-}BP_3$, mg/L	4.5	(0.8)	0.054	-0.057; 0.165	0.377
Vitamin D					
25-(OH) vitamin D_3, nmol/L	69.6	(18.8)	0.000	-0.004; 0.005	0.872
$1,25\text{-}(OH)_2$ vitamin D, pmol/L	136.4	(39.0)	0.007	0.005; 0.009	<0.001
Sex steroids					
Testosterone in boys, nmol/L [a]	0.7	(0.3-22.0)	0.257	0.127; 0.388	<0.001
Estradiol in girls, pmol/L [a]	37.0	(16.0-318.0)	0.178	0.018; 0.337	0.029

Values are presented as mean (sd); [a] values are presented as median (range). p values, regression coefficient (B) and 95% CI for B are based on multivariate linear regression analyses with natural log-transformed UCR as dependent variable, adjusted for gender, pubertal stage (early versus late) and weight. Log-transformed values of testosterone and estradiol were used in regression analyses. Direct interpretation of the coefficients requires back transformation to original units.

Table 3: **Associations between (changes in) bone mineral content and (changes in) vitamin K status**

		UCR baseline [a]	
BMC parameters at baseline	B	95% CI	p
BMC-TB	-10.9	-22.3; 0.44	0.060
BMC-LS	0.42	-0.13; 0.96	0.132
BMC-FN	0.07	0.010; 0.13	0.022
		UCR follow-up [b]	
BMC parameters at follow-up	B	95% CI	p
BMC-TB	-19.1	-36.2; -2.0	0.029
BMC-LS	-0.02	-0.81; 0.78	0.969
BMC-FN	0.03	-0.06; 0.12	0.560
		Δ UCR [c]	
Δ BMC	B	95% CI	p
Δ BMC-TB	-49.1	-63.0; -35.2	<0.001
Δ BMC-LS	0.19	-0.25; 0.64	0.392
Δ BMC-FN	-0.10	-0.15; -0.05	<0.001

[a] adjusted for bone size, age, gender and pubertal stage (early versus late) at baseline.

[b] adjusted for bone size, age, gender and pubertal stage (early versus late) at follow-up.

[c] adjusted for difference in bone size (follow up - baseline), difference in bodyweight, difference in body height, gender and differences in pubertal stage.

Values (B, 95% CI and p) are based on linear regression analyses with BMC parameters as dependent variables and the natural log-transformed UCR as the variable of interest. Direct interpretation of the coefficients requires back transformation to original units.

Δ UCR is the difference in natural log-transformed UCR at follow up versus baseline.

A negative figure for Δ UCR indicates a decrease in UCR over time. Vice versa, a positive figure for Δ UCR represents an increase in UCR over time. Higher UCR-values indicate an inferior vitamin K status.

3.5 Discussion

In the present study in healthy peripubertal children, we have found that gains in whole body-bone mass over two years are associated with changes in vitamin K status of bone, even after adjusting for potential confounders. Furthermore, vitamin K status was related to BMC, most evident at follow-up. In addition, vitamin K status was associated with markers of bone formation, vitamin D status and sex steroids at baseline.

The relationship between vitamin K status and bone health has already been studied extensively in the adult population (5;16;19). It is recognized that circulating undercarboxylated osteocalcin levels may be useful in predicting fracture rate and relate to bone mass in the elderly (15;30). Also in healthy children, evidence for the usefulness of the carboxylation of osteocalcin as biochemical marker for vitamin K status is available (21;22;24;31). In these studies, vitamin K status is expressed as the amount of undercarboxylated osteocalcin relative to the amount of total or carboxylated osteocalcin. In the present longitudinal study, better vitamin K status was associated with higher bone mass at baseline and after 2 years of follow-up, although this relation was more evident at follow-up. The associations found were more pronounced for bone mass of the whole body than for site-specific bone mass at femoral neck or lumbar spine. It has been suggested that in growing children, the total body BMC is a preferable outcome measure to monitor changes in overall bone mass over time because it takes bone size and shape of all skeletal regions into account (32).

The association of current vitamin K status and bone mass in healthy children has also been described in other observational studies (21;22). O'Connor et al. also found that better vitamin K status, expressed as %ucOC, was positively related to current BMC of total body and lumbar spine (21). Kalkwarf et al. found that %ucOC was related to markers of bone turnover in a group of healthy girls (22). In addition, indicators of vitamin K status were not consistently associated with 4-year changes in BMC (22). In our study, we have found similar associations of the UCR and markers of bone turnover. However, in contrast to the findings from the study by Kalkwarf, we have found that improvement of the vitamin K status over time, indicated by a decrease in UCR, is related to an additional increase in BMC. A possible explanation for this divergent findings is that the broad age range of the healthy girls (3-16 yrs) in the study cohort of Kalkwarf have obscured the relation between vitamin K

status and bone mass variables, despite the large number of participants (n = 245).

A previous study conducted by our group has shown that the majority of healthy children have a suboptimal vitamin K status, based on the extent of osteocalcin carboxylation. In the latter study, we found high circulating levels of undercarboxylated osteocalcin and high UCR levels in children in comparison to the adult population (24). Also in the present study, we have found high levels of UCR in children, suggestive for a relative vitamin K shortage in bone. Furthermore, the UCR was correlated to pubertal stages, indicating that in advanced pubertal stages, coinciding with highest growth velocities, higher UCR levels are found. Comparable associations of UCR and pubertal development were also observed in our previous study (24). It may be reasoned that the high levels of UCR result from an imbalance between dietary vitamin K intake and the metabolic requirement for vitamin K during growth. A gradual decline in vitamin K intake in children in recent years is described by Prynne et al. (33). In the U.S.A., the recommended daily allowance (RDA) for vitamin K in children (4-18 years) is 55-75 µg vitamin K (34). Bounds et al. found an average daily intake of 51 (sd 30) µg vitamin K in American children (2-8 years) whereas others found a median intake of 45 µg vitamin K per day in American girls (3-16 years) (22;35). In the latter study, wide ranges of the percentage of undercarboxylated osteocalcin (% ucOC) were found, indicating that intakes of vitamin K were not sufficient, despite dietary intakes approximating the RDA. It could also be that the high levels of UCR and ucOC are only a reflection of a physiological situation during normal growth and bone mass acquisition. However, from an evolutionary point of view, it seems unlikely that large amounts of non-functional undercarboxylated osteocalcin are meant to be synthesized. It could also be that a relative vitamin K shortage in this period has no adverse effects as long as levels of carboxylated osteocalcin (the functional form of osteocalcin) are sufficiently high. In the present study, the average cOC concentrations at baseline and after two years did remain constant.

In the present study, we found that vitamin K status was only related to the active form of vitamin D which is $1,25(OH)_2$ vitamin D levels, and not to 25(OH) vitamin D_3. In literature, the relationship between vitamin D and vitamin K remains subject of debate. Several studies suggest a contribution of vitamin D in osteocalcin expression (36). However, vitamin D is not involved in the carboxylation of osteocalcin. The synergistic effect of the combined supplementation of vitamin D and vitamin K on bone mass has been shown in some

intervention trials in adults (1;37;38).

Our report describes the associations of vitamin K status and bone mass in peripubertal healthy children. One of the limitations of this study may be the lack of longitudinal data on physical activity. Weight-bearing physical activity is another important determinant of bone mass (39). Nevertheless, it is not likely that physical activity is also a determinant of vitamin K status of bone and therefore not a true confounder. Also, other nutritional factors like calcium metabolism (e.g. urinary calcium excretion or milk intake) or carbonated beverage consumption were not taken into account (40).

Taking together the findings from the present and other observational studies on vitamin K and bone in children, one may conclude that a suboptimal vitamin K status in children probably has a negative impact on childhood bone health. Whilst (bone) growth continues, the relative lack of vitamin K may lead to inferior bone quality/strength or suboptimal bone mineralization resulting in increased fracture risk. Indeed, an increased fracture risk is observed in healthy children in the peripubertal years because the increase of bone mass fails to keep up with the increase in height (41-43). Furthermore, recent data in adults suggest that vitamin K supplementation results in improved bone geometry besides its effect on bone density (44). Hence, the question remains what happens to bone mass if vitamin K is supplemented in this period of life. We speculate that vitamin K supplementation in healthy adolescents will lead to improved bone health and decreased fracture risk, in analogy to the situation in another population characterized by high bone metabolism, i.e. postmenopausal women (14;20;30;45). An adequate vitamin K status during puberty may lead contribute to a higher peak bone mass, which is the maximal amount of bone mineral accrued during life. Achievement of optimal peak bone mass in adolescence may be a possible strategy in the prevention of osteoporosis in later life (46;47). The effect of vitamin K supplementation on bone mass will need to be studied in intervention studies in the adolescent population.

In conclusion, we found that an improved vitamin K status over time is associated with a more pronounced increase in bone mass over two years in healthy peripubertal children. In order to determine the significance of these findings, both longitudinal observational studies and placebo-controlled randomized intervention trials in children are needed.

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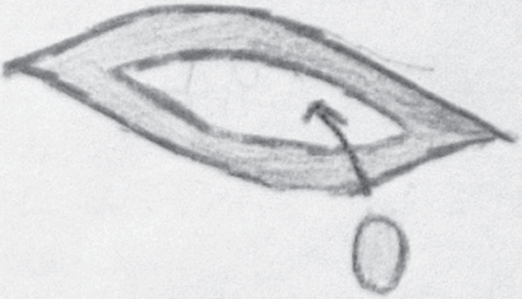

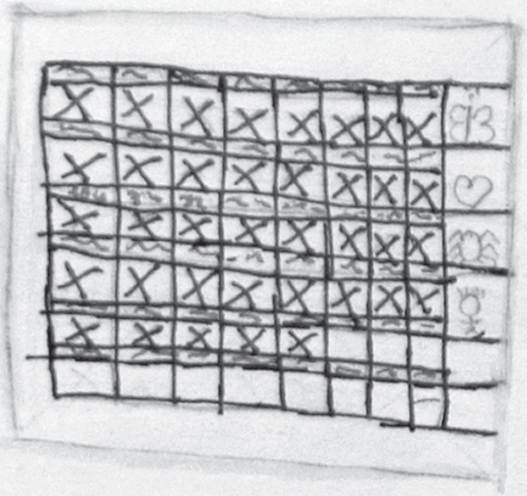

Na een week mag je
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Na het meten en wegen gingen ze naar je hart
en longen ~~luisteren~~ luisteren.

Gerlinde, 8 years

Chapter 4 The effect of vitamin K_2 supplementation on osteocalcin carboxylation in healthy children (the VitaKids study)

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4.1 Abstract

In adult bone, vitamin K contributes to bone health, probably through its role as co-factor in the carboxylation of osteocalcin. Intervention studies in adults have demonstrated that markedly higher osteocalcin carboxylation is obtained by intakes of vitamin K well above the current recommended dietary intake. However, the relation between increased vitamin K_2 intake and enhanced osteocalcin carboxylation has never been shown in healthy children.

The aim of our study was to study the effect of 45 µg MK-7, one of the vitamin K_2 species, on the circulating levels of undercarboxylated (ucOC) and carboxylated (cOC) osteocalcin in healthy prepubertal children. We hypothesized that MK-7 supplementation will reduce the ratio of ucOC and cOC (UCR), indicating an improved vitamin K status.

This study is a double-blind randomized placebo-controlled intervention study which examines the effect of 8 weeks MK-7 supplementation on the carboxylation of osteocalcin in healthy children (n=56). Levels of ucOC and cOC were measured at baseline and after 8 weeks. The ratio of ucOC and cOC (UCR) was used as an indicator of vitamin K status. In the MK-7 supplemented group (n=28), the circulating concentration of inactive undercarboxylated osteocalcin reduced whereas vitamin K status improved. Within the placebo group, ucOC, cOC and vitamin K status did not significantly change over time.

These findings support the hypothesis that in healthy children, dietary vitamin K intake alone is generally inadequate for optimal osteocalcin carboxylation and may lead to reconsideration of current recommended dietary intakes for vitamin K.

4.2 Introduction

Only fifty years after its discovery in the 1930s, the importance of vitamin K in physiologic processes other than blood coagulation has become increasingly clear. Several studies in adults suggest a beneficial role for vitamin K in bone mineral metabolism and bone fracture prevention, although precise mechanisms have not been entirely elucidated (1-3). A well recognized concept is the vital role of vitamin K as a co-factor in the posttranslational γ-carboxylation of osteocalcin, a protein synthesized by osteoblasts (4;5). In this carboxylation process, glutamate acid (Glu) residues are converted into γ-carboxyglutamate acid (Gla) (6). These Gla residues have a high affinity for calcium (Ca2+) which is an essential property of all Gla-proteins. A poor vitamin K status will lead to production of undercarboxylated (i.e. inactive) osteocalcin (7). In the healthy adult population, osteocalcin is carboxylated to a variable extent, suggesting that the dietary vitamin K intake is often insufficient for full osteocalcin carboxylation (8). Previous studies in postmenopausal women found a clear association between elevated undercarboxylated osteocalcin (ucOC) and increased fracture risk (9-11).

Recently, we reported that the majority of healthy children between 6 and 18 years of age have high circulating levels of ucOC compared to adults (12). In addition, the amount of ucOC relative to the carboxylated fraction of osteocalcin (cOC) was also high in these children. This ratio of ucOC and cOC (UCR) is used as indicator for the vitamin K status (5;13). An elevated UCR is indicative of an inferior vitamin K status of bone and relates to a suboptimal nutritional vitamin K intake (8;13). O'Connor et al. reported that in healthy girls aged 11-12 years, better vitamin K status was associated with increased bone mineral content (14). In an observational study in healthy peripubertal children, we showed that improvement of vitamin K status over 2 years was associated with a marked increase in bone mineral density (M. van Summeren, unpublished data, chapter 3 of this thesis).

These findings justify clinical intervention studies in children in whom bone quality is monitored as a function of long-term vitamin K-supplementation. Before undertaking such long-term intervention studies, the effect of increased vitamin K-intake in healthy children on osteocalcin carboxylation has to be established. Intervention studies in adults have already demonstrated that markedly higher osteocalcin carboxylation is obtained by intakes of vitamin K well above the current recommended dietary intake (15;16).

The two most important forms of vitamin K are phylloquinone (vitamin K_1) and the group of K_2 vitamins (menaquinones, MK-n). A recent report by Schurgers et al. showed that menaquinone-7 (MK-7), a form of vitamin K_2 which is particularly abundant in fermented soybeans (natto), has possible advantages in comparison to vitamin K_1, largely due to its long half-life time. In this study, supplementation with MK-7 in healthy volunteers resulted in increased circulating vitamin K levels and more complete osteocalcin carboxylation than vitamin K_1 (17).

The relation between increased vitamin K_2 intake and enhanced osteocalcin carboxylation has never been shown in healthy children. It is reasonable to infer that vitamin K_2 species will improve osteocalcin carboxylation in children but to what extent is unclear. Therefore, we set up a randomized controlled trial in healthy children using an MK-7 containing food supplement to study the effect of vitamin K_2 on the circulating levels of undercarboxylated (ucOC) and carboxylated fractions (cOC) of osteocalcin in healthy prepubertal children. We hypothesized that MK-7 supplementation reduces the UCR, indicating an improved vitamin K status.

4.3 Subjects and methods

This study is a double-blind randomized placebo-controlled intervention study. Subjects were recruited from two primary schools around Utrecht, the Netherlands. Inclusion criteria were apparently healthy prepubertal children between 6 and 10 years of age, normal (p3-p97) height and weight according to standard growth charts and parents giving written consent for the child to take part in the study. We chose this age category because these children are most likely to be in a prepubertal state when bone turnover is fairly stable. In this way, we were able to study the effect of vitamin K on osteocalcin carboxylation without possible confounding effects from rapid changes in bone metabolic activity which may occur during puberty and may influence vitamin K status (12;18). Exclusion criteria were current or previous medical diseases such as metabolic or gastro-intestinal disease, soy allergy, chronic inflammatory diseases and current use of systemic corticoid treatment, vitamin supplements containing vitamin K or oral anticoagulants. The exclusion criterion "soy allergy" was introduced because the vitamin K-containing capsules used in this study may contain traces of soy proteins originating from

the natto-extract. The study protocol was approved by the Central Committee on Research involving Human Subjects (CCMO, the Netherlands).

The total eligible group consisted of 240 children. A total of 55 children met the criteria for participation, and were included in our study. Enrollment took place in January 2007. One investigator was responsible for the intake of participants and supervision of the study. Participants were randomized into two groups by computer-generated random permutation procedures: those receiving a placebo (n=27) and those receiving a supplement containing 45 µg MK-7 (n=28) during 8 weeks. Based on previous observations from studies in adults in whom the maximal effect on osteocalcin carboxylation is seen after an intervention period ranging from 3 to 8 weeks, we chose for an intervention period of 8 weeks (15;16). The randomisation schedule was generated by an investigator not involved in the coordination of the study and kept apart during the trial. A block randomisation was performed for gender, in order to avoid unequal distribution of boys and girls in the two intervention groups. Placebo- and vitamin K-capsules (MenaQ7), provided by NattoPharma (Oslo, Norway), were similar in taste and appearance, thus indistinguishable for participants and investigators. Both capsules contained equal amounts of linseed oil (210 mg). The dosage of 45 µg and explicit choice for the vitamer MK-7 are based on results from a previous study in which the effects of MK-7 on bioavailability and osteocalcin carboxylation were compared to vitamin K_1 (17). In this study, adult volunteers received a daily dose of equimolar amounts (0.22 µM) of either MK-7 (150 µg) or vitamin K_1 (100 µg) during 6 weeks. The dosage of 100 µg vitamin K_1 was chosen based on the recommended daily allowance (RDA: 100 µg vitamin K_1) for adults. The extent of osteocalcin carboxylation was significantly higher in the MK-7 group compared to the K_1-group. At the end of the intervention period, a still increasing carboxylation of osteocalcin was seen in the MK-7 group, whereas the K_1-group had already reached a plateau phase. Furthermore, it was shown that the half-life time of vitamin K_1 is only 1-2 hours, in contrast to MK-7, which had a half-life time of approximately 3 days. The dosage of 150 µg MK-7 used for adults (estimated weight 80 kg) was calculated to the bodyweight of children (6-10 years; estimated average weight 25 kg) leading to a dosage of 45 µg MK-7 for children.

After randomization, subjects and their parents were invited for a study visit in which the study capsules were handed out. Participants were instructed to take one capsule per day during 8 weeks, preferably during the main meal. During this visit, a short physical examination was performed and blood was

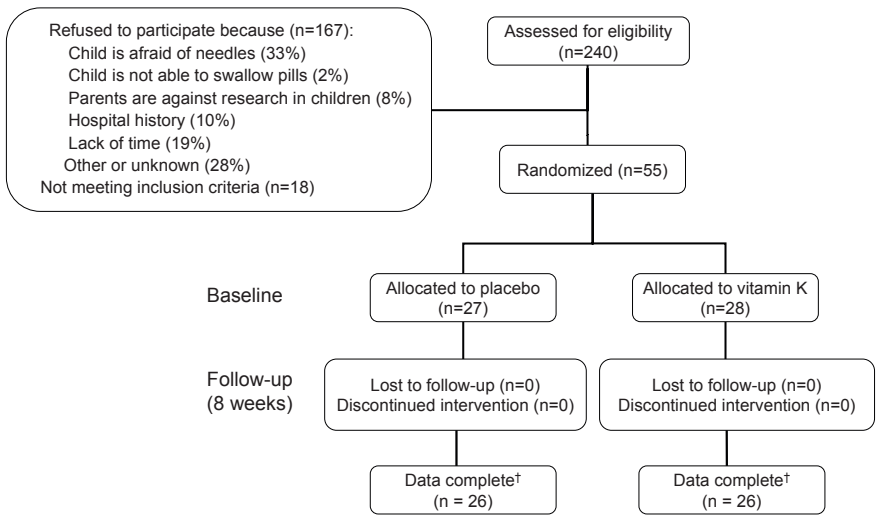
taken by venipuncture. Furthermore, body height and weight were measured in a standardized manner without shoes and heavy clothing, to the nearest centimeter and 100 g, respectively. From these values, the BMI (weight/height^2) was calculated. The values of height, weight and BMI were compared with reference values for healthy subjects matched for age and sex, and standard deviation scores (sds) were calculated (19). Participants and parents were advised to continue their usual activities and maintain their normal diets during the study period. Co-medication was allowed with the exception of other vitamin supplements, oral anticoagulants and corticosteroid treatment. At 2, 4 and 6 weeks, short phone calls were scheduled to inform about the occurrence of adverse events and possible side-effects of the study capsules. At 8 weeks, the final visit was planned in which the second venipuncture was performed. Compliance was monitored using a calendar on which the daily intake of the study supplement had to be recorded. Also, the parents were requested to hand in the remaining capsules and capsule container at the end of the study.

The primary outcome of the study was the percentages of change in UCR from baseline (0 weeks) to endpoint (8 weeks) in both treatment groups. Secondary outcomes were the percentage of change in the absolute concentrations of circulating ucOC and cOC. Non-fasting blood samples were obtained at baseline from 55 children and in 52 children at the end of the study after 8 weeks. After blood sampling and serum preparation, all samples were frozen and kept at -80°C until use. Undercarboxylated and carboxylated fractions of osteocalcin were measured in serum by enzyme-linked immunosorbent assay (Takara Shuzo Co Ltd., Shiga, Japan).
The desired effect of the vitamin K treatment in this study was to reduce the UCR in children to the average level of young adults. The expected difference in UCR between the treatment and control group is based on reference values found in a previous study in which the average UCR level was 0.8 in young adults and 2.3 in children(12). The sample size was calculated on the assumption that the desired effect was a minimum reduction of 20 % in UCR from baseline to endpoint (8 weeks) in the treatment group compared to the placebo group with a 90% power and a 0.05 level of significance. With the assumption of a drop-out rate of 10% over the total study period, we calculated that at least 25 subjects had to be included in each group. Normality of distribution for all subjects was checked for all study parameters, using histograms. Normally distributed baseline characteristics of both

placebo- and treatment group were compared using independent t-tests. A chi-square test was performed to compare the distribution of gender across groups. Differences at baseline as well as % of change in ucOC, cOC and UCR from baseline to endpoint between groups were assessed using Mann-Whitney tests. In the placebo- and treatment group separately, differences in ucOC, cOC and UCR from baseline to follow-up were examined using Wilcoxon signed ranks tests for paired variables. Statistical analyses were performed using SPSS Base 12.0.2 for Windows (SPSS Inc, Chicago, USA) with two-sided significance levels of 5%.

4.4 Results

Figure 1: Flow chart of participants in the VitaKids study



† Missing data because venapunction failed in three subjects who completed the total study.

Figure 1 shows the flowchart for treatment of participants in the study. All included children completed the intervention period of 8 weeks. However, we had missing data in three children, one in the placebo group and two in the treatment group, because the second venapunction failed. These subjects were included in the baseline analyses. The majority of children (66.7 % of the placebo group and 71.4 % of the treatment group) reported to have never

missed their daily capsule. In the placebo group, 9 children missed on average 2 capsules (range 1-3) during the study period. In the treatment group, 8 children missed on average 3 capsules (range 1-7) during the study period. The compliance as reported on the calendar by the children was in accordance with the remaining capsules in the containers, and confirmed by the parents. No side-effects were reported during the study.

The baseline characteristics for the placebo- and treatment groups are shown in table 1. The groups were comparable in age, height, weight, BMI and distribution of gender.

Table 1: Baseline characteristics of the placebo- and vitamin K group

	Placebo (n = 27)	Vitamin K (n = 28)	p
Anthropometry			
Male gender, n (%) * †	10 (37 %)	12 (43 %)	0.660
Age, years	8.4 (1.1)	8.2 (1.3)	0.665
Height, cm	133.8 (9.0)	132.2 (9.3)	0.515
Height-sds	-0.04 (0.96)	-0.14 (0.88)	0.679
Weight, kg	30.4 (7.1)	29.2 (5.5)	0.479
Weight for height-sds	0.18 (1.1)	0.23 (0.83)	0.860
BMI, kg/m^2	16.8 (2.5)	16.6 (1.6)	0.673
BMI-sds	0.21 (1.1)	0.20 (0.78)	0.983
Vitamin K parameters			
ucOC, ng/ml †	18.3 (3.4 – 73.2)	21.6 (4.7 – 96.9)	0.444
cOC, ng/ml †	25.8 (4.3 – 42.1)	23.1 (10.0 – 44.3)	0.355
UCR †	0.6 (0.2 – 2.7)	0.9 (0.2 – 4.5)	0.330

Values are presented as mean (sd); * male gender is presented as n (%); † values presented as median (range). p values are presented for differences in variables between placebo- and vitamin K-groups at baseline. p values are based on independent t tests, except for, based on chi-square test and for †, based on Mann-Whitney tests.

The baseline characteristics for the placebo- and treatment groups are shown in table 1. The groups were comparable in age, height, weight, BMI and distribution of gender. Markers representing the vitamin K status of bone (UCR, ucOC and cOC) were also similar across groups. A large range of ucOC, cOC and the UCR was found in both groups at baseline.

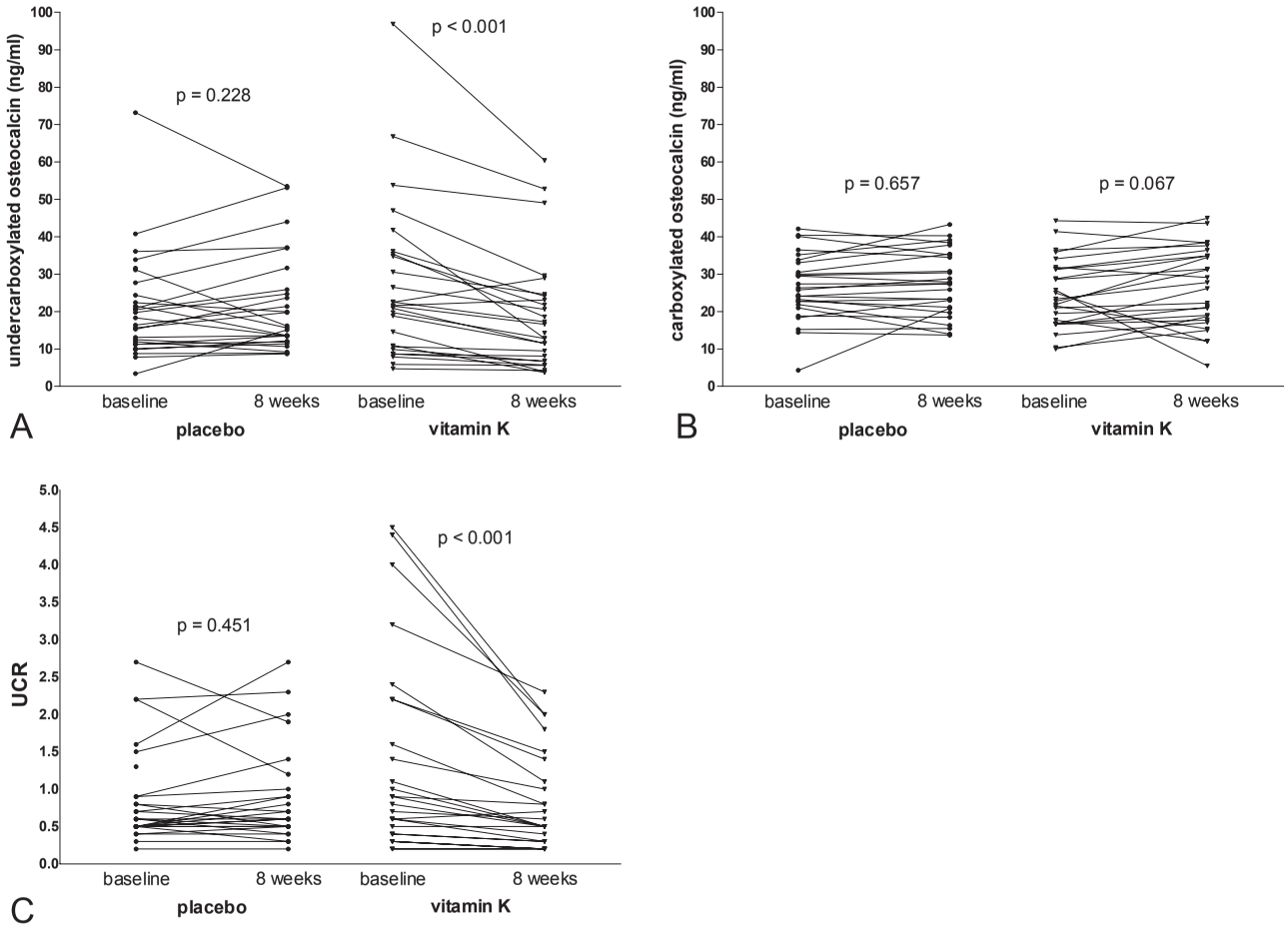
Figure 2 shows the absolute values of ucOC, cOC and UCR at baseline and after 8 weeks in the placebo- and treatment group. Within the treatment group, ucOC and UCR decreased significantly from baseline to endpoint. The cOC increased slightly but this was a near significant increase. Within the placebo group, ucOC, cOC and UCR did not change over time. In table 2, the median and range of the percentages of change (%Δ) in ucOC, cOC and UCR from baseline to endpoint in the placebo- and treatment group are shown. The %Δ in ucOC and UCR in the treatment group was significantly different from the placebo group whereas the difference in %Δ in cOC between groups was nearly significant.

Table 2: Changes in vitamin K parameters in placebo- and vitamin K-group

	Placebo (n = 26)	Vitamin K (n = 26)	p
Vitamin K parameters			
Δ ucOC, %	+12.2 (-48.1 – 344.1)	-25.6 (-73.3 – 28.9)	<0.001
Δ cOC, %	+1.2 (-33.3 – 386.1)	+12.2 (-78.6 – 87.0)	0.067
Δ UCR, %	+0.0 (-45.5 – 80.0)	-33.3 (-59.1 – 16.7)	<0.001

Values are presented as median percentage (%) of change from baseline to follow up at 8 weeks (range). A positive value indicates an increase from baseline to endpoint; a negative value indicates a decrease. p values are presented for differences in the % of change in variables between placebo- and vitamin K-groups. p values are based on based on Mann-Whitney tests.

Figure 2: Vitamin K parameters at baseline and after 8 weeks in placebo- and vitamin K-groups



4.5 Discussion

The present study shows that supplementation with 45 µg MK-7 during 8 weeks in healthy prepubertal children improves vitamin K status as indicated by the UCR. In the MK-7 supplemented group, the circulating concentration of the inactive, undercarboxylated fraction of osteocalcin decreased. This study represents the first randomized controlled trial using MK-7 and its effect on osteocalcin carboxylation in healthy children. These findings support the hypothesis that in healthy children, dietary vitamin K intake alone is generally inadequate for optimal osteocalcin carboxylation (12) and may lead to reconsideration of current recommended dietary intakes for vitamin K.

Until now, only three studies in children, all pediatric patients, have reported on vitamin K supplementation and all three showed a positive effect on osteocalcin carboxylation (20-22). In a 4-week cross-over study in 18 cystic fibrosis (CF) patients (13-35 years of age) using 5 mg vitamin K_1 weekly, Beker et al. noted an increase in serum ucOC when patients were off supplementation (21). Nicolaidou et al. studied 20 CF patients (6-17 years of age) who received 10 mg vitamin K_1 per week during 1 year and they reported a decrease in ucOC concentrations that was even under the ucOC-level of 25 healthy controls (8-17 years of age) (20). Findings from a randomized controlled trial in 40 children with classical galactosemia (3-18 years of age) by Panis et al. showed a significant increase in cOC and decrease in ucOC after 1 year in children receiving a daily dose of 1.0 mg vitamin K_1 together with calcium and vitamin D_3 (22). The dosages of vitamin K_1 used in these studies are quite high, probably because these children had an established risk of (subclinical) vitamin K-deficiency due to malabsorption associated with their underlying condition.

Figure 2: Vitamin K parameters at baseline and after 8 weeks in placebo- and vitamin K-groups

Figure 2A depicts the values of undercarboxylated osteocalcin (ucOC) at baseline and follow-up at 8 weeks in the placebo and vitamin K group.

Figure 2B depicts the values of carboxylated osteocalcin (cOC) at baseline and follow-up at 8 weeks in the placebo and vitamin K group.

Figure 2C depicts the values of the ratio of ucOC and cOC at baseline and follow-up at 8 weeks in the placebo and vitamin K group. p values are presented for the differences from baseline to follow-up in each group and are based on Wilcoxon signed ranks tests.

In the present study, we used a supplement containing an amount of vitamin K approximating the recommended dietary allowance (RDA) in children, in addition to habitual dietary vitamin K intakes. At present, the RDA of 30-75 µg vitamin K/day for children (1-18 years) in the USA is based on the maintenance of normal concentrations of the hepatic vitamin K-dependent protein, prothrombin (23). However, dietary vitamin K levels that are sufficient to maintain the hepatic production of normal blood coagulation factors may be too low for optimal carboxylation of the extra hepatic Gla-proteins (13). Subjects can also be defined as vitamin K deficient based on the absolute amount of circulating undercarboxylated osteocalcin (13;24;25). Vitamin K status in children is preferably based on the UCR or the quantity of ucOC relative to the total osteocalcin, in order to minimize possible influences of bone metabolism (12;18). In healthy adult volunteers, vitamin K supplementation reduced the circulating level of ucOC and increased the level of cOC (8;15;25;26). Also in our study, vitamin K supplementation resulted in reduction of ucOC levels. Hence, vitamin K intake from the habitual diet of prepubertal healthy children appears to be inadequate for optimal carboxylation of osteocalcin.

The children included in the present intervention study had a better vitamin K status at baseline in comparison to the children from another study in healthy children conducted by our department (12). In the latter study, a pronounced elevation (as compared to adults) of ucOC together with a marked elevation of the UCR was found in the majority of children. The UCR was on average 2.3 (range 0.3 - 6.8) in children versus 0.8 (range 0.1 - 2.2) in adults. In the present study, only 6 children (11%) had an UCR above 2.3 at baseline in comparison to 35% of children from the previous cohort. This observation can be explained by the fact that we studied vitamin K supplementation in prepubertal children. As was shown in previous studies in children, suboptimal vitamin K status is most evident in puberty when the bone metabolic activity is high, leading to increased vitamin K demand (M. van Summeren, unpublished data, chapter 3 of this thesis) (12). Our study illustrates that vitamin K supplementation has an effect on osteocalcin carboxylation, even in prepubertal children in the lowest UCR ranges. Most likely, adolescents will benefit even more from vitamin K supplementation.

In the present study, the increase in the circulating amount of carboxylated osteocalcin after 8 weeks of MK-7 supplementation was smaller than expected, although there was a nearly significant increase compared to the placebo-

group. Apparently, the decrease in ucOC is not linearly related with the increase in cOC. This observation may be explained by the fact that ucOC and cOC have a different binding affinity to hydroxyapatite in bone. The major part of newly synthesized osteocalcin is absorbed to hydroxyapatite in bone, but a part of it leaks into the circulation where it can be detected (27). UcOC has a lower binding capacity to hydroxyapatite in bone and therefore, may leak more easily to the circulation, whereas the large part of cOC will be bound in bone. Provided that the total osteocalcin production remains constant, supplementation of additional vitamin K will lead to a relative increase in cOC in bone. It may be assumed that only when the bone mineral matrix is fully saturated with cOC, indicative of optimal vitamin K supply to bone, the amount that leaks to the circulation will increase. An extended period of supplementation or increased dosage of MK-7 would probably have resulted in higher circulating levels of cOC in our study. Thus, serum ucOC and cOC levels are determined by both the total osteocalcin production and the fraction thereof that has been carboxylated by vitamin K action. Therefore, the ratio between ucOC and cOC (or total OC) is a more reliable marker for vitamin K status than the separate fractions of ucOC or cOC because they also reflect bone metabolic activity.

Studies in the elderly population indicate that supplementation with vitamin K has a beneficial effect on bone mass and bone strength, most likely by improved carboxylation of osteocalcin (1;28). A growing body of evidence on the relation between vitamin K, osteocalcin carboxylation and bone health is also emerging in healthy children. O'Connor et al. demonstrated that better vitamin K status, expressed as percentage undercarboxylated osteocalcin, was associated with increased bone mineral content in a large cohort of healthy girls aged 11-12 years (14). Although both vitamin K_1 and menaquinones serve as cofactor the carboxylation of osteocalcin (17), menaquinones (notably MK-7) may be preferable in food supplements because of their pleiotropic effects on bone. In vitro models have shown that MK-7 affects the function of the osteoblasts by inducing the expression of osteoblast-specific genes (e.g. osteocalcin, osteoprotegerin, RANK and RANKL). In addition, MK-7 promotes the differentiation of the osteoblasts, probably by interaction with the steroid and xenobiotic receptor (SXR) (29;30). Furthermore, MK-7 prevented ovarectomy-induced bone loss in rats (31;32).

A limitation of the present study may be that it is not designed as a dose-finding study. The results only indicate that vitamin K improves osteocalcin

carboxylation in healthy children and can therefore not answer the question "What is the amount of vitamin K that is needed for maximal or optimal carboxylation of osteocalcin in children?". Furthermore, another limitation may be that we did not collect information on vitamin K intake from habitual diet. However, food frequency questionnaires are not mandatory to study the effect of additional vitamin K on osteocalcin carboxylation when the study is designed as a randomized placebo-controlled trial.

In conclusion, our study indicates that supplementation with MK-7, one of the vitamin K_2 species, during 8 weeks reduces the amount of circulating ucOC and improves vitamin K status in healthy prepubertal children. Additional dose-finding studies are needed to investigate optimal osteocalcin carboxylation and redefine the recommended intakes for vitamin K in healthy children.

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Chapter 5 Extremes in vitamin K status of bone are related to bone ultrasound properties in children with juvenile idiopathic arthritis

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5.1 Abstract

Osteopenia is a common complication of juvenile idiopathic arthritis (JIA). In adults, low bone density and increased fracture risk are associated with low vitamin K status of bone. The vitamin K-dependent protein osteocalcin plays an important role in bone metabolism. Its activity depends upon post-translational carboxylation in which vitamin K is an essential co-factor. Hence, vitamin K deficiency leads to undercarboxylated (i.e. inactive) osteocalcin (ucOC). Little is known about the vitamin K status and bone health in children with juvenile idiopathic arthritis (JIA). We studied the vitamin K status of bone and its association with bone mass properties in children with JIA compared to healthy children. We performed a cross sectional study in 55 children with JIA and 54 healthy controls between 6-18 years of age. Bone markers, ultrasound bone mass properties and vitamin K status of bone were determined. Overall, no differences in vitamin K status of bone were found between the study groups. Among children with JIA, a high ratio of ucOC/cOC indicating low vitamin K status was associated with low bone ultrasound parameters, whereas children with a high vitamin K status had markedly higher bone properties. This association was independent of physical activity, age, gender and BMI. These results suggest that vitamin K may be one of multiple risk factors for low bone mass in children with JIA, in addition to other recognized determinants of bone mass. The question remains whether JIA patients would benefit from increased dietary vitamin K intake.

5.2 Introduction

Juvenile idiopathic arthritis (JIA) is the most prevalent rheumatic disease in childhood (1). In these patients, reduced generalized bone mass resulting in osteopenia or osteoporosis is frequently observed (2;3). In children with JIA, multiple risk factors for the development of systemic osteopenia are present. The main contributors are glucocorticoid treatment, systemic inflammation and physical inactivity resulting from disease severity (4-7). Osteopenia in JIA patients may lead to increased fracture risk in childhood (8). Low bone mass and increased fractures are noted in later (adult) life as well probably due to an inadequate build up of peak bone mass in adolescence (8;9).

Several studies in adults suggest a beneficial role for vitamin K in bone mineral acquisition and bone fracture prevention, although precise mechanisms have not been entirely elucidated (10-12). A recognized concept is the vital role of vitamin K as a co-factor in the posttranslational carboxylation of osteocalcin, a protein synthesized by osteoblasts (13;14). In this carboxylation process, glutamate acid (Glu) residues are converted into γ-carboxyglutamate acid (Gla) (15). The common property of all Gla-proteins is their high affinity for calcium which is essential for the function of these proteins. Osteocalcin is the most abundant non-collagenous protein found in human bone and consists of 49 amino-acids of which three are Gla (16;17). The Gla residues in osteocalcin are positioned in such a way that they point directly to the calcium ions in the crystal structure of hydroxyapatite, the mineral matrix in bone. In order to adequately carboxylate osteocalcin, the osteoblast requires sufficient vitamin K (18). In case of vitamin K deficiency, undercarboxylated osteocalcin (ucOC) will be produced. In the healthy adult population, osteocalcin is carboxylated to a variable extent, suggesting that the dietary vitamin K intake is insufficient for full osteocalcin carboxylation (19). Markedly higher osteocalcin carboxylation is obtained by increasing vitamin K intake (20). Bioavailable vitamin K is mainly derived from nutritional sources such as green leafy vegetables and cheese (21;22).

Research in the elderly population has revealed that a high vitamin K intake may improve bone quality and diminish fracture risk (23-25). The amount of ucOC relative to the total (or carboxylated) osteocalcin as well as absolute levels of circulating ucOC are used as indicators for the vitamin K status of bone (14;26). Serum vitamin K concentrations fluctuate with recent dietary vitamin K intake and are no reliable markers for tissue vitamin K status (22).

Also in children, several studies have confirmed the positive relationship between vitamin K and bone health (27-30). Kalkwarf et al. reported that in healthy girls better vitamin K status was associated with decreased bone turnover as indicated by the level of bone markers (29). A study by O'Connor et al. in healthy girls (11-12 years) showed that optimal vitamin K status was related to increased bone mineral content (27). There are no data on vitamin K status in children with juvenile idiopathic arthritis and its possible contribution to bone health in this group. Children in general seem to be at risk of a reduced dietary intake of vitamin K, as was recently reported in British children (31). Furthermore, children with JIA are at risk of malnutrition due to reduced food intake and increased energy expenditure (32). All this may lead to subclinical vitamin K deficiency of bone in children with JIA. This shortage may affect bone mineralization in a population already susceptible for osteopenia. The present study examines vitamin K status in children with JIA compared to vitamin K status in healthy children. Furthermore, we studied the association between vitamin K status of bone, bone markers and quantitative ultrasound properties of calcaneal bone in these children.

5.3 Patients and Methods

Study subjects

From October 2003 to January 2004, 55 JIA patients between 6-18 years of age, classified according to International League Against Rheumatism (ILAR) criteria were enrolled into this study (33). Patients were consecutively recruited from the outpatient clinic from the department of Pediatric Immunology (Utrecht, the Netherlands) at routine follow up visit.

Fifty-four healthy controls, between 6-18 years of age as well, were recruited from a population of children undergoing minor surgery (e.g. strabismus correction, phimosis correction), and friends or relatives of above mentioned patients. Exclusion criteria for controls were JIA or other inflammatory diseases and the use of systemic corticosteroids, non-steroidal anti-inflammatory drugs (NSAIDs) or anti-tumor necrosis factor (TNF) α medication.

This study was approved by the Central Committee on Research Involving Human Subjects (CCMO, the Netherlands) and by the Medical Ethical Committee of the University Medical Centre Utrecht (METC-UMCU, the Netherlands).

Written informed consent was obtained from the parents of all children and also from children being ≥12 years of age.

Data collection procedure

Body height and weight of all subjects were measured in a standardized manner without shoes and heavy clothing, to the nearest centimeter and 100 g, respectively. From these values the BMI (weight/height^2) was calculated. The values of height, weight and BMI were compared with the reference values for healthy subjects matched for age and sex, and Z-scores were calculated (34). Pubertal stage was determined according to Tanner's sexual maturity scale and divided into three categories: prepuberty (prepubertal stage), puberty (pubertal stages II-IV) and end of puberty (pubertal stage V). Leisure-time participation in sport activities (yes/no) and the amount of hours spend on sport activities per week were assessed with a short self-report questionnaire. No distinctions were made in the quantity of weight bearing and intensity of sport activities. Seven JIA patients had missing data for sport-activities and mean number of hours of sport. Duration of disease and current use of medication in JIA patients were derived from medical records. Disease-activity (active disease versus clinical (partial) remission) in JIA patients was based upon the preliminary criteria for clinical remission for different categories of JIA (35). These criteria define inactive disease as the absence of active arthritis; no fever, rash, serositis, splenomegaly, or generalized lymphadenopathy attributable to JIA; no active uveitis; normal erythrocyte sedimentation rate (ESR); and a physician's global assessment of disease activity rated at the best score possible for the instrument used. According to these criteria, partial clinical remission was defined as the absence of disease activity during 6 continuous months while using medication. Clinical remission off medication was defined as the absence of disease activity during 12 months.

From the Child Health Questionnaire (CHQ), the physical functioning (PF) and psychosocial summary scores (PSS) were obtained for JIA patients and controls (36). Four JIA patients did not fill out the complete questionnaire to compute the CHQ-PF; nine JIA patients and four controls did fill out the complete questionnaire to compute the CHQ-PSS. In the control group, information about medical history and current use of any medication was ascertained by a short interview.

Assessment of vitamin K status, bone turnover and inflammation markers

After blood sampling and serum preparation, all samples were frozen and kept at -80 °C until use. The inflammation marker high sensitivity C-reactive protein (hsCRP) was measured by nephelometry; ESR was measured using the Westergren technique. Five JIA patients did not have values for hsCRP; one JIA patient and one control did not have values for ESR. In serum, two biochemical markers of bone turnover were measured. Bone-specific alkaline phosphatase (BAP, Hybritech, Belgium) was measured as a marker for bone formation by radio immunoassay. As a marker for bone resorption, N-telopeptide cross-links of collagen breakdown (NTX, Ostex, Washington) were determined. Undercarboxylated (ucOC) and carboxylated (cOC) fractions of osteocalcin (Takara, Japan) were measured by enzyme-linked immunosorbent assay (ELISA). We used the ucOC/cOC ratio (UCR) because it is a sensitive indicator for the vitamin K status of bone and relatively independent of bone turnover (14;26;28). Elevated levels of UCR are indicative of an inferior vitamin K status of bone and relate to a suboptimal nutritional vitamin K intake (19).

Assessment of bone ultrasound parameters

To assess bone mineral density and bone structure, quantitative ultrasound measurements were performed using an ultrasonic device (Hologic QDR 4500, Hologic Inc, Waltham, MA). This equipment is an easy and rapid means of evaluating bone density without ionising radiation and it provides information about bone mass and architecture (37). It has been shown that bone density measured by bone ultrasound and DEXA are highly correlated (38;39). However, it is recommended that the definitive diagnosis of osteoporosis or osteopenia is established using DEXA (40).

For every bone ultrasound measurement, two parameters are determined automatically: broadband ultrasound attenuation (BUA, dB/MHz) and speed of sound (SOS, m/s). BUA is an indicator of bone quantity whereas SOS is used as parameter for bone stiffness. Two measurements at the right and left os calcis were performed for each participant. These four values were averaged to one outcome for BUA and SOS respectively. Comparison of these data to the mean of BUA and SOS of the left and right os calcis separately revealed no differences. For that reason, the overall means for BUA and SOS were used in data-analyses.

Acoustic phantoms provided by the manufacturer were scanned monthly and showed no drift over the time period of the study.

Statistical analysis

Normality of distribution for all subjects was checked for all study parameters. Z-scores for height, weight and BMI were calculated with the equation Z-score = (observed value – mean value)/SD. Characteristics of children with JIA and healthy children were compared using independent t-tests. A Chi-square test was performed to compare the distribution of gender across groups. ANOVA was used to compare the distribution of pubertal stages across groups. Differences in hsCRP and ESR were assessed using the Mann-Whitney test. Outcome parameters (bone turnover markers, bone parameters and vitamin K status) between groups were compared using multivariate regression analyses adjusting for the potential confounders pubertal stage, gender and BMI. In the analyses for the bone ultrasound parameters, we also added variables like sport activities, CHQ physical functioning and the inflammation marker hsCRP in order to investigate whether differences between groups could be explained by these factors.

We examined the association of vitamin K status and the bone turnover markers BAP and NTX in both groups using multivariate linear regression analyses, adjusting for potential confounders pubertal stage, gender and BMI. Secondly, we studied the relationship of vitamin K status and bone density in both groups with use of univariate regression analyses.

The plotting of vitamin K status against bone properties showed a non-linear relationship between these variables. Therefore, vitamin K status was categorized into three equal groups based on the UCR (low-median-high). The UCR groups were used as independent (dummy) variables in multivariate regression analyses studying the association of vitamin K status with the bone ultrasound properties BUA and SOS in JIA patients and healthy children separately. The analyses were adjusted for age, gender and BMI. We also introduced variables like sport activities, CHQ physical functioning and hsCRP in these models in order to investigate whether these factors could explain the association between BUA/SOS and low versus high UCR. For JIA patients only, we also introduced disease-variables into these models.

Finally, we studied potential associations between vitamin K status of bone and disease-variables in JIA patients only using linear regression analyses. All statistical tests were executed using a two-sided significance level of 5%.

SPSS Base 12.0.2 for Windows (SPSS Inc, Chicago, Illinois, USA) was used for all analysis.

5.4 Results

Subjects

The characteristics of the study subjects are shown in table 1. Age, height, weight, BMI and pubertal stages were comparable in patients and controls. There was a trend toward more girls in the JIA sample. As expected, the inflammation markers ESR and hsCRP were elevated in patients with JIA. The physical functioning (PF) score was significantly lower in the JIA group and a trend towards lower psychosocial summary score (PSS) was observed. These results, both derived from the CHQ, indicate that JIA patients are impaired in their daily physical functioning compared to controls. Participation in sports was similar across both groups, but patients with JIA spent fewer hours on sport activities than healthy controls. The clinical characteristics of JIA patients are shown in table 2. Most patients were classified as having oligoarthritis (n = 29) or poly-arthritis (n = 20) and only a minority with systemic disease (n = 6). Disease activity was absent in 40 patients, of whom 32 were in partial remission. Partial remission was defined as the absence of disease activity during 6 months while using medication. Active disease was present in 15 patients (table 2). Mean duration (sd) of disease for all subtypes was 59.1 (41.6) months.

Bone markers, bone mass and vitamin K status of bone

The outcome parameters for the JIA patients and the control group are shown in table 3. Patients with JIA showed an increased level of BAP (marker for bone formation) and a lower level of NTX (marker for bone resorption) compared to the control group. In addition, the patients had lower bone ultrasound variables, both for BUA as well as for SOS, even when adjusting for potential confounders. The differences in the bone ultrasound parameter SOS between the groups were not explained by sport activities, CHQ physical functioning or inflammation markers. CHQ physical functioning and inflammation markers did also not explain the difference in the bone ultrasound parameter BUA between groups. However, after adjusting for sport activities in the model for BUA, the difference between groups was not longer statistically significant.

Table 1: Characteristics of the study groups

		Control group (n = 54)	JIA group (n = 55)	p
Demographics				
Gender, male‖		21 (39)	12 (22)	0.052
Age, years		11.9 (3.0)	11.3 (3.2)	0.368
Height, cm		153.2 (17.2)	148.8 (18.2)	0.201
Height sds		0.05 (0.9)	-0.06 (0.9)	0.526
Weight, kg		46.6 (15.4)	42.3 (14.4)	0.135
Weight-height sds		0.46 (0.98)	0.23 (0.97)	0.231
BMI, kg/m^2		19.3 (3.1)	18.5 (3.0)	0.180
BMI sds		0.46 (0.97)	0.22 (0.94)	0.190
Pubertal stage *				0.626
	Prepuberty	27 (50)	26 (47)	
	Puberty	15 (28)	14 (25)	
	End of puberty	12 (22)	15 (28)	
Inflammation markers				
hsCRP, mg/l * §		0.43 (0.2-13.3)	1.3 (0.2 - 97.6)	0.001
ESR, mm/hour * §		4.3 (1-25)	6.5 (1-40)	0.014
Child Health questionnaire (CHQ)				
CHQ Physical Functioning † ‡ §		100 (33.3-100)	83.3 (33.3-100)	<0.0001
CHQ Psychosocial Summary Score † ‡ §		53.7 (37.9-61.2)	51.3 (24.9-59.1)	0.055
Sport				
Sport activities, yes‖		44 (81.5 %)	36 (75%)	0.427
Sport activities per week/year, hours † §		1.7 (0-13.7)	1.0 (0-17.5)	0.047

Values are presented as mean (sd). Pubertal stages, male gender and sport activities are presented as n (%). p values based on t tests, except for *, based on ANOVA; ‖, based on Chi square test; §, based on Mann-Whitney test.

† Variable presented as median (minimum-maximum).

* Variable presented as geometric mean (minimum-maximum).

‡ Lower values indicate greater functional impairment, maximal score is 100.

Table 2: **Characteristics of JIA patients**

	Total group JIA	Oligoarticular JIA	Polyarticular JIA	Systemic JIA
Demographics				
No. of subjects	55	29	20	6
Extended form		10 (34.5)		
Gender, male	12 (21.8)	8 (27.6)	4 (20.0)	0 (0)
Age, years ¶	11.3 (3.2)	11.0 (3.0)	11.1 (2.9)	13.4 (3.9)
Disease variables				
Duration of disease, months ¶	59.1 (41.6)	58.3 (38.7)	54.4 (38.9)	64.2 (48.3)
RF positive	5 (9.1)	2 (6.9)	3 (15.0)	0
ANA positive	22 (40.0)	12 (41.4)	9 (45.0)	1 (16.7)
Medication				
NSAID use	41 (74.5)	22 (75.9)	17 (85.0)	3 (50.0)
Methotrexate use	29 (52.7)	13 (44.8)	13 (65.0)	4 (66.7)
Corticosteroid use	1 (1.8)	0	0	1 (16.7)
Disease activity				
Complete remission	8 (14.5)	6 (20.7)	1 (5.0)	1 (16.7)
Partial remission	32 (58.2)	17 (58.6)	11 (55.0)	4 (66.7)
Active disease	15 (27.3)	6 (20.7)	8 (40.0)	1 (16.6)

Values are presented as n (%), except for ¶ age and duration of disease, presented as mean (sd). Disease-activity (active disease versus clinical (partial) remission) in JIA patients was based upon the preliminary criteria for clinical remission for different categories of JIA (35).

Markers representing the vitamin K status of bone (UCR, ucOC and cOC) were similar across groups. In the group of JIA patients, no associations were found between the vitamin K status of bone and disease-variables like duration of disease, disease activity, subtype of JIA, inflammation markers and use of medication (data not shown). A remarkably large variation of the level of ucOC and the UCR was found, suggesting a substantial interindividual difference in vitamin K status of bone in both JIA patients and controls. To further investigate the significance of these differences, we studied its association with (markers of) bone metabolism in both groups.

Table 3: Outcome variables of bone markers, bone ultrasound parameters and vitamin K status in JIA patients and controls

	Control group (n = 54)	JIA group (n = 55)	p *
Bone markers			
BAP, ng/ml	189.0 (155.7)	288.4 (147.2)	<0.001
NTX, ng/ml	89.8 (43.3)	58.6 (30.2)	<0.001
Bone ultrasound parameters			
BUA, dB/MHz	53.4 (12.9)	48.6 (10.0)	0.041
SOS, m/sec	1555.9 (22.9)	1545.0 (20.7)	0.017
Vitamin K status			
ucOC, ng/ml	34.8 (22.1)	28.2 (19.8)	0.167
cOC, ng/ml	17.1 (7.9)	15.3 (6.8)	0.267
UCR	2.3 (1.4)	2.1 (1.4)	0.490

BAP is used as indicator of bone formation; NTX is used as indicator of bone resorption. Elevated levels of UCR are indicative of an inferior vitamin K status of bone.
Values are presented as mean (sd). * p values are based on multivariate analyses with adjustment for gender, BMI and pubertal stage.

Association of bone markers and vitamin K status of bone

Although no actual differences in vitamin K status were found between the study groups, we examined whether the adequacy of vitamin K in bone, indicated by the UCR, was associated with markers of bone turnover (BAP and NTX). In the group of healthy children, no significant associations between the UCR and bone markers were found. In the JIA-sample, multivariate analysis showed a trend for the positive association of the bone formation marker BAP and UCR ($p = 0.068$), independent of the effects of pubertal stage, gender and BMI. A non-significant association was found for the bone resorption marker NTX and UCR in the JIA-group ($p = 0.116$).

Bone mass and vitamin K status of bone

Next, we studied the association of the bone ultrasound properties (SOS and BUA) with the UCR as indicator of vitamin K status of bone. Figure 1 shows the bone mass variables per subgroup of vitamin K status (low-median-high) in healthy children and patients with JIA. Using multivariate regression analyses, we investigated whether these extremes in vitamin K status (expressed as low versus high or median UCR) were associated with bone mass parameters in the JIA group. No significant differences for bone ultrasound parameters between the different UCR-groups were found in the healthy children. However, patients in the low UCR group had higher bone mass properties compared to the patients in the high UCR group (figure 1B/D).
In patients with JIA, those with a high UCR, indicating suboptimal vitamin K status, had a 3 % lower SOS and a 7 % lower BUA, independent of age, gender and BMI. The difference in SOS between the low and high UCR group was not explained by sport activities, CHQ-physical functioning or the inflammatory marker hsCRP. CHQ physical functioning and inflammation markers did also not explain the difference in the bone ultrasound parameter BUA between the low and high UCR group.

However, after adjusting for sport activities in the model for BUA, the difference between groups only showed a clear trend. Also, disease-variables like duration of disease, subdiagnosis, current disease activity, current medication or cumulative dosages of methotrexate or prednisone did not explain the difference in bone mass in JIA patients between the low and high UCR group.

Figure 1: The bone ultrasound parameters SOS and BUA per group of vitamin K status in healthy children and JIA patients

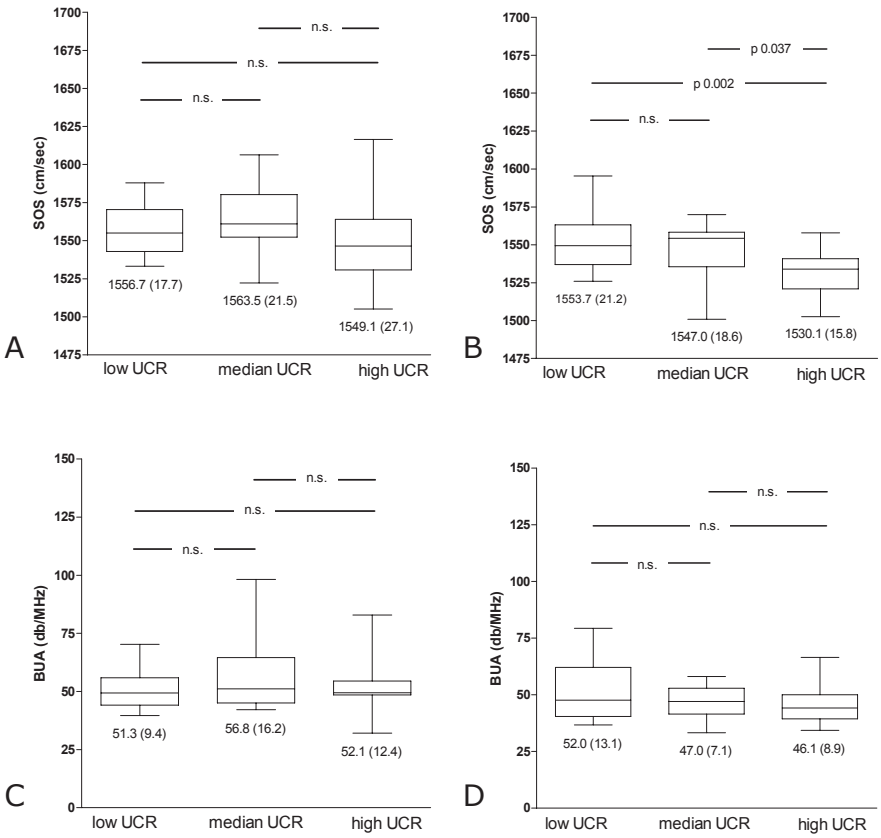

Figure 1A depicts box diagrams for SOS parameters in low, middle and high UCR-groups in healthy children. Figure 1B depicts box diagrams for SOS parameters in low, middle and high UCR-groups in JIA-patients. Figure 1C depicts box diagrams for BUA parameters in low, middle and high UCR-groups in healthy children. Figure 1D depicts box diagrams for BUA parameters in low, middle and high UCR-groups in JIA-patients. p values are based on multivariate analyses with the UCR as the independent variable of interest, comparing subjects with high UCR or median UCR to those with low UCR (reference category). Analyses were adjusted for age, gender and BMI. Box diagrams: the upper limit of the box is the 75th percentile; the lower limit of the box is the 25th percentile; the horizontal line within box represents the median; the whiskers show the range of values. Values per box are presented as mean (sd). Elevated levels of UCR are indicative of an inferior vitamin K status of bone.

5.5 Discussion

In this cross-sectional study, we describe the finding of low bone mineral density in children with JIA in comparison to healthy controls. No differences in vitamin K parameters between the study groups were found, although large interindividual variability in vitamin K status of bone was observed. Within the group of JIA patients, children with a high UCR indicating suboptimal vitamin K status had lower bone ultrasound parameters compared to children with a low UCR, independent of confounding factors.

Several other studies have reported the presence of low bone mineral density in children with JIA (3;9;41;42). Besides conventional DEXA measurements, quantitative ultrasound methods have also been used to detect low bone density in these patients (43-45). To our best knowledge, this is the first study in which vitamin K status in relation to bone health in JIA patients is examined. Although we did not find a linear relationship between bone properties and vitamin K status in JIA patients, it was demonstrated that in the extremes of vitamin K status, bone ultrasound properties were significantly different. We choose to divide the group into three equal groups based on the UCR because a clear definition of optimal vitamin K status based on osteocalcin carboxylation is lacking presently. The relationship between suboptimal vitamin K status and bone health was shown in another study in pediatric patients with long-standing vitamin K deficiency caused by the anti-coagulation drug warfarin; these children showed a reduced bone density compared to healthy children (46).

In addition to its role in bone mass accrual, vitamin K may also be important for bone structure and geometry as was recently shown by Knapen et al. (47). In the present study, we used calcaneal ultrasound to measure bone mass. Both ultrasound parameters reflect bone density as well as bone micro-architecture, whereas SOS is more indicative for bone quantity and BUA for bone strength (48). In the present study, the difference in bone ultrasound parameters between the low and high UCR-group in JIA patients was more evident for the SOS than for the BUA, suggesting that vitamin K might be more important in bone density than bone strength. In a small group of healthy children, Sugiyama et al. also found that carboxylation of osteocalcin was related to the bone ultrasound property SOS (30).

In contrast to a limited number of previous studies in healthy children, we did not find a relation between vitamin K status and bone density in

our healthy control group. A study in a large cohort of healthy girls aged 11-12 years showed that better vitamin K status, expressed as the % of undercarboxylated osteocalcin, was associated with increased bone mineral content (27). In comparison to the latter study, our control group had a broader range of age, which might explain the absent relation between vitamin K status and bone mass variables. This was also the case in a study by Kalkwarf et al. who did not find consistent associations between markers of vitamin K status and bone mass variables in a large cohort of healthy girls aged 3-16 years (29). Additionally, in this study, suboptimal vitamin K status was associated with increased levels of bone markers indicating bone metabolic activity (29). Again in our control group, we did not observe any association of bone turnover markers and vitamin K status. We merely observed a trend towards higher bone turnover markers in children with JIA with lower vitamin K status.

When considering the levels of the bone turnover markers, it was quite remarkable that the bone formation marker BAP was significantly higher in the patients than in the healthy subjects, whereas the bone resorption marker NTX was lower. This is in contrast to findings of other studies in children with JIA, reporting reduced markers of bone formation and increased markers of bone resorption (3;49). However, the majority of our patients are in partial or complete remission whereas these studies mainly described children with active JIA early in the course of disease. Most likely, a catch-up phenomenon of bone growth and bone mineral acquisition occurs in patients who are adequately treated. This hypothesis is supported by a study of Reed et al. who noted that improvement of disease activity leads to increased bone formation markers (50).

In order to appreciate the findings of the present study, some aspects of the cross sectional study design need to be discussed. Nowadays, most patients will achieve clinical remission due to prompt and adequate treatment. As a result, the majority of the JIA-patients in this study are in a relatively well clinical condition, resulting in few active disease-cases. This might explain the absent relationship of current clinical disease characteristics, bone ultrasound parameters and vitamin K status in the present study. In addition, we would like to make some additional remarks about the bone measurements using calcaneal ultrasound. At present, no reference ultrasound data are available to allow comparisons between our study groups and large age and sex matched cohorts. However, the groups in our study were quite comparable in

anthropomorphic data. Furthermore, the question may rise whether calcaneal ultrasound is representative for total body BMD (51). When considering the clinical relevance of calcaneal ultrasound measurements, other studies have shown that this method is a surrogate measure for fractures (52).

In conclusion, children with JIA with suboptimal vitamin K status of bone have lower bone ultrasound variables as compared to those with a better vitamin K status. These results suggest a possible contributory role for vitamin K to bone health in children with JIA, besides other recognized determinants of bone mass. The question remains whether JIA patients would benefit from increased dietary vitamin K intake. This will need to be confirmed in a prospective intervention study in JIA patients, preferably in a selected group of patients with severe active disease who are at highest risk of developing reduced bone health.

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	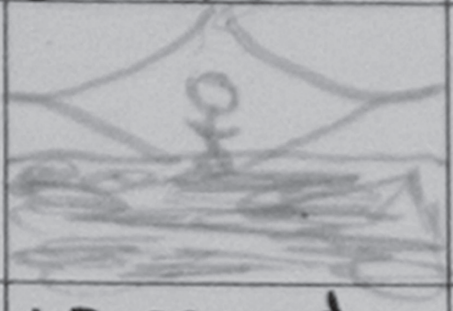	
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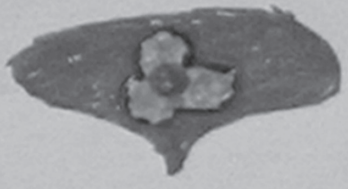		
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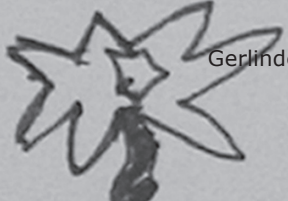		

Gerlinde, 8 years

Chapter 6 Circulating levels of calcification inhibitors and vascular properties in children after renal transplantation

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6.1 Abstract

Pediatric transplant patients are known to have vascular abnormalities. The calcification inhibitors matrix Gla protein (MGP) and fetuin-A play an important role in the pathophysiology of vascular calcification. This cross sectional study examines the circulating levels of fetuin-A and MGP in children after renal transplantation compared to healthy children and their association with vascular properties of the carotid artery. Levels of MGP and fetuin-A together with vascular properties of the carotid artery were determined in 29 pediatric renal transplant recipients and 54 healthy controls. The level of fetuin-A was decreased in the transplant group ($p=0.005$) whereas the level of MGP (both non-phosphorylated MGP and non-carboxylated MGP) did not differ between groups. Furthermore, the intima-media thickness ($p<0.001$) and the elasticity ($p=0.002$) of the carotid artery were significantly increased in children after renal transplantation compared to healthy children. No associations between vascular parameters and calcification inhibitors were found in either group. Circulating levels of MGP and fetuin-A could not be identified as independent predictors for vascular stiffness or other carotid artery parameters in pediatric renal transplant recipients. Future prospective studies in pediatric ESRD and transplant patients are needed to learn more about the role of calcification inhibitors in relation to the prevention of vascular damage.

6.2 Introduction

Young adults with chronic renal failure (CRF) are at risk of early cardiovascular morbidity and mortality (1). After renal transplantation, improvement of vascular function has been described (2), but an increased risk of early cardiovascular death remains (3). Also in children after renal transplantation, abnormal vascular properties are found (4). Long-term follow up of pediatric kidney transplant recipients shows that the main cause of death is cardiovascular disease (CVD) (5). The presence of the classical risk factors for atherosclerosis cannot fully explain this increased risk. Therefore, a better understanding of the mechanisms leading to CVD in end stage renal disease (ESRD) will facilitate the development of preventive and therapeutic measures in these patients.

Several lines of evidence point towards an important contribution of pathologic vascular calcification to CVD in patients with ESRD (6). Vascular calcification occurs at two sites in the arterial wall: the intima and media (7). Vascular media calcification is related to arterial stiffening in patients with ESRD (6). Arterial stiffening can be measured non-invasively using ultrasound techniques. The elasticity of the common carotid artery is an independent predictor of cardiovascular disease in adult renal transplant recipients (8) and ESRD (9). The intima-media thickness (IMT) of the common carotid artery (CCA) is considered to be a surrogate marker for the extent of atherosclerosis (10). Increased carotid artery IMT and increased arterial stiffening are found simultaneously in kidney transplantation and ESRD, suggesting coexistence of media calcification and intima calcification (atherosclerosis) in these patients (11).

In recent years, it has become clear that vascular calcification is an actively regulated process resembling the formation of bone (12). In the calcification process, bone and mineralization regulatory factors are expressed (7). Arterial calcification results from the imbalance between factors favoring calcium deposition and inhibitory mechanisms. Several regulatory factors involved in arterial calcification have been discovered (13). One of these factors is the arterial calcification inhibitor matrix Gla protein (MGP) (14). MGP is a 10-kdalton circulating protein containing 5 γ-carboxyglutamic acid (Gla) residues and 3 serine residues which may be phosphorylated (15). The Gla residues are formed in a posttranslational carboxylation process for which vitamin K is required (16). The Gla residues in MGP are essential for its function and have

a high affinity for calcium (17). Another calcification inhibitor is fetuin-A (also known as α2-Heremans Schmidt glycoprotein), a circulating protein which prevents precipitation of calcium and phosphate in serum (18) and may also play a role in the inhibition of arterial calcification (19).

Several in vivo and in vitro studies have shown that MGP as well as fetuin-A may be involved in the pathophysiology of vascular calcification in ESRD (20). Circulating levels of MGP and fetuin-A may reflect local vascular calcification processes (19;21). However, there is limited evidence linking serum levels of calcification inhibitors to clinical outcomes in ESRD. MGP gene polymorphisms in ESRD patients resulted in altered MGP synthesis and were correlated to cardiovascular outcome (22;23). In adult dialysis patients, low circulating levels of fetuin-A were associated with increased cardiovascular mortality and a high coronary artery calcification score (20;24;25).

Currently, no data have been published on the relationship between inhibitors of calcification and vascular health in children. We hypothesize that insufficient circulating levels of active MGP and fetuin-A play a role in vascular abnormalities in children after renal transplantation. The present study examines the circulating levels of these vascular calcification inhibitors in children after renal transplantation compared to healthy children. Furthermore, we studied the association between the calcification inhibitors and vascular properties of the carotid artery in these children.

6.3 Subjects and methods

Study subjects

We performed a cross-sectional study in 29 pediatric renal transplant recipients and 54 healthy controls between 6-18 years of age. From October 2003 to May 2006, renal transplant recipients were consecutively recruited from the outpatient clinic from the department of Pediatric Nephrology (University Medical Centre Utrecht, the Netherlands) at routine follow up visit. Underlying diseases were congenital abnormalities of the urinary tract (n = 17), congenital nephrotic syndrome (n = 2), focal segmental glomerulosclerosis (n = 2), hereditary syndrome with nephropathy (n=3), acute tubular necrosis (n = 1), chronic tubular interstitial nephritis (n =1), membranoproliferative glomerulonephritis (n = 1). In 2 children, underlying primary renal disease was unexplained. The median time to progress from CRF to ESRD was 3.0 years (range 0 - 14.7 years). The majority of the transplant

patients (n=27) had their first kidney transplantation. Two patients were transplanted for the second time. Five transplanted patients underwent pre-emptive kidney transplantation.
Exclusion criteria were use of anticoagulants, any clinically overt inflammatory disease at the time of the investigation, and any clinically severe condition.

Fifty-two healthy controls, between 6-18 years of age as well, were recruited from a population of children undergoing minor surgery (e.g. strabismus correction, phimosis correction), and friends or relatives of above mentioned patients. Exclusion criteria were renal disease, inflammatory diseases, diabetes mellitus, recent or current infection, and use of systemic corticosteroids, non-steroidal anti-inflammatory drugs (NSAIDs) or anti-tumor necrosis factor (TNF) α medication.

This study was approved by the Medical Ethical Committee of the University Medical Centre Utrecht (METC-UMCU, the Netherlands). Written informed consent was obtained from the parents of all children and also from children being ≥12 years of age.

Data collection procedure

At the day of examination, body height and weight of all subjects were measured in a standardized manner without shoes and heavy clothing, to the nearest centimeter and 100 g, respectively. From these values the BMI (weight/height^2) was calculated. The values of height, weight and BMI of all subjects were compared with the reference values for healthy children matched for age and sex, and Z-scores were calculated (26).
Serum high sensitivity-CRP (hs-CRP), ESR, calcium, phosphate, albumin, creatinine and cholesterol were measured at the day of the study examination using routine laboratory techniques. The glomerular filtration rate (GFR) was calculated using the formula of Schwartz.

The medical records of all patients were reviewed for clinical history, laboratory parameters and medications. The time-integrated means of calcium, phosphate, albumin and creatinine of the patients were calculated as the average of the routine values that had been recorded in the previous 2 years. In the control group, information about medical history and current use of any medication was ascertained by a short interview.

Vascular measurements

Vascular characteristics were measured non-invasively, using a conventional

ultrasound system ("Scanner 200", Pie Medical, Maastricht, the Netherlands) equipped with a 7.5 MHz linear array transducer and a vessel wall moving detector system (M-mode, "Wall Track System 2.0", Pie Medical, Maastricht, the Netherlands). This system is able to automatically detect relative changes in arterial diameter (artery wall-lumen displacements) over subsequent cardiac cycles using ECG-derived triggers as time reference (27;28). Furthermore, local intima media thickness (IMT) and estimates of the arterial end-diastolic diameter are determined automatically (29).

Measurements for all subjects were performed in supine position, after at least 10 minutes resting. First, an image of the right CCA was obtained in conventional two-dimensional B-mode to identify the region of interest. Secondly, an M-line was placed perpendicularly, at 1-2 cm from the carotid bifurcation. After switching to the M-mode, a recording of wall motion covering 3-5 seconds was acquired. The measurement procedure was repeated at least 5 times during one session. One investigator (MvS) performed all ultrasonic assessments. Reproducibility was evaluated for the measured common carotid distension, diameter and IMT. The intersession coefficient of variation (CV; between sessions for the same subject, in one observer) for distension, IMT and diameter were respectively 6.7 %, 7.1% and 2.9 %.

Blood pressure was recorded at the level of the brachial artery by means of a semiautomatic oscillometric device (DINAMAP, Criticon, Tampa, Florida, USA). From these values, blood pressure in the carotid artery was calculated (see appendix) in order to estimate the local pulse pressure under the assumption that in the central arterial system the difference between mean arterial pressure (MAP) and diastolic pressure is constant. Furthermore, standard deviation scores for blood pressure values for all study subjects were calculated taking age, gender and height into account (30). The averages of the acquired parameters (IMT, diameter, distension) for every session per individual were computed. From these data, elasticity (E) was calculated (see appendix).

Assessment of MGP (ucMGP and serMGP) and fetuin-A

After blood sampling, all samples were frozen and kept at -80 °C until use.

Non-phosphorylated MGP (serMGP) concentrations were quantified using the enzyme-linked immunosorbent assay (ELISA) method (Biomedica, Vienna, Austria). This method uses antibodies against a peptide homologous to the

non-phosphorylated amino sequence 3-15 in human MGP which are coated on the microtiter plate and does not discriminate between carboxylated and non-carboxylated MGP species (31).

Levels of circulating undercarboxylated MGP (ucMGP) were determined using a competitive ELISA using a monoclonal antibody against the non-carboxylated amino sequence 35-49 in human MGP (VitaK BV, Maastricht, the Netherlands). This method was recently developed, and its details are described by Cranenburg et al. ("The circulating inactive form of Matrix Gla Protein (ucMGP) as a biomarker for cardiovascular disease", submitted May 2007). Methods to specifically detect carboxylated forms of MGP are currently not available.

Serum fetuin-A levels were measured by nephelometry. The nephelometry method for fetuin-A employs the same high-specificity antibody as the ELISA method described previously (24). Serum samples were diluted 1:4 with 400 µl phosphate-buffered saline (N Diluent; Dade Behring Holding, Liederbach, Germany). Nephelometric assays were performed manually using an automatic nephelometer (BNII; Dade Behring Holding, Liederbach, Germany).

Statistical analysis

Normality of distributions was checked for all study parameters. Characteristics of patients and healthy children were compared using independent groups t-tests. A chi-square test was performed to compare the distribution of gender across groups. A Mann Whitney test was performed to compare the levels of hs-CRP across groups. In order to examine differences in the level of calcification inhibitors, we performed multivariable linear regression analyses with adjustment for possible confounders age, gender and BMI.

Univariable linear regression analyses were performed to investigate possible associations between levels of calcification inhibitors and other variables (both general and laboratory parameters) in the total group. Possible associations between levels of calcification inhibitors and disease variables (e.g. duration of dialysis, duration of progression to ESRD, use of growth hormone) were investigated in the group of renal transplant recipients only.

We used multivariable linear regression analysis in order to explore the association between the levels of the calcification inhibitors (ucMGP, serMGP and fetuin-A) and the vascular properties of the common carotid artery (IMT and E) in the renal transplant recipients and in the control group. The vascular properties were used as dependent variables and the levels of the calcification

inhibitors as independent variables. Analyses were adjusted for gender, age, pubertal stage, weight and height, but only when these variables were associated with the E or IMT outcome at $p < 0.05$. In the regression model for the renal transplant group, other potential disease-related confounders (e.g. duration of dialysis, duration of progression to ESRD, use of growth hormone) were also considered. Since none of these independent risk factors for vascular calcification were significantly associated with either E or IMT, they were omitted from the final regression models.

The statistical tests were executed using a two-sided significance level of 5%. A p value <0.05 was considered to be statistically significant. SPSS Base 12.0.2 for Windows (SPSS Inc., Chicago, Illinois, USA) was used for all analyses.

6.4 Results

Study groups

Baseline characteristics of both study groups are shown in table 1. Additional clinical data of the renal transplant patients are shown in table 2. Patients and controls differed in age but not in height, weight and BMI. There was a trend towards more boys in the transplant sample. The patients had remarkably small body statures as can be seen in the standard deviation score of height which was on average – 2. This was despite the fact that almost half of the patients (48 %) had used growth hormone in the past. The estimated GFR in transplant patients was lower compared to healthy controls. Although within normal ranges in both groups, the average level of cholesterol was elevated in the transplant group compared to the group of healthy children. Both calcium and phosphate were within normal ranges in both groups.

Table 1: Characteristics of the study groups

Values are presented as mean (sd); male gender is presented as number (percentage). [b] Variable is presented as geometric mean (minimum-maximum). p values are based on t tests, except for [a], based on Chi square test; except for [c], based on Mann-Whitney test.

Table 1: **Characteristics of the study groups**

	Controls (n=54)	RTX-patients (n=29)	p
Demographics			
Gender, male [a]	21 (38.9 %)	17 (58.6 %)	0.085
Age, years	11.9 (3.0)	13.8 (2.7)	0.005
Height, cm	153.2 (17.2)	149.1 (14.2)	0.258
Height-sds	0.05 (0.9)	-2.1 (1.2)	<0.0001
Weight, kg	46.6 (15.4)	45.2 (10.7)	0.633
Weight for height-sds	0.46 (1.0)	0.69 (1.5)	0.508
BMI, kg/m^2	19.3 (3.1)	20.1 (3.1)	0.240
BMI-sds	0.46 (1.0)	0.69 (1.5)	0.409
Blood pressure (BP)			
Systolic BP, mmHg	110.6 (12.0)	121.3 (13.3)	0.001
Systolic BP-sds [b]	0.2 (-1.5 ; 3.4)	1.3 (-1.2 ; 6.6)	0.001
Diastolic BP, mmHg	61.8 (6.2)	67.1 (8.5)	0.005
Diastolic BP-sds [b]	-0.2 (-1.1 ; 1.4)	0.5 (-1.5 ; 3.4)	0.008
Common carotid artery dimensions			
IMT, µm	437.2 (42.8)	497.1 (75.1)	<0.0001
E, MPa	0.29 (0.07)	0.35 (0.08)	0.002
Biochemistry			
Creatinine, µmol/L	59.5 (11.3)	134.6 (77.8)	< 0.0001
GFR, ml/min per 1.73 m^2	99.7 (12.8)	49.6 (16.9)	< 0.0001
hsCRP, mg/L b [c]	0.3 (0.2-3.3)	0.5 (0.2-12.0)	0.066
Total cholesterol, mmol/L	4.2 (0.74)	5.0 (1.2)	0.001
Albumin, g/L	42.0 (2.4)	41.9 (2.4)	0.845
Calcium (Ca), mmol/L	2.28 (0.07)	2.35 (0.09)	<0.0001
Phosphate (P), mmol/L	1.40 (0.2)	1.16 (0.3)	<0.0001
Ca x P product, (mmol/L)^2	3.20 (0.46)	2.74 (0.65)	0.001

Blood pressure and properties of the carotid artery

Table 1 also shows the blood pressure values and the properties of the common carotid artery of both study groups. Patients had significantly higher blood pressure values compared to the control group. The majority of the patients were using one or more antihypertensive drugs; 9 patients did not use any antihypertensive medication.

The geometrical and mechanical vascular characteristics of the kidney recipients were compared to those in healthy controls (table 1). The diameter (7.2 ± 0.6 versus 6.7 ± 0.5 mm; $p < 0.001$) and IMT (table 1) were significantly increased in the renal transplant recipients whereas the distension was significantly decreased (0.85 ± 0.20 versus 0.93 ± 0.15 mm; $p = 0.044$). The elastic modulus E was significantly increased in the patients indicating increased arterial stiffness in the pediatric transplantation group (table 1).

Table 2: Characteristics of renal transplant recipients (RTX) (n=29)

		RTX-patients (n=29)
Clinical data		
Time since transplant, months [a]		36 (6-139)
Live-related transplant, yes [b]		10 (35 %)
Cumulative time on dialysis, months [a]		17 (0-146)
Former dialysis method [b]	Hemodialysis	10 (35 %)
	Peritoneal dialysis	10 (35 %)
	Both	4 (13 %)
	None	5 (17 %)
Use of growth-hormone (ever), yes [b]		14 (48 %)
Use of antihypertensive drugs [b]		20 (69 %)
Biochemistry		
Time-integrated mean creatinine, µmol/L [a]		108 (65-242)
Time-integrated mean albumin, g/L		40.5 (3.6)
Time-integrated mean calcium (Ca), mmol/L		2.39 (0.09)
Time-integrated mean phosphate (P), mmol/L		1.21 (0.27)
Time-integrated Ca x P product, $(mmol/L)^2$		2.88 (0.58)

Values are presented as mean (sd); [a] variable is presented as median (range); [b] variable is presented as number (percentage).

Figure 1: Serum levels of undercarboxylated MGP (ucMGP), non-phosphorylated MGP (serMGP), and fetuin-A in controls and renal transplant recipients

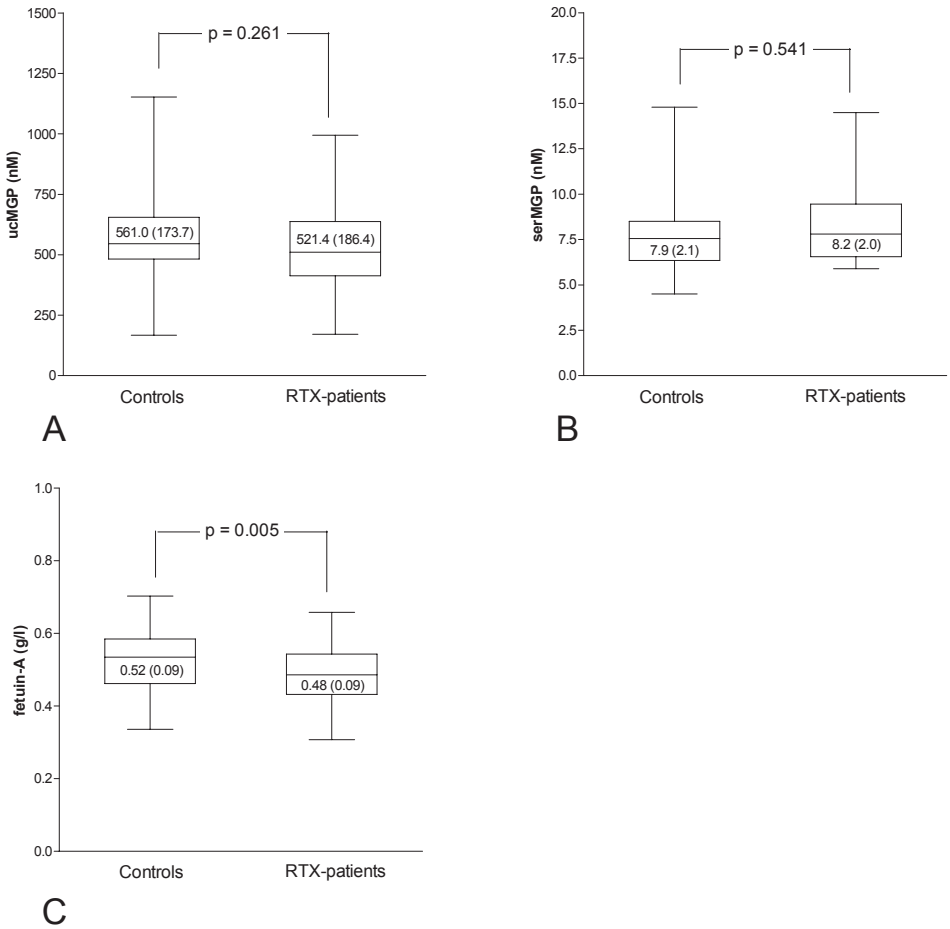

Figure 1A depicts box diagrams for ucMGP in controls and renal transplant patients.
Figure 1B depicts box diagrams for serMGP in controls and renal transplant patients.
Figure 1C depicts box diagrams for fetuin-A in controls and renal transplant patients.
p values for the difference in calcification inhibitors between groups are based on multivariate analyses, adjusted for age, gender and BMI.
Box diagrams: the upper limit of the box is the 75th percentile; the lower limit of the box is the 25th percentile; the horizontal line within box represents the median; the whiskers show the range of values. Values per box are presented as mean (sd).

Vascular calcification inhibitors

Figure 1 shows the serum levels of the vascular calcification inhibitors MGP and fetuin-A in both study groups. For MGP, the fractions of non-phosphorylated (serMGP) and undercarboxylated MGP (ucMGP) are depicted. SerMGP was circulating in lower amounts compared to uc-MGP in both groups ($p < 0.001$). UcMGP concentrations ranged widely, especially within the transplant group. The average levels of ucMGP and serMGP did not differ between groups.
The level of fetuin was significantly lower in the renal transplant recipients compared to the controls. The difference in fetuin between groups was independent of other factors like hsCRP, serum-calcium, serum-phosphate or CaxP product. In all subjects, levels of ucMGP were associated with serum-cholesterol ($B= 49.4$, $p= 0.010$) whereas levels of fetuin were associated with Hs-CRP ($B= -0.008$, $p= 0.047$) and CaxP product ($B= 0.037$; $p= 0.045$). No associations were found between levels of serMGP and any of the laboratory parameters.

Association of carotid artery properties and vascular calcification inhibitors

Table 3 shows the associations between vascular properties of the common carotid artery and circulating levels of ucMGP, serMGP and fetuin-A in both study groups. No significant linear associations between either IMT or E, and ucMGP, serMGP or fetuin-A were found in either group (table 3). In the regression models for IMT and E of the transplant group, adjustment for other possible independent risk factors for vascular calcification (e.g. duration of dialysis, time from diagnosis of CRF to progress to ESRD, use of growth hormone, cumulative Ca x P levels, etc.) also did not reveal significant linear associations between vascular properties and calcification inhibitors.

Table 3: Associations between characteristics of the common carotid artery and non-phosphorylated MGP (serMGP), undercarboxylated MGP (ucMGP) and fetuin-A

	B	95% CI	p
Controls			
IMT, µm			
serMGP, nM	-2.6	-8.4 ; 3.3	0.387
ucMGP, nM	-0.05	-0.12 ; 0.03	0.192
Fetuin-A, g/L	-78.3	-227.6 ; 71.0	0.297
E, MPa			
serMGP, nM	0.0	-0.010 ; 0.009	0.923
ucMGP, nM	3.0 * 10-5	0.0 ; 0.0	0.590
Fetuin-A, g/L	0.12	-0.012 ; 0.35	0.326
Renal transplant patients			
IMT, µm			
serMGP, nM	-0.81	-21.4 ; 19.8	0.936
ucMGP, nM	-0.08	-0.26 ; 0.11	0.398
Fetuin-A, g/L	-262.4	-609.8 ; 85.1	0.132
E, MPa			
serMGP, nM	-0.01	-0.04 ; 0.01	0.274
ucMGP, nM	-1.3 * 10-6	0.0 ; 0.0	0.990
Fetuin-A, g/L	-0.04	-0.46 ; 0.39	0.866

p values and regression coefficients (B) are based on multivariate linear regression analysis with IMT and E as dependent variables, adjusted for age, gender and BMI.

6.5 Discussion

This is the first study to examine the influence of calcification inhibitors on vascular health in children after renal transplantation. In this cross-sectional study, we describe the finding that the circulating level of the calcification inhibitor fetuin-A was decreased in the transplant group whereas the level of MGP (both serMGP and ucMGP) did not differ between groups. Furthermore, carotid artery properties in children after renal transplantation were abnormal compared to healthy children. However, no associations between vascular parameters and calcification inhibitors were found in either group.

Pediatric patients with ESRD are known to have vascular abnormalities which may persist after renal transplantation (4). This vascular damage is only partly reversible due to persistence of abnormalities associated with chronic renal failure preceding kidney transplantation (e.g. hypertension). On the other hand, renal transplant recipients use medications (immunosuppressive drugs, corticosteroids) which possibly enhance vascular damage (32).
The calcification inhibitors MGP and fetuin-A play an important role in the pathophysiology of vascular calcification (7). Vascular calcification is an actively regulated process, which depends on the balance between calcification inducers and calcification inhibitors like MGP and fetuin-A and is associated with low-grade chronic inflammation (12;33). In case of abnormal vascular calcification processes, MGP is thought to be upregulated locally in order to prevent calcification (34). Part of the newly synthesized MGP is released in the circulation. Fetuin-A is a circulating glycoprotein which is deposited at sites of vascular calcifications to inhibit the ectopic precipitation of calcium and phosphate (35). Furthermore, their circulating levels may be used as a biomarker for existing vascular damage or prediction of future cardiovascular events (24;36).

In pediatric patients with an increased risk of premature cardiovascular disease, no previous studies on calcification inhibitors in relation to parameters of vascular function were performed. We hypothesized that in pediatric renal transplant patients, who are known to have vascular abnormalities, circulating levels of calcification inhibitors would be different from those in healthy children. This was only the case for fetuin-A, but not for the fractions of MGP. The presence of low-grade inflammation in the transplant group may have contributed to the lower levels of fetuin-A in our study.

Fetuin-A is a negative acute phase reactant which was confirmed in

studies in adult ESRD patients (25). It was reported that decreased levels of fetuin-A were clearly linked to elevated inflammatory markers (24;25). The down-regulation of fetuin-A in inflammatory states is probably the underlying mechanism facilitating vascular calcification. In our study, we also found an association between inflammatory markers and fetuin-A. For MGP, no relation with inflammatory markers was found. Remarkably, the levels of ucMGP and serMGP were comparable in transplant patients and healthy controls. It was expected that in transplant patients, who have generally been exposed to longstanding disturbances in calcium and phosphate metabolism, MGP would be upregulated. It may therefore be that in transplant patients, the protective mechanism of MGP fails because it is insufficiently upregulated (14).

Furthermore, the present study in pediatric renal transplant recipients could not demonstrate an association between circulating levels of MGP or fetuin-A, and elastic properties of the carotid artery. Several studies in adults did show associations of MGP and fetuin-A with vascular calcification, determined by electron-beam computed tomography (37;38). An explanation for the absent link of calcification inhibitors and vascular health in pediatric kidney transplant patients may be that advanced vascular calcification is not yet present in our cohort of patients. However, our pediatric transplant group did have deviant vessel wall parameters when compared to healthy children, showing the presence of both atherosclerotic changes and increased stiffness. Studies in adults have shown that increased stiffness of the carotid artery, as found in the present study, is associated with increased vascular calcification (6). In adolescents with ESRD since childhood, increased vascular stiffness has been demonstrated (39-41). Goodman et al. have studied vascular calcification in a young adult ESRD cohort (age 19 ± 7 years) on dialysis with the use of electron-beam computed tomography and found only coronary artery calcification in patients over 20 years of age (41). Using a comparable method, Civilibal et al. did show the presence of coronary artery calcification in younger patients, but in these patients, the average time on dialysis was extremely long (39). Electron-beam computed tomography is a more direct and robust assessment of the extent of vascular calcification and is likely to detect advanced stages of arterial calcification. Measurements of vascular function like arterial stiffness and general morphologic changes of the arterial wall will probably signal detect early stages of vascular damage. The abnormal elasticity in pediatric kidney recipients as we have found in the present study may probably be interpreted as signs of (early) vascular dysfunction. To what

extent (micro)calcification contributes to this beginning vascular damage remains speculative.

In comparison to levels of serMGP, levels of undercarboxylated MGP were rather high in both study groups in the current study. This is of possible importance because circulating ucMGP levels may reflect the local vitamin K status in the arterial wall (21). MGP belongs to the family of the vitamin K-dependent proteins (16). These proteins all require vitamin K as a co-factor in the so-called carboxylation process in which γ-carboxyglutamate acid (Gla) residues are formed (42). MGP contains 5 of these Gla residues and 3 serine residues which may be phosphorylated (15). The Gla residues in MGP have a high affinity for calcium and are essential for its function (17). Hence, in case of suboptimal vitamin K status, undercarboxylated non-functional MGP may induce ectopic calcification. Evidence for this hypothesis comes from animal models using the anti-coagulant drug warfarin to induce vitamin K deficiency. The warfarin-treated rats showed calcification of elastic lamellae and heart valves, resembling the findings of the MGP-knockout mice (43;44). Population studies have also confirmed the assumption that vitamin K is beneficial for vascular health (45). Recently, subclinical vitamin K deficiency was shown in adult dialysis patients (46). Via the carboxylation of MGP, optimal (local) vitamin K status may be an additional method to prevent vascular damage in (pediatric) ESRD patients. However, our study did not show a significant relation between levels of ucMGP and vascular parameters.

We have studied the relation of circulating calcification inhibitors and arterial stiffness, a surrogate parameter of vascular calcification. A limitation of our study is that the complex multifactorial process of vascular calcification is studied in a small group of pediatric transplant patients. The study may have been underpowered to detect subtle associations between calcification inhibitors and vascular abnormalities. Furthermore, circulating levels of MGP in children may not reflect local vascular MGP activity. MGP is also produced in chondrocytes, so (accelerated) skeletal growth may contribute to serum MGP levels as well. Finally, the previous exposure to factors favoring vascular calcification during preceding stages of renal failure may override the influence of current levels of calcification inhibitors on vascular stiffness in children after renal transplantation.

In conclusion, circulating levels of MGP and fetuin-A could not be identified as independent predictors for vascular stiffness or other carotid artery

parameters in pediatric renal transplant recipients. Nevertheless, vitamin K may be a modifiable factor of vascular health through its influence on the local functionality of MGP in the vascular wall. Future prospective studies in large cohorts of pediatric ESRD and transplant recipients are needed to learn more about the role of calcification inhibitors in relation to the prevention of vascular damage.

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Appendix

Equations

Δd (change in carotid diameter):	dsystolic – d
Δp (carotid pulse pressure):	CF * Δd
CF (conversion factor):	(MAP – DBP)/(dmean – d)
MAP (mean arterial pressure):	DBP + (SBP – DBP)/3
Δ A (change in arterial cross-sectional area):	$\pi/4 * (d + \Delta d)^2 - d^2)$
DC:	$(\Delta A / A) / \Delta p = (2 \Delta d * d + \Delta d^2) / (\Delta p * d^2)$
E:	(d/IMT) / DC

$d_{systolic}$:	mean end-systolic lumen-IMT diameter (mm)
d:	mean end-diastolic lumen-IMT diameter (mm)
A:	arterial cross-sectional area (mm^2)
DC:	distensibility coefficient (1/MPa)
E:	elasticity, Young's modulus (MPa)
IMT:	intima-media thickness, end-diastolic (mm)
SBP:	systolic blood pressure in brachial artery (mmHg)
DBP:	diastolic blood pressure in brachial artery (mmHg)
MAP:	mean arterial pressure in brachial artery (mmHg)

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Imke, 7 years

Chapter 7 Calcinosis in juvenile dermatomyositis: a possible role for the vitamin K-dependent protein Matrix Gla protein (MGP)

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7.1 Abstract

The aim of the present study was to investigate whether the calcification inhibitor matrix Gla protein (MGP) is expressed in muscle biopsies of patients with juvenile dermatomyositis (JDM), and whether different forms of MGP are differentially expressed in JDM patients with and without subcutaneous calcifications. Muscle tissue from 6 JDM patients (3 without calcinosis, 2 with calcinosis and 1 recently diagnosed patient), 4 patients with muscular dystrophy, 3 patients with inclusion-body myositis (IBM) and 5 control subjects was used for immunohistochemistry staining using novel antibodies to different conformations of MGP.

In the JDM patients, all forms of MGP were more intensely stained in the perifascicular compared to the central muscle fibers. In addition, the different MGP species were demonstrated in the pathological muscle fibers of IBM and dystrophy patients, but hardly in normal muscle tissue. In JDM patients with calcifications, only the phosphorylated form of MGP was increased compared to those without calcifications. The different forms of MGP were also found in various staining intensities in the microvasculature and macrophages of normal and disease biopsies. MGP was expressed at the site of muscle damage in JDM patients as well as in the other patients. The difference in staining intensity of phosphorylated MGP appeared to distinguish between JDM-patients with and without calcifications, whereas carboxylated MGP, the other functional form, was equally expressed.

7.2 Introduction

Patients with juvenile dermatomyositis (JDM) may develop subcutaneous calcification in the course of the disease (1). The pathophysiology of calcinosis in JDM is still largely unknown (2;3). A remarkably increased urinary γ-carboxyglutamic acid (Gla) output was found in JDM patients, especially those with calcifications (4). Furthermore, Gla-containing proteins have been demonstrated in several types of pathologic calcifications including calcium-containing material extruded from skin and subcutaneous plaques from patients with dermatomyositis (5).

Matrix Gla protein (MGP) is a vitamin K-dependent protein which contains 5 Gla residues. In recent years, the role of MGP as an inhibitor of calcification in vascular and cartilage calcification has become overt (6). Vitamin K serves as a cofactor in the posttranslational γ-carboxylation of MGP. In this process the conversion of selective protein-bound glutamate acid (Glu) residues into γ-carboxyglutamic acid (Gla) takes place, which is essential for protein function (7). Besides its five Gla residues, mature MGP contains three serine residues which may be phosphorylated. Several conformation-specific antibodies of MGP for immunohistochemistry have been developed (8).

We hypothesized that MGP may be involved in the pathogenesis of calcinosis in JDM and investigated the localization of the different forms of MGP in muscle biopsies from JDM patients with and without calcification.

7.3 Methods

Patient material

For tissue samples, we had access to a database for human tissue collected and registered by the Department of Pathology of the University Medical Center Utrecht. Specimens were collected and classified according to histological examination. Since muscle biopsies are not obligatory to confirm the diagnosis of juvenile dermatomyositis, we obtained muscle tissue from 6 JDM patients only (period 2001-2007). All but two biopsies were taken at time of the onset of disease. Two of six JDM patients had developed severe calcifications during the disease course, starting 1.5 and 2 years after disease onset. The patients with calcifications had a biopsy at the time when calcifications had already developed. No biopsies were taken from the areas of calcinosis due to concerns about the risk of poor healing.

The average clinical follow-up time of JDM-patients after biopsy was 4.0 years (range 0.1 – 6 years). Since the biopsy of one patient was recently taken, it is uncertain whether calcinosis will develop in this patient.

As negative control material, muscle biopsies from 5 patients with myopathic complaints, but normal histology and without definitive clinical diagnosis were used. These samples showed no muscle fiber abnormalities or signs of inflammation. Muscle biopsies from patients with confirmed muscle disease were used as disease-controls; 3 patients had inclusion-body myositis (IBM) and 4 patients had muscular dystrophy.

This study was approved by the Medical Ethical Committee of the University Medical Centre Utrecht (METC-UMCU, the Netherlands).

MGP-antibodies

Monoclonal antibodies against the different MGP confirmations used for immunohistochemistry were provided by VitaK BV (Maastricht, The Netherlands). In brief, monoclonal antibodies were raised against the non-carboxylated human MGP Gla-domain (residues 35-49; designated as mAb-ucMGP), the carboxylated human MGP Gla-domain (residues 35-54; designated as mAb-cMGP), the non-phosphorylated human MGP (residues 3-15; designated as mAb-serMGP) and phosphorylated human MGP (residues 3-15; designated as mAb-pserMGP). The monoclonal antibodies were selected for their specificity against ucMGP, cMGP, serMGP and pserMGP respectively. The method to obtain the antibodies for ucMGP, cMGP and serMGP has been described before (8). The conformation-specific antibody for pserMGP has not yet been described but was raised according to the method as described by Schurgers et al. (8).

Immunohistochemistry of ucMGP, cMGP, serMGP and pserMGP

All tissues were stained for hematoxylin and eosin to demonstrate tissue integrity. For immunostaining with any of the four mAb MGP antibodies, sections were incubated with anti-ucMGP (0.9 µg/ml), anti-cMGP (1.0 µg/ml), anti-serMGP (1.0 µg/ml) or anti-pserMGP (0.75 µg/ml). All antibodies were diluted in blocking reagent (Roche Diagnostics, Mannheim, Germany). Biotinylated sheep anti-mouse IgG (Amersham Biosciences, Little Chalfont, UK) was used a second antibody, followed by incubation with avidin-linked alkaline phosphatase complex (Dako, Golstrup, Denmark); staining was

performed by the alkaline phosphatase kit I (Vector Laboratories, Burlingame, CA). All specimens were counterstained using hematoxylin and sections were mounted using imsol-mount.

Qualitative and semi-quantitative analysis of MGP-staining

The overall pattern of immunostaining and the staining intensity was examined microscopically in the microvasculature, fibroblasts, macrophages and muscle-fibers of the biopsies by an experienced pathologist. The staining intensity was scored from 0 (no staining), + (low), ++ (medium) or +++ (intense). Because the typical muscular abnormalities of JDM-patients are localized in the perifascicular muscle fibers, a distinction between central and perifascicular muscle fibers was made when comparing the biopsies of the JDM patients with the negative control tissue. When comparing the biopsies of the JDM patients with the disease-controls, a distinction between pathological and normal muscle fibers was made.

7.4 Results

The immunostaining results for the different MGP-antibodies in JDM patients with and without calcifications, negative-control patients, IBM-patients and muscular dystrophy-patients are shown in table 1. Figure 1 shows the immunohistochemical localization of MGP species in muscle biopsies of a negative-control patient and JDM patient with subcutaneous calcifications. In negative-control biopsies, no staining of cMGP and ucMGP was found anywhere in muscle tissue. In two negative-control patients only, pserMGP and serMGP were faintly stained in perifascicular and central muscle fibers.

In JDM-patients, MGP was mainly localized within degenerated atrophied (perifascicular) muscle fibers. The imunostained product of cMGP and pserMGP was seen in perifascicular fibers in all JDM patients. UcMGP was present in perifascicular muscle fibers in most JDM-patients. PserMGP was present in central muscle fibers of all JDM-patients. The staining pattern in the JDM patient who was recently diagnosed, was quite different from the other JDM patients. This biopsy showed the presence of perifascicular-localized serMGP together with cMGP and serMGP in central muscle fibers. In JDM-patients, all forms of MGP showed increased staining intensity in perifascicular muscle fibers

Table 1: Quantitative MGP-immunostaining in JDM-patients and control patients

		Pathological muscle fibers [a]				Normal muscle fibers [a]				Macrophages				Microvasculature			
Biopsy	Histology	uc	c	ser	pser	uc	c	ser	pser	uc	c	ser	pser	uc	c	ser	pser
1	normal	0	0	0	+	0	0	0	+	+	+	++	++	+	+	0	++
2	normal	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
3	normal	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
4	normal	0	0	0	0	0	0	0	0	++	++	++	++	++	++	++	++
5	normal	0	0	++	+	0	0	0	+	++	++	++	++	++	++	++	++
6	JDM+	+	+	0	++	0	0	0	++	++	++	0	++	++	++	0	++
7	JDM+	++	+++	0	+++	0	0	0	++	++	++	0	++	++	++	0	++
8	JDM-	0	+	0	+	0	0	0	+	+	++	0	+	+	++	0	+
9	JDM-	++	+	0	+	0	0	0	+	++	++	0	+	++	+	0	+
10	JDM-	++	+	0	+	0	0	0	+	++	++	0	+	++	+	0	+
11	JDM? [b]	++	++	+++	+++	0	+	++	+	++	++	++	++	++	++	+	++
12	dystrophy	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
13	dystrophy	0	+	+	+	0	0	+	0	++	++	++	++	++	++	+	++
14	dystrophy	+	++	++	++	0	0	0	0	++	++	++	++	++	++	+	++
15	dystrophy	+	+	+	++	0	0	0	+	++	++	++	++	++	++	++	++
16	IBM	+	++	++	+	0	0	0	0	++	++	++	++	++	++	++	++
17	IBM	+	+	++	+	0	0	0	0	++	++	++	++	++	++	+	++
18	IBM	+	+	++	++	0	0	0	+	++	++	++	++	++	++	++	++

in comparison to the negative-control biopsies. When comparing the JDM patients with and without calcifications, an increased staining intensity of pserMGP was noted in the JDM patients with calcifications in perifascicular and central muscle fibers. The staining of all types of MGP was not clearly co-localized or upregulated within inflammatory infiltrates (figure 1).
In most disease-control biopsies, all types of MGP were found in the abnormal muscle fibers. In normal muscle fibers of these biopsies, no cMGP or ucMGP was found. In three patients only, pserMGP and serMGP were weakly stained in normal muscle fibers.

In the majority of biopsies (of JDM-, disease control- and negative control-patients), all species of MGP were found in macrophages and microvasculature, although subtle differences in staining intensities within and between patient groups were noted.

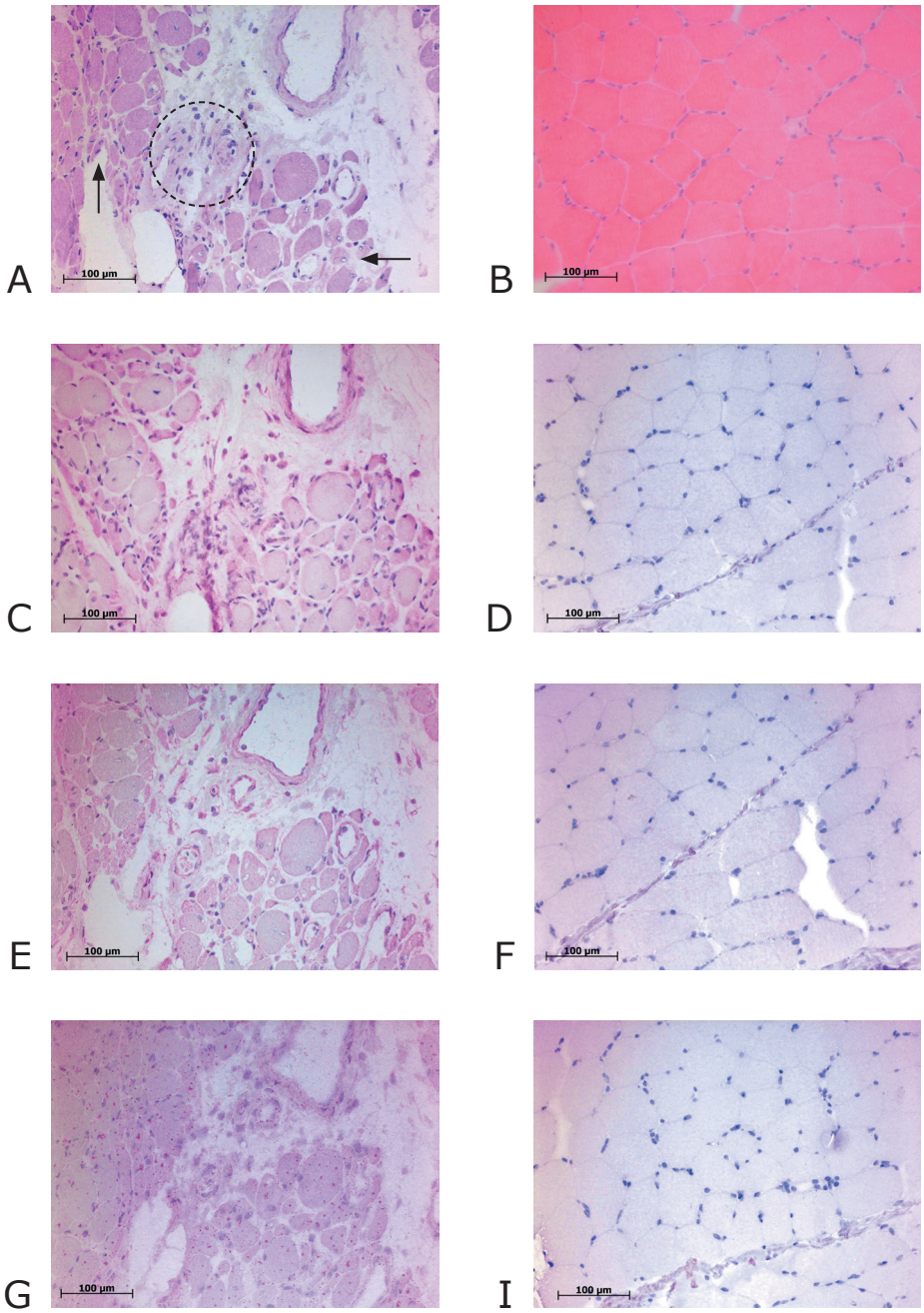
Table 1: Quantitative MGP-immunostaining in JDM-patients and control patients

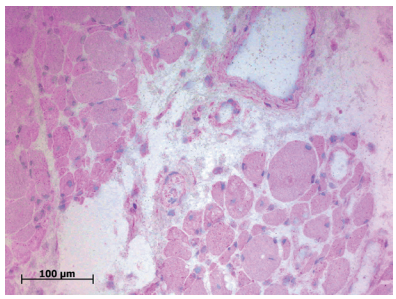
This table shows the staining intensity of non-carboxylated MGP (uc), carboxylated MGP (c), non-phosphorylated MGP (ser) and phosphorylated MGP (pser) in muscle biopsies of JDM patients with (JDM+) and without (JDM-) calcification, IBM-patients (IBM), patients with muscular dystrophy (dystrophy) and negative-control patients (normal).
The staining intensity of pathological and normal muscle fibers, macrophages and microvasculature was scored from 0 (no staining), + (low), ++ (medium) to +++ (intense).

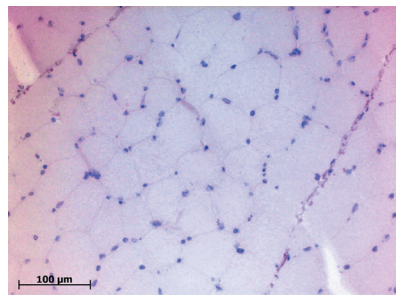
[a] in biopsies of JDM patients and negative-control patients, the pathological muscle fibers refer to the perifascicular muscle fibers whereas the normal muscle fibers refer to the central muscle fibers.

[b] this biopsy was recently taken and presently it is uncertain whether calcinosis will develop in this patient.

Figure 1: Immunohistochemical localization of MGP species in muscle biopsies of a JDM patient and a negative-control patient



J 

K 

A and B represent hematoxylin/eosin (HE) staining in a JDM patient and negative-control patient, respectively. A shows the typical perifascicular atrophy of muscle cells (indicated by the arrows) as well as a small inflammatory infiltrate in the epimysial connective tissue (indicated by the circle) as can be found in JDM biopsies. B shows normal muscle tissue. C and D represent non-carboxylated MGP staining in a JDM patient and negative-control patient, respectively. E and F represent carboxylated MGP staining in a JDM patient and negative-control patient respectively. G and H represent non-phosphorylated MGP staining in a JDM patient and negative-control patient respectively. I and J represent phosphorylated MGP staining in a JDM patient and negative-control patient respectively. In the biopsy of the JDM patient, staining of different species of MGP is demonstrated (in pink/red color) in perifascicular muscle cells whereas in the negative-control muscle tissue, no MGP is demonstrated.

7.5 Discussion

Our study describes the identification of the calcification inhibitor MGP at the site of muscle damage in JDM patients with and without calcifications using novel conformation-specific MGP-antibodies. In addition, MGP was found in the microvasculature and macrophages of normal and disease biopsies. Others have also reported the presence of mRNA and protein for MGP in macrophages and the arterial medial wall (8;9) whereas the presence of MGP in striated muscle tissue was not demonstrated before. The possible role of MGP in (dermal) calcinosis was studied previously in another auto-inflammatory disease (10). This study in scleroderma patients demonstrated the presence of MGP in skin biopsies, using only the serMGP antibody.

In JDM patients, chronic inflammation is presumably a necessary condition for the formation of dystrophic calcification (3). The destruction of muscle fibers in JDM results from the deposition of complement in endomysial capillaries which leads to swelling of endothelial cells and capillary necrosis and ultimately to perivascular inflammation and local ischemia (11). The resulting tissue injury is a trigger for the calcification process. Increased local calcium concentration, resulting from cell damage, leads to enhanced MGP-expression (12). A proposed mechanism of MGP to prevent (progression of) calcinosis is to bind tightly and selectively to calcium both in solution and in calcium crystal nuclei in order to prevent their growth and ability to seed daughter crystals (13). In ectopic calcifications of JDM patients, hydroxyapatite was found to be present, indicating that osteoblast-like cells may also be involved (14). Besides direct binding to calcium, Gla-containing MGP (cMGP) also inhibits cell differentiation into an osteoblastic phenotype, induced by bone-morphogenetic protein 2 (BMP-2) (7). Because the binding to BMP-2 is dependent on the presence of Gla-residues in MGP, insufficient levels of carboxylated MGP may predispose to calcification. Inadequate (local) vitamin K reserves or supply may lead to impaired carboxylation of MGP (15).
The question is thus raised whether supplementation with vitamin K in patients with JDM will increase the functionality of MGP and thereby may positively influence the development of calcinosis.

As only cMGP is presumed to fully exert its role as inhibitor of calcification, we had expected that level of cMGP expression would be differentiating between JDM-patients with and without calcifications. However, this was not the case in our study. The fact that the biopsies of the JDM-patients

with calcinosis were not taken at the site of the calcification may explain the similar expression patterns of MGP. Given the focality of the calcification process, it may be that altered levels of cMGP can only be found at the site of the calcifications in the JDM-patients. Surprisingly, the intensity of pserMGP staining appeared to distinguish between JDM-patients with and without calcifications. Besides the presence of Gla-residues, the activity of MGP may also be determined by the presence of phosphorylated serines (16). The precise function of the phosphoserines in MGP is presently unknown. The extent of serine phosphorylation is probably regulated by a Golgi-associated protein kinase which can rapidly react to changes in the extracellular environment (17). It remains unclear whether both posttranslational modification processes (γ-carboxylation and phosphorylation) are simultaneously required for MGP to fully exert its function.

In the present study, MGP was also expressed in pathological muscle tissue of the other myopathies studied. Since the occurrence of calcification in IBM and dystrophy is not common, the expression of MGP at the site of muscle damage most likely represents a general protective mechanism. When the protective action of MGP fails due to insufficient expression or decreased functionality, this may contribute to the development of calcifications. The defensive mechanism of MGP is probably not disease-specific but related to the extent of inflammation and concomitant tissue damage. In JDM, muscular destruction, presenting as perifascicular atrophy, is more prominent compared to IBM (18). The extensive inflammatory responses and tissue damage are likely to predispose JDM patients to calcinosis. Accordingly, clinical studies have shown that calcinosis in JDM-patients is related to severity of disease and delays in diagnosis or delayed initial treatment (19).

To conclude, the expression of MGP in damaged muscle tissue probably represents a general protective mechanism against unwanted calcification that may fall short in some patients with JDM. Local availability of carboxylated functional MGP may be increased by supplementation of vitamin K. Additional studies are warranted to learn more about regulating factors in MGP serine phosphorylation, another potential determinant of MGP functionality.

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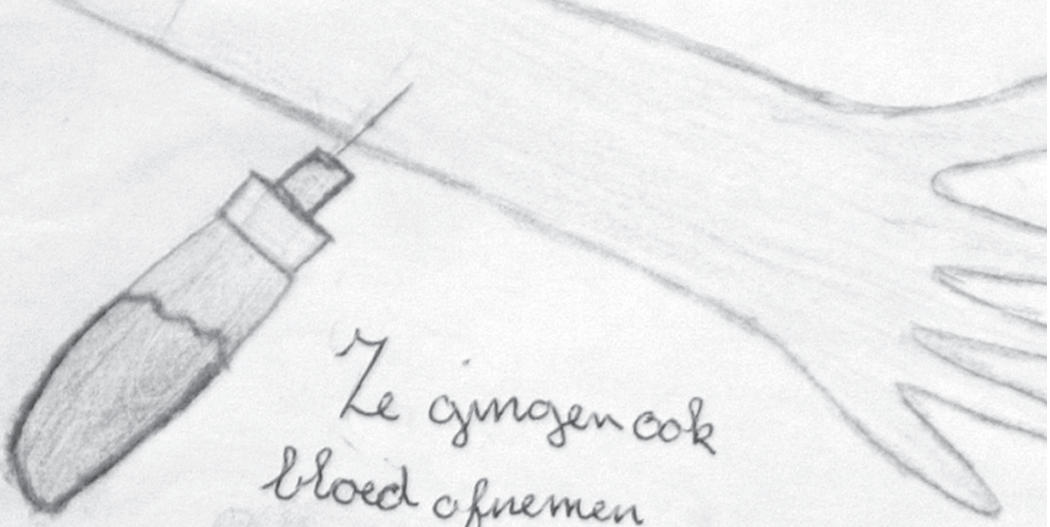

Ze gingen ook bloed afnemen

Eerst hebben ze je gemeten en gewogen.

Gerlinde, 8 years

Chapter 8 Summary and discussion

Osteoporosis and cardiovascular disease (CVD) are conditions commonly associated with aging and senescence. However, evidence is available suggesting that the pathophysiological foundation of these conditions is already laid in childhood (1-4). Osteoporosis in later life can be due to inadequate build-up of peak bone-mass (PBM) in adolescence (1;2). PBM is defined as the maximal amount of bone mineral accrued during life; eighty to ninety percent of the PBM is acquired between birth and adolescence (5;6). Subclinical signs of atherosclerosis are found at young age in children with conditions that are related to an increased prevalence of CVD in adults (e.g. severe obesity, hypertension) (3;4).

In chronic diseases of childhood, osteoporosis and CVD may also be of importance. The increased survival of pediatric patients with chronic illnesses is paralleled with an increased morbidity, which is a major concern. Late sequela may arise as a result of the disease itself or from medication used in treatment. A number of chronic disorders (e.g. rheumatic diseases) will put children at risk of generalized diminished bone mass, leading to increased fracture prevalence (7;8). Other pediatric patients (e.g. those with chronic inflammatory disorders or chronic renal failure) have an established risk of early CVD in adulthood (4;7;9).
Increased knowledge about the pathophysiology of osteoporosis and CVD in children with chronic diseases will lead to therapeutic strategies and preventive measures. Subsequently, intervention may already start in childhood which may be advantageous for both children and adults. In the adult population, the role of vitamin K in bone and vascular health is quite well described (10). However, this field of research in children has remained fairly unexplored up to now.

Vitamin K, a pluripotent vitamin?

Because of its vital role as a co-factor in the γ-carboxylation of the calcium-binding Gla-proteins, vitamin K is involved in several physiological processes; the most important being blood coagulation, bone mineral metabolism and (the prevention of) ectopic calcification, especially in the vascular wall (11). Evidence in adults suggests that optimal carboxylation of osteocalcin is likely to contribute to improved bone quality, both by increased bone mineral density and improved bone morphology (12;13). The calcification inhibiting properties of matrix Gla protein (MGP) also depend on γ-carboxylation and

thus the presence of vitamin K (14;15). Several other vitamin-K dependent proteins (e.g. growth arrest specific gene-6 protein (GAS-6); see appendix I) have been discovered in recent years (16-19). Since the functions of these Gla proteins are presently unidentified, it is feasible that in next decades, the involvement of vitamin K in other physiological processes will be recognized. Given its necessity for the γ-carboxylation of all Gla-proteins, it is likely that general nutritional requirement of vitamin K will be determined by overall "metabolic activity" of physiological processes involving the different Gla- proteins. As a result, suboptimal vitamin K status may be an independent risk factor for both osteoporosis and ectopic calcification, also in children. Overall metabolic activity in growing children is elevated compared to that in adults, suggesting that nutritional demand for vitamin K may be high as well. The aim of this thesis was to investigate the role of vitamin K and osteocalcin in childhood bone health. In addition, the role of MGP in relation to ectopic calcification in pediatric disease was examined.

Vitamin K status in children and adults

Since the metabolic activity and osteocalcin production (20) are high during bone development and skeletal growth, we hypothesized that the vitamin K requirement in these circumstances is also high. The most accurate way to monitor whether the nutritional intake of vitamin K is adequate is by assessing undercarboxylated and carboxylated amounts of the different Gla-proteins in the systemic circulation or in tissue. The serum vitamin K concentration fluctuates with recent dietary vitamin K intake and is no reliable marker for vitamin K status. Presently, the circulating fractions of the Gla-proteins prothrombin and osteocalcin can be detected using conformation-specific antibodies. Prothrombin is a marker for the hepatic γ-carboxylation capacity, whereas osteocalcin reflects that of bone (21-23). The fractions of osteocalcin are also considered to be sensitive markers of general vitamin K nutritional status (24). **Chapter 2** presents the results of a cross-sectional study on vitamin K status, expressed as the ratio of undercarboxylated and carboxylated osteocalcin (UCR). Here we compared healthy children (with high bone metabolic activity) and adults (with stable bone metabolic activity). Indeed, the UCR was significantly higher in children than in adults, suggesting a suboptimal vitamin K status during growth. Large variations in UCR were observed, especially in children, indicating that the extent of vitamin K deficiency varies between individuals. Previously, it was reported that in

the healthy adult population osteocalcin is carboxylated to a variable extent (25). Also in children a marked variation in vitamin K status, indicated by the percentage of ucOC related to the total amount of osteocalcin, was reported before (26).

In adults, the level of circulating ucOC is most often used as indicator for the vitamin K status of bone (10;21;27). However, in children circulating ucOC alone may not be an adequate marker of vitamin K status. The level of ucOC is determined by both the total osteocalcin production and the fraction thereof that has been carboxylated by vitamin K action. Therefore, the UCR, which is the amount of undercarboxylated osteocalcin relative to the carboxylated osteocalcin, is a more reliable marker for vitamin K status of bone in children. Its use allows comparisons in vitamin K status between children with different levels of bone metabolic activity.

During puberty, rapid changes in bone metabolic activity take place that may negatively influence vitamin K status, if not compensated for by increased nutritional intake of vitamin K. In other words, the demand for vitamin K increases because the total osteocalcin production increases. When dietary intake of vitamin K fails to keep up with increasing demands, this will result in an higher UCR, indicating a lower vitamin K status. This was illustrated in **chapter 2**, where levels of ucOC and cOC were highest during puberty and declined at the end of puberty, when bone metabolic activity decreases. In **chapter 3**, the UCR was found to be significantly (positively) associated with pubertal stage.

To conclude, suboptimal vitamin K status is common in healthy children, and is most prominent in adolescence.

Vitamin K in childhood bone health

The question arises whether the UCR, which is considered to be a marker for general vitamin K nutritional status (24), is also representative for bone vitamin K status in children as is suggested in adults (22;23). Since osteocalcin is the major non-collagenous constituent of bone and its optimal function is dependent on vitamin K (28), one would expect that the UCR is related to outcome measures of bone metabolism. For that reason, we studied possible correlations of vitamin K status, indicated by the UCR, with the level of bone turnover markers as well bone mineral parameters in children. The UCR and the bone markers were found to be inconsistently associated. In a cohort of 86 children between 3 and 18 years of age, the bone markers BAP

and NTX did not correlate with the UCR (**chapter 2**). However, in another cohort of 307 children between 8 and 14 years of age, the UCR was found to have a positive correlation with markers of bone formation (BAP and PINP) and the marker for bone resorption (ICTP) (**chapter 3**). In the same cohort of healthy peripubertal children, we have found that gains in whole body-bone mass over two years are associated with changes in vitamin K status, even after adjusting for potential confounders. In another study in 54 healthy children between 6 and 18 years of age, we did not find a relation between the UCR and bone density, as determined by calcaneal ultrasound (**chapter 5**).

In conclusion, the UCR, an indicator of vitamin K status, is associated with markers of bone metabolism and bone mass parameters, but only in a large cohort of children with a small range of age. Supplementation of vitamin K in case of subclinically deficient but otherwise healthy children may be a strategy to optimize peak bone mass in childhood in order to prevent osteoporosis in later life.

...and childhood bone disease

Patients with juvenile idiopathic arthritis (JIA) are known to be at risk of reduced generalized bone mass resulting in osteopenia or osteoporosis (29;30). We hypothesized that a suboptimal vitamin K status may also be a contributing factor in the development of osteopenia in these patients. In **chapter 5**, vitamin K status in patients with JIA was compared to that in healthy children, and the association between vitamin K status, as expressed by the UCR, and quantitative ultrasound properties of calcaneal bone were studied in these children. Calcaneal ultrasound is a method that measures bone density without ionizing radiation and provides information about bone mass and architecture (31). Bone density measured by bone ultrasound correlates well to dual X-ray absorptiometry (DEXA) which is currently the gold standard to diagnose osteopenia (32;33). In our cross-sectional study in 54 healthy controls and 55 JIA patients, we found no differences in average vitamin K status between the JIA- and control group, whereas calcaneal ultrasound properties were significantly lower in the JIA cohort. Again, a large variation in vitamin K status between individuals was found. Vitamin K status was not linearly related to bone ultrasound properties in either group. When the groups were categorized according to high, median and low UCR-values, it was shown that patients in the low UCR group had higher bone ultrasound properties compared to the patients in the high UCR group, independent

of age, gender and BMI. These results suggest a possible contributory role for vitamin K to bone health in children with JIA, besides other recognized determinants of bone mass.

Limited studies on the subject of vitamin K and bone health have been performed in other (pediatric) patients that may develop osteoporosis or osteopenia as long-term complication of their disease. The majority of studies on this subject have been performed in cystic fibrosis (CF) patients, who have a known risk of (subclinical) vitamin K deficiency due to malabsorption together with an increased prevalence of osteopenia. A cross-sectional study in 106 patients with CF showed that both fractions of osteocalcin were associated with bone turnover markers (34). In this study, no association of ucOC levels and bone mineral density (BMD) was found while 70% of patients had a suboptimal vitamin K status, based on PIVKA-II levels. Another study in 18 CF patients demonstrated that supplementation with vitamin K1 for 4 weeks reduced both PIVKA-II and ucOC levels (35). In 20 CF patients who received a weekly dose of 10 mg vitamin K_1 during one year, BMD z-scores at follow-up remained unchanged from baseline (36). The dose of vitamin K in the latter study may have been too low to demonstrate beneficial effects on bone mass. A randomized controlled trial was performed in 40 children with classical galactosemia (3-18 years of age) by Panis et al. which showed a significant increase in cOC and decrease in ucOC after 1 year in children receiving a daily dose of 1.0 mg vitamin K_1 together with calcium and vitamin D_3, together with an increase in bone mineral content (BMC) of the lumbar spine (37). Adult patients with Crohn's disease (n = 44) were found to have higher ucOC levels compared to healthy controls and these levels were related to bone turnover (38). Another study in 32 patients with longstanding Crohn's disease, poor vitamin K status was associated with low BMD of the lumbar spine (39).

Given the possible importance of vitamin K status for bone health, it seems worthwhile to investigate the relationship between vitamin K status, osteocalcin carboxylation and bone health in other (pediatric) patient groups, e.g. children with a primary bone disease, children receiving chronic oral anticoagulants or children with unexplained osteoporosis and recurrent fractures. To date, data on this subject are fairly scarce.

Vitamin K in childhood ectopic calcification

Ectopic calcification is the process of pathological calcium deposition in tissues which can occur at several places in the human body. Pathological calcifications

are also found in children, although its occurrence is rare.

One form of ectopic calcification is vascular calcification, which develops at two anatomic sites: in the intima and the tunica media of the arterial wall. Arterial intima calcification represents an advanced stage of atherosclerosis. Atherosclerosis is an actively regulated, cell-mediated process in which local inflammation of the arterial wall plays a pivotal role (40). Early signs of atherosclerosis have been described in children (41), e.g. in those with dyslipidemia (42) or severe obesity (43). Medial calcification, also known as arteriosclerosis, is associated with uremia and abnormalities in calcium-phosphate metabolism, and is common in end stage renal disease (ESRD) (44-46). In the medial vascular calcification process, bone and mineralization regulatory factors are expressed (47). Arterial calcification results from the imbalance between factors favoring calcium deposition and inhibitory mechanisms. Several regulatory factors involved in arterial calcification have been discovered (48). One of these factors is the arterial calcification inhibitor matrix Gla protein (MGP) (49). The presence of MGP was demonstrated in medial calcification as well as in calcified atherosclerotic plaques (50;51). The Gla residues in MGP have a high affinity for calcium and are essential for its function (15). Hence, in case of suboptimal vitamin K status, a deficiency of the carboxylated, functional MGP may induce ectopic calcification. The non-carboxylated fraction of the vitamin K-dependent protein MGP can be detected in the circulation and in tissue (ref Cranenburg et al., submitted for publication). The carboxylated fraction of MGP can presently be detected in tissue only, using immunohistochemical staining.

The propensity to develop early arterial calcifications is present especially in children with end stage renal disease (ESRD) (4). Vascular calcification is a strong prognostic marker of cardiovascular disease and stroke which are the leading causes of death in young patients with ESRD. In **chapter 6**, the circulating levels of the vascular calcification inhibitors MGP and fetuin-A in children after renal transplantation are compared to healthy children and possible associations with vascular properties of the carotid artery in these children were studied as well. In this study, we used two conformation-specific antibodies of MGP, namely non-phosphorylated MGP (serMGP) and non-carboxylated MGP (ucMGP). The circulating level of the calcification inhibitor fetuin-A was decreased in the transplant group whereas the level of MGP (both serMGP and ucMGP) did not differ between groups. It was expected that in transplant patients, who have generally been exposed

to longstanding disturbances in calcium and phosphate metabolism, MGP would be upregulated. It may be that in transplant patients, the protective mechanism of MGP fails because it is insufficiently upregulated (49). Levels of non-carboxylated MGP were rather high in comparison to levels of serMGP in both study groups, indicating that insufficient vitamin K was present for optimal carboxylation. Carotid artery properties (elasticity and IMT) in the renal transplant recipients were abnormal compared to the healthy children. However, no associations between vascular parameters and calcification inhibitors were found in either group. In conclusion, circulating levels of MGP and fetuin-A could not be identified as independent predictors for vascular stiffness or other carotid artery parameters in pediatric renal transplant recipients. In adult ESRD patients on dialysis, MGP levels were found to be decreased (ref Cranenburg et al., submitted for publication). So, an alternative explanation for the normal levels of MGP in transplant patients compared to controls as found in our study may be that renal transplantation leads to normalization of MGP levels but preexistent vascular calcification persists. In animal experiments, high doses of vitamin K induced regression of warfarin-induced vascular calcification (52). In analogy to these experiments, it may be hypothesized that high doses of vitamin K may improve vascular health in renal transplant patients by increasing local functionality of MGP.

Another form of ectopic calcification is calcinosis, which is the massive deposition of insoluble calcium salts in the skin and the subcutis. These calcifications occur in patients with juvenile dermatomyositis (JDM) and can cause significant disability with severe pain, muscle atrophy and joint contracture (53). The presence of Gla-containing proteins has been demonstrated in skin and subcutaneous plaques from patients with dermatomyositis (54). Because of the calcification inhibitory properties of MGP, this vitamin K-dependent protein may play an important role in the counteraction of calcinosis in JDM, but also in other rheumatologic disorders presenting with calcinosis (55;56). In chapter 7, the localization of the different forms of MGP in muscle biopsies from JDM patients with and without calcification are studied, using novel conformation-specific MGP-antibodies. The various forms of MGP were found to be present at the site of muscle damage in JDM patients in different staining intensities. MGP was also expressed in pathological muscle tissue of the other myopathies (i.e. dystrophy and inclusion-body myositis) studied. Since the occurrence of calcification in IBM and dystrophy is not common, the expression of MGP

at the site of muscle damage most likely represents a general protective mechanism. The defensive mechanism of MGP is probably not disease-specific but related to the extent of inflammation and concomitant tissue damage. When the protective action of MGP fails due to insufficient expression or decreased functionality, this may contribute to the development of calcifications. Local deficiency of vitamin K in tissue may occur due to inflammatory processes generating free radicals that may rapidly degrade vitamin K. As only carboxylated MGP (cMGP) is presumed to exert its role as inhibitor of calcification, we had expected that level of cMGP expression would be differentiating between JDM-patients with and without calcifications. However, this was not the case in our study. Surprisingly, the intensity of phosphorylated MGP staining appeared to distinguish between JDM-patients with and without calcifications. The precise function of the phosphoserines in MGP is presently unknown. It may be that both posttranslational modification processes (γ-carboxylation and phosphorylation) are simultaneously required for MGP to fully exert its function.

The question may rise whether supplementation with vitamin K in renal transplant recipients and patients with JDM will increase the functionality of MGP and thereby may positively influence the development of ectopic calcifications. Until now, no other papers have reported about the vitamin K-dependent protein MGP and its relationship to ectopic calcification in children.

Revision of the recommended dietary allowance for vitamin K in children?

Several studies in this thesis describe that in healthy children as well as in pediatric patients, varying circulating amounts of undercarboxylated forms of osteocalcin and MGP can be found. This suggests that in both populations, suboptimal vitamin K status is common. At present, the RDA of 30-75 µg vitamin K/day for children (1-18 years) in the USA is based on the maintenance of normal concentrations of prothrombin, the hepatic vitamin K-dependent protein (57). However, dietary vitamin K levels that are sufficient to maintain the hepatic production of blood coagulation factors may be too low for optimal carboxylation of the extra-hepatic Gla-proteins, e.g. osteocalcin and MGP (21). Intervention studies in adults have demonstrated that markedly higher osteocalcin carboxylation is obtained by intakes of vitamin K well above the current recommended dietary intake (58;59). **Chapter 4** describes the findings of a randomized controlled trial in which the effect of vitamin K on

osteocalcin carboxylation in healthy prepubertal children was studied. In this study, we used a food supplement containing one of the vitamin K_2 species, menaquinone-7 (MK-7). In the supplemented group, vitamin K status, as indicated by the UCR, improved significantly over the intervention period of 8 weeks. Vitamin K supplementation had an effect on osteocalcin carboxylation, even in children in the lowest UCR ranges. In addition, the circulating concentration of the inactive, undercarboxylated fraction of osteocalcin decreased. The increase in the circulating amount of carboxylated osteocalcin after 8 weeks supplementation was smaller than expected. Apparently, the decrease in ucOC is not linearly related with the increase in cOC. This observation may be explained by the fact that ucOC and cOC have a different binding affinity to hydroxyapatite in bone. The major part of newly synthesized osteocalcin is absorbed to hydroxyapatite in bone, but a part of it leaks into the circulation where it can be detected (60). UcOC has a lower binding capacity to hydroxyapatite in bone and therefore, may leak more easily to the circulation, whereas the large part of cOC will be bound in bone. Provided that the total osteocalcin production remains constant, supplementation of additional vitamin K will lead to a relative increase in cOC in bone. It may be assumed that only when the bone mineral matrix is fully saturated with cOC, indicative of optimal vitamin K supply to bone, the amount that leaks to the circulation will increase.

The children included in the present intervention study had a better vitamin K status at baseline in comparison to the children from the two other studies described in this thesis (**chapter 2 & 3**), probably due to their prepubertal status. Hence, vitamin K intake from the habitual diet of healthy children appears to be inadequate for optimal carboxylation of osteocalcin. The findings from this thesis support the hypothesis that in healthy children, dietary vitamin K intake alone is generally inadequate for optimal osteocalcin carboxylation and may lead to reconsideration of current recommended dietary intakes for vitamin K. Unfortunately, methods to detect the circulating form of carboxylated MGP are presently not available so studies on the effect of vitamin K supplementation on carboxylation of MGP are not possible yet.

Future directions

In pediatric medicine, vitamin K and vitamin K-dependent proteins are best known for their role in blood coagulation. The works as presented in this thesis are a substantial addition to the body of evidence on different aspects

of the vitamin K-dependent proteins, i.e. the role of osteocalcin in childhood bone health and the role of MGP in relation to ectopic calcification in pediatric disease. The findings from this thesis suggest that vitamin K may be of possible benefit in preventing ectopic calcification and osteoporosis in pediatric patients. Furthermore, also healthy children may benefit from elevated vitamin K intakes leading to increased osteocalcin carboxylation, which may result in improved bone health. However, this thesis also puts forward several other issues that may be subject of future studies. For instance, the effect of various conditions (e.g. prolonged vitamin K deficiency during growth or chronic inflammation) on carboxylated and undercarboxylated fractions of osteocalcin in bone may be studied in animal models. In addition, concurrent changes in the circulating ucOC and cOC and possible changes in bone mass and structure can be documented. This will learn us more about the physiological function of both fractions in bone, and in what way circulating levels of osteocalcin reflect the process in bone. Another interesting issue is the enhanced expression of osteocalcin under influence of vitamin D, which may theoretically raise vitamin K demand and even induce general vitamin K deficiency at other sites (61). Research in growing animals can also be used to study the carboxylation (and phosphorylation) of MGP in relation to ectopic calcification in vascular and muscle tissue under different circumstances. A central question in these experiments is whether local deficiency of vitamin K in tissue can be induced by inflammatory processes generating free radicals that may rapidly degrade vitamin K. If inflammation results in local vitamin K deficiency, is this reversed by supplementation of high dosages of vitamin K? When this is indeed the case, it may be of importance to monitor vitamin K status in other pediatric patients with (low-grade) inflammatory diseases (e.g. those with inflammatory bowel diseases, obesity or diabetes). Generally, children with chronic diseases are known to have an increased risk of malnutrition (62). An optimal vitamin K status may also be of importance in these children. Dose-finding studies in different pediatric patient populations as well as in healthy children are needed to determine how much vitamin K is required to achieve optimal osteocalcin and MGP carboxylation. These studies will help answering the question "What is optimal vitamin K status?" and possibly lead to redefinition of the recommended intakes for vitamin K in children. The direct cause-effect relationships between vitamin K deficiency and bone health can be demonstrated in large prospective randomized trials in pediatric patients and healthy children. Other trials may be designed to study the effects of increased

vitamin K intake on vascular health and the prevention of ectopic calcifications in pediatric patients. Together with increased insights in other functions of vitamin K and the possible discovery of novel Gla proteins (16-18;63), these studies will show the true pluripotence of vitamin K.

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Appendix Vitamin K

In pediatric medicine, vitamin K and vitamin K-dependent proteins are best known for their role in blood coagulation. For example, vitamin K is used as prophylaxis after birth and during breastfeeding to prevent vitamin K-deficiency bleeding disorders in neonates. This hemorrhagic abnormality is due to the fact that the newborn's poor vitamin K status results in the synthesis of inactive coagulation factors VII, IX and X, and prothrombin, the so called "proteins induced in vitamin K absence" (PIVKA's). Hence the name vitamin K, from Koagulations vitamin, brought up by Henrik Dam (figure 1) at its discovery in the early 1930s (1;2).

Figure 1: H. Dam, the discoverer of vitamin K.

(Carl Peter) Henrik Dam (1895-1976) discovered vitamin K in the 1930's.
For this discovery, he received the Nobel price for medicine in 1943.

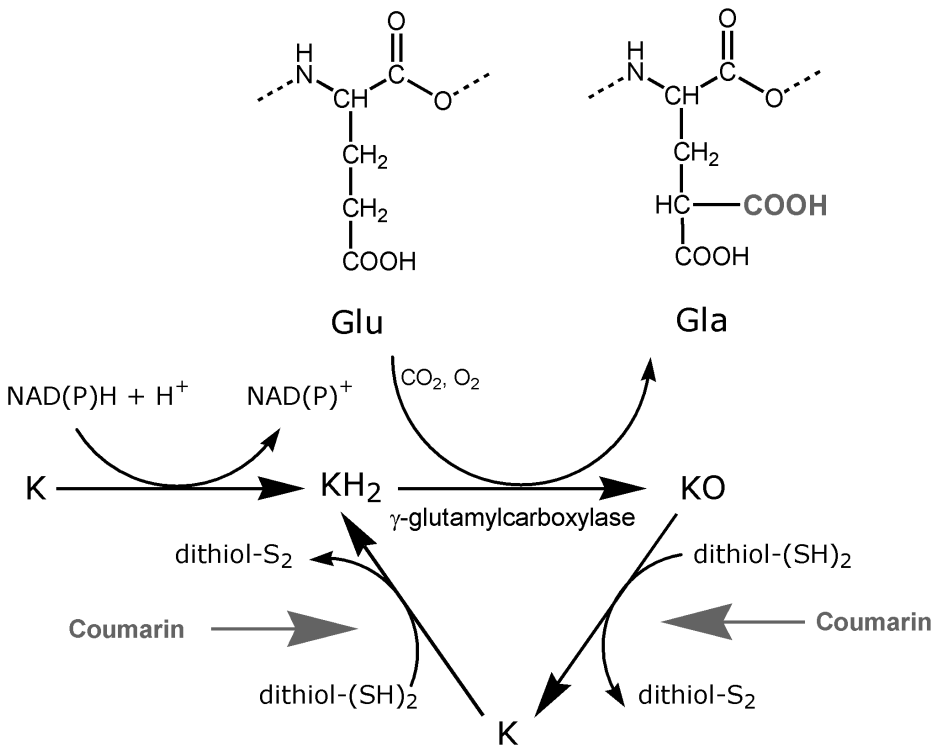
The importance of vitamin K in children is also shown when considering the teratogenic effects of the vitamin K-antagonist warfarin in early pregnancy. This warfarin embryopathy results in the syndrome chondrodysplasia punctata. The phenotype is characterized by punctate calcifications of the cartilage of the epiphyses, larynx and trachea, leading to skeletal abnormalities (3;4).

Vitamin K is a co-factor in carboxylation of Gla proteins

Vitamin K acts as a co-factor in the posttranslational carboxylation of all Gla proteins (figure 2). In the carboxylation process, glutamate acid residues (Glu) are converted into γ-carboxyglutamate acid (Gla) residues (5). These Gla residues have a high binding capacity to calcium (Ca^{2+}) which is an essential property of Gla-proteins. A shortage of vitamin K will lead to production of under-carboxylated (i.e. inactive Gla-proteins) proteins. Besides coagulation

factors, other vitamin K-dependent proteins or Gla proteins have been discovered in recent years (table 1). All these proteins are characterized by one or more Gla residues. The vitamin K-dependent proteins osteocalcin and matrix Gla protein (MGP) are involved in regulation of bone formation and the inhibition of calcification.

Figure 2: The vitamin K-cycle



Vitamin K (dietary form) is first reduced to vitamin K-hydroquinone (KH^2) and than oxidized to vitamin K-epoxide (KO). During the oxidation step, glutamic acid (Glu) is reduced to γ-carboxyglutamic acid (Gla). Finally, KO is reduced to K.
The enzymes involved are dithiol-dependent K-reductase, γ-glutamyl carboxylase, dithiol-dependent KO-reductase and NAD(P)H-dependent reductase (also called DT-diaphorase). Oral anticoagulants (coumarin-derivatives) block the KO-reductase and thereby inhibit the recycling process of vitamin K. The NAD(P)H-dependent reductase is the alternative pathway in case of blockade of KO-reductase; no recycling is possible.

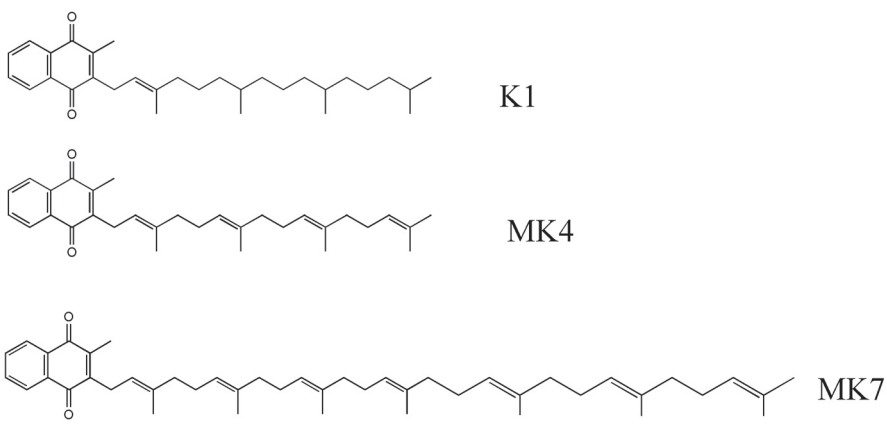
Table 1: **The vitamin K dependent proteins**

Site of synthesis	Function	Localization
Hepatic (42-44)		
Factor VII	procoagulant	plasma
Factor IX	procoagulant	plasma
Factor X	procoagulant	plasma
Prothrombin (factor II)	procoagulant	plasma
Protein C	anticoagulant	plasma
Protein Z	anticoagulant	plasma
Protein S	cofactor for protein C	plasma
Extra-hepatic (31;45-47)		
MGP	inhibitor of ectopic mineralization	cartilage, bone, most soft tissues
Osteocalcin	regulator of orderly crystallization	bone, dentin
GAS-6 (growth arrest specific gene-6 protein)	regulator of cell growth	most soft tissues
Membrane-bound (48-50)		
γ-glutamyl-carboxylase	Enzyme for the conversion of Glu to Gla	most soft tissues
TMG (transmembrane Gla-proteins) 3 & 4	unknown	most soft tissues
PRGP (proline rich Gla-protein) 1 & 2	unknown	most soft tissues

Forms & nutritional sources of vitamin K

Vitamin K is a generic term for a group of compounds consisting of two naturally occurring forms: vitamin K_1 (phylloquinone) and vitamin K_2 (menaquinones, MK-n) (figure 3). All vitamin K species have in common the methylated naphthoquinone ring structure and it is generally accepted that this part represents the functional group. The varying side chains may lead to substantial differences in intestinal absorption, transport, tissue distribution and bioavailability (6). Nutritional intake constitutes the major source of the K-vitamins. The most common form in the diet is vitamin K_1 (>90%). Vitamin K_1 is found in green leafy vegetables whereas vitamin K_2 is produced by bacteria and can be found in fermented products like dairy (table 2) (6;7). The gut flora also produces vitamin K_2 but at the site of synthesis (colon) its absorption is probably negligible (8-10).

Figure 3: **Chemical structure of vitamin K**



Vitamin K is a group name for several molecular forms that contain a common 2-methyl-1,4-naphthoquinone ring but the side chains which are attached to the 3 position are different. Vitamin K_1 or phylloquinone is a single compound containing one unsaturated and three saturated isoprenoid residues.

Vitamin K_2 or menaquinones (MK-n) form a group of related forms with differing numbers of isoprenyl groups in the side chain (MK-1 – MK-14).

Table 2: Vitamin K content of various food (µg/100 g)

Nutrient	K_1	K_2
Meat	0.5 - 3	1 - 8
Fish	0.1 - 1	0.1 -1.6
Milk	0.5 - 1	0.2 – 2
Yoghurt	0.2 – 0.5	0.1- 1
Cheese	2.5 - 15	0.5 – 80
Butter	9 -20	10 – 20
Margarines (from plant oil)	80 – 110	0
Green vegetables	100 -750	0
Natto (fermented soybeans)	20 - 30	800 – 1000
Fruit	0.1 – 3	0
Bread	0.5 - 1	0

Adapted from (6;7;51)

Absorption of vitamin K & transport to liver and extrahepatic tissues

The fat-soluble vitamin K is taken up in the small intestine (ileum and jejunum) in the form of mixed micelle complexes together with bile salts (11). The food matrix is an important determinant of the absorption rate of vitamin K. Fatsolubilized vitamin K is more efficiently absorbed in comparison to vitamin K_1 from green leafy vegetables (5-15% only) (12;13).

The absorptive enterocytes in the intestinal mucosa will pack vitamin K together with cholesterol, lipids and lipoproteins into chylomicrons. These chylomicrons enter the blood circulation via the lymphatic system and are degraded into chylomicron remnants. Most of the absorbed vitamin K is delivered to the liver by these chylomicron remnants which are cleared form the circulation via apolipoprotein E (apoE) receptor mediated uptake (14).

The clearance rate is dependent on the apoE genotype (E2 < E3 < E4) resulting in different plasma vitamin K concentrations (15;16). The remaining part of vitamin K is carried by the triglyceride rich lipoprotein fraction (TGRLP) (14;17). The low-density lipoprotein- (LDL) and high-density lipoprotein- (HDL) fractions also carry vitamin K, but in smaller amounts (14).

Little is known about the vitamin K transport to and uptake by extra-hepatic tissues like the arterial vessel wall and bone. Cultured osteoblasts can internalize vitamin K form LDL, chylomicron remnants, very low density lipoprotein (VLDL) and HDL by an apoE-dependent mechanism (18;19). In vitro studies have demonstrated that MK-4 is more rapidly absorbed and metabolized into human cell lines compared to phylloquinone (20).

Tissue distribution of vitamin K

Experiments in rats have shown that vitamin K1 accumulates preferentially in hepatic tissue (21). Despite this fact, phylloquinone comprises only about 10% of the human hepatic store; the remaining 90% are menaquinones, mostly higher homologues (> MK10) (22). The biological relevance of this large hepatic menaquinone store is unclear. The most prevalent form of the menaquinones, MK-4, is preferentially taken up by extra-hepatic tissues (23). The MK-4 levels exceed those of phylloquinone in the pancreas, the salivary gland and the brain (23). Furthermore, vitamin K_1 can probably be converted into MK-4 in at least some extra-hepatic tissues (24).

Tissue-dependent vitamin K need: difference between hepatic and extra hepatic Gla-proteins

The recommended dietary (daily) allowance (RDA) for vitamin K is based on the maintenance of normal plasma prothrombin concentrations. Current recommended dietary intakes for vitamin K in the USA are 30-75 µg/d for children 1-18 years of age and 90-120 µg/d for adults (25). Based on their prothrombin levels, healthy subjects are normally not vitamin K deficient thus showing that sufficient amounts of vitamin K are present for carboxylation of the hepatic coagulation proteins (26). However, dietary vitamin K levels that are sufficient to maintain normal blood coagulation may be suboptimal for carboxylation of the extra hepatic Gla-proteins. For example, subjects can also be defined as vitamin K deficient based on the amount of undercarboxylated osteocalcin present in bone (26;27).

In healthy volunteers, vitamin K treatment induces an increase in the amount

of carboxylated osteocalcin (26;28-30). Hence, vitamin K intake well above the RDA value is required for adequate carboxylation. This has led to the concept of tissue-specific vitamin K-demand (31) in which the RDA-values for vitamin K are defined as the amount of vitamin K needed for maximal carboxylation of the different vitamin K-dependent proteins.

Different biochemical measures to monitor vitamin K status

Several biochemical measures to monitor vitamin K status in human populations are available: circulating vitamin K-levels, urinary free Gla residues, determination of urinary metabolites of vitamin K and assessment of the γ-carboxylation status of specific Gla proteins.

Circulating vitamin K levels can be used as an indicator of tissue stores (32;33). This method has several drawbacks. Firstly, vitamin K concentrations are instable markers due to a large day-to-day variation in intake of vitamin K. Secondly, serum-lipid and apolipoprotein concentrations have to be taken into account because they may influence the association between dietary intake and plasma concentrations (34). Furthermore, assays for the various forms of vitamin K_2 are not sensitive enough to detect very low levels of the menaquinones. Through detection of urinary metabolites of vitamin K (both K_1 and K_2) using a HPLC redox-mode electrochemical detection, an overall measure for vitamin K degradation is obtained (35).

Another way to monitor vitamin K status is by assessment of the γ-carboxylation status of Gla proteins. The overall γ-carboxylation of all Gla proteins can be determined by measuring the urinary content of free Gla residues (36;37). This method cannot discriminate between the different Gla proteins. The γ-carboxylation of individual circulating Gla proteins can be measured by using conformation-specific antibodies. Prothrombin is a marker for the hepatic γ-carboxylation capacity, whereas osteocalcin reflects that of bone (38-40). The amount of undercarboxylated osteocalcin (ucOC) relative to total (or carboxylated) osteocalcin as well as circulating ucOC levels are used as indicators for the vitamin K status of bone (30;31;38).
The ratio of ucOC/cOC (UCR) is probably the most sensitive marker for bone vitamin K status because of its relative independence of bone metabolism (31). The fractions of osteocalcin are also considered to be sensitive markers of general vitamin K nutritional status (41). Furthermore, the extent of carboxylation of the vitamin K-dependent protein MGP can be determined

(ref Cranenburg et al., submitted for publication). This assay may be used as a biomarker for vascular vitamin K status, possibly also for various forms of cardiovascular diseases.

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Nederlandse samenvatting

Osteoporose en hart- en vaatziekten worden vaak gezien als ouderdomsziekten. Er zijn echter steeds meer aanwijzingen dat de basis voor deze aandoeningen juist op de kinderleeftijd gelegd wordt (1-4). Osteoporose op latere leeftijd kan o.a. ontstaan door een suboptimale opbouw van piekbotmassa (PBM) tijdens de adolescentie (1;2). De PBM is de maximale opgebouwde hoeveelheid botmassa; 80-90% van de PBM wordt opgebouwd tussen geboorte en adolescentie (5;6). Vroege tekenen van atherosclerose worden al gezien op de kinderleeftijd, voornamelijk bij kinderen met aandoeningen waarvan bekend is dat deze een duidelijk verhoogd risico geven op hart- en vaatziekten bij volwassenen (bijv. hypertensie, ernstig overgewicht) (3;4).

Osteoporose en hart- en vaatziekten zijn eveneens van toenemend belang in relatie tot chronische ziekten op de kinderleeftijd. Door de jaren heen hebben kinderen met een chronische ziekte steeds betere overlevingskansen, echter dit gaat gepaard met een toegenomen morbiditeit. Ten gevolge van de onderliggende ziekte of de gebruikte medicatie kunnen late complicaties ontstaan. Een aantal chronische aandoeningen (bijv. jeugdreuma) worden geassocieerd met een afname van de botmassa waardoor de kans op botbreuken toeneemt (7;8). Andere kinderen (met bijv. chronisch nierfalen) hebben een sterk verhoogd risico op hart- en vaatziekten, hetgeen al tot ziekteverschijnselen kan leiden rond de leeftijd van 30 jaar (4;7;9).
Door verbeterde kennis over de ontstaanswijze van osteoporose en hart- en vaatziekten bij kinderen met chronische ziekten kunnen nieuwe behandelingen en preventieve maatregelen worden ontwikkeld. Zodoende kan al op de kinderleeftijd worden ingegrepen, hetgeen van voordeel kan zijn voor zowel kinderen als volwassenen. Bij volwassenen werd al veel onderzoek verricht naar de rol van vitamine K bij het voorkómen van osteoporose en hart- en vaatziekten (10). Bij kinderen is dit onderzoeksveld nog vrijwel onontgonnen.

Vitamin K, een pluripotent vitamine?

Vitamine K functioneert als co-factor voor een enzym dat betrokken is bij de γ-carboxylering van de zogenaamde Gla-eiwitten waarbij glutaminezuur (Glu) wordt omgezet in γ-carboxyglutaminezuur (Gla). De aanwezigheid van de Gla-residuen bepaalt de functionaliteit (activiteit) van het eiwit doordat deze calciumbindende eigenschappen bezitten. Vitamine K is betrokken bij verschillende fysiologische processen en speelt een rol bij de bloedstolling,

de regulatie van botgroei en (het voorkomen van) ectopische verkalking, m.n. in de vaatwand (11). Voorbeelden van Gla-eiwitten zijn osteocalcine en matrix Gla proteïne (MGP). Studies bij volwassenen suggereren dat optimale carboxylering van osteocalcine bijdraagt aan de kwaliteit van bot, zowel door een verhoogde botmassa als verbeterde morfologie van het bot (12;13). MGP heeft verkalkingsremmende eigenschappen die eveneens afhankelijk zijn van carboxylering en dus de aanwezigheid van vitamine K (14;15).

In de afgelopen jaren zijn verschillende andere Gla-eiwitten (ook wel genaamd vitamine K-afhankelijke eiwitten) ontdekt, ondermeer GAS-6 (growth arrest specific gene-6 protein) (zie ook appendix: Vitamin K) (16-19). Gezien het feit dat de functie van deze Gla-eiwitten tot op heden grotendeels onbekend is, is het denkbaar dat vitamine K nog bij andere fysiologische processen betrokken is. Een literatuuroverzicht over vitamine K wordt gegeven in de **appendix** van dit proefschrift.

Omdat vitamine K een noodzakelijke factor is voor de γ-carboxylering van alle Gla-eiwitten kan worden aangenomen dat de metabole activiteit van de processen waarbij deze eiwitten betrokken zijn de totale behoefte aan vitamine K bepaalt. Onvoldoende inname van vitamine K (suboptimale vitamine K status) zou dan een onafhankelijke risicofactor voor zowel osteoporose als ectopische verkalking kunnen zijn, eveneens bij kinderen. De metabole activiteit bij groeiende kinderen is groter dan bij volwassenen, hetgeen waarschijnlijk betekent dat de behoefte aan vitamine K uit de voeding ook groter is. Het doel van dit proefschrift was om de rol van vitamine K en osteocalcine in relatie tot botmetabolisme bij kinderen te onderzoeken. Verder werd de rol van MGP in relatie tot het ontstaan van ectopische verkalkingen bij een tweetal chronische ziekten bij kinderen onderzocht. In **hoofdstuk 1** van dit proefschrift wordt een literatuuroverzicht gegeven over osteocalcine, MGP en vitamine K bij kinderen.

Vitamine K status bij kinderen en volwassenen

Gezien het feit dat ten tijde van bot- en skeletgroei er een grote productie van osteocalcine is (20), veronderstelden we dat de behoefte aan vitamine K onder deze omstandigheden ook hoog zou zijn. De meest betrouwbare manier om te beoordelen of de inname aan vitamine K adequaat is, is door het bepalen van de gecarboxyleerde en ondergecarboxyleerde fracties van de verschillende Gla-eiwitten in het bloed of in de lichaamsweefsels. Momenteel zijn er technieken beschikbaar die de verschillende fracties van de Gla-eiwitten

protrombine en osteocalcine kunnen meten. Er wordt gebruik gemaakt van conformatie-specifieke antilichamen. De mate waarin protrombine, een van de stollingseiwitten, is gecarboxyleerd wordt beschouwd als een maat voor de vitamine K status van de lever, terwijl de mate waarin osteocalcine gecarboxyleerd is een maat is voor de vitamine K status van bot (21-23). De verhouding tussen de gecarboxyleerde en ondergecarboxyleerde fracties osteocalcine (afgekort tot UCR) wordt eveneens beschouwd als een gevoelige maat voor de algehele vitamine K status van het lichaam (24).
In **hoofdstuk 2** worden de resultaten van een observationeel onderzoek naar mogelijke verschillen in vitamine K status, uitgedrukt als de UCR, tussen 86 gezonde kinderen (3-18 jaar) en 30 volwassenen beschreven. Uit dit onderzoek blijkt dat de UCR significant hoger was bij kinderen hetgeen een suboptimale vitamine K status tijdens groei suggereert. M.n. in de groep van de kinderen werd een grote variatie in de UCR gezien. Dit betekent dat de mate van vitamine K tekort per individu kan verschillen. Deze bevinding bevestigt resultaten uit eerdere onderzoeken bij volwassenen en kinderen waaruit eveneens blijkt dat er veel variatie in de mate van carboxylering van osteocalcine bestaat (25;26).

Bij volwassenen wordt het circulerend ondergecarboxyleerd osteocalcine (ucOC) meestal gebruikt als indicator voor de vitamine K status van bot (10;21;27). Bij kinderen is het circulerend ucOC alleen waarschijnlijk geen goede maat voor de vitamine K status. Het niveau van ucOC wordt bepaald door zowel de totale osteocalcine productie en de fractie daarvan dat gecarboxyleerd wordt in aanwezigheid van vitamine K. Bij kinderen is het mogelijk beter om de UCR te gebruiken. De UCR geeft de verhouding tussen ucOC en gecarboxyleerd osteocalcine (cOC) weer en houdt zodoende rekening met verschillen in botturnover. Door het gebruiken van de UCR is het mogelijk om de vitamine K status tussen kinderen met een verschillende graad van botturnover te vergelijken.

Tijdens de puberteit treden snelle veranderingen op het in het botmetabolisme die de vitamine K status negatief zouden kunnen beïnvloeden, tenzij de inname van vitamine K via de voeding toeneemt. Met andere woorden, de behoefte aan vitamine K neemt toe omdat de totale osteocalcine productie toeneemt. Als verhoogde behoefte aan vitamine K niet gecompenseerd wordt door verhoogde inname van vitamine K, dan zal de UCR toenemen, hetgeen betekent dat de vitamine K status afneemt. Uit de resultaten van de eerdergenoemde studie in **hoofdstuk 2** blijkt dat de hoogste waarden van

ucOC en cOC gevonden tijdens de puberteit. Aan het einde van de puberteit werd er een daling gezien van de gemiddelde waarden van ucOC en cOC, hetgeen samenvalt met een daling in de metabole activiteit van het bot. **Hoofdstuk 3** beschrijft de resultaten van een studie naar de relatie tussen vitamine K status, uitgedrukt als UCR, en botmassa in een groep van 307 gezonde kinderen (gemiddelde leeftijd 11,2 jaar) rond de puberteit. Uit deze studie blijkt eveneens dat de UCR een significant positief verband heeft met het puberteits- stadium, hetgeen betekent dat naar mate de puberteit vordert, de vitamine K status lijkt te verslechteren.

Geconcludeerd kan worden dat een suboptimale vitamine K status vaak voorkomt bij gezonde kinderen, en meest opvallend is tijdens de puberteit.

Vitamine K en botmetabolisme bij gezonde kinderen

Bij volwassenen wordt de UCR beschouwd als een maat voor zowel de vitamine K status van bot (22;23) als de algemene vitamine K status van het lichaam (24). Het is echter de vraag of dit ook opgaat voor kinderen. Gezien het feit dat osteocalcine het belangrijkste niet-collagene bestanddeel van bot vormt en de functie van osteocalcine afhankelijk is van vitamine K (28), is het te verwachten dat de UCR als maat van vitamine K status gerelateerd is met markers van botmetabolisme en botmassa, ook bij kinderen. De verschillende studies uit dit proefschrift tonen echter tegenstrijdige resultaten. Uit de studie in **hoofdstuk 2** blijkt dat er geen samenhang is tussen de UCR en de botmarkers, bot-alkalische fosfatase (BAP) en N-telopeptide cross-links van collageen afbraak (NTX). De studie in **hoofdstuk 3** laat echter wel een (positief) verband zien tussen tussen de UCR en de markers voor botopbouw en botresorptie. Uit laatstgenoemde studie blijkt verder dat toename in "total-body" botdichtheid over 2 jaar, gemeten met DEXA, gerelateerd was met veranderingen in vitamine K status waarbij gecorrigeerd werd voor mogelijke confounders. In een studie bij 54 gezonde kinderen (6-18 jaar), beschreven in **hoofdstuk 5**, werd geen relatie tussen botmassa, gemeten met echografie van de calcaneus, en de UCR gevonden. Echografie van de calcaneus is een methode om de botdichtheid en botmorfologie te bepalen zonder stralingsbelasting (31).

Geconcludeerd kan worden dat de UCR als indicator voor vitamine K status geassocieerd is met botmarkers en botparameters. Toekomstig onderzoek zou zich moeten richten op de vraag of vitamine K suppletie bij kinderen met

hoge UCR en dus een suboptimale vitamine K status een strategie kan zijn om de piekbotmassa te optimaliseren en op deze wijze mogelijk osteoporose op latere leeftijd te voorkomen.

...en botmetabolisme bij zieke kinderen

Patiënten met juveniele idiopathische arthritis (JIA) hebben een verhoogd risico op osteoporose (29;30). We veronderstelden dat bij deze patiëntengroep een suboptimale vitamine K status een mogelijke bijdragende factor kan zijn in de ontwikkeling van een verminderde botmassa.

In **hoofdstuk 5** worden de resultaten beschreven van een studie waarin de vitamine K status van 55 kinderen met JIA werd vergeleken met die bij 54 gezonde kinderen. Verder werd het mogelijke verband tussen vitamine K status, uitgedrukt als UCR, en botparameters, gemeten met echografie van de calcaneus, onderzocht. De vitamine K status was niet verschillend tussen de kinderen met JIA en de gezonde kinderen, terwijl de botparameters duidelijk verlaagd waren in de laatstgenoemde groep. Ook nu werd een grote variatie in de UCR in beide groepen gezien. In beide groepen werd geen lineaire relatie tussen vitamine K status en botparameters vastgesteld. Echter, nadat de groepen werden gecategoriseerd op grond van hoge, gemiddelde en lage UCR-waarden, bleken de patiënten in de "lage UCR"-groep (dus betere vitamine K status) betere botparameters te hebben in vergelijking met de patiënten in de "hoge UCR"-groep. Dit verschil in botmassa was onafhankelijk van verschillen in leeftijd, geslacht en BMI. Deze resultaten suggereren dat vitamine K inderdaad een mogelijke bijdrage levert aan de opbouw van bot bij kinderen met JIA.

Gezien het mogelijke belang van vitamine K in de opbouw van bot lijkt het van belang om verder onderzoek te verrichten naar het verband tussen vitamine K status, carboxylering van osteocalcine en opbouw van bot bij andere pediatrische patiëntengroepen, zoals bijvoorbeeld kinderen met primaire botziekten en kinderen met onverklaarde osteoporose met herhaalde botbreuken.

Vitamin K en ectopische verkalkingen op de kinderleeftijd

Ectopische verkalking is het proces van pathologische calcium-afzetting in weefsels. Dit proces kan overal in het lichaam voorkomen en wordt ook bij kinderen gevonden, alhoewel zeer zeldzaam.

Ectopische verkalking komt voor in de arteriële vaatwand, zowel in de intima als de tunica media. Verkalking van de intima wordt beschouwd als een vergevorderd stadium van atherosclerose. Atherosclerose is een cel-gemedieerd proces waarin een locale ontstekingsreactie van de vaatwand een belangrijke rol speelt (40). Vroege tekenen van atherosclerose zijn ook bij kinderen beschreven, bijvoorbeeld bij kinderen met ernstige obesitas of afwijkingen in het vetmetabolisme (42;43). Verkalking van de tunica media, ook wel arteriosclerose genoemd, wordt geassocieerd met uremie en afwijkingen in de calcium-fosfaat-stofwisseling, en komt veelvuldig voor bij terminale nierinsufficiëntie (44-46). Ook bij kinderen en adolescenten met terminale nierinsufficiëntie kunnen arteriële verkalkingen worden aangetoond (4). Het verkalkingsproces in de tunica media wordt gekenmerkt door de aanwezigheid van stoffen die ook in bot tot expressie komen en geassocieerd worden met het mineralisatieproces (47). Verkalking in de arteriële vaatwand komt tot stand door de disbalans tussen factoren die de calciumafzetting bevorderen en inhibiërende mechanismen. Matrix Gla proteïne (MGP) is een van de regulerende factoren die in de afgelopen jaren is ontdekt (48;49).

In voorgaand onderzoek werd de aanwezigheid van MGP zowel in verkalkingen van de tunica media als in atherosclerotische plaques aangetoond (50;51). De Gla residuen van MGP hebben, zoals eerder gesteld, een hoge affiniteit voor calcium en bepalen de functionaliteit van het eiwit (15). Suboptimale vitamine K status kan leiden tot een tekort aan gecarboxyleerd MGP en dit zou kunnen bijdragen aan het ontstaan van ectopische verkalkingen. **Hoofdstuk 6** beschrijft de resultaten van een cross-sectionele studie bij 29 kinderen na niertransplantatie en 54 gezonde kinderen waarin naast MGP, ook fetuin A, een andere calcificatieremmer werd gemeten. Tevens werden bij deze kinderen de vaatwandeigenschappen van de a.carotis gemeten middels echo-onderzoek, en werden mogelijke relaties tussen deze eigenschappen en de calcificatieremmers onderzocht. In dit onderzoek werden twee vormen van MGP bepaald, het niet-gefosforyleerd MGP (serMGP) en het niet-gecarboxyleerd MGP (ucMGP). De resultaten van het onderzoek laten zien dat het fetuin-A verlaagd was in de transplantatie-groep terwijl het MGP (zowel serMGP als ucMGP) niet verschilde.

De verwachting was dat het MGP bij kinderen na niertransplantatie verhoogd zou zijn omdat deze kinderen doorgaans veel verstoringen in het calcium-fosfaatmetabolisme hebben gehad. Het feit dat MGP juist niet verhoogd is, zou kunnen wijzen op onvoldoende upregulatie waardoor het

beschermend mechanisme van MGP faalt (49). In deze studie waren de gemiddelde waarden van ucMGP in beide groepen hoog in vergelijking met de serMGP waarden, hetgeen mogelijk wijst op een onvoldoende vitamine K toevoer voor optimale carboxylering. Verder waren de vaatwandparameters (elasticiteit en intima-media-dikte) bij de kinderen na niertransplantatie zoals verwacht hoger in vergelijking met de gezonde kinderen. Er werden geen verbanden gevonden tussen de calcificatieremmers fetuin-A en MGP enerzijds en de vaatwandparameters elasticiteit en IMT anderzijds.

Een andere vorm van ectopische verkalking is calcinose, waarbij kalkafzetting plaatsvindt in de huid en het onderhuidse bindweefsel. Calcinose kan voorkomen bij patiënten met juveniele dermatomyositis (JDM) en leidt dan vaak tot pijnklachten, spieratrofie en contracturen (53). In eerder onderzoek bij dermatomyositis patiënten werden in deze kalkafzettingen Gla-bevattende eiwitten aangetoond (54). We onderstelden dat MGP vanwege de verkalkingsremmende eigenschappen en de aanwezige Gla-residuen een rol zou kunnen spelen in het vóorkomen van calcinose bij JDM (55;56).
In **hoofdstuk 7** worden de resultaten van een studie naar de aanwezigheid en localisatie van verschillende vormen van MGP in spierbiopten van JDM-patiënten met en zonder calcinose beschreven. Voor dit immuunhistochemisch onderzoek werd gebruik gemaakt van nieuwe antilichamen tegen MGP.
MGP bleek aanwezig te zijn in de spierbiopten van JDM-patiënten, m.n. op de plek van spierschade. Ook in spierweefsel van patiënten met andere spieraandoeningen, spierdystrofie en inclusion-body myositis (IBM), werd MGP aangetoond. Omdat bij deze aandoeningen normaal gesproken geen verkalkingen optreden, is de aanwezigheid van MGP ter plekke van de spierschade waarschijnlijk een aspecifiek beschermend mechanisme. Alleen wanneer MGP niet functioneel is of onvoldoende tot expressie komt, dan zou dit kunnen bijdragen aan het ontstaan van verkalkingen zoals calcinose. Uit het onderzoek blijkt verder dat een van de vormen van MGP, te weten de gefosforyleerde vorm van MGP (pserMGP) mogelijk een onderscheid maakt tussen de JDM-patiënten met en zonder calcinose. Echter, op dit moment is de functie van deze vorm van MGP nog onduidelijk.

De bevindingen uit bovenstaande studies werpen de vraag op of vitamine K suppletie leidt tot een verbeterde functionaliteit van MGP en zodoende het ontstaan van verkalkingen kan worden voorkomen.
Dit zal toekomstig onderzoek moeten uitwijzen.

Herziening van de aanbevolen dagelijkse hoeveelheid voor vitamine K bij kinderen?

Verschillende studies in dit proefschrift laten zien dat bij zowel gezonde kinderen als pediatrische patiënten veel variatie in de ondergecarboxyleerde vormen van osteocalcine en MGP wordt gezien. Deze bevindingen suggereren dat een suboptimale vitamine K status in beide groepen een veelvoorkomend verschijnsel is. De aanbevolen hoeveelheid vitamine K voor kinderen (1-18 jaar) in de VS is 30-75 microgram per dag; dit is gebaseerd op het handhaven van adequate prothrombine concentraties (57). Uit eerder onderzoek bij volwassenen blijkt dat vitamine K inname, voldoende voor een normale stolling, vaak tekort schiet voor optimale carboxylering van de andere Gla-eiwitten als MGP en osteocalcine (21;58;59). In **hoofdstuk 4** worden de bevindingen van een gerandomiseerd placebo-gecontroleerd interventieonderzoek (8 weken) naar het effect van vitamine K toediening op de carboxylering van osteocalcine bij gezonde prepuberale kinderen beschreven. Voor deze studie werd een voedingssupplement gebruikt met daarin een vorm van vitamine K_2, te weten menaquinone-7 (MK-7). Er was een duidelijke verbetering van de vitamine K status (uitgedrukt als de UCR) te zien in de groep kinderen die het vitamine K-supplement kreeg. Tegelijkertijd daalde de ondergecarboxyleerde fractie van het osteocalcine in deze groep. Het gecarboxyleerde osteocalcine liet een toename zien alhoewel minder groot dan verwacht. De kinderen die deelnamen aan deze interventiestudie hadden een betere vitamine K status bij start van het onderzoek in vergelijking met de kinderen uit de twee andere onderzoeken die in dit proefschrift werden beschreven (**hoofdstuk 2 en 3**), waarschijnlijk omdat zij in het prepuberale stadium verkeren.

De resultaten zoals beschreven in dit proefschrift ondersteunen de hypothese dat ook bij gezonde kinderen de inname van vitamine K via de voeding vaak onvoldoende is voor optimale carboxylering van de Gla-eiwitten. Dit betekent dat de aanbevolen dagelijkse hoeveelheid voor vitamine K mogelijk herzien moet worden. Momenteel ontbreekt de techniek om ook de ondergecarboxyleerde vorm van MGP in serum aan te tonen. Hierdoor kan helaas het effect van vitamine K suppletie op de carboxylering van MGP nog niet worden aangetoond.

De toekomst van vitamine K...

In de vakgebied van de kindergeneeskunde zijn vitamine K en de vitamine K-afhankelijke eiwitten vooral bekend vanwege hun rol in de bloedstolling. Met

dit proefschrift wordt een bijdrage geleverd aan de kennis over de minder bekende aspecten van de vitamine K-afhankelijke eiwitten, te weten de rol van osteocalcine bij het botmetabolisme en de rol van MGP bij het vóórkomen van ectopische verkalkingen. De bevindingen uit dit proefschrift suggeren dat vitamine K van belang kan zijn in het vóórkomen van osteoporose en ectopische verkalkingen bij verschillende pediatrische patiëntgroepen. Ook gezonde kinderen zouden mogelijk kunnen profiteren van verhoogde inname van vitamine K.

Echter, dit proefschrift roept ook nieuwe vragen op die de basis kunnen vormen voor toekomstig onderzoek. Het is van belang om meer inzicht te krijgen in de functie van osteocalcine en MGP op moleculair-fysiologisch gebied en de betrokken regulatiemechanismen. Met behulp van diermodellen kan worden bestudeerd in welke mate veranderingen in de circulerende fracties van beide eiwitten samenhangen met door langdurig vitamine K-tekort geïnduceerde veranderingen in het botmetabolisme en (de vorming van) ectopische verkalking in vaatwand en huid. In deze onderzoeksopzet zou tevens de invloed van vitamine K-deficiëntie en -suppletie op de botvorming en ectopische verkalkingen, zowel tijdens de groei als bij chronische inflammatie, kunnen worden onderzocht. Een centrale vraag is dan of een (lokaal) vitamine K tekort geïnduceerd wordt door vrije radicaalvorming ten gevolge van ontstekingsprocessen, en zo ja, of dit tekort dan aangevuld kan worden door inname van grote hoevellheden vitamine K. Mocht dit inderdaad het geval zijn, dan zou het monitoren van vitamine K status ook bij kinderen met andere chronische inflammatoire aandoeningen van belang kunnen zijn (bijv. kinderen met IBD (inflammatory bowel diseases), obesitas of diabetes). "Dose-finding" onderzoek, zowel bij gezonde kinderen als bij verschillende pediatrische patiëntengroepen, is nodig om de vraag te kunnen beantwoorden hoeveel vitamine K nodig is voor optimale carboxylering van osteocalcine en MGP, en dit onderzoek kan leiden tot nieuwe aanbevelingen voor de aanbevolen dagelijkse hoeveelheid vitamine K. Hierna kan het verband tussen vitamine K inname en botmassa worden bevestigd in prospectief interventieonderzoek in diverse patiëntengroepen en bij gezonde kinderen. Ook kan het mogelijke effect van vitamine K-suppletie op de preventie van vaatwandverkalking en andere ectopische verkalkingen in klinische trials worden onderzocht. Het daadwerkelijke bewijs dat vitamine K een pluripotent vitamine is, zal voortkomen uit de ontdekking van nieuwe Gla-eiwitten en verdere studies naar

vitamine K (16-18;63). Dit proefschrift hoopt alvast een bescheiden bijdrage aan dit bewijs te leveren.

Referenties

zie **hoofdstuk 8**

Dankwoord in drie delen

Hoe heeft het ooit zover kunnen komen?
Beladen met een kinderlijke bewondering voor de magie van de wetenschappelijke wereld, waarin een eigen bijdrage ver weg lijkt; met dat gevoel begon ik mijn onderzoekstijd. Het proefschrift, als bewijs van zelfstandig beoefend wetenschappelijk onderzoek, is af. Achteraf bezien is deze periode een wetenschappelijke odyssee, waarin het doel (lees: proefschrift) eigenlijk ondergeschikt is aan de afgelegde weg, de beleefde avonturen (lees ook: opgedane kennis en vaardigheden) en reisgenoten (lees: de hulp en steun van vele personen zonder wie ik dit onderzoek niet tot een goed einde had kunnen brengen).

Ideas for Innovators – Get inspired

"Zonder inspiratie, geen motivatie". Dit geldt zeker voor het volbrengen van wetenschappelijk onderzoek. Mijn motivatie werd aangewakkerd door het vitamine K-enthousiasme van dr. Kees Vermeer (co-promotor en ooit mijn begeleider tijdens mijn student-assistentschap), en op volle toeren gebracht onder bezielende leiding van prof.dr. Wietse Kuis (promotor) en dr. Marc Lilien (co-promotor). Bedankt voor "creating energy from opposing forces" want ondanks jullie uiteenlopende wetenschappelijke achtergronden, was de chemie voor het onderzoek mij duidelijk.

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Connecting people

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Design your own life

Je eigen leven vormgeven klinkt gemakkelijk, maar kan pas echt als je je gesteund voelt door je naasten. Bart, dank voor jou! Pipa en mima, dank voor het stevige fundament! Sanne, Ivonne, Joyce en andere vrienden, en ook mijn maatjes uit het WKZ, dank voor het instandhouden van de balans werk-ontspanning! Familie, dank voor de warme belangstelling!

Marieke

Curriculum Vitae

Marieke van Summeren werd geboren op 8 augustus 1976 te Gemert. In 1994 behaalde zij het VWO-diploma op het Macropedius college te Gemert. Datzelfde jaar begon zij met de studie Geneeskunde aan de Rijksuniversiteit Limburg (thans Universiteit Maastricht) te Maastricht, waar zij in 1998 het doctorale examen haalde. Tijdens haar studie was zij van 1996 tot en met 1998 werkzaam als student-assistent bij de divisie Vitamine K, vakgroep Biochemie te Maastricht onder begeleiding van dr. C. Vermeer. Zij verrichtte haar wetenschapsstage in 1998 in de United Bulawayo Hospitals (Zimbabwe) onder begeleiding van dr. D.A. Verkuyl, hetgeen resulteerde in de scriptie "A descriptive study in a Zimbabwian population: comparison between women with cervix-carcinoma with and without HIV/aids; prognosis and survival". Na haar artsexamen in 2000 werkte zij gedurende enkele maanden als S.E.H.- en I.C.-arts in het Sint Anna ziekenhuis te Geldrop, en als arts-assistent kindergeneeskunde in het Elkerliek ziekenhuis te Helmond. In mei 2001 begon zij als arts-assistent kindergeneeskunde in het Wilhelmina Kinderziekenhuis (UMC Utrecht), waar zij in oktober 2001 werd aangenomen voor de opleiding tot kinderarts (opleider prof.dr. J.L.L. Kimpen). In het kader van de perifere opleidingsstage was zij gedurende 1,5 jaar werkzaam in het Sint Antonius ziekenhuis te Nieuwegein (opleider dr. J.A. Schipper). Vanaf januari 2003 heeft zij de klinische opleiding afgewisseld met promotieonderzoek (promotor: prof.dr. W.Kuis; co-promotoren: dr. C. Vermeer en dr. M.R. Lilien) dat resulteerde in dit proefschrift.

List of publications

Marieke J.H. van Summeren, Lavienja A.J.L.M Braam, Florence Noirt, Wietse Kuis, Cees Vermeer.
Pronounced elevation of undercarboxylated osteocalcin in healthy children. Pediatric Research 2007;61:366-70

Marieke J.H. van Summeren, Silvia C.C.M. van Coeverden, Leon J. Schurgers, Lavienja A.J.L.M. Braam, Florence Noirt, Cuno S.P.M. Uiterwaal, Wietse Kuis, Cees Vermeer.
Vitamin K status is associated with childhood bone mineral content. Submitted

Marieke J.H. van Summeren, Lavienja A.J.L.M Braam, Marc R. Lilien, Leon J. Schurgers, Wietse Kuis, Cees Vermeer.
The effect of vitamin K_2 supplementation on osteocalcin carboxylation in healthy children. In preparation

Marieke J.H. van Summeren, Cees Vermeer, Raoul H.H. Engelbert, Leon J. Schurgers, Tim Takken, Kathelijn Fischer, Wietse Kuis.
Extremes in vitamin K status of bone are related to bone ultrasound properties in children with JIA. Accepted (Clinical and Experimental Rheumatology)

Marieke J.H. van Summeren, Jeroen M. Hameleers, Leon J. Schurgers, Arnold P.G. Hoeks, Cuno S.P.M. Uiterwaal, Thilo Krueger, Cees Vermeer, Wietse Kuis, Marc R. Lilien.
Circulating levels of calcification inhibitors and vascular properties in children after renal transplantation. Submitted

Marieke J.H. van Summeren, Wim G.M. Spliet, Annet van Royen-Kerkhof, Cees Vermeer, Marc Lilien, Wietse Kuis, Leon J. Schurgers.
Calcinosis in juvenile dermatomyositis: a possible role for the vitamin K-dependent protein matrix Gla protein (MGP). Submitted

Abstracts

Marieke J.H. van Summeren, Raoul H.H. Engelbert, Danielle M. de Vries, Cees Vermeer, Berent J. Prakken, Wietse Kuis.
Quantitative ultrasound properties of calcaneal bone in children with juvenile idiopathic arthritis (JIA). Clinical and Experimental Rheumatology 2004; 22(4)

Marieke J.H. van Summeren, Jan M. Meinders, Danielle M. de Vries, Cees Vermeer, Raoul H.H. Engelbert, Marc R.Lilien, Berent J. Prakken, Wietse Kuis.
Isobaric carotid vessel wall properties (M-mode) in children with juvenile idiopathic arthritis (JIA). Clinical and Experimental Rheumatology 2004; 22(4)

Marieke J.H. van Summeren, Raoul H.H. Engelbert, Berent J. Prakken, Wietse Kuis, Cees Vermeer.
Poor vitamin K status is associated with bone quantitative ultrasound properties in children with juvenile idiopathic arthritis (JIA). Annals of the Rheumatic Diseases 2006; 65:supplement II.

Marieke J.H. van Summeren, Leon J. Schurgers, Arnold P.G. Hoeks, Cees Vermeer, Wietse Kuis, Marc R. Lilien.
Matrix Gla protein (MGP) and vascular properties in children after renal transplantation. Pediatric Transplantation 2007; 11(S1):6-33