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PREVENTION OF VITAMIN K DEFICIENCY IN INFANCY

E.A.M. CORNELISSEN

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Dit onderzoek werd gesubsidieerd door het Praeventiefonds (no. 28-1563).

Publikatie van dit proefschrift is mede mogelijk gemaakt door giften van het Fonds Wetenschapsbeoefening van de Afdeling Kindergeneeskunde (Academisch Ziekenhuis Nijmegen), Hoffmann-La Roche bv, Chefaro Nederland bv en Nutricia Nederland bv.

PREVENTION OF VITAMIN K DEFICIENCY IN INFANCY

EEN WETENSCHAPPELIJKE PROEVE OP
HET GEBIED VAN DE MEDISCHE WETENSCHAPPEN,
IN HET BIJZONDER DE GENEESKUNDE

PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD VAN DOCTOR
AAN DE KATHOLIEKE UNIVERSITEIT NIJMEGEN,
VOLGENS BESLUIT VAN HET COLLEGE VAN DECANEN
IN HET OPENBAAR TE VERDEDIGEN OP

DINSDAG 8 DECEMBER 1992
DES NAMIDDAGS TE 1.30 UUR PRECIES

DOOR

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Geboren op 8 december 1962 te Terheijden

1992
Drukkerij Benda BV, Nijmegen

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CIP-GEGEVENS KONINKLIJKE BIBLIOTHEEK, DEN HAAG

Cornelissen, Elisabeth Adriana Maria

Prevention of vitamin K deficiency in infancy / Elisabeth Adriana Maria Cornelissen. - [S.l. : s.n.]. - Ill.
Proefschrift Nijmegen. - Met lit. opg. - Met samenvatting in het Nederlands.
ISBN 90-9005406-5
Trefw.: vitamine K.

Aan mijn ouders

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ABBREVIATIONS

AC	Anticonvulsant(s)
ALAT	Alanine aminotransferase
AU	Arbitrary unit
BrdU	Bromodeoxyuridine
CA	Chromosome aberrations
ch	Chapter
DTT	Dithiothreitol
EIA	Enzyme-immuno asssay
Factor II	Prothrombin
Factor VII	Proconvertin
Factor IX	Haemophilia B factor, Christmas factor
Factor X	Stuart-Prower factor
Gla	y-carboxyglutamic acid residue
Glu	Glutamic acid residue
G-P	Gla-containing proteins
HPLC	High-performance liquid chromotography
HDN	Haemorrhagic disease of the newborn
i.m.	Intramuscular(ly)
IS	Internal standard
i.v.	Intravenous(ly)
K_1	Vitamin K_1, phylloquinone, phytomenadione
K_2	Vitamin K_2, menaquinone
K_3	Vitamin K_3, menadione
KH_2	Vitamin K hydroquinone
KO	Vitamin K-2,3-epoxide
MK-*n*	Menaquinone-*n*
n	number of samples
NMTT	N-methyl-thiotetrazole
NS	Not significant ($p>0.05$)
PIVKA	Proteins induced by vitamin K absence
PIVKA-II	Des-carboxylated prothrombin
p.o.	Per os, oral(ly)
PPSB	Prothrombin, Proconvertin, Stuart and Haemofilia B factors
PT	Prothrombin time
PTT	Partial thromboplastin time
RDI	Recommended dietary intake
Ref	Reference
SCE	Sister chromatid exchanges
TT	Thrombotest

CHAPTER 1

INTRODUCTION

1. INTRODUCTION

1.1 HISTORY

The discovery of vitamin K was the result of serendipity. In 1929 Dam observed that chicks fed on a lipid-free diet developed a haemorrhagic condition (1). A few years later he proposed that the active compound was a new fat-soluble anti-haemorrhagic vitamin that he called vitamin K (koagulation). At that time the belief was that the bleeding tendency of animals fed on vitamin K-deficient diets was solely caused by a lack of prothrombin. A few decades later factors VII, IX and X were discovered. In the meanwhile vitamin K_1 was isolated and characterized. The compounds isolated from putrefied fishmeal differed from vitamin K_1 in structure and properties, and were called vitamins K_2. For their discoveries of vitamin K_1 and K_2, Dam and Doisy received the Nobel prize in physiology and medicine (1). The role of vitamin K in the synthesis of clotting factors was elucidated in the 1970s with the demonstration that prothrombin contains a number of residues of γ-carboxyglutamic acid, a hitherto unidentified amino acid, (2). These residues are added to the hepatic precursor proteins in a vitamin K-dependent step. The molecular role of vitamin K is clear; it acts as a cofactor for an enzyme that carboxylates peptide-bound glutamic acid residues to γ-*carboxy*glutamic acid residues, see Fig. 1.1.

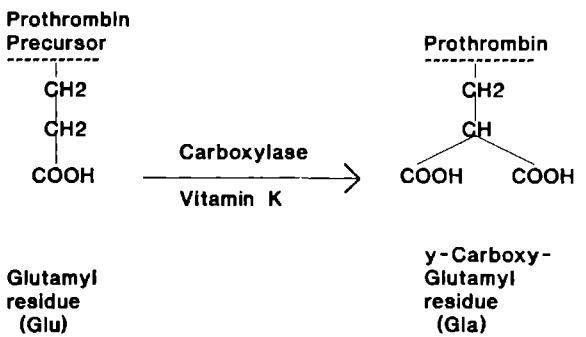

Fig. 1.1 Vitamin K-dependent carboxylation.

As early as 1894, Townsend reported that haemorrhages with a self-limiting and definite character occurred in newborns. He was the first to classify them all together under the name 'Haemorrhagic Disease of the Newborn (HDN)' (3). An infectious aetiology was assumed. After the discovery of vitamin K it was soon established that HDN was caused by a deficiency of vitamin K and that administration of vitamin K was effective in therapy as well as prevention of HDN (4). In 1961 the Committee on Nutrition of the American Academy of Pediatrics recommended to administer vitamin K prophylaxis to all newborn infants (5). Since then, many countries have adopted this recommendation, although controversies concerning the optimal dose, route and frequency of administration still exist.

1.2 STRUCTURE AND NOMENCLATURE OF VITAMIN K

The nomenclature of compounds possessing vitamin K activity has been modified a number of times. The term vitamin K is used as a generic descriptor of 2-methyl-1,4-naphtoquinone and all derivatives of this compound that exhibit an antihaemorrhagic activity in animals fed on a vitamin K-deficient diet. The naphtoquinone nucleus and 2-methyl group are essential. Without them anti-haemorrhagic activity is precluded. The nomenclature of some compounds is presented in Table 1.1; their structure is depicted in Fig. 1.2.

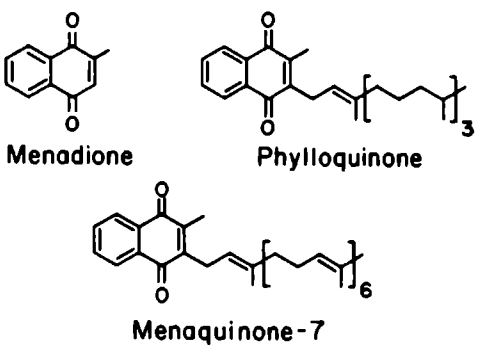

Fig. 1.2 Structure of vitamin K compounds

Table 1.1 Nomenclature of vitamin K. All compounds have a 2-methyl-1,4-naphtoquinone nucleus but differ in their side chain.

	Side chain	IUPAC[a]	Abbreviation
(I)	--	menadione	K_3
(II)	3-phytyl	phylloquinone	K_1 / $K_{1(20)}$
(III)	3-(prenyl)n^b	menaquinone-n^b	MK-n^b / $K_{2(n)}$[c]
e.g.	3-(prenyl)$_7$	menaquinone-7	MK-7 / $K_{2(35)}$

[a], according to the International Union of Pure and Applied Chemistry (6).
[b], *n* represents the number of prenyl units in the side chain.
[c], n represents the number of carbon atoms in the side chain.

I. Vitamin K_3 or menadione is not a natural vitamin K. It is more water-soluble than the other vitamin K compounds. *In vitro* menadione is not biologically active; *in vivo* it is metabolized to MK-4 (7). Because of the lack of a side chain, menadione can react with sulfhydryl groups of the erythrocyte membrane and can thus cause haemolysis and severe hyperbilirubinaemia (8).
II. Vitamin K_1 or phylloquinone is also called phytomenadione. Natural vitamin K_1 is the *trans* isomer at the 2'3' position of the phytyl side chain. *Cis* isomers are inactive. Konakion^R, which is the vitamin K_1 solution used for vitamin K prophylaxis in this study, contains both *trans* en *cis* vitamin K_1 in a ratio of 88:12 (9).
III. Vitamins K_2 or menaquinones are a series of vitamin K compounds with unsaturated side chains found in animal tissues and in bacteria. Menaquinones with up to 13 prenyl-groups have been identified, as well as several partially saturated kinds (10). The length of the 3-isoprenoid side chain and its saturation account for differences in biological activity. *In vitro* studies have demonstrated that menaquinones 2 to 6 show cofactor activity which is more or less comparable to that of vitamin K_1. Menaquinones with longer side chains are less active (11).

1.3 PHYSICAL AND CHEMICAL PROPERTIES OF VITAMIN K

Vitamin K_1 or phylloquinone is a yellow coloured oil at room temperature, while the menaquinones have melting points ranging from 35 to 60 °C. Phylloquinone has a molecular weight of 450.7 Da, MK-4 of 444 Da and MK-13 of 1056 Da. In normal light the vitamins are subject to ultraviolet decomposition, therefore handling should be performed in subdued lighting. Vitamin K is sensitive to alkali, but is relatively stable to heating and freezing. The oxidized forms of the K vitamins exhibit an ultraviolet

spectrum that is characteristic of the naphtoquinone nucleus. The vitamins have no native fluorescence, but the reduced hydroquinones are highly fluorescent. Vitamin K_1 is hydrophobic and insoluble in aqueous solutions. In serum it is transported by lipoproteins (12) and in milk in the lipid core of the milk fat globules (13).

1.4 SOURCES OF VITAMIN K AND RECOMMENDED DIETARY INTAKE

1.4.1 Adults

Sources in adults

Reported *vitamin K_1* contents of various commonly consumed foods vary widely. To what extent this variation is due to an actual variation in vitamin content of the product, or differences in bioavailability, or error in laboratory methods cannot be determined from the existing data. In general, green and/or leafy vegetables are the best source of vitamin K_1. For example, fruits and potatoes contain less than 10 μg/100g, while spinach and broccoli contain more than 100 μg/100 g (7). The vitamin seems stable in food processing and meal preparation.

To what extent *vitamin K_2* is important in adult vitamin K supply has not been established yet. Menaquinones account for some 90% of total hepatic stores of vitamin K (14, 15), but it is unknown whether these are the result of bacterial synthesis in the gut or due to food contamination (16).

RDI for adults

Recommended dietary intake (RDI) for adults is calculated to be about 1 μg/kg/day or 80 μg for men and 65 μg for women (17). Many other estimates, however, are reported; *e.g.* 0.5 μg/kg/day, 45 μg for men and 35 μg for women (18). A normal mixed diet consumed daily by a healthy adult has been estimated to contain an average of 200-400 μg of vitamin K_1 (19). Therefore, in normal healthy adults vitamin K deficiency is rare. Nevertheless, vitamin K deficiency has been reported in hospitalized patients on antibiotic therapy (20, 21, 22), and in postmenopausal women with osteoporosis (23, 24).

1.4.2 Infants

Sources in breast-fed infants

In neonates and young infants *vitamin K_1* supply is provided by milk-intake solely. Human milk is relatively low in vitamin K_1: the mean concentration is about 2 μg/l, while the mean for cows' milk is about 4.9 μg/l (25). Vitamin K_1 concentrations reported in human milk vary, due to differences in:

1. - Sampling procedure. The composition of milk varies with the method of expression (hand *v* electric). Contamination of menaquinones from skin bacteria must be avoided (26).
2. - Moment of sampling;
   a. day of lactation. Colostrum (0 - 7 days) seems to have a higher concentration of vitamin K_1 than mature milk (25, 27). However, since volumes of colostrum are limited, the total dose of vitamin K_1 ingested in the first week of life is small (13). Thereafter, vitamin K_1 concentration in human milk remains constant (13, 19), and since daily volumes of milk also remain constant after the first few weeks of lactation, vitamin K_1 intake per kg body weight decreases during growth (19).
   b. moment of the day. Many lipids show diurnal variation (26).
   c. moment during feeding. Hind milk is higher in vitamin K_1 than fore milk (27).
3. - Determination technique. Differences in extraction and detection method account for differences in sensitivity and specificity.

Some reported concentrations are represented in Table 1.2. The concentration cannot be predicted by dietary intake of vegetables or fat by the mother (13, 19). Oral administration of extra vitamin K_1 (100 μg-20 mg) to the mother increases the vitamin K_1 content of her breast milk significantly (13,19,25,27,28,29).

Table 1.2 Vitamin K_1 concentratrion in human milk (μg/l).

Author	(Ref)	mature milk	$(n)^a$	colostrum	$(n)^a$
Haroon 1982	(25)	2.1	(20)	2.3	(9)
Motohara 1984	(30)	3.8 ± 0.9	(337)	--	
von Kries 1987	(27)	1.2	(9)	1.8	(9)
Canfield 1991	(13)	2.9 ± 2.4	(60)	3.4 ± 2.7	(15)
Greer 1991	(19)	0.9 ± 0.5	(23)	0.6 ± 0.4	(10)

[a], number of samples studied

The amount of *vitamin K_2* in human milk is of no importance. MK-4 to 7 were detected in very low concentrations (31), although this could not be confirmed when sample contamination by skin bacteria was ruled out (26).

Sources in formula-fed infants

The minimum vitamin K_1 concentration for adapted infant formula is 4 μg/100 kcal, corresponding to 26 μg/l (32). Although an upper limit cannot be indicated with certainty, there is no reason to exceed 20 μg/100 kcal (100 μg/l) (33). According to manufacturers' statements, Dutch formulas (Nutrilon^R, Frisolac^R) contain about 7.5-10 μg/100 kcal (50-65 μg/l) vitamin K_1. In other words, the milk consumed by the Dutch breast-fed infants studied in this thesis is about 25 fold lower in vitamin K_1 content than the milk consumed by Dutch bottle-fed infants.

In neonates and young infants *vitamin K_2* is of limited importance. It takes some time before gut colonization by vitamin K_2 - producing bacteria is established. Correspondingly, menaquinones are not detectable in human liver until the age of 14 days (34). Intestinal flora of breast-fed infants is thought to be different from formula-fed infants, resulting in less production of vitamin K_2 (35). However, Benno *et al.* (36) noted that the numbers of *Bacteroides fragilis* and *Escherichia coli*, who are responsible for most of the vitamin K_2 production, were higher in the faeces of infants with vitamin K deficiency compared to faeces of healthy breast-fed infants. Therefore, vitamin K deficiency seems to occur regardless of the presence of menaquinone-producing bacteria in the gut.

RDI for infants

Determining vitamin K requirements for infants is difficult. The American National Research Council (17) recommends that infants up to 6 months of age receive 5 μg/day vitamin K, infants 6 to 12 months of age 10 μg/day, and older children 1 μg/kg/day. Since human milk provides approximately 2 μg/l vitamin K_1, breast-fed infants may ingest only 1 μg/day, which amounts only to a mere 20% of the RDI. In a longitudinal study, Greer *et al.* (19) measured a vitamin K_1 intake in breast-fed infants of approximately 0.1 μg/kg/day during the first 6 months of life. Since vitamin K_2 supply is minimal, the needs of the breast-fed human infant are scarcely met. Conversely, bottle-fed infants are nourished with more than 25 μg/day vitamin K_1, which is a rather generous intake related to the RDI of 5 μg. Greer *et al.* (19) calculated an intake of 8 μg/kg/day in formula-fed infants. Vitamin K_1 intake by formula-fed infants was approximately 100 times higher than by breast-fed infants.

1.5 PHARMACOKINETICS

1.5.1 Absorption

Vitamin K is absorbed by incorporation in mixed micelles from the intestine into the lymphatic system. Bile and pancreatic juice are required. With the addition of phosphatidylcholine the amount of solubilized vitamin K increases dramatically (37).

Thus, exogenous phospholipid may enhance assimilation of vitamin K_1. This may be important to improve preparations for oral vitamin K prophylaxis.

Vitamin K_1 is absorbed by an energy-dependent, saturable process from the proximal portion of the small intestine (38). Its resorption is not influenced by the presence of vitamin K_2 or K_3 molecules (38). Intestinal absorption of vitamin K_2 displays no saturation kinetics. It is absorbed by a passive non-carrier mediated process (38). However, whether or not menaquinones synthesized by colonic bacterial flora are absorbed remains controversial. Some authors conclude that vitamin K deficiency is rare in adults because of resorption of vitamin K_2 (38), while others suggest that the concentration of bile salts in the lower intestinal tract is not sufficient to allow physiologically significant absorption of menaquinones (39). Ichihashi *et al.* (40) reported that colonic absorption of radioactive MK-4 and 9 was very limited in rats.

Absorption in adults

Shearer *et al.* (39) and Blomstrand *et al.* (41) studied absorption of radioactive vitamin K_1 in normal adults and in patients with impaired fat absorption. Both studies indicated that absorption varies widely interindividually. Maximum absorption was calculated to be 80%.

Hagstrom *et al.* (42) reported that plasma concentrations of vitamin K_1 reach a peak in 8 hours after an oral dose and in 10 to 30 hours after an intramuscular injection.

Absorption in infants

Few studies on the intestinal absorption of vitamin K in the newborn have been reported. Lipids are the sole vehicle for fat-soluble vitamins. Infants absorb lipids inefficiently, with the preterm infant normally absorbing as little as 60 to 75% of intake. The full-term infant may absorb 85 to 90% of intake, but adult values (95%) are not achieved until about 4 to 6 months of age (43). Breast-fed infants have the advantage of human milk lipase, a nonspecific lipase which may support digestion of lipovitamins (44).

McNinch *et al.* (45) measured plasma concentrations of vitamin K_1 after oral doses of 1 mg phylloquinone, given either at birth or with the first feed. The absorption curve suggested that the peak plasma concentration was attained after 4 hours (median 73 ng/ml). Thereafter, levels gradually declined. Twenty-four hours after ingestion median plasma concentrations had fallen to values of about 30 ng/ml. There was no difference between the group who had received the vitamin directly after birth and the group who had ingested the vitamin with their first feed. As was the case in adults, absorption varied widely interindividually (45). In low-birth-weight infants an intestinal absorption rate of 30% has been reported (46).

After an intramuscular injection of 1 mg vitamin K_1 plasma concentrations were much higher (45). The peak median concentration was 1781 ng/ml at 12 hours. Twenty-four

hours after administration concentrations were still about 444 ng/ml. The higher concentrations after parenteral administration indicate an inefficient intestinal absorption of pharmacological doses.

In Japan, in contrast to most other countries, an oral syrup of menaquinone-4 is used for oral vitamin K prophylaxis instead of phylloquinone. Shinzawa *et al.* (47) reported that, similar to vitamin K_1, plasma concentrations of MK-4 varied widely after an oral dose of 4 mg MK-4. When plasma MK-4 concentrations were corrected for infant's weight, a significant negative correlation ($r=-0.32$, $p<0.01$) was found between the absorption index and plasma concentration of PIVKA-II, which is a biochemical sign of vitamin K deficiency (see chapter 2). These results suggest that individual differences in vitamin K absorption might contribute to vitamin K deficiency. Subclinical malabsorption may be a causative factor in late onset HDN, as confirmed by the reports of von Kries *et al.* (48) and Matsuda *et al.* (49). In the latter study, reduced plasma concentrations of 25-hydroxy-vitamin D and elevated bile acids were found in vitamin K-deficient breast-fed infants. This supports the concept of an impaired intestinal absorption of vitamin K caused by subclinical cholestasis as a predisposing factor for clinical vitamin K deficiency.

1.5.2 Transport

Plasma transport

Vitamin K_1 is transported by chylomicrons in the lymphatic system (41) and by lipoproteins in plasma (12). In adults, clearance of an injected dose of radioactive phylloquinone from plasma shows a two-exponential decline, with a first half-life of 20-30 min and a second half-life of 120-165 min (50, 51). The disappearance curve in newborns seems to be similar to that observed in adults (52). The volume of distribution (34 ml/kg) suggests that during the first decline vitamin K_1 is mainly distributed in plasma. During the second decline the mean volume of distribution (345 ml/kg) is similar to the extracellular volume (52). The plasma clearance was calculated to be 78 ml/kg in the first hour and 96 ml/kg during the next 5 hours.

Transplacental transport

Placental transport of vitamin K_1 requires a large maternal-fetal concentration gradient (53). A median maternal-cord ratio of 30:1 has been reported, being the highest ratio recorded for any fat-soluble vitamin (54). There is no correlation between paired maternal and neonatal plasma concentrations. Consequently, pharmacological doses of vitamin K_1 have to be administered to the mother to increase the fetal plasma concentration slightly. In addition, there is a lag before vitamin K_1 crosses the placenta; administration of a single oral dose of vitamin K_1 to pregnant rats resulted in peak maternal plasma concentrations after 2 hours and peak fetal concentrations after 8 hours (53). This is in accordance with the clinical finding that administration of vitamin

K_1 to the pregnant mother must be performed more than 4 hours before delivery to be beneficial in the prevention of symptoms of vitamin K deficiency in the neonate (55, 56).

Similar to vitamin K_1, there seems to be a placental barrier for vitamin K_2. While vitamin K_1 is barely detectable in cord blood, long chain menaquinones are undetectable. Only MK-4 can be detected in umbilical cord plasma (31). Administration of pharmacological amounts of MK-4 to pregnant women resulted in increased neonatal MK-4 plasma concentrations, hence MK-4 can also be applied for prenatal vitamin K prophylaxis (28).

1.5.3 Storage

Phylloquinone storage

Although phylloquinone is rapidly concentrated in the liver, it disappears quickly from this organ again. In rat liver peak concentrations lagged behind serum values by 1.5-3 hours, but then rapidly declined with a half-life of about 10 hours (57). Whole-body radiography has demonstrated that vitamin K_1 is concentrated by organs other than the liver: adrenal glands, lungs, bone marrow, kidneys and lymph nodes have this capacity too (58). Whether this distribution is related to a physiological function of the vitamin is not elucidated yet.

Hepatic stores of phylloquinone in neonates appear to be small. Vitamin K_1 was detected in livers of human fetuses as early as 10 weeks, at concentrations of 1-2 ng/g liver (fresh weight). Similar concentrations were measured in preterm (median 1.4 ng/g) and term (1.0 ng/g) neonates (14). These hepatic concentrations are significantly less compared to adult levels, which are reported to be 5.5 ng/g (14) or 12.7 ng/g (15). Thus, the ratio of adult versus neonatal hepatic levels is 5:1 or higher. From the liver weights, total liver stores of approximately 0.1 μg for term neonates, 8 μg (14) and 18 μg (15) for adults were calculated, respectively.

In a postmortem study, it was demonstrated that vitamin K prophylaxis by intramuscular injection raises liver stores dramatically (14). After injection of 0.5-1 mg vitamin K_1, values for hepatic stores increased from 0.1 μg to 20 μg in 10 hours. After 1 to 4 days stores were in the range of 50-200 μg. During these 4 days the proportion of the injected dose in the liver ranged from 1 to 52% (median 14%). Two infants who lived for 13 and 28 days, had hepatic stores of 24 and 15 μg, respectively (14). Thus, it seems that hepatic stores remain elevated for at least 2 weeks. Whether this is similar after an oral application is unknown.

Menaquinone stores

Besides vitamin K_1, different forms of vitamin K_2 (MK-4 to MK-13) can be detected in adult liver (29). Menaquinones even accounted for some 90% of total hepatic stores

of vitamin K on a molar basis (14, 15). In the fetus and neonate menaquinones were not detectable until the age of 14 days. Thereafter a gradual build-up was noticed (14, 29).

1.5.4 Metabolism and excretion

Shearer *et al.* (51) studied metabolism of radioactive phylloquinone in adults. About 20% of an intravenously injected dose was excreted in the urine and 30-40% was excreted in the faeces via the bile. Comparable results were achieved after oral administration (12). The vitamin is excreted as more polar metabolites. Phylloquinone is metabolized to various oxygenated derivatives, mostly by shortening the side chain to 5 or 7 carbon atoms, yielding carboxylic acids which are conjugated with glucuronic acid. Only a few of the oxidation products have been identified (39). It is unknown whether neonatal vitamin K metabolism differs from adult metabolism.

1.6 BIOCHEMICAL FUNCTION

1.6.1 Vitamin K-dependent coagulation proteins

Hemker *et al.* (59) were the first to state that a precursor-protein of prothrombin is synthesized in the liver cells, which is converted to prothrombin in a vitamin K-dependent step. When this conversion is inhibited, these precursor-proteins are shed in the blood and become detectable. They are called proteins induced by vitamin K's absence (PIVKA). The rapid appearance of normal prothrombin in the circulation when vitamin K is administered to severly hypoprothrombinaemic vitamin K-deficient patients, proves that a significant pool of precursor-protein is present in the liver cells and that these proteins can be converted to prothrombin following vitamin K administration (60). This response is insensitive to the protein synthesis inhibitor cycloheximide (61).

In 1974 the nature of the vitamin K-dependent modification was elucidated; the glutamic acid (Glu) residues of the precursor proteins are modifed to γ-carboxyglutamic acid (Gla; 3-amino-1,1,3-propanetricarboxylic acid) residues (Fig. 1.1) (2). All 10 Glu residues in the first 33 residues at the aminoterminal end of prothrombin are modified in this fashion, while in the remainder of the molecule the Glu residues are not carboxylated (61). The Gla residues confer metal binding properties on the vitamin K-dependent proteins. This allows them to undergo a conformational change in the presence of calcium ions, leading to the expression of membrane binding properties. In the presence of membrane-bound cofactors, the proteins assemble on membrane surfaces and act on membrane-bound substrates. Thus, the Gla rich domain of the vitamin K-dependent proteins allows for reversible calcium-dependent protein complex formation on cell surfaces (62). Proteins which are uncompletely carboxylated, i.e. the PIVKA's, are functionally defective.

All vitamin K-dependent coagulation proteins are synthesized in a precursor form that consists in a signal sequence, a propeptide and the mature protein (62). A schematic presentation of structural domains is shown in Fig. 1.3. The signal peptide serves to translocate the polypeptide to the rough endoplasmic reticulum for further post-translational modification, including signal peptide cleavage, propeptide cleavage, disulfide bond formation, glycosylation and γ-glutamylcarboxylation (61, 62). The propeptide designates specific precursor polypeptides for vitamin K-dependent carboxylation; in particular, the carboxylation recognition site on the propeptide binds tightly to the carboxylase-enzyme (62), see Fig. 1.4. For more information on function and structure of other regions the reader is referred to the literature (62,63,64,65,66).

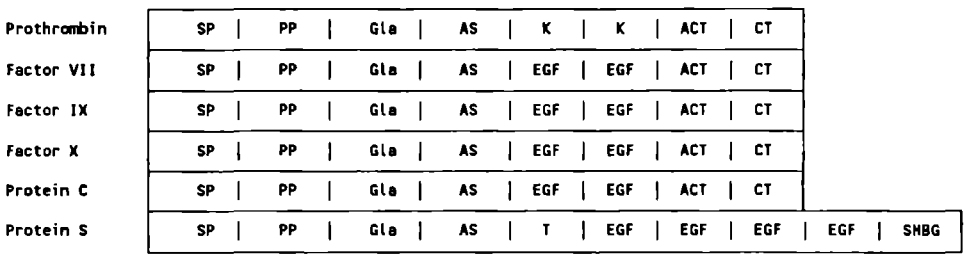

Fig. 1.3 Structural domains in the human vitamin K-dependent coagulation factors. SP, signal peptide; PP, propeptide; Gla, γ-carboxyglutamic acid-rich region; AS, aromatic amino acid stack; K, kringle domain; ACT, activation region; CT, catalytic triad; EGF, domain homologous to the epidermal growth factor precursor; T, thrombin-sensitive region; SHBG, domain similar to the sex homone binding globulin. Modified after Hessing (63).

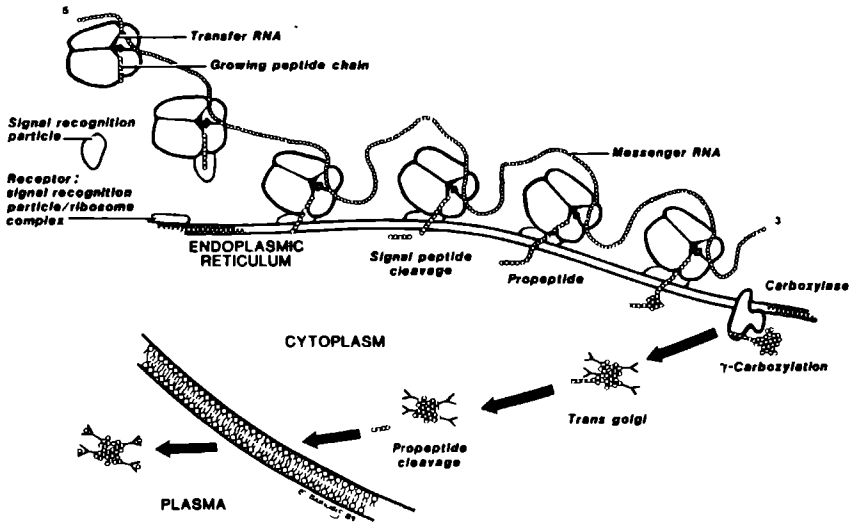

Fig. 1.4 Posttranslational carboxylation of vitamin K-dependent proteins during protein synthesis. The signal recognition particle binds to the signal peptide, leading to the formation of a ribosome-particle-messenger RNA complex on the endoplasmic reticulum. The signal peptide is translocated to the luminal aspect of the rough endoplasmic reticulum. After signal peptide cleavage, the propeptide is expressed on the nascent polypeptide chain. The propeptide, containing the γ-carboxylation recognition site, binds to the vitamin K-dependent carboxylase associated within the endoplasmic reticulum. Specific glutamic acids are converted to γ-carboxyglutamic acids, then the protein is transported to the Golgi apparatus where the propeptide is cleaved. The fully processed protein is secreted into the circulation. (From Furie & Furie (62), with permission).

Table 1.3 Vitamin K-dependent plasma proteins in adults.

Factor	II	VII	IX	X	C	S	Z
Plasma conc (mg/l)	90	0.5	4	6	4	30	1
Mol. mass (kDa)	72	50	57	59	62	71	55
Number of Gla residues	10	10	12	11	9	11	13
Half-life (h)	78	4	22	40	7	?	?

Data derived in part from (63, 66).

Some properties of the vitamin K-dependent plasma proteins are given in Table 1.3. These proteins have a crucial role in both the intrinsic and extrinsic pathway of coagulation, which is a cascade system of reactions, initiated upon exposure of blood to tissue factor or negatively charged surfaces, leading to the formation of thrombin and fibrin, Fig. 1.5. Protein S serves as a cofactor for activated protein C in the inactivation of coagulation factors Va and VIIIa. The function of protein Z is still unknown.

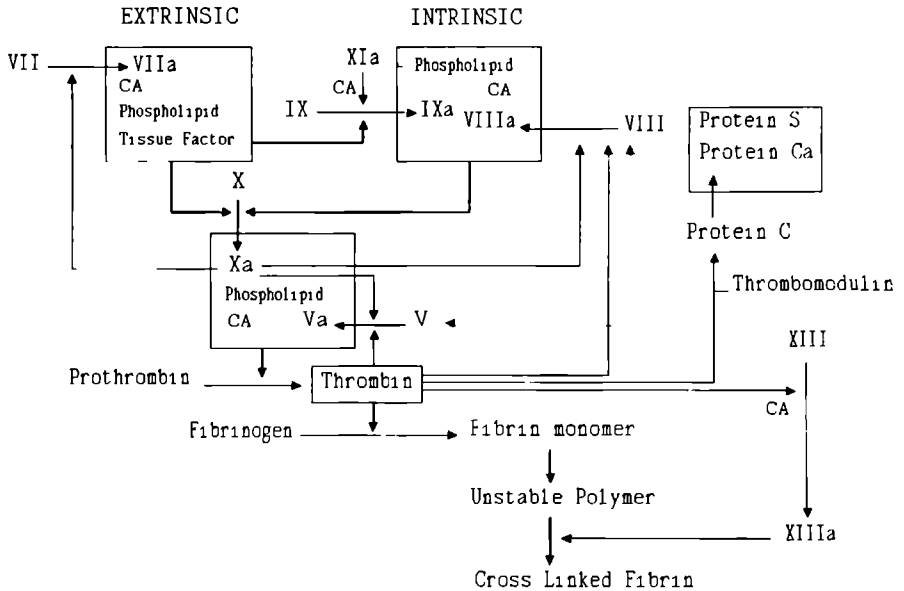

Fig. 1.5 The coagulation cascade. CA, calcium ions. Activated vitamin K dependent proteins (eg. VIIa, Xa) form complexes, as shown by boxes.
— activation, ... inhibition.

Vitamin K deficiency results in incomplete carboxylation of the coagulation proteins that no longer form complexes with calcium and phospholipids. A deficiency of functional factor II, VII, IX and X is most apparent, resulting in a generalized bleeding tendency.

1.6.2 Other vitamin K-dependent proteins

Vitamin K-dependent carboxylase belongs to the standard machinery of almost all types of eukaryotic cells (67). Therefore, it was to be expected that a wide variety of Gla-containing proteins would be discovered. Nevertheless, the number of well characterized Gla-proteins (G-P) has remained low until now. They include the plasma-Gla-proteins mentioned previously, and Gla-proteins found in bone and dentine (osteocalcin or bone-G-P, and matrix-G-P), in atherosclerotic plaques (atherocalcin or plaque-G-P), in urine and renal stones (nefrocalcin or urinary-G-P), in sperm, and in snake and snail venom (16, 64).

All Gla-proteins seem to be involved in calcium mediated processes. The importance of these proteins to the fetus is demonstrated by the so-called warfarin embryopathy, which occurs when a fetus is exposed to vitamin K antagonists during the first trimester of pregnancy. The defects are characterized by excessive calcification of the epiphyses and irregular growth of facial and long bones (68). Identical clinical features are observed in infants with congenital deficiency of vitamin K epoxide reductase (69). It is hypothesized that the bone defects are due to abnormalities in carboxylation of osteocalcin.

1.6.3 Carboxylase and vitamin K cycle

Carboxylase

As stated previously, vitamin K acts as a cofactor for the microsomal γ-glutamylcarboxylase, which γ-carboxylates Glu residues on the amino-terminal end of vitamin K-dependent proteins (Fig. 1.1). Some properties of this carboxylase enzyme are represented in Table 1.4 (70). The molecular mechanism of carboxylation is recently reviewed by Vermeer (64).

Table 1.4 Properties of vitamin K-dependent carboxylase.

Required:	vitamin K hydroquinone / vitamin K + NADH
	O_2
	CO_2
Stimulators:	dithiothreitol
	dexamethasone
Inhibitors:	chloro-vitamin K
	warfarin
Substrates:	precursors of vitamin K-dependent proteins
	pentapeptides, *eg.* Phe-Leu-Glu-Glu-Val/Leu

The vitamin K-dependent carboxylase is an integral membrane protein concentrated at the luminal side of the rough endoplasmic reticulum (62). A model of the interaction of the carboxylase with the precursor-peptides is depicted in Fig. 1.6.

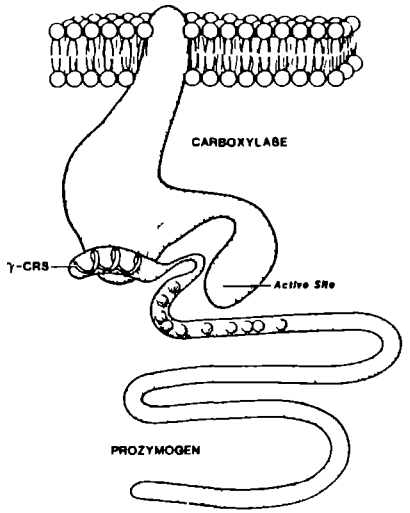

Fig. 1.6 Model of interaction of the vitamin K-dependent carboxylase with the precursor form of the vitamin K-dependent proteins. The carboxylase is an integral membrane protein. One domain includes the active site, responsible for the γ-carboxylation reaction. Another adjacent section of the extended substrate binding site is a complementary surface to the γ-carboxylation recognition site (γ-CRS) on the propeptide. (From Furie & Furie (62), with permission).

Vitamin K cycle

The energy to drive the carboxylation reaction is provided solely by oxidation of reduced vitamin K (KH_2) to vitamin K-2,3-epoxide (KO), see Fig. 1.7. Vitamin K epoxidase and carboxylase activities are coupled. Subsequently KO can be recycled to vitamin K (K) by vitamin K-epoxide reductase. This enzyme is dithiothreitol (DTT)-dependent *in vitro* and is inhibited by warfarin. The physiological counterpart of DTT has not been identified yet. The cycle is completed by reduction of K to KH_2 by a reductase. Several enzymes have been shown to be able to perform this reduction. One is DTT-dependent and sensitive to warfarin, hence it may be identical to vitamin K-epoxide reductase (64, 70). Other reductases are NAD(P)H-dependent and are relatively insensitive to warfarin. In other words, during coumarin anticoagulation the recycling of KO is blocked, and the supply of KH_2 is rapidly exhausted. However, exogenous vitamin K can still be reduced to KH_2 by the NAD(P)H-dependent reductases. Therefore, high doses of extra vitamin K can be used to bypass the blockade and act as an antidote to coumarin intoxication. Under these conditions KH_2 can only be used once, resulting in an accumulation of KO in the liver and plasma (16, 50).

NMTT-cephalosporins or NMTT (N-methyl-thiotetrazole) alone are also thought to be inhibitors of hepatic vitamin K-epoxide reductase (71) and hence can induce vitamin K deficiency.

The importance of the vitamin K-cycle being operative *in vivo* is demonstrated by the fact that on a molar basis urinary Gla-excretion exceeds the dietary intake of vitamin K 200-500 times. Each molecule of vitamin K must be recycled several thousand times before it is metabolized into inactive degradation products (16).

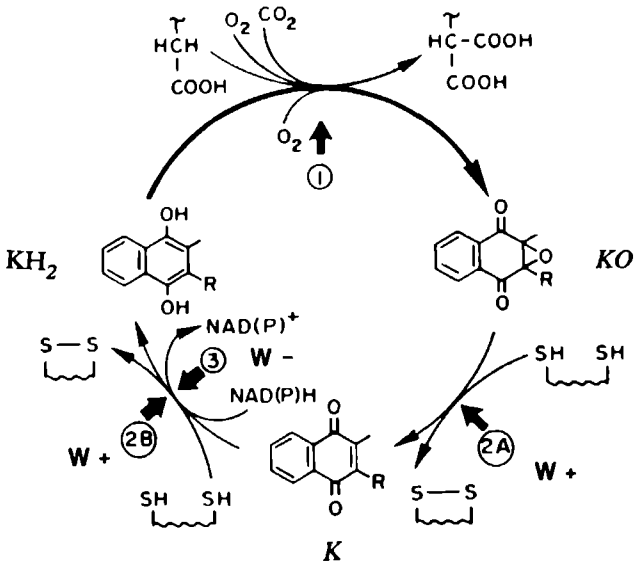

Fig. 1.7 Vitamin K cycle. Vitamin K hydroquinone (KH_2) is oxidated by vitamin K epoxidase (1) to vitamin K-2,3-epoxide (KO), while Glu is carboxylated to Gla. KO can be reduced by vitamin K-epoxide reductase (2A) in vitamin K quinone (K). This enzyme is Warfarin sensitive (W+). K can be reduced back to KH_2 by multiple reductases (2B/3).

Neonatal carboxylase-system

Wallin (72) reported data on maturation of the carboxylation system in fetal and neonatal rat liver microsomes. The system exhibited low γ-carboxylation activity before birth. Around the time of birth there was a 1.5 fold increase in the carboxylation of synthetic peptide Phe-Leu-Glu-Glu-Leu. In 2-day-old rats carboxylase activity dropped to 50% of activity measured at birth, but in 7-day-old rats activity had risen again to a level that was 2.5 fold higher than adult levels. This 'dip' in activity found in 2-day-old rats coincided with a similar dip for carboxylation of endogenous protein substrates and a lower prothrombin precursor concentration in the livers of these rats. Thus, all parameters measured suggest that a less efficient carboxylase system exists shortly after birth. This may be related to the prolonged prothrombin time 2-3 days after birth in human neonates. At birth vitamin K-dependent coagulation factors are about 40-60% of adult values (73), due to immature production. However, the presence of PIVKA-II in neonatal blood cannot solely be due to hepatic immaturity, as administration of vitamin K at birth resulted in a significant decrease in the detection of PIVKA-II at the age of 3 and 5 days (74). A combination of vitamin K deficiency and hepatic immaturity must be responsible for the appearance of PIVKA-II in neonatal blood.

While carboxylase activity reached adult levels in 7-day-old rats, the activities of the two pathways that provide carboxylase with reduced vitamin KH_2 cofactor (vitamin K reductase and vitamin K-epoxide reductase) were never as high as in adult liver (72). For this reason, it might be hypothesized that an increased requirement of vitamin K exists in early infancy, due to immature re-utilization of vitamin K-epoxide.

Wallin also reported that dexamethasone stimulated carboxylase activity 6 days after injection of the drug in newborn rats (75). Currently, the available knowledge about regulation of the carboxylase system is limited.

1.7 CLINICAL MANIFESTATIONS OF VITAMIN K DEFICIENCY - HAEMORRHAGIC DISEASE OF THE NEWBORN

Vitamin K deficiency in infancy can cause haemorrhagic disease of the newborn (HDN), of which three patterns have been differentiated: early HDN, classic HDN and late HDN (see Table 1.5) (76, 77);

1.7.1 Early haemorrhagic disease of the newborn

Early haemorrhagic disease occurs at the time of delivery or during the first 24 hours after birth. They are often life-threatening. The extent of bleeding varies from skin bruising or umbilical bleeding to widespread and fatal intracranial, intrathoracic and intra-abdominal haemorrhage. This disease is almost confined to neonates whose mothers have taken drugs that affect vitamin K metabolism (78,79,80). It was hypothesized that these drugs, *eg.* barbiturates, phenytoin, carbamazepine, isoniazid and rifampicin, cause induction of microsomal enzymes in the fetal liver and hence accelerate

degradation of vitamin K (81). The exact risk for this complication is unknown. Mountain *et al.* (82) reported clinical bleeding tendency in 12.5% of fullterm neonates born to mothers on anticonvulsants, while 50% had abnormal coagulation tests. In the survey of Deblay *et al.* (83), 7% of neonates exposed antenatally to anti-epileptic drugs had severe internal bleedings and 27% had subnormal prothrombin assays.

Table 1.5 Classification of haemorrhagic disease of the newborn.

	Age	Common bleeding site	Cause
Early HDN	0-24 h	Cephalhaematoma Umbilical Intracranial Intrathoracic Intra-abdominal Gastrointestinal	Maternal drugs coumarins anti-epileptics antibiotics Idiopathic
Classic HDN	2-7 d	Skin Nasal Gastrointestinal Circumcision	Idiopathic Maternal drugs
Late HDN	1-52 wk	Intracranial Skin Gastrointestinal	Idiopathic Secondary diarrhea malabsorption

1.7.2 Classic haemorrhagic disease of the newborn

Classic HDN usually presents itself on the 2nd to 7th day of life with gastro-intestinal, nasal, skin and circumcision bleeding. Most cases are idiopathic. Breast-feeding is an important pathogenetic factor (84). Estimates of the incidence of classic HDN prior to the initiation of routine vitamin K prophylaxis vary widely. An incidence as high as 1.7% in full-term infants has been reported (84). Other estimates are somewhat lower, ranging from 1:200 to 400 in term neonates (76).

1.7.3 Late haemorrhagic disease of the newborn

Late HDN is observed after the first week of life, predominantly during weeks 3 to 8. Intracranial haemorrhage accounts for at least half of the bleeding episodes (77, 85). Mortality is more than 30% and those who survive frequently suffer from severe

neurological sequelae. In one series, vitamin K deficiency was the major cause of intracranial haemorrhage in infants after the first week of life, leading to death or severe neurological sequelae in 75% of these patients (86). Late HDN is almost confined to exclusively breast-fed infants (77, 85, 86). The male : female ratio is approximately 1:2 (85). The incidence is particularly high in the Far East. In a survey in Japan the incidence of late HDN was found to be 1:3500 in unselected infants and 1:1700 in breast-fed infants (86). In the former Federal Republic of Germany an incidence of 1:27,000 infants who had not received vitamin K prophylaxis at birth was calculated (85). In the British Isles a recent two year prospective study revealed an incidence of 1:22,000 (87).

Late HDN may be idiopathic or occur as a secondary manifestation of an underlying disorder, like diarrhea, cystic fibrosis, biliary atresia, α_1-antitrypsin deficiency, hepatitis, a-beta-lipoproteinaemia, coeliac disease, and chronic exposure to coumarin-derivatives (76). In 550 case-reports, 189 cases (34%) had other risk-factors, including vomiting, jaundice and antibiotic therapy (88). HDN may be the initial manifestation of serious underlying disease (76). Aetiology of idiopathic late HDN is unknown. Some contributing factors previously discussed are:

- dietary deficiency of vitamin K (1.4.2)
- subclinical fat malabsorption (1.5.1)
- disturbed bacterial gut colonisation (1.5.1)
- diminished liver stores of vitamin K (1.5.3)
- immature or impaired carboxylase system in the liver (1.6.3).

It is likely that a combination of different factors which reduce the ability of babies either to absorb or to utilize the vitamin are responsible for late HDN.

1.8 TREATMENT OF HAEMORRHAGIC DISEASE OF THE NEWBORN

An infant suspected of having vitamin K deficiency-haemorrhage should be treated immediately with 1 mg vitamin K_1 while awaiting laboratory confirmation. Except from conventional coagulation tests, PIVKA-II should be determined. Due to a long disappearance time PIVKA-II can be detected in plasma hours after correction of the deficiency and therefore can confirm diagnosis after treatment has been initiated. Even in the instance of minor bleedings vitamin K_1 should be administered, to prevent progression of the deficiency with the hazard of intracranial haemorrhage. When the deficiency is severe, it may be prudent to administer the vitamin intravenously, because intramuscular injection may be associated with haematoma formation and delayed absorption. Intravenous injection should be given slowly, since cases of anaphylactoid reactions after rapid injection have been reported in adults (89). The coagulopathy is usually corrected within a mere few hours, since the pool of noncarboxylated precursors is readily carboxylated and excreted after the resurgence of vitamin K (see 1.6.1). For life-threatening haemorrhage, parenteral administration of vitamin K_1 should be

followed by 10 to 20 ml/kg of fresh-frozen plasma, to increase the levels of vitamin K-dependent factors immediately. Administration of prothrombin complex concentrates (PPSB; concentrates of factors II, VII, IX and X) is rarely needed and has the potential risk of viral infection.
The use of vitamin K_1 (phytomenadione) in infancy is safe. Doses as high as 10 to 25 mg have not been associated with toxic symptoms or hyperbilirubinaemia (5). The use of menadione (vitamin K_3) or its water-soluble derivatives, however, is obsolete, since it may cause haemolytic anaemia and subsequent hyperbilirubinaemia and kernicterus in the neonate (8).

1.9 CURRENT CONTROVERSIES ABOUT PREVENTION OF VITAMIN K DEFICIENCY

1.9.1 Safety of vitamin K prophylaxis

Although no serious side effects have been reported so far despite widespread use of vitamin K, some concern exists about the innocence of vitamin K administration to young infants. Israels *et al.* (90) have reported that increased levels of vitamin K_1 enhance the mutagenic and carcinogenic effect of benzopyrenes in mice. They also reported an increase in sister chromatid exchanges (SCE) in lymphocytes after *in vitro* and *in vivo* exposure to vitamin K_1 (91). The authors concluded that maintaining vitamin K_1 at a cellular concentration just sufficient to meet the needs of the carboxylase system, presents a biological advantage to the fetus by reducing the risk of mutagenic events. Conversely, vitamin K_1 was found to be non-mutagenic in the Ames/ *Salmonella typhimurium* mutagenicity test (92). Golding *et al.* (93) unexpectedly discovered a statistical association between childhood cancer and vitamin K administration (among other drugs) in the first week of life. This concern with a possible genotoxic or carcinogenic risk of the administration of vitamin K during early life should not be neglected; further studies are needed. In chapter 3 this problem is discussed more extensively, in combination with own results of mutagenicity tests of vitamin K prophylaxis in the human neonate.

1.9.2 Classic haemorrhagic disease of the newborn

Vitamin K prophylaxis was introduced to prevent classic HDN and its efficacy has been established beyond doubt. Reduction in bleeding episodes (84, 94) as well as prevention of neonatal hypoprothrombinaemia (55, 94) have been reported after administration of 1-2 mg vitamin K_1 at birth. Abandonment of routine vitamin K prophylaxis and high rates of breast-feeding have led to a resurgence of HDN in the U.K. (95). Currently, many countries recommend vitamin K prophylaxis (96). Nevertheless, controversy still exists as to whether all neonates require prophylaxis. Some have suggested, partly with the aim of reducing costs, to recommend selective prophylaxis in those at risk: low-birth-weight infants, breast-fed infants, infants with inadequate food

intake, infants on antibiotics, infants needing surgery, etc. (97). Some children, however, develop vitamin K deficiency-haemorrhage without any perinatal risk-factor. In addition, a recent cost-benefit analysis revealed that the total costs for the society without a prophylaxis regimen exceed the costs for an oral or intramuscular (i.m.) prophylaxis programme (98). It was recommended to adopt the oral route as it is cheaper, is more consumer acceptable and has a lower risk of iatrogenic disease (98). Indeed, severe neonatal complications after accidental injection of maternal ergometrine and oxytocin have been reported (99). On the other hand, there is some debate whether oral and i.m. application are comparable. Studies of conventional coagulation tests, vitamin K_1 and PIVKA-II concentrations revealed that oral administration of vitamin K is as effective as i.m. injection in the prevention of classic HDN (45,55,94,100,101,102). Dunn (103) recorded no proven case of classic HDN in 31,000 newborns given 1 mg vitamin K_1 with their first feed. Whether oral vitamin K prophylaxis at birth is also capable of preventing late HDN will be discussed in the next paragraph.

1.9.3 Late haemorrhagic disease of the newborn

Whether prophylactic measures designed to protect against classic HDN are able to prevent late HDN has been questioned with respect to the short plasma half-life of vitamin K_1. The plasma half-life, however, is irrelevant with respect to liver stores, which have been reported to remain elevated for at least 2 weeks after an i.m. administration at birth (see 1.5.3). Clinical experience suggests that parenteral vitamin K prophylaxis at birth offers some protection against late HDN since most case-reports concern breast-fed children whithout prophylaxis (77). Nevertheless, several cases of late HDN have been reported despite vitamin K prophylaxis at birth (Table 1.6).

It seems to be that failures are more frequent after oral than after i.m. application of the vitamin at birth, but this must be corrected for the relative frequency of oral administration compared to i.m. injection. Hathaway *et al.* (104) compared oral and i.m. prophylaxes at the age of 4 weeks and concluded that a single dose of vitamin K prevents most instances of late HDN. However, vitamin K prophylaxis should prevent *all* cases of HDN. Isarangkura *et al.* (105) reported that 1 mg i.m. and 2 mg oral doses of vitamin K_1 increased mean Thrombotest values up to the age of 2 months, while the effect of a 1 mg oral dose was limited to 1 month. Others, however, dispute the need for any vitamin K supplementation (106).

Alltogether, the discussion about the best route of administration of vitamin K and whether repeated administrations are needed in breast-fed infants is still ongoing. Prospective studies evaluating different regimens of vitamin K prophylaxis are lacking. In this thesis 3 such studies are reported (chapters 4, 5, and 6) comparing oral and i.m. administration and evaluating a weekly and daily regimen of vitamin K prophylaxis for breast-fed infants.

Table 1.6 Failures of oral and i.m. vitamin K prophylaxis at birth, resulting in late HDN.

Author	(Ref)	Oral	I.m.
Verity 1983	(107)		3
Chaou 1984	(108)		1
Chanvitan 1986	(109)		1
Matsuzaka 1987	(86)	1	
Priestley 1987	(110)	1	
Hanawa 1988	(111)	18	
Sutor 1988	(112)	1	1
Tönz 1988	(113)	8	
Kries 1988	(114)	3	
Lange 1988	(115)	1	
Matsuda 1989	(49)	1	
Matsuzaka 1989	(116)	2	
Hanawa 1990	(117)	26	1
Göbel 1991	(118)	3	1
Sutor 1991	(85)	4	3
McNinch 1991	(87)	7	
Ekelund 1991	(119)	17	
Total cases		93	11

1.9.4 Early haemorrhagic disease of the newborn

More than 40 case-reports have been published on neonatal haemorrhage in infants born to mothers treated with anti-epileptic drugs which induce liver enzymes (79). Most of the neonates had not received vitamin K prophylaxis at birth, although some had (79). A fatal case despite prompt i.m. administration of vitamin K at birth and additional doses at the onset of bleeding, has been reported (120). Since women with epilepsy account for approximately 0.5% of all pregnancies (121), clear guide-lines for prevention of this disease should be available. Maternal ingestion of 20 mg vitamin K_1 during the last 2 to 4 weeks of pregnancy has been proposed (79,82,121) and was found to prevent neonatal hypoprothrombinaemia in a preliminary study (83). Alternatively, vitamin K_1 can be administered intravenously to the mother during labour (79,82,120). Complicating aspects of transplacental transport have been discussed in paragraph 1.5.2. Another suggestion for prevention of early HDN is to change the treatment of pregnant women with epilepsy to drugs that are not enzyme-inducive, such as clonazepam (79). In this thesis first an observational study is reported, to establish

the incidence of (biochemical) vitamin K deficiency in mothers and neonates exposed to anticonvulsant therapy (chapter 7), and secondly a case-control study evaluating the effects of daily supplementation of 10 mg vitamin K_1 during the last month of pregnancy (chapter 8).

1.10 AIM OF THE STUDY

Vitamin K deficiency is associated with HDN, which may be fatal or cause serious morbidity. After having studied the detection and incidence of vitamin K deficiency in normal infants (thesis Widdershoven, 122), our group was interested in the prevention of vitamin K deficiency. At that time vitamin K prophylaxis was not uniformly practised in the Netherlands or abroad due to many controversies.
The aim of this thesis is to provide some answers to the following questions concerning appropriate vitamin K prophylaxis for young infants:

1. Is the administration of 1 mg vitamin K_1 i.m. at birth genotoxic? (chapter 3)
2. Is i.m. administration more effective than oral administration? (chapter 4)
3. Does vitamin K prophylaxis at birth prevent late HDN? (chapter 4)
4. What is the optimal mode of vitamin K supplementation for breast-fed infants *after* the neonatal period, in order to prevent late HDN? (chapters 5 and 6)
5. Can early HDN be prevented by administration of vitamin K_1 to the mother prenatally? (chapters 7 and 8)

In other words, what is the best recommendation for prevention of vitamin K deficiency and thereby haemorrhagic disease of the newborn?

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CHAPTER 2

LABORATORY PARAMETERS FOR VITAMIN K DEFICIENCY

2. LABORATORY PARAMETERS FOR VITAMIN K DEFICIENCY

2.1 CONVENTIONAL COAGULATION TESTS

The diagnosis of vitamin K deficiency is suggested if decreased concentrations of vitamin K-dependent coagulation factors normalize after vitamin K administration. However, in full-term neonates normal levels of these factors are about 40-60% of adult values (1). In preterm neonates the levels are even lower, about 25-60% of adult values and they are further decreased when hypoxia or respiratory distress syndrome is present (2). After birth, concentrations of factor VII rise to near adult values in 5 days, but factors II, IX and X only reach adult values after 3 to 6 months. The vitamin K-dependent coagulation inhibitors proteins C and S are about 35% of adult values at birth. Protein C is still markedly low at 6 months of age. In clinical bleeding from vitamin K deficiency, levels of vitamin K-dependent clotting factors are usually below 15%.
Measurement of vitamin K-dependent coagulation factors or screening tests reflecting the levels of vitamin K-dependent factors such as prothrombin time (PT), partial thromboplastin time (PTT) and thrombotest (TT), are not sensitive enough to detect mild vitamin K deficiency (3). In this thesis, apart from TT and activities of factors VII and X, abnormal des-carboxy-prothrombin (PIVKA-II) and vitamin K_1 concentrations were used to assess vitamin K status.

Thrombotest
TT, which reflects activities of factors II, VII and X and is sensitive to PIVKA, was performed according to the method of Owren (4), using Thrombotest Reagents (Nyegaard & Co, Oslo, Norway).

Assays for clotting factors VII and X
Activities of factors VII and X were measured by chromogenic substrate assays, using commercially available test-kits (Coa-set FVII and Coatest FX, Kabi Diagnostica, Mölndal, Sweden). Determination of factor VII is based on a two-stage principle. In stage one factor X is activated to Xa via the extrinsic pathway by factor VII and Tissue Factor (Fig. 1.5). During this proces factor VII is completely converted to VIIa. In stage two the generated factor Xa hydrolyses the chromogenic substrate, thus liberating the chromophoric *p*-nitroaniline. The colour is read photometrically at 405 nm. The determination of factor X is based on a single step assay, in which factor X is activated by Russell's Viper Venom.

2.2 PIVKA-II DETERMINATION

The determination of uncompletely carboxylated coagulation factors (PIVKA) is suitable to detect biochemical vitamin K deficiency (3). Different methods are available to determine PIVKA-II. In one method the ratio between factor II activated by physiological activators (II_{act}) and factor II activated by viper's venom (II_{ag}) is used. Because the latter activates both normal and abnormal factor II, a clear difference, defined as ratio $II_{act} / II_{ag} < 0.86$, indicates vitamin K deficiency. Other methods use adsorption of native prothrombin, electrophoresis or antibodies specific to PIVKA-II. Widdershoven *et al.* (5) compared four methods to determine PIVKA-II and concluded that direct detection by monoclonal antibody was the most sensitive method. This method was found to be fine to study the incidence of subclinical vitamin K deficiency and the effect of different prophylactic regimens on this incidence (5).

PIVKA-II assay in this thesis

Monoclonal antibody against PIVKA-II was prepared by Motohara *et al.* (6). This antibody quantitatively reacts with PIVKA-II and does not cross-react with native prothrombin. The antibody has a higher affinity to PIVKA-II possessing less Gla residues. A sandwich enzyme-immuno assay (EIA) was established (Eitest mono P-II kit, Eisai Co, Tokyo, Japan). In this assay the wells of a polyvinyl microplate are coated with the monoclonal antibody. Standard antigen solutions and 100 μl of the test samples are added to the cups and incubated overnight at 4°C. After washing 100 μl of peroxidase-labelled antihuman prothrombin anti-IgG is added to the wells and incubated for 1 hour at 4°C. After washing again, peroxidase activity of the immunocomplexes fixed onto the cups is assayed with 2,2'-azino-bis-(3-ethyl-benzothiazoline-6-sulfonic acid) as a chromogen. After stopping the reaction the colour is measured by spectrophotometry at 405 nm.

The results are expressed in arbitrary units (AU); 1 AU corresponds to 1 μg of purified prothrombin. The limit of detection is the PIVKA-II level obtained when the difference between the absorbance of the specimen and the blank is 15 mAbs. The detection limit was 0.10 AU/ml. Recovery of PIVKA-II added to normal plasma was 101 ± 9%.

PIVKA-II levels in 50 healthy adults, 13 pregnant women and 67 adults with liver cirrhosis were all below 0.10 AU/ml, which was considered as the upper normal limit (7). PIVKA-II values higher or lower than 0.10 AU/ml were defined as positive or negative, respectively (7). In three infants with intracranial haemorrhage caused by vitamin K deficiency, PIVKA-II amounted to very high levels of about 50 AU/ml (6). In adults receiving warfarin PIVKA-II levels ranged from 3 to 43 AU/ml (6).

The plasma half-life of PIVKA-II in adults was reported to be 60 hours (7, 8), though others reported a shorter half-life of about 20 hours (9, 10). This long disappearance time of PIVKA-II allows detection of vitamin K deficiency after the deficiency itself has been corrected.

2.3 VITAMIN K_1 DETERMINATION

By high-performance liquid chromatographic (HPLC) separation vitamin K_1 can be determined. Early studies used ultra-violet detection to quantify the vitamin. Recently, more selective and sensitive detection techniques have been developed, e.g. electrochemical reduction, mass spectrometric detection and fluorescence detection after electrochemical or chemical reduction.
In this thesis the method developed by Lambert *et al.* (11, 12) was applied, involving post-column chemical reduction and fluorescence detection. Vitamin $K_{1(25)}$, which was kindly supplied by Hoffmann-La Roche, was used as internal standard (IS). All sample manipulations were performed in the absence of daylight and by use of low actinic (brown) glassware to prevent isomerization and photodegradation of the K vitamins. Information about the equipment used is stated in paragraph 4.3.

Consecutive steps in the determination of trans-vitamin K_1 were as follows;
(see Fig. 2.1)

1. Addition of a defined amount of internal standard (IS).
2. Denaturation of proteins by ethanol.
3. Extraction of vitamin K_1 and IS by hexane.
4. Pre-separation on the first HPLC system by a silica column (100 x 4.6 mm, Microspher-SI, Chrompack, the Netherlands).
5. Collection of the effluent fraction containing vitamin K_1 as well as IS. Retention times of these vitamins were determined daily by injecting standards which could be monitored by UV detection. Fractions were usually collected between 3.0 and 5.5 min.
6. Separation on the second HPLC system by reversed-phase column (100 x 4.6 mm, Microspher-C18, Chrompack).
7. Chemical reduction. The reductive agent (tetramethylammoniumoctahydridotriborate) was already added to the eluent of the second HPLC system. On line post-column reduction was performed at the high temperature present in the knitted-coil-reactor situated in an oil-bath set at 85°C. Each day the first runs were performed with standards to check the reduction process.
8. Fluorescence detection at an emission-wavelength of 450 nm after excitation at 325 nm. An example of a chromatogram is depicted in Fig. 2.2.
9. Calculation of vitamin K_1 concentration by measurement of fluorescence peak-heigth of vitamin K_1 hydroquinone and correction for peak-height of

IS. The correlation coefficient between corrected peak-heights and known amounts of vitamin K_1 added to plasma or water is 0.9985. After calibration with known amounts of vitamin K_1, next equation was found for calculating vitamin K_1 concentrations:

$$\left(\left(\frac{\text{peak-height } K_1}{\text{peak-height IS}}\right) \times 100 \times 17.626\right) - 24.93$$

Recovery of vitamin K_1 added to normal serum in four different concentrations was 85 ± 5%. The minimal detection limit was 45 pg/ml at a signal-to-noise ratio of 3. However, the detection limit of individual samples can vary, dependent on the recovery of IS during extraction and on the amount of serum extracted. Within-day coefficient of variation was calculated 2.2 ± 1.0% (n=6, x=1250 pg/ml). Day-to-day coefficient of variation was 3.9% (n=24, x=1250 pg/ml).

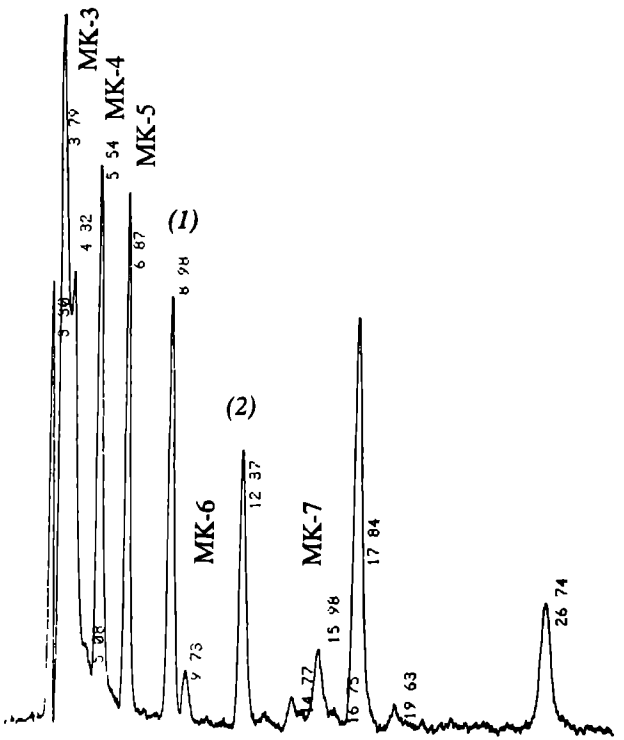

Fig. 2.2 Representative chromatogram of a serum extract. Peak identification: (1) vitamin $K_{1(20)}$, concentration 2657 pg/ml; (2) vitamin $K_{1(25)}$, used as internal standard.

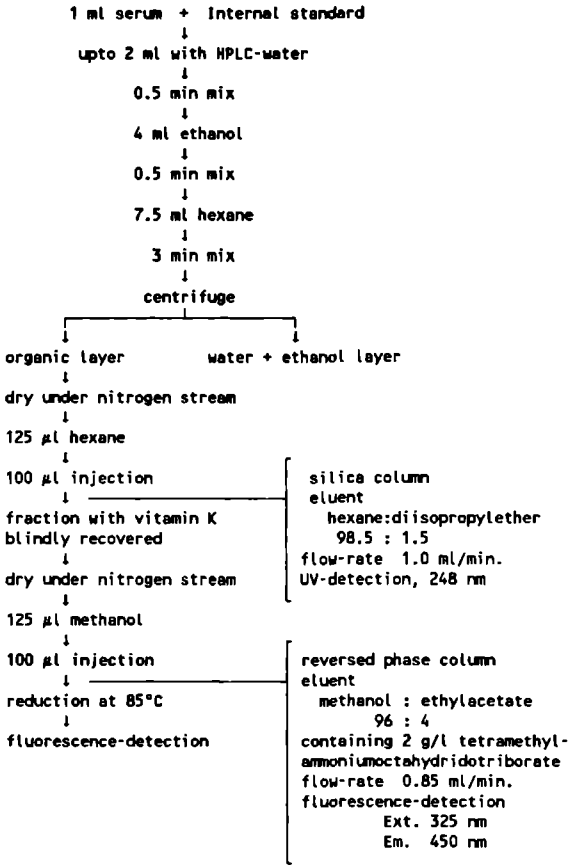

Fig. 2.1 Schematic representation of vitamin K_1 determination.

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CHAPTER 3

ANALYSIS OF CHROMOSOME ABERRATIONS AND SISTER CHROMATID EXCHANGES IN PERIPHERAL BLOOD LYMPHOCYTES OF NEWBORNS AFTER VITAMIN K PROPHYLAXIS AT BIRTH

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Pediatric Research 1991;30:550-553

3.1 ABSTRACT

In many countries vitamin K prophylaxis at birth is recommended in order to prevent bleeding in infants due to vitamin K deficiency. Because the incidence of clinical vitamin K deficiency is very low, such a vitamin K administration should be completely safe. However, an increase in sister chromatid exchanges in lymphocytes of fetal sheep 24 hours after injection of vitamin K_1 has been reported. Therefore, a study concerning genotoxicity of vitamin K_1 in man was conducted. Sister chromatid exchanges and chromosome aberrations were analysed in peripheral blood lymphocytes of 6 newborns 24 hours after intramuscular administration of 1 mg vitamin K_1 and in 6 control neonates. The mean number of sister chromatid exchanges per metaphase in the vitamin K group was 8.88 ± 1.22 as compared with 9.05 ± 1.14 in the control group (NS). The mean number of chromosome aberrations per 100 mitoses was 3.00 ± 2.61 in the vitamin K group and 2.50 ± 1.87 in the control group (NS). Vitamin K_1 plasma concentrations ranged from 115 to 1150 ng/ml (255 to 2555 x 10^{-9} M) in the supplemented group, a 5000-fold rise as compared with the control group ($p<0.01$). We did not find any evidence for genetic toxicity due to the administration of 1 mg vitamin K_1 intramuscularly to the newborn child.

3.2 INTRODUCTION

The problem of bleeding in infants due to vitamin K deficiency has been recognized worldwide. In many countries vitamin K prophylaxis at birth is recommended to prevent this hazard. Introduction of such a prophylaxis appeared to have spectacular results on the incidence of this so-called Haemorrhagic Disease of the Newborn (1). Because the incidence of clinical vitamin K deficiency is very low, this vitamin K supplementation should be completely safe. However, in 1987 Israels *et al.* (2) reported a dose-dependent increase of the frequency of sister chromatid exchanges (SCE) in lymphocytes after *in vitro* exposure to vitamin K_1. *In vivo* exposure, tested in five fetal sheep given 1 mg of vitamin K_1 i.v., also resulted in an increase in SCE 24 hours after injection. SCE represent interchanges of DNA at apparently homologous chromosomal sites after replication. These exchanges presumably involve DNA breakage and reunion. Although there is no perfect agreement between the ability of a compound to produce SCE and its mutagenicity or carcinogenicity, there is a high degree of correlation (3). Israels *et al.* (2) postulated that the relative vitamin K deficient state of the newborn may in fact play a role in the protection of the fetus *in utero* by reducing the risk of mutagenic events during this period of rapid cell proliferation. The question arises whether the results of the study of Israels *et al.* indicate a genotoxic or carcinogenic risk to man, specifically when the vitamin is administered during early life. Moreover, Golding *et al.* (4) reported a statistical association between childhood cancer and vitamin K administration

(among other drugs in the first week of life). This association was found unexpectedly and fitted no prior hypothesis. The need for further research was stressed by the authors. To our knowledge, cytogenetic studies concerning genotoxicity of vitamin K_1 in man have as yet not been performed.

Apart from SCE assays, another way of testing for cytogenetic mutagenicity is to analyse chromosome aberrations (CA). CA reflect damage of the chromosome- or chromatid-type and can be observed as breaks, acentric fragments, minutes, ring chromosomes, and gaps. Gaps are defined as regions in the chromatid structure that are achromatic, thus, the chromosome structure is not really discontinued (5). Because CA and SCE are two different cytogenetic end points of mutagenic action, they are complementary (6). Neither CA nor SCE have been studied before in human neonates exposed to vitamin K_1.
The aim of this case-controlled clinical trial is to determine whether the administration of 1 mg vitamin K_1 i.m. after birth influences the occurrence of SCE and CA in cultured peripheral blood lymphocytes.

3.3 METHODS

Subjects
The study was approved by the local medical ethical committee. Informed consent was obtained from the parents. Twelve healthy breast-fed newborns, born in our hospital, were recruited for this study. Selection criteria were delivery at term (37-42 wk), birth weight over 2500 g, and Apgar score at 5 min of seven or more. The mother could not be on a special diet, smoke, abuse alcohol, or take any medication during the last month of pregnancy, except for iron or folic acid. Arterial cord blood pH, mode of delivery, and medication of the mother during labour were recorded. All infants had an arterial cord blood pH over 7.24, except for subject 7, who had a pH of 7.17. All children were born vaginally, except for subject 1 who was born by Caesarean section. In two cases oxytocin was given to the mother during labour (subjects 3 and 11).
The infants were randomly divided into two groups; six received 1 mg vitamin K_1 i.m. (Konakion^R, Hoffmann-La Roche), the other 6 received no medication. Some characteristics of the neonates are given in Table 3.1. No item differed significantly between the groups. After 24 hours 5 ml of blood were drawn by aseptic venepuncture: 2 ml of heparinized blood and approximately 3 ml of blood with no additive. The latter was kept in the dark immediately after sampling, centrifuged (1000 x *g* for 5 min), and stored at -20°C until vitamin K_1 determination. All samples were coded to provide blind cytogenetic analysis.

Table 3.1 Study population characteristics (mean ± SD).

	Vitamin K	Control
Number	6	6
Male : female	2 : 4	3 : 3
Gestational age (wk)	39.5 ± 1.8	40.4 ± 0.7
Birth weight (g)	3338 ± 608	3817 ± 299
Apgar score at 5 min	8.8 ± 1.2	9.7 ± 0.5
Arterial cord blood pH	7.27 ± 0.03	7.29 ± 0.09
Age at venepuncture (h)	24.6 ± 0.4	27.8 ± 5.8

Vitamin K_1

Vitamin K_1 was measured in 1 ml serum samples by a two-step HPLC procedure, according to the method of Lambert *et al.* (7, 8). A few modifications were applied. The assays were performed with two Spectra Physics SP8800 systems (Spectra Physics, Santa Clara, USA) equipped with Rheodyne 7125 manual injectors (Rheodyne, Cotati, USA), with 100 μl sample loops. Vitamin $K_{1(25)}$ was used as an internal standard. The first HPLC step was used for pre-separation of vitamin K_1 and $K_{1(25)}$ on a microspher-Si column (Chrompack, Middelburg, the Netherlands). The locations of vitamin K_1 and internal standard were detected with a variable wavelength detector model 770 (Spectra Physics) set at 248 nm. Final separation and quantification were performed during the second HPLC step, using a microspher-C18 column (Chrompack) for separation and post-column chemical reduction with tetramethylammonium-octahydridotriborate for fluorescence detection (λ_{ex}= 325nm, λ_{em}=450 nm) with a Waters 470 fluorescence detector (Waters, Millipore Corp., Milford, USA). Concentrations were calculated from relative peak heights of vitamin K_1 *versus* known amounts of internal standard. The lower limit of detection was 0.045 ng/ml; however, the detection limit is dependent on the recovery of internal standard and the amount of serum extracted.

Cytogenetics

Peripheral lymphocyte cultures were initiated within two hours after blood collection. For each culture two containers with medium (8 ml RPMI 1640 medium, supplemented with 15% fetal calf serum, heparin, phytohemagglutinin, penicillin and streptomycin) were each supplied with 0.2 ml neonatal whole blood. From all infants two cultures were set up: one with and the other without bromodeoxyuridine (BrdU, final concentration 10 μg/ml). BrdU was present in the medium during the whole culture period. All cultures were grown in the dark at 37°C for exactly 72 hours. Colcemid (final concentration 0.2 μg/ml) was added 90 min before harvesting.

Chromosome spreads were made according to routine procedures. The standard cultures without BrdU were applied for aberration screening by one technician. From every child 100 mitoses were studied after conventional Giemsa staining and all CA were recorded and photographed. The cultures with BrdU were used for SCE studies. From every infant 25 complete mitoses were photographed in fluorescence microscopy after the chromosomes had been stained with acridine orange (9). In this way the number of SCE could easily be counted from the photograph.

Statistics

For statistical evaluation the unpaired *t* test was applied for study-population characteristics, SCE, and CA results. Vitamin K_1 concentrations were compared by Wilcoxon's two-sample test. All values are given as means ± SD.

3.4 RESULTS

The vitamin K_1 concentrations and the number of SCE and CA of each subject are presented in Table 3.2. The SCE results are missing for subjects 4 and 8 because of insufficient growth of BrdU cultures. Less than 20 mitoses were suitable for SCE analysis. Conventional growth was adequate and CA were counted. During determination of vitamin K_1 in subject 4, a technical error was encountered. Because of the small volumes of serum available from the neonates, the procedure could not be repeated.

Twenty-four hours after injection of 1 mg vitamin K_1, plasma concentrations ranged from 114.6 to 1150.5 ng/ml (255 to 2555 x 10^{-9} M). Vitamin K_1 concentrations in the supplemented group were raised some 5000-fold as compared with the control group ($p<0.01$). In two subjects of the control group, the vitamin K_1 concentration was not even detectable. Because of differences in volume of serum available, the detection limit ranged from 0.045 to 0.198 ng/ml in these samples.

In the vitamin K supplemented group, the mean number of SCE was 8.88 ± 1.22 per metaphase compared with 9.05 ± 1.14 in the control group. The groups did not differ significantly ($p=0.82$).

When the total number of chromosome and chromatid breaks as well as gaps was taken into account, a mean number of 3.00 ± 2.61 CA per 100 mitoses was detected in the vitamin K group. In the control group a mean number of 2.50 ± 1.87 per 100 mitoses was found. (NS, $p=0.71$). When gaps were excluded, the mean numbers amounted to 1.50 ± 1.05 and 2.50 ± 1.87, respectively. Still, no significant difference was present ($p=0.28$).

Table 3.2 Number of sister chromatid exchanges (SCE) and chromosome aberrations (CA) in metaphases of cultured lymphocytes of newborns who did or did not receive 1 mg vitamin K_1 i.m. after birth.

	SCE			CA[1]			Vit K_1
Subject	n^2	mean	SD	chrbr	chrmatbr	gap	(ng/ml)
VITAMIN K							
1	25	9.96	2.92	0	1	0	114.6
2	24	7.96	2.46	0	0	0	203.9
3	26	8.03	3.13	0	2	5	545.0
5	20	10.45	3.68	0	3	0	443.4
8	--	--	--	0	1	4	1150.5
11	24	8.00	2.67	2	0	0	757.4
mean	23.8	8.88	2.97	0.33	1.17	1.5	535.8
CONTROL							
4	--	--	--	0	2	0	--
6	20	10.30	2.92	0	6	0	0.188
7	21	8.52	2.69	0	1	0	<0.045
9	24	7.80	2.77	1	2	0	0.130
10	29	8.41	2.10	1	0	0	0.132
12	25	10.24	3.20	0	2	0	<0.198
mean	23.8	9.05	2.74	0.33	2.17	0	--

1, per subject 100 mitoses were screened.
2, number of cells investigated for SCE.
--, missing value (see text).
chrbr, number of chromosome-breaks; chrmatbr, number of chromatide-breaks.

3.5 DISCUSSION

As would be expected, 24 hours after the administration of 1 mg vitamin K_1 i.m. serum concentrations vitamin K_1 were extremely high, the mean being 536 ng/ml. This finding is in accordance with the reported mean values of 444 ng/ml by Mc Ninch *et al.* (10) and of 348 ng/ml by Lucock *et al.* (11). Mc Ninch *et al.* (10) detected a peak concentration of 1781 ng/ml after 12 h. This shows that the blood lymphocytes in the vitamin K group certainly have been exposed to extremely high

concentrations of vitamin K before sampling. Previous studies have shown that the plasma concentration declines rapidly (12).

Several short-term mutagenicity test sytems have been developed to identify agents which can adversely affect the genetic material of cells (The American Industrial Health Council, 13). This property may indicate the potential for a number of adverse effects, one of which is carcinogenicity. There is a clear and justifiable rationale for the use of genetic toxicology assays, but they are less than perfect. Accuracy, which is a combination of sensitivity and specificity, is about 60%. Therefore, one assay will be insufficient (13, 14). The Ames/*Salmonella typhimurium* assay is the test most commonly used for evaluation of chemicals *in vitro* (15). Vitamin K_1 was not mutagenic in this test (16). A negative Ames test, however, does not exclude carcinogenicity, inasmuch as a negative predictive value of mere 51% was calculated (14). In other words, performance of an Ames test alone is inadequate to determine whether vitamin K_1 is genotoxic. Moreover, Israels *et al.* (2) did report an increase of SCE in lymphocytes after *in vitro* and *in vivo* exposure to vitamin K_1. *In vitro* exposure was analysed in cultured lymphocytes of human placental blood and adult female blood with added vitamin K_1 at a concentration of 1×10^{-6} M (450 ng/ml). Consequently, the lymphocytes that were stimulated to replicate by phytohemagglutinin were exposed for at least 48 hours to a high concentration of vitamin K_1. This is quite different from *in vivo* situations, where high concentrations of vitamin K_1 are only temporary and lymphocytes normally do not divide. *In vivo* exposure was tested in fetal sheep *in utero* given 1 mg of vitamin K_1 i.v.. SCE were measured before and 24 hours after injection. Each fetus showed an increased number of SCE after vitamin K_1.

Because the aim of the present investigation was to determine whether there are indications that vitamin K may have genotoxic effects in the human neonate, we opted for an *in vivo* study in humans with the intention to reflect the clinical situation, especially because several unknown factors may hamper generalization of *in vitro* and animal studies to clinical practice. SCE and CA analyses were performed on cultured lymphocytes after exposure of the neonate *in vivo*. Accordingly, the vitamin will be subjected to the spectrum of activating and deactivating enzymes as far as these are developed in the newborn (17). Detoxification or formation of toxic degradation products might be different in fetal lambs as compared with human neonates. In fetal mice SCE response was found to decline with gestational age (18). Administration of mitomycin C, a mutagen that does not require metabolic activation, and of cyclophosphamid, a drug activated by the liver into a potent alkylating agent, both resulted in a decline in SCE with gestational age. Repair capacities may vary with gestational age.
Fortunately, no carcinogenic metabolites are reported for vitamin K_1. In the liver vitamin K is first reduced to vitamin K hydroquinone and subsequently oxidized to

vitamin K-2,3-epoxide. This oxidation is connected with the carboxylation reaction in which glutamic acid residues of vitamin K dependent coagulation factors are carboxylated. Subsequently, vitamin K-2,3-epoxide is converted back into natural vitamin K and the cycle is complete. In adults vitamin K_1 is excreted in urine and bile as more polar metabolites (carboxylic acids) conjugated with glucuronic acid. An enterohepatic circulation is present (19). After supplementation with vitamin K_1 urinary and faecal excretion is virtually complete after three days and amounts to about 60% of the dose (19). Whether fetal vitamin K metabolism differs from adult metabolism is unknown but conceivable.

Besides advantages, *in vivo* testing carries the problem that unknown confounding factors may disturb the conclusions. Age, sex, tobacco smoking, medication, and alcohol abuse may be confounders (20). In the present study these items were excluded or otherwise randomized. We were unable to detect an increase in SCE 24 hours after the administration of 1 mg vitamin K_1 i.m..
Because additional negative results were achieved in the CA analysis, which is a less sensitive but more specific short-term mutagenicity test (14), a genotoxic effect is improbable.

When the effects of supplementation with vitamin K are considered, not only plasma concentrations (and lymphocyte exposure) have to be taken into account. Concentrations in other tissues may differ. It might be possible that because of accumulation hepatic concentrations which are higher than plasma concentrations are reached. The hepatic concentration of vitamin K_1 in unsupplemented term neonates is reported to be about 1 ng/g liver (fresh weight) with a wide range (21). This is 10-fold higher than the plasma concentration of about 0.1 ng/ml in our control group. Shearer *et al.* (21) have reported hepatic vitamin K_1 concentrations 10 hours after i.m. administration of 0.5 to 1.0 mg vitamin K_1 of approximately 200 ng/g, and after 1 to 4 days of 500-2000 ng/g. These levels are comparable to the plasma concentrations in our supplemented group, so there are no indications that higher concentrations are reached in the liver than in the blood after a single i.m. dose of vitamin K_1.

In conclusion, as yet there is no evidence for cytogenetic toxicity due to the administration of 1 mg vitamin K_1 i.m. to the newborn infant.

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CHAPTER 4

EFFECTS OF ORAL AND INTRAMUSCULAR VITAMIN K PROPHYLAXIS ON VITAMIN K_1, PIVKA-II AND CLOTTING FACTORS IN BREAST-FED INFANTS

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Accepted Archives of Diseases in Childhood

4.1 ABSTRACT

A randomised clinical trial was conducted to establish the effects of oral and intramuscular administration of vitamin K at birth on plasma concentrations of vitamin K_1, proteins induced by vitamin K absence (PIVKA-II), and clotting factors. Two groups of about 165 healthy breast-fed infants who received at random 1 mg vitamin K_1 orally or intramuscularly after birth, were studied at the age of 2 weeks and 1 and 3 months. Although vitamin K_1 concentrations were statistically significantly higher in the intramuscular group, Thrombotest, activities of factors VII and X and PIVKA-II concentrations did not reveal any difference between the two groups. At the age of 2 weeks vitamin K_1 concentrations were raised as compared with reported unsupplemented concentrations and PIVKA-II was not detectable. At the age of 3 months vitamin K_1 levels were back at unsupplemented values and PIVKA-II was detectable in 11.5% of infants. Therefore, a repeated oral prophylaxis will be necessary to completely prevent (biochemical) vitamin K deficiency beyond the age of 1 month.

4.2 INTRODUCTION

Vitamin K deficiency is associated with haemorrhagic disease of the newborn (HDN). Three patterns of bleeding have been differentiated: early HDN within 24 hours after birth, classical HDN on days 1 to 7, and late HDN after the first week of life (1). Late HDN is often located intracranial. These haemorrhages may be fatal or cause serious morbidity. Breast-feeding has an important role in the pathogenesis of classical and late disease (1). Many countries recommend vitamin K prophylaxis after birth to prevent this hazard of vitamin K deficiency. Nevertheless, there are still controversies concerning the best way of providing an effective prophylaxis, resulting in different policies. The safety of oral and parenteral vitamin K prophylaxis in the prevention of classical HDN has been established beyond doubt, whereas the relationship between a single vitamin K prophylaxis at birth and late HDN has not been clearly determined (1, 2). Several studies have indicated that oral prophylaxis might be as effective as intramuscular (i.m.) administration, but these studies lack follow-up beyond the first week of life (3-5). Epidemiologically, i.m. vitamin K prophylaxis appears to have a lower incidence of failure (6-8), probably because of the more reliable absorption (4). Oral administration, however, has the appealing characteristics that the injury of injection is avoided and that administration is simple, resulting in better parental acceptance.

The aim of this study was to evaluate whether oral administration of vitamin K is as effective as i.m. administration in the prevention of vitamin K deficiency beyond the first week of life in breast-fed infants. Determination of proteins induced by vitamin

K absence (PIVKA-II) was applied to detect biochemical vitamin K deficiency. Vitamin K is necessary for production in the liver of coagulation factors II, VII, IX and X. The vitamin K-dependent carboxylation of glutamic acid residues to γ-carboxyglutamic acid residues promotes calcium binding to these proteins which is essential for effective haemostatic function. When carboxylation is impaired because of deficiency or antagonism of vitamin K, inert precursors of prothrombin (factor II) are detected in the blood (9). These are known as PIVKA-II. In formula-fed infants and adults PIVKA-II is not detectable, but it is found relatively frequently in breast-fed infants without vitamin K prophylaxis at birth (10). Although biochemical markers of vitamin K deficiency are only of limited value in assessing clinical relevance, they provide a most sensitive way to determine which group of infants is at risk for HDN.

4.3 SUBJECTS AND METHODS

Subjects

A total of 331 infants, born spontaneously vaginally at the University Hospital of Nijmegen or at home under midwife guidance, were enrolled. Inclusion criteria were; a gestational age of 37 wks or more, a birth weight over the 2.3rd centile, and an Apgar score of 7 or more at 5 min. The mother had to be healthy and not be taking vitamin K, anticoagulants, antibiotics or anti-epileptics. All mothers intended to breast-feed their child. After the parents had given informed consent, the neonates received vitamin K prophylaxis on the first or second day of life. The newborns were randomly allocated to one of the two treatment groups. One group (n=165) received 1 mg vitamin K_1 orally (1 mg/ml phytomenadione, Konakion^R, Hoffmann-La Roche). The other group (n=166) received 1 mg vitamin K_1 i.m. (Konakion^R 2 mg/ml). Some characteristics of the infants are represented in Table 4.1. No item was significantly different between the groups at start, nor at other moments of study (χ^2 and Student t test).

If the child was still exclusively breast-fed, blood was sampled at the age of 2 weeks and 1 and 3 months. In other words, when the mother stopped nursing follow-up was terminated. A sample of 5 ml of blood was drawn by venepuncture: 2 ml of citrated blood (in silicone coated tubes containing 10% (v/v) of sodium citrate 3.8%) and 3 ml of coagulated blood with no additive. After measuring blood coagulability by Thrombotest (TT) (Nijegaard & Co, Oslo, Norway), the citrated blood was centrifuged (5000rpm for 10 min) and the removed plasma stored at -70^{o}C until coagulation parameters were determined. The coagulated blood was protected from daylight immediately after sampling, centrifuged (3000rpm for 5 min) and the serum stored at -20^{o}C for vitamin K_1 determination. Samples from one child were assayed in one run. All samples were coded to provide blind analysis.

Table 4.1 Comparison of the two study-groups of healthy breast-fed infants (mean ± SD)

	Oral group	Intramuscular group
Number of infants	165	166
Sex (% male)	55.8	47.0
Gestational age (wk)	39.8 ± 1.3	40.1 ± 1.2
Birth weight (g)	3424 ± 439	3417 ± 440
Apgar score at 5 min	9.7 ± 0.5	9.7 ± 0.6
Arterial cord blood pH	7.26 ± 0.07	7.26 ± 0.07
Age at vit K administr (h)	19.9 ± 12.7	13.6 ± 13.5
Follow-up assessments:		
Age at 1st venepuncture (days)	14.1 ± 1.2	14.0 ± 1.3
Age at 2nd venepuncture (days)	30.5 ± 1.8	30.5 ± 1.8
Age at 3rd venepuncture (days)	88.6 ± 5.6	89.5 ± 5.6

Laboratory determinations

Activities of *clotting factors VII and X* were measured by chromogenic substrate assays, with substrate S2765 (Coa-set FVII kit, Kabi Diagnostica, Mölndal, Sweden) and substrate S2337 (Coatest FX kit, Kabi), respectively. These methods are not sensitive to PIVKA factors.

PIVKA-II was assayed by an enzyme-linked immunosorbent assay, using a monoclonal antibody previously described (Eitest mono P-II, Eisai, Tokyo, Japan) (11). This antibody quantitatively reacts with descarboxylated prothrombin (PIVKA-II) and does not cross react with native prothrombin. PIVKA-II concentrations are expressed in arbitrary units (AU)/ml, so that 1 AU corresponds with 1 μg of purified prothrombin. In severe vitamin K deficiency PIVKA-II can amount to more than 20 AU/ml. The detection limit of 0.10 AU/ml was used as the upper normal limit, defining PIVKA-II concentrations higher or lower than that value as PIVKA-II positive or negative, respectively.

Vitamin K_1 was extracted from 1 ml serum samples by a two-step high performance liquid chromatographic (HPLC) procedure, according to the method of Lambert *et al.* (12, 13). A few modifications were applied. The assays were performed with two Spectra Physics SP8800 systems (Spectra Physics, Santa Clara, USA) equipped with Rheodyne 7125 manual injectors (Rheodyne, Cotati, USA), with 100 μl sample loops. Vitamin $K_{1(25)}$ was used as an internal standard. The first HPLC step was used for preseparation of vitamin K_1 and $K_{1(25)}$ on a microspher-Si column

(Chrompack, Middelburg, Netherlands). Locations of vitamin K_1 and internal standard were detected with a variable wavelength detector model 770 (Spectra Physics) set at 248 nm. Final separation and quantification were performed during the second HPLC step, using a microspher-C18 column (Chrompack) and post-column chemical reduction with tetramethylammonium-octahydridotriborate for fluorescence detection (λ_{ex}=325nm, λ_{em}=450nm) with a Waters 470 fluorescence detector (Waters, Millipore Corp., Milford, USA). Concentrations were calculated from relative peak heights of vitamin K_1 *versus* known amount of internal standard. Recovery of vitamin K_1 from standard solutions added to normal serum was 85 ± 5%, with a detection limit of 45 pg/ml.

In all PIVKA-II positive samples and in about 30% of other samples *Alanine aminotransferase* (ALAT) was determined (normal <40 U/l).

Statistical analyses

χ^2, Student *t*, Mann-Whitney U, and Wilcoxon's one-sample tests and Spearman's rank correlation coefficient were used where appropriate.

The study was approved by the local medical ethical committee.

4.4 RESULTS

None of the infants had clinical symptoms of bleeding diathesis or severely disturbed coagulation parameters.
The decrease in number of infants studied during follow-up is caused by frequent stopping of exclusive breast-feeding before the age of three months. Some values are missing due to sampling errors. The number of samples studied is stated where different.

Results of *coagulation tests* are shown in Fig. 4.1. TT and activities of clotting factors VII and X revealed no difference between the two groups at any of the ages (M-W, $p>0.05$). As to be expected, a rise in activity between 14 and 30 days of age was found for these coagulation parameters (Wilcoxon, $p<0.01$).

Results of *PIVKA-II* determination are represented in Table 4.2. Two weeks after birth PIVKA-II could not be demonstrated in any of the 285 infants studied. At 1 month PIVKA-II was detectable in 4 out of 262 infants; 1 in the i.m. group (0.8%) and 3 in the oral group (2.2%). PIVKA-II concentrations ranged from 0.10 to 0.47 AU/ml (see Table 4.3). The difference in percentages of positive samples after oral compared with i.m. administration is not statistically significant (χ^2, $p=0.34$). The 95% confidence limits of the difference were -1.5 to 4.3%. At the age of 3 months

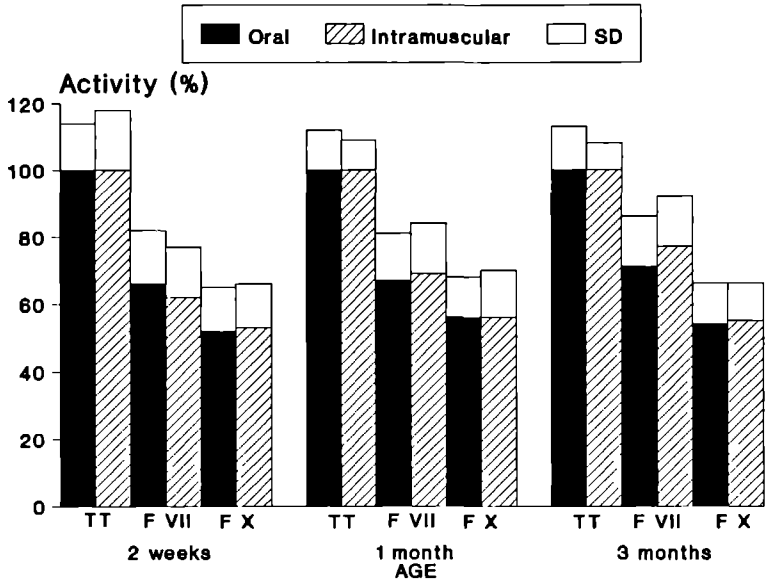

Fig. 4.1 Median (SD) of Thrombotest (TT) values and activities of clotting factors VII and X in breast-fed infants at the age of 2 weeks and 1 and 3 months after either oral or i.m. vitamin K prophylaxis at birth. Values are expressed as a percentage of normal adult pooled plasma.

Table 4.2 Presence of PIVKA-II ($\geq$ 0.10 AU/ml) in breast-fed infants of different ages after either oral or i.m. vitamin K prophylaxis at birth (n).

	TIME AFTER BIRTH		
	2 weeks	1 month	3 months
Oral group	0 (145)	3 (135)	7 (68)
Intramuscular group	0 (140)	1 (127)	8 (63)

PIVKA-II could be detected in 15 out of 131 infants; 7 in the oral group (10.3%) and 8 in the i.m. group (12.7%). The 95% confidence interval of the difference between the oral and i.m. group was -13.3 to 8.5% (p=0.67). PIVKA-II concentrations ranged from 0.10 to 0.32 AU/ml

Vitamin K_1 plasma concentrations decreased significantly during follow-up in both groups (Wilcoxon, p<0.001), see Fig. 4.2. At the age of 2 weeks the mean ± SD of 1608 ± 873 pg/ml in the i.m. group (n=64) was significantly higher than that of 815 ± 414 pg/ml in the oral group (n=74) (M-W, p<0.0001). At the age of 1 month the level was still significantly higher in the former group: 615 ± 272 pg/ml (n=84) *v* 391 ± 207 pg/ml (n=94); p<0.0001. Remarkably, at 3 months concentrations were still slightly different: 329 ± 186 pg/ml (n=57) *v* 268 ± 174 pg/ml (n=62); p=0.03. Plasma concentrations of vitamin K_1 did not correlate with sex, gestational age, birth weight, Apgar score, or arterial cord blood pH, nor with Thrombotest, activities of factors VII or X, or PIVKA-II concentration.

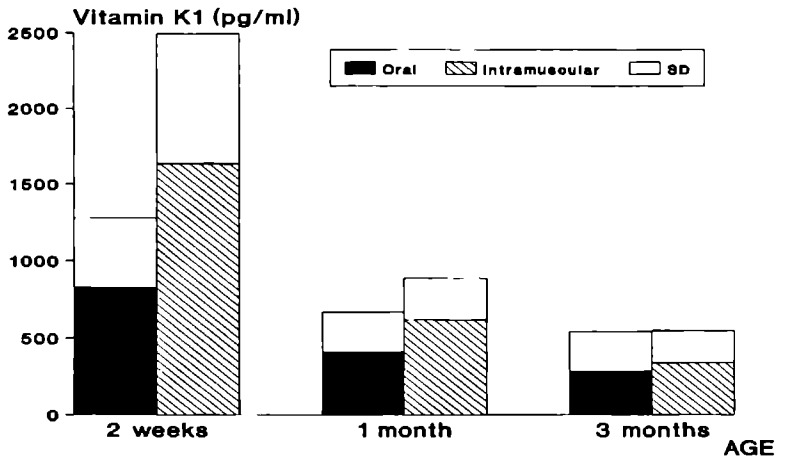

Fig. 4.2 Vitamin K_1 plasma concentrations in breast-fed infants at 2 weeks and 1 and 3 months of age after either oral or i.m. vitamin K prophylaxis at birth.

In Table 4.3 individual results of PIVKA-II, Thrombotest, factors VII and X, vitamin K_1, and ALAT determinations are shown for the infants positive for PIVKA-II. Values for TT and factors VII and X were not different from infants negative for

PIVKA-II. Similarly, vitamin K_1 concentrations were not extremely low. At the agee of 3 months mean ± SD vitamin K_1 concentration in the infants positive for PIVKA-II was 221 ± 74 pg/ml, compared with 312 ± 191 pg/ml in the infants negative for PIVKA-II (M-W, p=0.16). Results of ALAT determinations indicate that liver dysfunction is not a major cause for the appearance of PIVKA-II. No relevant correlations between the concentration of PIVKA-II and other parameters could be detected.

Table 4.3 Results of PIVKA-II, Thrombotest, clotting factors VII and X, vitamin K_1 and ALAT in the PIVKA-II positive infants.

Group	Age (mo)	PIVKA-II (AU/ml)	TT[a] (%)	F VII[a] (%)	F X[a] (%)	Vit K_1 (pg/ml)	ALAT[b] (U/l)
Oral	1	0.47	90	44	57	--[c]	21
Oral	1	0.16	70*	77	47	189	15
Oral	1	0.12	>100	60	43	243	6
I.m.	1	0.10	>100	74	51	436	23
I.m.	3	0.32	>100	99	52	190	20
I.m.	3	0.31	100	85	61	220	58
I.m.	3	0.29	>100	61	52	238	31
Oral	3	0.28	>100	70	47	140	16
Oral	3	0.22	>100	66	51	125	13
I.m.	3	0.19	>100	95	74	328	17
I.m.	3	0.14	65*	78	48	357	26
I.m.	3	0.14	>100	62	54	235	8
Oral	3	0.13	70*	74	48	126	20
Oral	3	0.12	57*	66	49	263	12
I.m.	3	0.11	>100	89	43	242	44
I.m.	3	0.11	>100	72	67	--	16
Oral	3	0.11	>100	89	46	184	11
Oral	3	0.10	>100	67	63	357	18
Oral	3	0.10	--	64	37	--	19

[a], TT (Thrombotest) and factors VII and X are expressed as a percentage of normal adult pooled plasma.

[b], ALAT (Alanine aminotransferase), normal value < 40 U/l.

[c], -- missing value due to insufficient volume of serum.

*, activity less than mean - 2 SD of PIVKA-II negative infants of that age.

4.5 DISCUSSION

Concentrations of vitamin K_1 in blood vary widely, according to the diet of the person and the determination method used. The reference interval reported for fasting adults, measured by a technique comparable with ours, ranges from 62 to 980 pg/ml (14). The reference interval for neonates and infants is still unknown. Mean plasma concentrations in healthy breast-fed infants of 1 month of age without vitamin K prophylaxis are reported to be 500 - 700 pg/ml (10, 15). Formula-fed infants have much higher concentrations of 3000 - 4500 pg/ml, because of the relatively high concentration of vitamin K_1 in formula (60 µg/l) compared with human milk (2 µg/l) (10, 15). Mc Ninch *et al.* (4) reported vitamin K_1 plasma concentrations in breast-fed infants after 1 mg vitamin K_1 orally or i.m. at birth. In the oral group the peak median concentration occurred earlier, but was lower. In both groups vitamin K_1 concentrations declined rapidly, after 24 hours the mean was 23 ng/ml in the oral group and 444 ng/ml in the i.m. group. The present study demonstrates that after 2 weeks the vitamin K_1 concentrations are still raised; concentrations of about 1600 pg/ml after i.m. injection and of 800 pg/ml after oral administration were found. At the age of 1 month concentrations were declined to about 600 and 400 pg/ml, respectively. Other reports confirm that 4 to 6 weeks after an i.m. injection of 1 mg vitamin K_1, plasma levels are back at unsupplemented values (10, 16). Nevertheless, at all ages vitamin K_1 concentrations were in the oral group. To our knowledge, vitamin K_1 concentrations beyond the first week of life after a single oral prophylaxis were not reported before. To determine whether the lower vitamin K_1 plasma concentration after oral application also entails a worse protection against vitamin K deficiency, we have to compare coagulation parameters.

TT and activities of clotting factors VII and X revealed no difference between the two groups. However, these coagulation tests are not sensitive enough to detect biochemical vitamin K deficiency (17). Accordingly, TT and factors VII and X were not different between infants positive and negative for PIVKA-II. In contrast to vitamin K_1 plasma concentrations and activities of clotting factors, PIVKA-II detection is a more direct reflection of vitamin K-dependent carboxylation of clotting factors in the liver. As mentioned previously, detection of PIVKA-II has no clinical consequences, but it does indicate whether enough vitamin K has been available to carboxylate all vitamin K-dependent proteins. Prolongation of low vitamin K intake may lead to serious complications.

In cord blood, using the same method as we did, Motohara *et al.* (18) detected PIVKA-II in 21.5% of 102 samples. At the age of 3 to 5 days 50 to 60% of infants were PIVKA-II positive if they were breast-fed and had not received vitamin K prophylaxis at birth (18). At the age of 1 month still 12.3% were positive (19). So, biochemical vitamin K deficiency occurs rather frequently in unsupplemented breast-

fed infants. Widdershoven *et al.* (10) compared PIVKA-II concentrations in breast-fed and formula-fed infants. At the age of 4 days about 10% of both groups had PIVKA-II in their blood. At 1 month of age PIVKA-II was not detected in any formula-fed infant, compared with in 5.5% of breast-fed infants. At 3 months of age no formula-fed infant was positive, compared with 7.5% of breast-fed infants. Thus, formula-feeding seems an effective way to prevent the appearance of PIVKA-II in the blood of young infants, probably due to the high intake of vitamin K_1. PIVKA-II was not detectable in any of our infants of 2 weeks old. This corresponds with the still raised vitamin K_1 concentrations in both groups. Surprisingly, in 4 one-month-old infants PIVKA-II was detected. However, the concentration in the only positive infant of the i.m. group was at the limit of detection. So, in the i.m. group hardly any one-month-old infant had PIVKA-II detectable, while a few in the oral group had. Exact comparison of our PIVKA-II results with those of Motohara *et al.* (19) and Widdershoven *et al.* (10) is hampered by the fact that our method is more sensitive. Their detection limit amounted to 0.13 compared with 0.10 AU/ml in our study. If we applied their detection limit as a selection criterium just 2 one-month-old infants would remain positive, thus strenghtening the clinically relevant difference in PIVKA-II detectability between our supplemented and their unsupplemented one-month-old breast-fed infants (χ^2, $p<0.01$). Even without correction, PIVKA-II was less frequently detected in our i.m. group than in the unsupplemented infants of Widdershoven *et al.* (10), (1/127 *v* 4/73, $p<0.05$). Our oral group did not differ significantly from the unsupplemented infants of Widdershoven *et al.* (3/135 *v* 4/73, $p=0.21$). This demonstrates that i.m. vitamin K seems still effective at the age of 1 month, while oral vitamin K is not.

Surprisingly, at the age of 3 months a rather high percentage of children in both the oral and i.m. groups had PIVKA-II detectable, indicating that neither prophylaxes was completely effective by that age. Vitamin K_1 concentrations were declined to values of 300 pg/ml. Although vitamin K_1 concentrations in the i.m. group were slightly higher than in the oral group, both levels seem insufficient to prevent biochemical symptoms of vitamin K deficiency in all infants. Other reports confirm the reappearance of PIVKA-II after a single vitamin K administration at birth. Motohara *et al.* (18) reported a decrease in PIVKA-II detectability on the third and fifth day of life after a single oral dose of 5 mg vitamine K_2 at birth. At the age of 1 month, however, no significant reduction in PIVKA-II detectability was demonstrated unless a second oral dose was administered at the age of 14 days (19). Widdershoven *et al.* (10) detected PIVKA-II in none of 48 one-month-old infants, in 1 of 29 two-month-old infants, and in 1 of 23 three-month-old infants after the administration of 1 mg vitamin K_1 i.m. at birth.

We failed to detect an association between PIVKA-II and vitamin K_1 plasma concentrations. Vitamin K_1 concentrations were not different in children positive for

PIVKA-II compared with infants negative for PIVKA-II. This may be caused by the fact that PIVKA-II has a half-life of about 70 hours and hence can still be present in plasma when the vitamin K deficiency has been corrected (9). Moreover, due to frequent feedings, plasma concentrations of vitamin K_1 in infants vary widely. The plasma half-life of tritiated vitamin K_1 has been reported to be 120-150 min (20). Information about hepatic vitamin K stores in infants is limited. Shearer *et al.* (21) reported that in unsupplemented term neonates hepatic vitamin K_1 concentrations were no more than about 1 ng/g liver (fresh weight). Total liver stores amounted to 0.1 μg. In adults a much higher mean concentration of 5.5 ng/g was measured, resulting in total stores of 8 μg. When the newborn had been given an i.m. injection of vitamin K_1 (0.5-1.0 mg) endogenous hepatic values remained raised for at least one week. Besides vitamin K_1 different forms of vitamin K_2 (menaquinones 6 to 12) can be detected in the liver (21). In adults menaquinones even accounted for some 75-97% of total hepatic stores of vitamin K on a molar basis. In the neonate, however, menaquinones were not detectable until about 14 days post-partum. Thereafter a gradual build-up was indicated and adult concentrations were attained about one month after birth (21). However, the extent of vitamin K_2 utilisation remains controversial. Nevertheless, besides vitamin K_1 vitamin K_2 has to be considered when assessing vitamin K status. Altogether, the plasma vitamin K_1 concentration may not adequately represent the total amount of vitamin K that is available as a cofactor for the carboxylase enzyme in the liver.

Wallin (22) has reported evidence to maturation of the vitamin K-dependent carboxylation system in fetal-neonatal rats. At the age of 7 days adult values of carboxylase activity were reached. But activities of the two pathways that provide carboxylase with reduced vitamin KH_2 cofactor (vitamin-K-epoxide reductase and vitamin K reductase) were never as high as in adult liver. In other words, it might be possible that an increased requirement of vitamin K exists in early infancy, due to immature re-utilisation of vitamin-K-epoxide. Again, the vitamin K_1 reference interval for fasting adults cannot automatically be applied to (non-fasting) young infants. Moreover, individual difference in enzyme maturation and therefore individual difference in vitamin K requirement could exist.

To summarise, single oral or i.m. administration of 1 mg vitamin K_1 postnatally may not offer complete protection against late biochemical vitamin K deficiency. Correspondingly, except for one infant with classical HDN (23), most case reports of failures of vitamin K prophylaxis concern late HDN (1, 24). Epidemiologically, i.m. vitamin K prophylaxis appears to have a lower incidence of failure (6-8). In a recent survey in the British Isles, 27 cases of HDN were recorded (8). Seven of them occurred in spite of oral vitamin K prophylaxis at birth. No failures were recorded after i.m. administration, although there was uncertainty about i.m. vitamin K in one case. The relative risk for babies who had received oral vitamin K compared with

babies who had received i.m. vitamin K was 13:1. The relative risk without prophylaxis was 81:1 (8). A schedule of repeated oral doses was considered (25). Taking our results as well as epidemiological evidence into account, we suggest that for complete protection of breast-fed infants against late HDN, vitamin K administration should be repeated. Whether a monthly, weekly, or daily administered oral dose should be recommended, deserves further investigation.

Acknowledgments

This study was supported by grant 28-1563 of the Dutch 'Praeventiefonds'. We are indebted to all parents and infants for participation in this study; to A. Teeuwes for statistical assistence; to Eisai Ltd for kind supply of PIVKA-II assay kits and to Hoffmann-La Roche for donation of vitamin $K_{1(25)}$.

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CHAPTER 5

PREVENTION OF VITAMIN K DEFICIENCY IN INFANCY BY WEEKLY ADMINISTRATION OF VITAMIN K

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Accepted Acta Paediatrica

5.1 ABSTRACT.

Vitamin K prophylaxis has been developed to prevent classic haemorrhagic disease of the newborn (HDN). Single vitamin K administration after birth has been reported to fail, resulting in late HDN. The preventive effect of an oral administration of 1 mg vitamin K_1 repeated weekly during the first three months of life, was studied in 48 healthy breast-fed infants by determination of Thrombotest (TT), PIVKA-II and vitamin K_1 concentrations at the age of 4, 8 and 12 weeks. All infants showed normal TT values and PIVKA-II was not detectable. Vitamin K_1 concentrations were negatively correlated with the number of days elapsed since the most recent vitamin K administration. Six to seven days after the latest application mean levels were 1223, 927 and 748 pg/ml at the age of 4, 8 and 12 weeks, respectively. In conclusion, weekly administration of 1 mg vitamin K_1 offers complete protection against vitamin K deficiency and does not result in an accumulation of vitamin K_1 in the blood.

5.2 INTRODUCTION

Vitamin K deficiency can cause haemorrhagic disease of the newborn (HDN), of which three patterns have been differentiated: early HDN within 24 hours after birth, classic HDN in the first week of life and late HDN after the first week (1, 2). These haemorrhages may be fatal or may cause severe morbidity. Breast-feeding plays an important role in the pathogenesis of classic and late HDN (2).
Vitamin K prophylaxis was introduced to prevent classic HDN and its efficacy has been established beyond any doubt (3, 4). At the moment many countries recommend vitamin K prophylaxis after birth, but it is still controversial whether this also protects infants against late HDN (2). Clinical experience suggests that parenteral vitamin K prophylaxis at birth offers some protection against late HDN, since most case-reports concern breast-fed children who had not received vitamin K postnatally (2). Nevertheless, more than 100 cases of late HDN despite either oral or intramuscular vitamin K prophylaxis at birth, have been reported worldwide (5, 6, other references on request). Intracranial haemorrhage accounted for at least half of the bleeding episodes and the mortality rate was over 30%. In accordance with these case-reports, we demonstrated that a single administration of vitamin K_1 after birth did not completely prevent biochemical signs of vitamin K deficiency in breast-fed infants beyond the age of one month (7).

Vitamin K acts as a cofactor for the vitamin K-dependent carboxylase enzyme, which is involved in the production of coagulation factors II, VII, IX and X, and proteins C and S. The carboxylation of specific glutamic acid residues to γ-carboxyglutamic acid residues confers unique calcium binding properties upon these

proteins which are essential for an effective haemostatic function. When vitamin K is lacking, descarboxylated proteins which are not functional appear in the blood. These are known as proteins induced by vitamin K absence (PIVKA). The determination of PIVKA-II (descarboxylated prothrombin) is a sensitive way of establishing biochemical vitamin K deficiency (8). The detection of PIVKA-II in a single patient may not be associated with bleeding symptoms, but the assay is of value in defining groups of infants risking vitamin K deficiency.

In this chapter the results are reported of a study of 48 healthy breast-fed infants who received a weekly oral dose of 1 mg vitamin K_1 during the first three months of their lives. To determine the efficacy in the prevention of late vitamin K deficiency Thrombotest, PIVKA-II and vitamin K_1 concentrations were measured monthly. The results were compared with previous results of infants who had received a single oral or intramuscular vitamin K administration at birth (7).

5.3 SUBJECTS AND METHODS

This study was approved by the local medical ethical committee and all parents signed a written informed consent.
Forty-eight infants, born vaginally and spontaneously at the University Hospital Nijmegen or at home under midwife guidance, were recruited. Inclusion-criteria were as previously reported (7); a gestational age of 37 weeks or more, a birth weight over the 2.3rd percentile and an Apgar score of 7 or more at 5 minutes. Arterial cord blood pH was measured in 25 neonates and ranged from 7.13 to 7.39. Some characteristics of these infants, as well as of our previously reported infants without weekly supplements (controls), are shown in Table 5.1. None of the parameters differed statistically between the groups. The mothers were healthy as well and did not use vitamin K, anti-coagulants, antibiotics or anti-epileptics. They intended to breast-feed their child for at least three months.
After the parents had given informed consent, the infants received 1 mg vitamin K_1 orally (20 mg/ml phytomenadione, Konakion^R, Hoffmann-La Roche) on the first or second day of life. This administration was repeated weekly by the parents just prior to a feeding. When the solution was spat out the administration was repeated. Date and time of administration were recorded.

If the child was still exclusively breast-fed, blood was sampled at the age of 4, 8 and 12 weeks, if possible just before the next vitamin K administration. The mean ages at assessments were 29 ± 2.5, 57 ± 1.6 and 85 ± 2.6 days. Five ml of blood were drawn by venepuncture: 2 ml of citrated blood (in silicone-coated tubes containing 10% (v/v) of sodium citrate 3.8%) and 3 ml of coagulated blood with no additive. After measuring a Thrombotest (Reagents, Nijegaard & Co, Oslo, Norway) the

citrated blood was centrifuged (3500*xg* for 10 min) and the removed plasma was stored at -70°C until PIVKA-II assay. The coagulated blood was screened from the light immediately after sampling, centrifuged (1000*xg* for 5 min), and the serum was stored at -20°C for vitamin K_1 determination. Samples from one child were assayed in one run.

Table 5.1. Characteristics of study and control breast-fed infants.

	Study	Control
Number	48	331
Male (%)	46	51
Gestational age (wk)	39.8 ± 1.5	39.9 ± 1.3
Birth weight (g)	3381 ± 450	3420 ± 439
Apgar score at 5 min	9.5 ± 0.8	9.7 ± 0.6
Arterial cord blood PH	7.25 ± 0.06	7.26 ± 0.07

PIVKA-II concentrations were determined by an enzyme-linked immunosorbent assay previously described, using a monoclonal antibody (Eitest mono P-II, Eisai, Tokyo, Japan) (9). This antibody quantitatively reacts with descarboxylated prothrombin (PIVKA-II) and does not cross react with native prothrombin. PIVKA-II levels are expressed in arbitrary units (AU) per ml, so that 1 AU corresponds with 1 μg of purified prothrombin. In case of severe vitamin K deficiency PIVKA-II can rise to more than 20 AU/ml. The detection limit was 0.10 AU/ml.
Vitamin K_1 was determined by a two-step high performance liquid chromatographic (HPLC) procedure, according to the method of Lambert *et al.* (10, 11). Few modifications were applied, as stated elsewhere (7). The detection limit was 45 pg/ml.

For *statistical evaluation* Student *t* test was applied on infants' characteristics; Fisher's exact test on PIVKA-II results; Wilcoxon's one and two sample tests on vitamin K_1 concentrations. Spearman's rank correlation coefficient was used for correlation detection.

5.4 RESULTS

All infants remained healthy during follow-up. No side-effects of vitamin K treatment were reported. None of the infants showed a bleeding diathesis.
The lowest Thrombotest value measured was 55%. At all ages median Thrombotest values were 100%.
PIVKA-II concentrations were measured in 42, 41 and 43 infants at the age of 4, 8 and 12 weeks, respectively. No PIVKA-II was detected in any sample. Comparison with the control group revealed a statistically significant difference at the age of 12 weeks (Table 5.2). In fifteen breast-fed infants without weekly supplements PIVKA-II was detectable at the age of 12 weeks; in 7 out of 68 infants after oral and in 8 out of 63 after intramuscular administration of vitamin K_1 at birth (7).

Vitamin K_1 plasma concentrations depended on the number of days elapsed since the latest vitamin K_1 administration ($r=-0.50$, $p=0.000$, $n=115$). Even after considering this time dependency, concentrations varied widely, see Fig. 5.1. Samples obtained 6 to 7 days after the latest vitamin K administration showed mean values of 1223 ± 647 ($n=22$), 927 ± 504 ($n=32$) and 748 ± 352 pg/ml ($n=27$) at the age of 4, 8 and 12 weeks, respectively. The lowest level found was 278 pg/ml. Vitamin K_1 concentrations at the age of 4 weeks were significantly higher than at 8 and 12 weeks ($p<0.05$). Values at 8 and 12 weeks did not differ significantly from each other. There were definitely no indications of an accumulation of vitamin K_1 in the blood due to the repeated doses.
In the study group vitamin K_1 concentrations were significantly higher than in the control group. For the control group there are no data available at the age of 8 weeks; the mean levels at 4 and 12 weeks are shown in Table 5.2.

Table 5.2. Number of samples with PIVKA-II ≥ 0.10 AU/ml, and vitamin K_1 concentrations (mean ± SD) at the age of 4 and 12 weeks in weekly supplemented[1] and control breast-fed infants (n).

	PIVKA-II		Vitamin K_1	
AGE (wk)	4	12	4	12
Study group	0 (42)	0 (43)	1223 ± 647 (22)	748 ± 352 (27)
Control group	4 (262)	15 (131)*	497 ± 264 (178)#	297 ± 182 (119)#

[1], Samples obtained 6 to 7 days after the latest administration.
*, Study *v* control group: Fisher's exact test, $p<0.05$.
#, Study *v* control group: Mann-Whitney test, $p<0.001$.

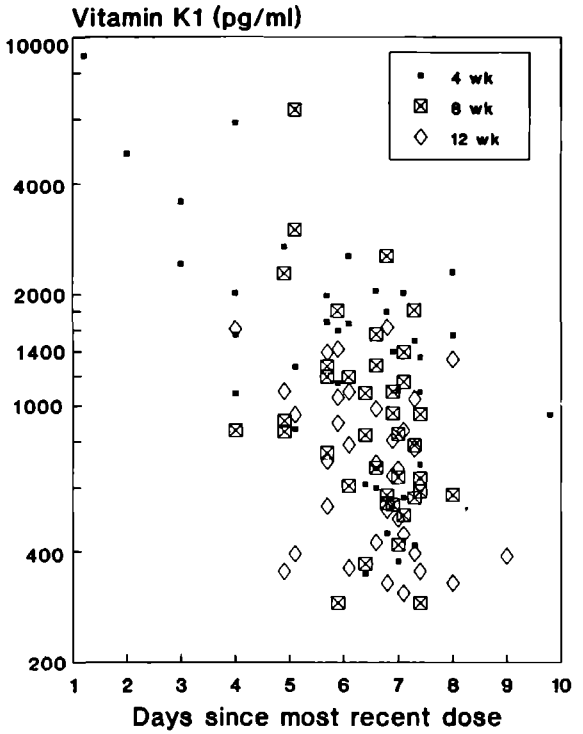

Fig. 5.1 Vitamin K_1 plasma concentrations at the age of 4, 8 and 12 weeks in relation to the number of days elapsed since the latest vitamin K administration, in 48 infants receiving an oral dose of 1 mg vitamin K_1 weekly.

5.5 DISCUSSION

In one-month-old unsupplemented healthy breast-fed infants mean vitamin K_1 plasma concentrations are reported to be 150 to 700 pg/ml (12-14). Formula-fed infants have much higher levels of 3000 to 6000 pg/ml (12-14). When a single oral dose of vitamin K_1 was administered at birth, after 2 weeks concentrations were about 800 pg/ml. From then the concentrations declined rapidly to unsupplemented levels (7). After an intramuscular injection concentrations at the age of 2 weeks were higher than compared with an oral dose (1600 pg/ml), but these concentrations also quickly declined to unsupplemented levels (7). In other words, a single administration of vitamin K_1 at birth has a limited effect on vitamin K_1 plasma con-

centrations beyond the neonatal period. Weekly repetition of the administration prevented the plasma concentration from diminishing to unsupplemented levels. Mean concentrations at the age of 4, 8 and 12 weeks - obtained just before the next administration - were about 1200, 900 and 750 pg/ml.

Despite a rather huge administration of vitamin K_1, plasma concentrations were moderate and still much lower than in formula-fed infants. This again illustrates the rapid disappearance of vitamin K_1 from the circulation. Tritiated vitamin K_1 was reported to have a plasma half-life of 120-150 min (15). Bottle-fed infants continually receive a small extra dose of vitamin K_1, thus the plasma level remains at a high level. The prescribed minimum concentration for formula is 26 μg/l vitamin K_1 (16), but they usually contain about 50 μg/l. The daily intake of formula-fed infants was reported to be about 50 μg vitamin K_1 (14). Unsupplemented breast-fed infants daily ingest less than 1 μg vitamin K_1 (14), while the recommended daily intake (RDI) amounts to 5 μg (16). Accordingly, in formula-fed infants PIVKA-II is not detectable (8) and haemorrhagic disease is almost confined to breast-fed infants (2). Prothrombin times were reported to be longer in three-month-old breast-fed infants than in formula-fed infants, even despite vitamin K prophylaxis at birth (14). Administration of 1 mg vitamin K_1 per week corresponds to approximately 150 μg per day. This is considerably more than the RDI and lower doses for supplementation might be equally effective.

Unfortunately, hepatic vitamin K_1 concentrations are unknown. However, as no PIVKA-II was detectable, a sufficient store of vitamin K must have been available for the carboxylation of prothrombin. As stated before, PIVKA-II was detected rather frequently in infants who had only received vitamin K_1 at birth (7). Thus, weekly oral administration of vitamin K_1 prevented the appearance of signs of vitamin K deficiency. This is in agreement with results of other groups. Motohara *et al.* (17) reported a decrease in PIVKA-II detectability on the third and fifth day of life following a single oral dose of 5 mg vitamin K_2 at birth. At the age of one month, however, no significant reduction in PIVKA-II detectability was demonstrated unless a second oral dose was administered at the age of 14 days (18).

To our knowledge this is the first report of vitamin K_1 and PIVKA-II blood levels determined in infants receiving weekly vitamin K supplementation. This regimen turned out to be reliable for the prevention of vitamin K deficiency in breast-fed infants. Current Dutch recommendations on vitamin K prophylaxis include oral or intramuscular vitamin K_1 administration (1 mg) at birth for all neonates, followed by weekly (1 mg) or daily (0.025 mg) supplementation of vitamin K_1 during a period of three months for breast-fed infants (19). The effect of daily administration is still to be evaluated. A special feature of these recommendations is that the administration

of vitamin K is performed by the parents. Since they want the best for their child, compliance is good.

Acknowledgements

This study was supported by grant 28-1563 of the Dutch 'Praeventiefonds'.
We are indebted to all parents and infants for their permission to perform this study; to Eisai Co Ltd for supplying PIVKA-II assay kits and to Hoffmann-La Roche for the donation of vitamin $K_{1(25)}$.

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CHAPTER 6

EVALUATION OF A DAILY DOSE OF 25 μg VITAMIN K_1 TO PREVENT VITAMIN K DEFICIENCY IN BREAST-FED INFANTS

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Accepted J Pediatric Gastroenterology and Nutrition

6.1 ABSTRACT

Vitamin K prophylaxis is recommended to prevent the hazard of haemorrhage caused by vitamin K deficiency in young infants. A single administration after birth seems inadequate to completely prevent late haemorrhagic disease in breast-fed infants. The preventive effect of a daily oral dose of 25 μg vitamin K_1, which is comparable to about half the dose ingested by formula-fed infants, was evaluated in 58 breast-fed infants. No clinical or biochemical signs of vitamin K deficiency occurred; PIVKA-II was not detectable and vitamin K_1 concentrations were moderately elevated. Vitamin K_1 concentrations were negatively correlated with the number of hours elapsed since the latest gift. Twenty to 28 hours after the administration mean concentrations ($\pm$ SD) were 1972 ($\pm$ 2028), 1371 ($\pm$ 1139) and 1068 ($\pm$ 713) pg/ml at the age of 4, 8 and 12 weeks, respectively. Vitamin K_1 concentrations in formula-fed infants (n=10) were around 7000 pg/ml. In conclusion, daily supplementation of 25 μg vitamin K_1 can be recommended for breast-fed infants to prevent vitamin K deficiency beyond the neonatal period.

6.2 INTRODUCTION

The term 'haemorrhagic disease of the newborn' (HDN) was first used in 1894 by Townsend to describe an acquired bleeding disorder in newborns (1). After the discovery of vitamin K in 1929, it was soon established that HDN was caused by a deficiency of vitamin K and that administration of vitamin K was effective in therapy and prevention of HDN. In 1961 the Committee on Nutrition of the American Academy of Pediatrics recommended to administer vitamin K prophylaxis to all newborn infants (2). Many countries adopted this recommendation, although controversies concerning the best dose, route and frequency of administration still exist.

Several cases of HDN after the first week of life despite vitamin K prophylaxis at birth have been reported (3-6). This so called late HDN is frequently located intracranial and is often fatal (3). All victims appear to be breast-fed (3). In accordance with these case-reports, we demonstrated that a single administration of vitamin K_1 after birth did not completely prevent laboratory evidence of vitamin K deficiency in breast-fed infants beyond the age of one month; in more than 10% of breast-fed infants proteins induced by vitamin K absence for factor II (PIVKA-II) were detectable in the blood at the age of 3 months (7). These proteins indicate that not all prothrombin was carboxylated completely due to a deficiency or an antagonism of vitamin K. Determination of PIVKA-II is a sensitive way to establish biochemical vitamin K deficiency (8) and can be used to determine which group of infants is at risk for HDN.

Formula-fed infants have higher plasma levels of vitamin K_1 than human milk-fed infants, and PIVKA-II is not detectable in their blood (9). Case-reports of late HDN in formula-fed infants are rare; of 198 infants with late HDN 3 were formula-fed (3). Therefore, to prevent symptoms of vitamin K deficiency in breast-fed infants, daily supplementation of an oral dose of vitamin K_1 comparable to the amount ingested by formula-fed infants would be logical from a theoretical point of view. The effects of such a regimen, however, have never been studied.

In the present study we evaluated the reliability of a daily administration of 25 μg vitamin K_1 in the prevention of vitamin K deficiency in healthy breast-fed infants. PIVKA-II and vitamin K_1 concentrations were measured monthly and were compared with previous results of infants who received a single oral or intramuscular vitamin K administration at birth (7). In addition, plasma levels of vitamin K_1 were measured in 10 formula-fed infants.

6.3 SUBJECTS AND METHODS

The study was approved by the local medical ethical committee and all parents signed a written informed consent.

Breast-fed infants

Fifty-eight newborns who were born vaginally at the University Hospital Nijmegen or at home under midwife guidance, were enrolled. Inclusion-criteria were as reported previously (7); a gestational age 37 weeks or more, a birth weight over the 2.3rd centile and an Apgar score 7 or more at 5 minutes. Arterial cord blood pH was measured in 37 neonates and ranged from 7.05 to 7.44. Some characteristics of these infants, as well as of our previously reported infants without daily supplementation (controls), are shown in Table 6.1. None of the parameters was statistically different between the groups. All 58 study newborns received routine vitamin K prophylaxis (1 mg) on the first day of life; 34 by oral route (20 mg/ml phytomenadione, Konakion^R, Hoffmann-La Roche) and 24 by i.m. injection (2 mg/ml Konakion^R). The mothers were healthy as well and did not take vitamin K, anticoagulants, antibiotics or anti-epileptics. All mothers intended to breast-feed their child for at least 3 months. During this period of 3 months the parents administered daily 25 μg vitamin K_1 (4 drops 0.25 mg/g Konakion^R) to the infant, starting at the age of 1 week. Mean age at start was 7.5 days, range 6 to 11 days. When the solution was spat out, administration was repeated.

If the child was still exclusively breast-fed, it was visited at home at the age of 4, 8 and 12 weeks. Mean ages at assessments were 28 $\pm$ 1.8, 56 $\pm$ 3.0 and 83 $\pm$ 4.1 days. Date and time of the two most recent vitamin K administrations were

recorded, as well as physical condition, medication and bleeding tendency of the baby. Five ml of blood were drawn by venepuncture: 2 ml of citrated blood (in silicone-coated tubes containing 10% (v/v) of sodium citrate 3.8%) and 3 ml of coagulated blood with no additive. The citrated blood was centrifuged (3500xg for 10 min) and stored at -70°C until PIVKA-II assay. The coagulated blood was screened from the daylight immediately after sampling, centrifuged (1000xg for 5 min) and stored at -20°C for vitamin K_1 determination. Samples from one child were assayed in one run.

Table 6.1 Characteristics of study and control breast-fed infants.

	Study	Control
Number	58	331
Sex (% male)	48	51
Gestational age (wk)	40.1 ± 1.2	39.9 ± 1.3
Birth weight (g)	3487 ± 495	3420 ± 439
Apgar score at 5 min	9.6 ± 0.8	9.7 ± 0.6
Arterial cord blood PH	7.28 ± 0.08	7.26 ± 0.07
Oral vitamin K at birth (%)	59	50

Formula-fed infants

Ten formula-fed infants (5 males, 5 females) who were all fed on a formula containing at least 50 μg/l vitamin K_1 (Nutrilon premium^R, Nutricia, Zoetermeer, Netherlands), were studied for vitamin K_1 plasma concentrations at the age of 4, 8 and 12 weeks. The same inclusion criteria were applied as for the breast-fed infants. Mean ± SD gestational age was 40.1 ± 1.2 weeks, mean birth weight was 3229 ± 466 gram and mean Apgar score at 5 min was 9.8 ± 0.4. Arterial cord blood pH was measured in 7 neonates and ranged from 7.07 to 7.40 (mean 7.25). These formula-fed infants also received a single dose of 1 mg (oral or intramuscular) vitamin K_1 at birth. Blood sampling for vitamin K_1 determinations was performed at 28 ± 1.9, 57 ± 2.5 and 85 ± 3.6 days of age. None of the characteristics mentioned was different for the formula-fed infants compared with the breast-fed infants ($p>0.05$).

Laboratory techniques

PIVKA-II concentrations were determined by an enzyme-linked immunosorbent assay previously described, using a monoclonal antibody (Eitest mono P-II, Eisai, Tokyo, Japan) (10). This antibody quantitatively reacts with descarboxylated prothrombin (PIVKA-II) and does not cross react with native prothrombin. The detection limit was 0.10 AU/ml.

Vitamin K_1 was extracted from 1 ml serum samples and determined by a two-step HPLC procedure, according to the method of Lambert *et al.* (11, 12). Few modifications were applied, as stated elsewhere (7). The detection limit was 45 pg/ml.

Statistical analysis

For statistical evaluation Student *t* test was applied on infants' characteristics, Fisher's exact test on PIVKA-II results, and Wilcoxon's one and two sample tests on vitamin K_1 concentrations. Spearman's rank correlation coefficient was used for correlation detection.

6.4 RESULTS

All infants remained healthy during follow-up. No side-effects of vitamin K treatment were reported in the breast-fed infants. None of the infants showed a bleeding tendency.

PIVKA-II concentrations were measured in 48, 49 and 50 breast-fed infants of 4, 8 and 12 weeks of age, respectively. No PIVKA-II was detected in any sample. Comparison with the control group revealed a statistically significant difference at 12 weeks of age, Table 6.2. In fifteen breast-fed infants of the control group PIVKA-II was detectable at the age of 12 weeks; in 7 out of 68 infants who had received oral vitamin K_1 at birth and in 8 out of 63 infants who had received intramuscular vitamin K_1 at birth (7). The reduction in detection of PIVKA-II after daily administration compared to single oral or intramuscular administration is statistically significant (0/50 *v* 7/68, $p=0.02$; 0/50 *v* 8/63, $p<0.01$).

Mean *vitamin K_1 plasma concentrations* in the 10 formula-fed infants were 7044 ± 2954, 7885 ± 1716 and 6040 ± 2304 pg/ml at the age of 4, 8 and 12 weeks, respectively. The total range was 3028 to 10,884 pg/ml. The concentrations were significantly higher as compared with the breast-fed infants ($p<0.001$).

Vitamin K_1 concentrations in the breast-fed infants depended on the time elapsed since the latest vitamin K_1 administration ($r=-0.59$, $p<0.001$, $n=132$). Even after correction for the number of hours elapsed since the most recent vitamin K supple-

mentation, concentrations varied widely (Fig. 6.1). There was no correlation between the total number of days of vitamin K therapy and vitamin K_1 plasma concentration (r=-0.10, p=0.14, n=126).
Samples obtained 20 to 28 hours after the latest vitamin K administration had mean values of 1972 ± 2028 (n=31), 1371 ± 1139 (n=32) and 1068 ± 713 pg/ml (n=33) at the age of 4, 8 and 12 weeks, respectively. Wilcoxon's one sample test applied on paired samples obtained 20 to 28 hours after the latest administration, revealed that the vitamin K_1 concentrations at the age of 4 weeks were higher than at 8 weeks, although not statistically significant (p=0.06, n=18 pairs). Levels at 4 weeks were significantly higher than at 12 weeks (p<0.01, n=21). Values at 8 and 12 weeks did not differ (p=0.33, n=20).
Whether vitamin K prophylaxis *at birth* was given by oral route or by intramuscular injection was not a discriminating factor (p=0.28).
Remarkably, one child had the lowest vitamin K_1 plasma concentration at all three assessments; 163, 112, and 205 pg/ml, respectively. His mother insisted that the vitamin was administered correctly. PIVKA-II was not detectable and the Thrombotest amounted to >100% at all ages.

Vitamin K_1 concentrations in the study group were significantly higher than in the control group, Table 6.2. For the latter group there are no data available at the age of 8 weeks; the mean values at 4 and 12 weeks are shown in Table 6.2.

Table 6.2 Number of samples with PIVKA-II ≥ 0.10 AU/ml, and vitamin K_1 concentrations (mean ± SD) at the age of 4 and 12 weeks in daily supplemented[1] and control breast-fed infants (n).

	PIVKA-II		Vitamin K_1	
AGE (wk)	4	12	4	12
Study group	0 (48)	0 (50)	1972 ± 2028 (31)	1068 ± 713 (33)
Control group	4 (262)	15 (131)*	497 ± 264 (178)#	297 ± 182 (119)#

[1], Samples obtained 20 to 28 hours after the latest administration.
*, Study *v* control group: Fisher's exact test, p=0.01.
#, Study *v* control group: Mann-Whitney test, p<0.001.

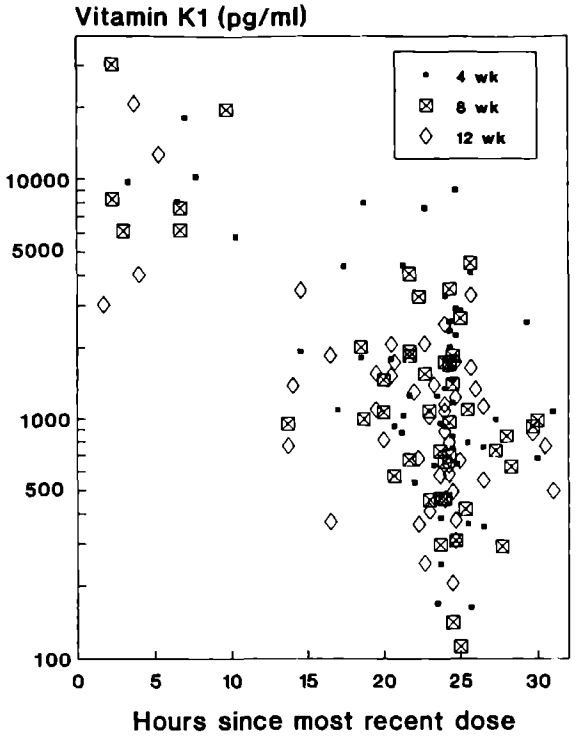

Fig. 6.1 Vitamin K_1 plasma concentrations at 4, 8 and 12 weeks of age in relation to the number of hours elapsed since the most recent vitamin K administration, in 48 breast-fed infants who received a daily oral dose of 25 μg vitamin K_1. Levels in 10 formula-fed infants ranged from 3,000 to 11,000 pg/ml (data not presented).

6.5 DISCUSSION

Neonates and young infants depend on their diet for vitamin K supply, since gut colonisation by vitamin K-producing bacteria has still to be established. Bacterial vitamin K_2 was not detectable in neonatal liver until the age of about 2 weeks (13). Thereafter a gradual build-up was noticed, but it is unknown whether this is the result of absorption from the colon or of food contamination.

Recommended dietary intake (RDI) for infants up to 6 months is 5 μg/day (14). Vitamin K_1 content of human milk is relatively low; concentrations of about 2 μg/l are reported (15). Conversely, the minimum vitamin K_1 content of formula is 4 μg/100 kcal, corresponding to 26 μg/l (16). A maximum concentration is not established, but an upper limit of 100 μg/l is suggested (17). Most formulas contain at least 60 μg/l vitamin K_1. Greer *et al.* (15) reported a vitamin K_1 intake of about 0.6 μg/day by human-milk-fed infants and of 50 μg/day in formula-fed infants. As a consequence, a large difference in vitamin K_1 intake exists between breast-fed and formula-fed infants and, more importantly, the RDI is not met by breast-feeding. The present suggested maintenance dose of 25 μg/day increases the vitamin K_1 intake of breast-fed infants significantly. An intake of 25 μg/day is 5 fold as much as the RDI, but only half as much as the intake of formula-fed infants.

Our mean vitamin K_1 level of about 7000 pg/ml in the formula-fed infants, confirms that vitamin K_1 levels in formula-fed infants are much higher than in breast-fed infants. Mean levels reported for bottle-fed infants are 3000-6000 pg/ml, and for unsupplemented human-milk-fed infants 150-700 pg/ml (9,15,18). Consequently, formula-fed infants rarely develop symptoms of vitamin K deficiency; these are almost confined to breast-fed infants (3). Accordingly, prothrombin times were reported to be longer in three-month-old breast-fed infants than in formula-fed infants, even despite vitamin K prophylaxis at birth (15). This is in accordance with the fact that PIVKA-II was rather frequently detectable in three-month-old breast-fed infants who had received a single dose of vitamin K_1 at birth (7), while it was not detectable in supplemented infants. Repetitive administration seems necessary to completely prevent (biochemical evidence of) vitamin K deficiency in young infants.

Daily supplements of 25 μg vitamin K_1 resulted in plasma vitamin K_1 levels which were still significantly lower than those of formula-fed infants. As mentioned previously, formula-fed infants probably ingest twice as much vitamin K_1. Moreover, they get their vitamin supplements in 5 to 6 portions per day, which may prevent decrease of the plasma concentration. The plasma half-life of tritiated vitamin K_1 was reported to be 120-150 min (19).

Plasma concentrations of vitamin K_1 did not accumulate due to the repeated supplements. On the contrary, vitamin K_1 levels declined during follow-up. Several explanations are conceivable. First, while the intake of vitamin K_1 remains constant, liver weight and volume of distribution increase with age. Secondly, degradation of vitamin K_1 may become more efficient. Nevertheless, the mean vitamin K_1 concentration of about 1000 pg/ml at the age of 12 weeks is still 3.5 times as high as the mean of the control group.

To our knowledge this is the first report of vitamin K_1 and PIVKA-II plasma concentrations determined in breast-fed infants receiving daily 25 μg vitamin K_1. This maintenance dose turned out to be effective in the prevention of biochemical evidence of vitamin K deficiency, and therefore it will probably protect against late HDN. Because there is serious concern about a potential association of childhood cancer and intramuscular administration of 1 mg vitamin K at birth (20), a low maintenance dose might be preferable.

Current Dutch recommendations on vitamin K prophylaxis include oral or i.m. vitamin K_1 (1 mg) at birth for all neonates, followed by weekly (1 mg) or daily (0.025 mg) administration of vitamin K_1 during three months for breast-fed infant (21). A preparation of vitamin K_1 for daily application is currently available in the Netherlands (Davitamon K^R, Chefaro). We suggest to recommend daily administration of 25 μg vitamin K_1 from one week until three months of age in breast-fed infants as an alternative to weekly supplements. The limit of 3 months, however, is still arbitrary. Late HDN is rarely reported beyond this age (3). A low rate of exclusive breast-feeding at that age and introduction of solid food, which may account for additional vitamin K intake, might be responsible for this. Whether this low dose is also suitable to prevent vitamin K deficiency in sick children with cholestasis or patent hepatic disease can not be concluded from this study.

Acknowledgments

This study was supported by grant 28-1563 of the Dutch 'Praeventiefonds'. We are indebted to all parents and infants for their participation; to Eisai Ltd for kind supplying PIVKA-II kits and to Hoffmann-La Roche for donating vitamin $K_{1(25)}$.

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CHAPTER 7

INCREASED INCIDENCE OF NEONATAL VITAMIN K DEFICIENCY DUE TO MATERNAL ANTICONVULSANT THERAPY

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Accepted American Journal of Obstetrics and Gynaecology

7.1 ABSTRACT

Objective - The null hypothesis of this study is that the incidence of vitamin K deficiency in mother-infant pairs exposed to anticonvulsant drugs is not higher than in controls.
Study design - Multicenter observational case-control study. 25 pregnant women on anticonvulsant therapy and 25 pregnant controls were studied for PIVKA-II and vitamin K_1 concentrations at 32 weeks pregnancy and at delivery.
Results - PIVKA-II was detectable in 54% of cord samples of the anticonvulsant group and in 20% of controls (χ^2, p=0.01). In both groups vitamin K_1 concentrations in cord blood were predominantly below the detection limit. Maternal vitamin K_1 concentrations were lower in epileptic women than in controls (Wilcoxon, $p<0.05$), but PIVKA-II was rarely present.
Conclusions - The incidence of vitamin K deficiency is increased in neonates exposed to anticonvulsants prenatally. Their mothers, however, are rarely vitamin K deficient.

7.2 INTRODUCTION

Maternal anticonvulsant (AC) therapy during pregnancy can affect the fetus and cause neonatal complications. Haemorrhagic disease of the newborn (HDN) related to AC therapy was first reported in 1958 (1). Hitherto, at least 40 cases of neonatal bleedings associated with maternal AC therapy have been reported (2-4). The haemorrhagic diathesis is caused by depletion of vitamin K-dependent coagulation factors II, VII, IX and X. Onset of bleeding is usually within 24 hours after birth. Bleeding can occur at any site, but predominantly in the skin, the gastro-intestinal tract or the brain, and it is often fatal (4).
In prospective studies the incidence of coagulation defects due to neonatal vitamin K deficiency was proved to be relatively high after intra-uterine exposure to AC drugs. Mountain *et al.* (5) reported abnormal coagulation tests in 50% of 16 fullterm neonates born to mothers on AC therapy. Deblay *et al.* (6) found subnormal prothrombin assays within 12 hours of birth in 27% of neonates born to women on AC medication. Seven percent suffered from severe bleedings. A more specific test for vitamin K deficiency is detection of proteins induced by vitamin K absence (PIVKA). Vitamin K is a cofactor necessary for posttranslational γ-carboxylation of glutamic acid residues in prothrombin and other vitamin K-dependent proteins, thereby providing these factors with effective calcium and phospholipid binding sites. In vitamin K deficiency, incompletely carboxylated proteins that are functionally defective appear in the circulation. These abnormal coagulation factors are called PIVKA. PIVKA-II (uncompletely carboxylated prothrombin) is not detected in plasma unless there is a defect in carboxylation attributable to impairment of

carboxylase enzyme and/or deficiency or antagonism of vitamin K. Determination of PIVKA-II by monoclonal antibody is a very specific and sensitive method (7).

Although several authors stress the need for antenatal vitamin K supplementation to pregnant women on AC therapy to prevent HDN (2,4-6), the true incidence of vitamin K deficiency in these neonates is not known. For this reason, we performed an observational study to establish the incidence of vitamin K deficiency in mothers and infants exposed to AC drugs compared to controls. In 25 mother-infant pairs on AC therapy and in 25 control pairs, concentrations of vitamin K_1 and PIVKA-II were determined.

7.3 MATERIAL AND METHODS

Study population

The study was approved by the medical ethical committee and after a full explanation of the study protocol informed consent was obtained from all participants.
Twenty-five pregnant epileptic women on AC treatment (mean duration 11 yrs, range 1-23 yrs) attending multicentre obstetric out-patient's departments and 25 pregnant healthy controls attending a midwife practice, were recruited. Selection criteria were: an uncomplicated pregnancy so far, no medication except for anticonvulsants in the epileptic group and folic acid, iron, and vitamins other than vitamin K in both groups. Complications and medication during follow-up, mode of delivery, and neonatal outcome were registered. Vitamin K was administered to the neonates after delivery according to local routine procedures.

Laboratory determinations

Maternal blood was sampled from the cubital vein at 32 weeks pregnancy and within two hours after delivery. Venous or mixed cord blood was sampled immediately after cord clamping. At each sampling, 5 ml of citrated blood (in silicone coated tubes containing 10% (v/v) of sodium citrate 3.8% (w/v)) and 5 ml of coagulated blood were collected. The citrated blood was centrifuged (3500 x g for 10 min) and the removed plasma stored at -70^{o}C until PIVKA-II was measured. The coagulated blood was protected from the light immediately after sampling, centrifuged (1000 x g for 5 min) and the serum stored at -20^{o}C for vitamin K_1 determination. Samples from a mother-child pair were assayed in one run. All samples were coded to provide blind analysis.

PIVKA-II concentrations were determined by an enzyme-linked immunosorbent assay, using a monoclonal antibody (Eitest mono P-II, Eisai, Tokyo, Japan) (8). This antibody quantitatively reacts with descarboxylated prothrombin (PIVKA-II) and does not cross-react with native prothrombin. PIVKA-II levels are expressed in

arbitrary units (AU) per ml, so that 1 AU corresponds with 1 μg of purified prothrombin. PIVKA-II levels in healthy adults, adults with liver cirrhosis and pregnant women are reported to be below 0.10 AU/ml, which is considered as the upper normal limit (9). In severe vitamin K deficiency PIVKA-II concentration can rise to more than 20 AU/ml (8).
Vitamin K_1 was extracted from 1 ml serum samples by a two-step high performance liquid chromatographic (HPLC) procedure, according to the method of Lambert *et al.* (10, 11). A few modifications were applied. Recovery of vitamin K_1 from standard solutions added to normal serum was 85 ± 5% with a detection limit of 30 pg/ml. However, the detection limit of individual samples can vary from 30 to 130 pg/ml, dependent on the recovery of internal standard during extraction and the amount of serum extracted.

Statistical analyses
χ^2, Fisher's exact and Student *t* tests were used for comparison of study population characteristics and PIVKA-II results. Wilcoxon's one and two sample tests were applied to vitamin K_1 results. Spearman's rank correlation coefficient was used for correlation detection.

7.4 RESULTS

The two groups of mother-infant pairs were well comparable (Table 7.1). One pregnancy of the epileptic group resulted in unexplained fetal death at the 36th week of gestation. Unfortunately, it was not possible to collect cord blood of this

Table 7.1 Characteristics of the study and control groups.

	Epileptics	Controls
Maternal age (y)*	28.2 (22-34)	29.4 (21-35)
Nulliparae	12	12
Gestational age (wk)*	40.1 (36.6-42.4)	40.1 (36.7-42.0)
Caesarean section	1	1
Blood loss >1000 ml	1	3
Neonatal sex, m:f	15:9	12:13
Birth weight (g)*	3396 (2670-4750)	3511 (2300-4500)
Apgar at 1, 5 min <7	2, 2	2, 0

* mean (range)

child, so he is not included in the results. His mother had no evidence of vitamin K deficiency.
During follow-up two epileptic women were treated with Ritordine to prevent premature labour. They also developed pregnancy induced hypertension and PIVKA-II was detectable in their cord blood samples. Only one neonate showed indication of a bleeding diathesis, a child whose mother used 300 mg Phenytoin (DPH) daily and who developed a haematoma at the site of vitamin K injection. His Thrombotest one hour postpartum was 22% (normal 20-40). The PIVKA-II concentration in his cord blood was 1.47 AU/ml, while the vitamin K_1 concentration was below the detection limit of 30 pg/ml.

In epileptic women and controls PIVKA-II was not detectable at 32 weeks gestational age, Table 7.2. At delivery, in 2 mothers who took Carbamazepine (CBZ) PIVKA-II was present in concentrations of 0.15 and 0.17 AU/ml.
In the neonates who were intra-uterine exposed to AC drugs, PIVKA-II was detectable in 13 out of 24 samples (54%), including the neonates of the two PIVKA-II positive mothers; 3.20 and 0.60 AU/ml, respectively. In 5 (20%) control neonates PIVKA-II was detectable, but concentrations were low (Table 7.3). The higher rate of PIVKA-II detectability in the epileptic as compared with the control cord samples was statistically significant ($p=0.01$). Subgroups of AC are presented in Table 7.2. Remarkably, PIVKA-II was not detected in 4 neonates exposed to Valproic acid (VPA) monotherapy. This is statistically different from the other neonates of the epileptic group (0/4 against 13/20, $p<0.05$).
In the control group all PIVKA-II positive cord samples were derived from primigravidae ($p<0.01$), who were significantly younger than the mothers of the PIVKA-II negative neonates ($p<0.05$). In the epileptic group such an association with parity or maternal age was not detected.

The results of vitamin K_1 determinations are depicted in Fig. 7.1. At 32 weeks of gestation vitamin K_1 concentrations (mean ± SD) amounted to 828 ± 548 pg/ml in the epileptic group and 1225 ± 787 pg/ml in the control group ($p<0.05$). At delivery maternal concentrations were decreased compared with levels at 32 weeks pregnancy; this was highly significant in the epileptic group (491 ± 256 pg/ml, $p<0.01$), but not significant in the control group (887 ± 401 pg/ml, $p=0.07$). At delivery maternal concentrations in the epileptic group were again significantly lower than in the control group ($p<0.001$).
In cord blood vitamin K_1 concentrations were predominantly below the detection limit: 22 out of 24 in the AC group and 17 out of 25 in the control group (NS). The other values are shown in Fig. 7.1. Vitamin K_1 concentrations in the PIVKA-II positive cord blood samples are given in Table 7.3. The two epileptic mothers with PIVKA-II detectable at delivery had vitamin K_1 levels of 512 and 189 pg/ml, respectively.

Table 7.2 PIVKA-II detectability (≥ 0.10 AU/ml) in mother-infant pairs with anticonvulsant (AC) therapy and in controls (n).

	Mother		Neonate
GROUP	32 wk	delivery	cord blood
CBZ	0 (8)	2 (11)	6 (11)
CBZ + DPH	0 (1)	0 (1)	1 (1)
CBZ + VPA	0 (2)	0 (2)	1 (2)
DPH	0 (1)	0 (2)	1 (2)
VPA	0 (4)	0 (5)	0 (4)
PHB	0 (4)	0 (4)	4 (4)
Total AC	0 (20)	2 (25)	13 (24)*
CONTROL	0 (25)	0 (25)	5 (25)

CBZ=carbamazepine, DPH=phenytoin, VPA=valproic acid, PHB=phenobarbital.
* AC *v* control group, $p < 0.05$.

Table 7.3 PIVKA-II and corresponding vitamin K_1 concentrations in cord blood samples with PIVKA-II detectable.

GROUP	PIVKA-II (AU/ml)			Vitamin K_1 (pg/ml)		
CBZ	0.14	0.60	0.89	b.d.	b.d.	b.d.
	3.20	3.99	4.54	b.d.	b.d.	b.d.
CBZ + DPH	0.42			b.d.		
CBZ + VPA	1.35			b.d.		
DPH	1.47			b.d.		
PHB	0.20	0.26	0.31	b.d.	b.d.	b.d.
	1.02			82		
CONTROLS	0.14	0.14	0.18	b.d.	120	174
	0.19	0.28		b.d.	b.d.	

b.d., below detection limit.

Neither a significant correlation was found between vitamin K_1 and PIVKA-II concentrations, nor between these data and neonatal sex, gestational age, birth weight, Apgar score or maternal dose of AC. There was no significant difference in maternal vitamin K_1 plasma concentrations in the PIVKA-II positive and negative neonates for either groups. Vitamin K_1 levels were not significantly higher in women treated with VPA, as compared with those treated with other AC drugs.

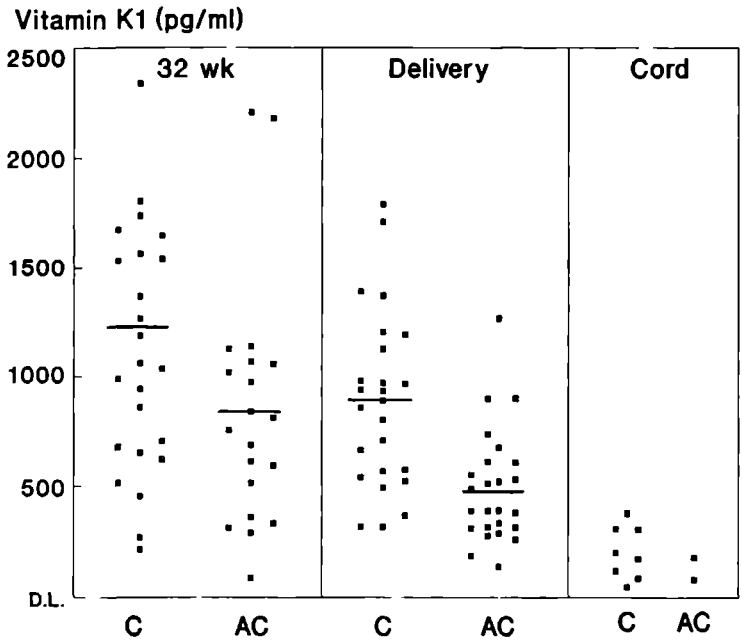

Fig. 7.1 Individual vitamin K_1 plasma concentrations and means (—) in maternal blood at 32 weeks pregnancy and after delivery, and in cord blood, of mother-infant pairs on anticonvulsant (AC) therapy and controls (C). In the control group an outlier of 3997 pg/ml at 32 weeks gestation is not depicted.

7.5 COMMENT

CBZ, DPH, PHB and VPA readily cross the placenta. Cord and maternal blood levels are almost the same or, as in VPA, the level is even higher in cord blood (12). DPH, PHB and CBZ induce microsomal mixed-function oxidase enzymes in the fetal liver and it is hypothesized that these hepatic enzymes increase the rate of oxidative degradation of vitamin K resulting in vitamin K deficiency (12, 13). Which of the many cytochrome P450 isozymes is involved in vitamin K metabolism is still not known. Bouwman *et al.* (14) reported an association between cytochrome P450 IIB activity and vitamin K dependent coagulation, so induction of this cytochrome may be important in the pathogenesis of vitamin K deficiency. In this study VPA did not increase the rate of detectability of PIVKA-II in cord blood of exposed neonates. This is in agreement with the fact that VPA does not induce hepatic enzymes (15).

The finding that PIVKA-II was present in 20% of control cord blood samples is in agreement with the incidence of 21.5% in 102 normal cord blood samples reported by Motohara *et al.* (16) and 31% in 16 samples reported by Widdershoven *et al.* (17). They both used the same method as we did. Two other studies, applying other methods of detecting PIVKA-II, reported an incidence in control cord blood of 28% (18) and 36% (19). These percentages are quite higher than measured by Hulac *et al.* (20), who reported a rate of 3% in general population and 27% in newborns exposed to AC therapy. Despite this lower frequency, what might be due to the less sensitive method to determine PIVKA-II, their conclusion is similar; PIVKA-II is more frequently present in cord blood of neonates intra-uterine exposed to AC drugs than in control newborns. They agreed that PIVKA-II is rarely detectable in the mothers.

Our results confirm that vitamin K_1 concentrations in normal cord blood are very low, usually below the detection limit of the method used (17, 21). This extremely low level is probably caused by a placental barrier. Shearer *et al.* (22) reported concentrations of 144 to 2420 pg/ml in mothers and 4 - 45 pg/ml in cord blood, resulting in a median maternal-cord ratio of 30:1. An experimental study in pregnant rats has shown that diffusion across the placenta requires a high mother-fetus concentration gradient (23). After oral administration of vitamin K_1 to pregnant rats, the amount of vitamin K_1 in the fetal liver is 1-2% of that in the maternal liver (24). Together with the frequent detection of PIVKA-II in cord blood, this shows that many neonates are in a state of borderline vitamin K deficiency. Few of these deficiencies proceed to clinical disease. In a subsequent study we have shown that administration of extra vitamin K_1 via the mother prevented the appearance of PIVKA-II in cord blood (25). So, there is a direct relation between the availability of vitamin K and the presence of PIVKA-II.

Vitamin K_1 plasma concentrations during normal pregnancy are reported to be normal (22) or slightly decreased (21). This study is the first to report levels for pregnant women on AC therapy. The concentrations appear to be decreased compared with those in control pregnant women, especially at delivery. This is supporting the hypothesis of an increased vitamin K turnover due to induction of hepatic enzymes by the AC drugs. The decrease of maternal vitamin K_1 plasma levels, however, is probably in the majority of cases not severe enough to impair maternal carboxylation of vitamin K dependent coagulation factors. In addition, adults can utilize abundant hepatic stores of vitamin K_2 (menaquinones) as cofactor for the carboxylation process. On the contrary, vitamin K_2 is not detectable in the first two weeks of life in the neonatal liver (26). With the placental barrier hampering transport of vitamin K_1, it is conceivable that fetuses of mothers on AC therapy are even more prone to develop vitamin K deficiency than normal newborns, as confirmed by our PIVKA-II results.
The finding that maternal vitamin K_1 plasma concentrations immediately after delivery are lower than at late-trimester pregnancy, may be related to fasting. Usually, women scarcely eat several hours during labour and it has been established that fasting concentrations of vitamin K_1 are lower than non-fasting levels (22).

The present paper is the first documentation of simultaneous measurements of both vitamin K_1 and PIVKA-II in mother-infant pairs on AC medication. Both parameters are necessary for a precise evaluation of vitamin K status. In conclusion, the results of this study point to an increased incidence of subclinical vitamin K deficiency in neonates of mothers who are treated during pregnancy with enzyme-inducive AC drugs. The women themselves have decreased vitamin K_1 plasma concentrations, but are not frequently vitamin K deficient. Whether vitamin K prophylaxis after birth can prevent early haemorrhagic disease in neonates intra-uterine exposed to AC drugs is doubted, though it may not be dispensed with (3). In a subsequent study we investigated whether antenatal supplementation of vitamin K to pregnant women on AC therapy, can reverse the increased incidence of vitamin K deficiency in their neonates (25).

Acknowledgments
This study was supported by grant 28-1563 of the 'Praeventiefonds'. We thank Mrs A. Koster for statistical advices; the research-nurses of the "P.P.A.A." and midwives Mrs I. Willems and Mrs J. Kervezée for practical assistance; Hoffmann-La Roche for supply of vitamin $K_{1(25)}$; Eisai Co. for supply of PIVKA-II kits; and the obstetrical departments, located in 14 different hospitals, for participation.

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CHAPTER 8

SUPPLEMENTATION OF VITAMIN K IN PREGNANT WOMEN ON ANTI-CONVULSANT THERAPY PREVENTS NEONATAL VITAMIN K DEFICIENCY

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Accepted American Journal of Obstetrics and Gynecology

8.1 ABSTRACT

Objective - The null hypothesis of this study is; extra vitamin K administered to pregnant women on enzyme-inducive anticonvulsant therapy will not decrease the frequency of signs of vitamin K deficiency in their neonates.
Study design - Multicenter case-control study. Sixteen pregnant women on anticonvulsant therapy received daily 10 mg vitamin K_1 from 36 weeks of gestation onwards. PIVKA-II and vitamin K_1 concentrations were determined in cord blood and compared with 20 controls.
Results - In 0/17 cord samples PIVKA-II was detectable compared with 13/20 in controls (χ^2, $p<0.001$). Median cord vitamin K_1 concentration was 530 pg/ml compared with concentrations below the detection limit in most controls.
Conclusions - Antenatal vitamin K_1 treatment decreases the frequency of vitamin K deficiency in neonates of mothers on enzyme-inducive AC therapy.

8.2 INTRODUCTION

The activity of vitamin K-dependent coagulation factors in neonates is lower than in adults. Severe haemorrhagic disorders have been reported in young infants suffering from vitamin K deficiency (1). Therefore, the Committee on Nutrition of the American Academy of Pediatrics has recommended that prophylactic vitamin K be administered to all newborns (2). The incidence of haemorrhagic disease of the newborn declined dramatically in Japan due to vitamin K prophylaxis (3).
Newborns exposed in utero to anticonvulsant (AC) drugs are most at risk for vitamin K deficiency (4). We reported that proteins induced by vitamin K absence (PIVKA-II; precursor prothrombin; descarboxylated prothrombin), which are a biochemical marker of subclinical vitamin K deficiency, were significantly more frequently detectable in cord blood of newborns prenatally exposed to AC drugs than in controls, especially if enzyme-inducive drugs were involved. In addition, cord blood concentrations of vitamin K_1 were predominantly below the detection limit (4). Many reports have been published on haemorrhages in infants whose mothers took AC medication during pregnancy. Some of these occurred despite vitamin K prophylaxis at birth (5-7). Intrapartum or early neonatal haemorrhages may not be prevented by postnatal administration of vitamin K. In this regard, prenatal administration of vitamin K might be worthwhile. Therefore, we studied the effect of vitamin K_1 supplementation by measuring plasma vitamin K_1 and PIVKA-II concentrations in mother-neonate pairs on AC therapy who were supplemented with extra vitamin K_1 during the last month of pregnancy and compared these concentrations with controls.

8.3 MATERIAL AND METHODS

Study population

The study was approved by the medical ethical committee and after full explanation of the study protocol written informed consent was obtained from all participants.
Thirty-six pregnant epileptic women on AC therapy (median duration 12 years, range from 1 to 31) attending multicentre obstetric out-patient's departments were involved. Only AC drugs known to induce microsomal hepatic enzymes, like Phenobarbital (PHB), Phenytoin (DPH), Carbamazepine (CBZ) and Primidone (Prim) were included. Other selection criteria were: an uncomplicated pregnancy so far and no medication except for anticonvulsants, folic acid, iron and vitamins other than vitamin K. The subjects were divided into two groups: 16 patients were supplemented with vitamin K_1 (Konakion^R, Hoffmann-La Roche) 10 mg/day orally from the 36th week of gestation until delivery (mean 29 days, range from 10 to 46). Date and time of intake were recorded. The final dose was ingested before delivery (mean 20 hours, range from 6 to 39). The other group of 20 women did not receive extra vitamin K and served as controls. The groups were studied consecutively, as the latter group was derived from 25 patients previously described (4). Valproic acid (VPA) was excluded because VPA does not induce microsomal enzymes (8), and hence does not increase the frequency of neonatal vitamin K deficiency (4). Characteristics of the two groups of mother-infant pairs are given in Table 8.1. One mother delivered a twin. Maternal age at delivery was higher in the supplemented group than in the control group ($p<0.05$). The other parameters did not differ significantly between the groups.

Laboratory determinations

Venous or mixed cord blood was sampled immediately after cord clamping. Within two hours after delivery blood was sampled from the cubital vein of the mother. Five ml of citrated blood (in silicone-coated tubes containing 10% (v/v) of sodium citrate 3.8% (w/v)) and 5 ml of coagulated blood were collected. The citrated blood was centrifuged (3500 x g for 10 min) and the removed plasma stored at -70°C until PIVKA-II was measured. The coagulated blood was protected from the light immediately after sampling, centrifuged (1000 x g for 5 min) and the serum stored at -20°C for vitamin K_1 determination. Samples from a mother-child pair were assayed in one run. All samples were coded to provide blind analysis.

PIVKA-II concentrations were determined by an enzyme-linked immunosorbent assay, using a monoclonal antibody (Eitest mono P-II, Eisai, Tokyo, Japan) (9). This antibody quantitatively reacts with descarboxylated prothrombin (PIVKA-II) and does not cross-react with native prothrombin. The detection limit was 0.10 AU/ml.
Vitamin K_1 was extracted from 1 ml serum samples by a two-step high performance liquid chromatographic (HPLC) procedure, according to the method of Lambert *et*

al. (10, 11). A few modifications were applied, as stated elsewhere (4). Recovery of vitamin K_1 from standard solutions added to normal serum was 85 ± 5% with a detection limit of 30 pg/ml. However, the detection limit of individual samples can vary, dependent on the recovery during extraction and the amount of serum extracted.

Statistical analyses

χ^2, Fisher's exact and Student *t* tests were used for comparison of study population characteristics and PIVKA-II results. Wilcoxon's one and two sample tests were applied to the vitamin K_1 results. Spearman's rank correlation coefficient was used for correlation detection.

Table 8.1. Characteristics of the study and control groups.

	Study group	Control group
Mothers (n)	16	20
Maternal age (y)*	31.0 (21-38)	28.6 (25-34)
Nulliparae	9	7
Gestational age (wk)*	39.7 (36.6-41.9)	40.1 (36.6-42.0)
Caesarean section	2	1
Blood loss >1000 ml	4	1
Neonates		
Neonatal sex, m:f	11:6 #	13:7
Birth weight (g)*	3161 (2410-3720)	3452 (2690-4750)
Apgar at 1, 5 min <7	3, 1	2, 2

* mean (range) # one pair of twins included

8.4 RESULTS

Except for one neonate with fetal hydantoin syndrome, all neonates were healthy and no bleeding tendencies were recorded.

PIVKA-II results are shown in Table 8.2. In both supplemented and control pregnant women on AC therapy, PIVKA-II was detectable rarely. In cord blood of control neonates PIVKA-II was detected in 13 out of 20 samples (65%), with concentrations ranging from 0.14 to 4.54 (median 0.89) AU/ml. On the contrary, in the supplemented group 0 out of 17 samples had PIVKA-II detectable ($p<0.001$). Various

subgroups of AC are distinguished in Table 8.2. The difference in the presence of PIVKA-II in cord blood between the supplemented and control group was statistically significant for CBZ monotherapy ($p<0.05$), CBZ with other medication ($p<0.01$) and PHB monotherapy ($p<0.05$). The number of individuals in the other subgroups was too small for statistical analysis.

Table 8.2 PIVKA-II detectability ($\geq$ 0.10 AU/ml) in vitamin K supplemented (suppl) and control mother-infant pairs on AC therapy during pregnancy (n).

	Mother		Neonate	
GROUP	control	suppl	control	suppl
CBZ	2 (11)	0 (5)	6 (11)	0 (6)#*
CBZ + DPH	0 (1)	0 (2)	1 (1)	0 (2)
CBZ + VPA	0 (2)	0 (1)	1 (2)	0 (1)
CBZ + PHB	--	0 (1)	--	0 (1)
DPH	0 (2)	0 (2)	1 (2)	0 (2)
PHB	0 (4)	0 (4)	4 (4)	0 (4) *
PHB + VPA	--	-- +	--	0 (1)
Total	2 (20)	0 (15)	13 (20)	0 (17) *

CBZ=carbamazepine, DPH=phenytoin, VPA=valproic acid, PHB=phenobarbital.
#, one pair of twins included.
*, suppl. *v* control group, $p<0.05$.
+, one value missing due to sampling error.

The median maternal *vitamin K_1 concentration* in the control group was 389 pg/ml (range from 139 to 1265) as compared with 22,760 pg/ml (range from 5630 to 111,460) in the supplemented group, ($p<0.001$).
In cord blood of control neonates vitamin K_1 was hardly detectable. The concentration was below the detection limit in all but one sample with 82 pg/ml. In contrast, in the supplemented neonates the vitamin K_1 concentration was always above the detection limit and ranged from 218 to 894 pg/ml, with an outlier of 1618, median 530 pg/ml. Maternal supplementation thus increased cord vitamin K_1 concentration at least 15 times. The ratio maternal/neonatal concentrations ranged from 10 to 128,

with an outlier of 511, median 44. The twin's vitamin K_1 concentrations were 530 and 716 pg/ml, while their mother's concentration was 21,640 pg/ml.
Although maternal vitamin K_1 concentrations were negatively correlated with time elapsed since the last vitamin K_1 intake ($r=-0.64$, $p=0.005$, $n=14$), they were not correlated with total duration of treatment ($r=0.10$, $p=0.36$, $n=16$). Cord blood concentrations tended to correlate with the time elapsed since the last vitamin K_1 ingestion ($r=-0.42$, $p=0.07$, $n=14$), but were not correlated with the total duration of therapy ($r=-0.12$, $p=0.33$, $n=17$).

8.5 COMMENT

Placental transfer of vitamin K is hampered. A high maternal-fetal concentration gradient is required for diffusion across the placental barrier (12). Shearer *et al.* (13) reported concentrations of 144 to 2420 pg/ml in healthy mothers and 4-45 pg/ml in cord blood, resulting in a median maternal-cord ratio of 30:1. Our concentrations of 139 to 1265 pg/ml in unsupplemented mothers on AC therapy are comparable, or as demonstrated in our previous publication, slightly lower than in controls (4). In unsupplemented neonates exposed in utero to AC drugs, cord vitamin K_1 concentrations were predominantly below the detection limit, like in control newborns (4). Maternal supplementation with an oral dose of 10 mg vitamin K_1 daily raised the median maternal vitamin K_1 concentration some 60-fold and the median cord concentration at least 15-fold. Our median maternal-cord ratio was 44. The outlying ratio of 511 was derived from a mother-neonate pair of whom the mother had ingested the last vitamin K dose only 6 hours prior to sampling. Correspondingly, the mother had the highest maternal concentration and the cord the lowest cord concentration of all.
Other studies confirm placental transfer of vitamin K_1 after supplementation of the mother, unless it is administered at least 4 hours before delivery (12,14,15). Owen *et al.* (16) concluded from a double blind study of 204 infants that prenatal oral administration of vitamin K_1 (5 mg/day for 12 days) exerted a beneficial effect on the relative deficiency of prothrombin that normally exists in the newborn infant. No undesirable side effects were encountered. Correspondingly, Deblay *et al.* (17) reported a decrease from 27 to 0% of neonatal subnormal prothrombin concentrations assayed by Quick's method, after treatment of pregnant epileptic women with 20 mg vitamin K_1 daily for 2 weeks. Mandelbrot *et al.* (18) performed a study of antenatal supplementation in non-epileptic mother-infant pairs. The mean maternal concentration increased to 45,190 pg/ml and the mean neonatal concentration to 783 pg/ml after administration of 20 mg vitamin K_1 daily for 3-7 days. The maternal-cord ratio was 44, similar to our ratio. Despite the resulting higher vitamin K_1 concentrations, there are as yet no reasons to prefer the administration of 20 mg over 10 mg daily. Kazzi *et al.* (15) demonstrated that cord vitamin K_1 concentrations

were higher in infants whose mothers received oral vitamin K_1 daily, than in infants whose mothers received an intramuscular injection every four days. Because neither Kazzi *et al.* (15), nor Mandelbrot *et al.* (18), nor we, could detect a correlation between maternal or cord vitamin K_1 concentrations and total duration of treatment, duration of therapy seems not important to elevate vitamin K_1 concentrations in cord blood. The correlations between the time elapsed since last vitamin K ingestion and maternal or cord vitamin K_1 lconcentrations, demonstrate that vitamin K_1 plasma levels decline rapidly after administration of pharmacological doses of vitamin K_1.

Another parameter for defining vitamin K status is determination of PIVKA-II. Detection of this protein indicates that not all prothrombin is carboxylated completely. Due to a long disappearance time PIVKA-II can still be present after the deficiency has been corrected. This study shows that vitamin K supplementation abolishes the presence of PIVKA-II, so detection of PIVKA-II seems a specific indicator of vitamin K deficiency. Low amounts of PIVKA-II have no clinical consequences, but they tell us which group of infants is most at risk for vitamin K deficiency. An additional burden may lead to bleeding diathesis and haemorrhages.
The present study is the first to report PIVKA-II concentrations in mother-infant pairs on AC therapy with and without supplementation of vitamin K_1. A comparable study in non-epileptic mother-infant pairs has been reported: PIVKA-II was not detected in any of the supplemented neonates as compared with 20% of the control newborns (14). In a previous paper we also found in 20% of control newborns PIVKA-II present (4). However, in neonates exposed to AC drugs the incidence was increased to 54%. Excluding VPA medication, the frequency was even 65%. Except for an higher frequency of PIVKA-II detectability, PIVKA-II concentrations were higher in infants exposed to enzyme-inducive AC drugs than in controls (4). The present study demonstrates that after antenatal vitamin K_1 supplementation PIVKA-II was not detectable in cord plasma. Consequently, under these conditions all prothrombin seems to be completely carboxylated. Antenatal vitamin K prophylaxis will probably diminish the risk of neonatal haemorrhages related to vitamin K deficiency. Administration of vitamin K resulted in high maternal vitamin K_1 blood concentrations, but adverse effects were not reported. It remains unknown what would be the best dosage to prescribe.

The existence of elevated vitamin K_1 cord blood concentrations and disappearance of PIVKA-II after maternal ingestion of vitamin K_1 confirms that prenatal prophylaxis of vitamin K deficiency is possible. Such a treatment is indicated to protect fetuses and newborns whose mothers receive medication that induce vitamin K deficiency. The pathogenetic mechanism by which anticonvulsants induce vitamin K deficiency is not established yet. PHB, DPH and CBZ induce microsomal mixed-function oxidase enzymes in the fetal liver and it is hypothesized that these enzymes

increase degradation of vitamin K (19). In the rabbit administration of PHB increased plasma clearance of vitamin K_1 (20), but this was not confirmed in a small group of human adults (21). However, the degree of enzyme induction might be higher in fetuses, who normally have low activity of microsomal enzymes, as compared with adults. Since fetal plasma levels of vitamin K_1 are already low, it is conceivable that some extra degradation of vitamin K results in vitamin K deficiency. Which of the cytochrome P450 isozymes is involved in vitamin K's metabolism is not known. Bouwman *et al.* (22) reported an association between cytochrome P450 IIB activity and vitamin K-dependent coagulation in rats. In our previous study we showed that VPA did not increase the rate of PIVKA-II detection in cord blood of exposed neonates (4). This is in agreement with the fact that VPA is not enzyme inducive (8). Other hypotheses how anticonvulsants could induce vitamin K deficiency, are hampering of placental transfer of vitamin K and inhibition of the carboxylase system (6, 21).

Although recommendations on antenatal vitamin K therapy will stand for unnecessary treatment of many, each intracranial haemorrhage with fatal or serious sequela in otherwise normal children must be prevented. Therefore, we recommend vitamin K_1 supplementation during the last days of pregnancy in mothers on enzyme-inducive AC therapy to prevent vitamin K deficiency in their neonates.

Acknowledgments

This study was supported by grant 28-1563 of the "Praeventiefonds". We thank Mrs A. Koster for statistical advices, and all collaborators from participating obstetrical departments, especially H. Bruinse MD, from the University Hospital Utrecht. We are indebted to Hoffmann-La Roche, who generously supplied vitamin $K_{1(25)}$ and to Eisai Co Ltd for donation of PIVKA-II assay kits.

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CHAPTER 9

SUMMARY AND GENERAL DISCUSSION

9. SUMMARY AND GENERAL DISCUSSION

Vitamin K serves as a cofactor for the vitamin K-dependent carboxylase enzyme, which is involved in the production of coagulation factors II, VII, IX, X, and coagulation inhibitors protein C and protein S (chapter 1). The carboxylation of specific glutamic acid residues to γ-carboxyglutamic acid residues confers unique calcium binding properties upon these proteins which are essential for an effective haemostatic function. When carboxylation is impaired because of a deficiency or antagonism of vitamin K, inert precursors of the coagulation factors appear in the blood. These are called "proteins induced by vitamin K absence (PIVKA)". PIVKA-II (des-carboxylated prothrombin) is a sensitive indicator of vitamin K deficiency, chapter 2 (1). Although biochemical vitamin K deficiency is of no clinical importance, it is of value in assessing which group of infants risks developing haemorrhage due to vitamin K deficiency.

Vitamin K deficiency results in a depletion of functional coagulation factors II, VII, IX and X, leading to a generalized bleeding tendency. In infancy three bleeding syndromes are differentiated: early haemorrhagic disease of the newborn (HDN) within 24 hours after birth, classical HDN at the age of 2 to 7 days, and late HDN after the first week of life (2, 3). Especially early and late HDN are often located intracranially, with a fatal or adverse late outcome.

Administration of vitamin K to neonates for the prevention of HDN has become widespread. However, controversies still exist over the need for a general vitamin K prophylaxis and over the best route, frequency and dose of vitamin K to be used (4). **The aim of this study was to establish recommendations for the prevention of vitamin K deficiency in otherwise healthy, normal infants.**

9.1 VITAMIN K PROPHYLAXIS IN GENERAL

The best prophylaxis is to eliminate the cause of vitamin K deficiency. Unfortunately, this is impossible for several reasons. First, in many cases the cause of the deficiency is unresolved (2). Next, in cases with an underlying disease, the cause frequently cannot be eliminated (5) or is only discovered *after* the bleeding episode. Some contributing factors in the aetiology of idiopathic HDN are discussed in chapter 1. Most important factors seem to be a dietary deficiency of vitamin K_1, especially in breast-fed infants, and subclinical fat malabsorption. Motohara *et al.* (6) as well as von Kries *et al.* (7) reported that the total amount of milk intake during the first few days of life was significantly lower in infants who were positive for PIVKA-II than in those negative for PIVKA-II. Administration of 15 μg vitamin K_2

after birth prevented the appearance of PIVKA-II during the first few days of life (6). This corresponds with the recommended dietary intake (RDI) of 5 μg/day for children up to the age of 6 months (8). Since the vitamin K_1 content of human milk is about 2 μg/l (paragraph 1.4.2) the needs of the breast-fed human infant are obviously not met. Greer *et al.* (9) reported that the vitamin K_1 intake of breast-fed infants is approximately 0.6 μg/day, while formula-fed infants ingest as much as 50 μg daily. In other words, vitamin K_1 intake of breast-fed infants is some 100 times lower than that of formula-fed infants. The latter rarely develop symptoms of vitamin K deficiency beyond the neonatal period (3,7,10). In some breast-fed infants with late HDN, the vitamin K_1 content of their mothers' milk was found to be subnormal (11,12,13), in others however it was normal (13). Supplementation of extra vitamin K for breast-fed infants therefore seems reasonable. Some prefer supplementation via breast-milk by dietary advise to the mother. However, more research is needed to establish the efficacy of such a regimen.

The presence of dioxins and related xenobiotics in breast-milk in industrial countries was hypothesized as a cause of late HDN. Koppe *et al.* (14) reported that the mean dioxin content in maternal milk of 4 vitamin K-deficient infants with clinical bleeding was higher than in that of 10 healthy infants. Comparable to the situation for enzyme-inducive anticonvulsants, which cause early HDN, dioxins may induce microsomal enzymes in the neonatal liver and hence increase degradation of vitamin K (14, 15). Additionally, these xenobiotics might disturb the intestinal flora and impair the production of vitamin K_2. More research into the causes of HDN is wanted.

9.2 SAFETY OF VITAMIN K PROPHYLAXIS

Before recommendations on vitamin K prophylaxis are justified, it must be certain that the administration of extra vitamin K is safe. As discussed in paragraph 1.9.1, there is some concern about a possible carcinogenic risk of the intramuscular administration of vitamin K during early life (16). In fetal sheep lymphocytes an increase in sister chromatid exchanges (SCE) was seen after injection of vitamin K_1 (17). In order to determine whether there are indications that vitamin K_1 may have genotoxic or carcinogenic effects in the human neonate, we performed two short-term mutagenicity tests (chapter 3). Neither SCE nor chromosome aberrations showed an increase in the peripheral blood lymphocytes of newborns 24 hours after the administration of 1 mg vitamin K_1 intramuscularly. Thus, we did not find any evidence of genetic toxicity due to administration of 1 mg vitamin K_1 to the newborn infant, but long-term tests are needed as well.

9.3 PREVENTION OF CLASSIC AND LATE HAEMORRHAGIC DISEASE OF THE NEWBORN

Vitamin K prophylaxis was introduced to protect infants against classic HDN and its efficacy has been established beyond doubt (18, 19). Jörgenson *et al.* (20) and O'Connor *et al.* (21) demonstrated that oral administration of vitamin K is as effective in the prevention of classic HDN as intramuscular injection, but follow-up was too limited to conclude about prevention of late HDN. It is doubtful whether all cases of late HDN are prevented by a single administration after birth (3). In Table 1.6 (chapter 1) 104 cases of late HDN despite vitamin K prophylaxis at birth are cited: 93 cases following oral vitamin K administration and 11 after intramuscular administration. Nevertheless, it must be emphasized that most case-reports of late HDN occurred in infants who had not received prophylaxis. To establish recommendations for the prevention of late HDN, we conducted three prospective studies (chapters 4, 5 and 6). Results of PIVKA-II and vitamin K_1 determinations from these studies and from the literature are summarized in Tables 9.1 and 9.2.

Table 9.1 Presence of PIVKA-II ($\geq$0.10 AU/ml) in serum of infants at different ages (n); influence of feeding regimen and vitamin K supplementation.

		Weeks after birth			
	(Ref)	2	4	8	12
FORMULA no vit K	(9)[a]	--	0 (49)	0 (48)	0(52)
BREAST no vit K	(9)[a]	--	4 (73)	4 (44)	3 (40)
BREAST 1 mg oral	(ch 4)	0(165)	3(135)	--	7 (68)
BREAST 1 mg i.m.	(ch 4)	0(166)	1(127)	--	8 (63)
BREAST 1 mg weekly	(ch 5)	--	0 (42)	0 (41)	0 (43)
BREAST 25 μg daily[b]	(ch 6)	--	0 (48)	0 (49)	0 (50)

[a] Reported by Widdershoven *et al.*, who applied a detection limit of 0.13 AU/ml.
[b] In addition to 1 mg vitamin K_1 orally or intramuscularly (i.m.) after birth.

Table 9.2 Mean vitamin K_1 concentrations (pg/ml) in formula-fed and breast-fed infants with and without vitamin K supplementation.

			Weeks after birth			
	(Ref)	*n*	2	4	8	12
FORMULA, no vit K	(ch 6)	10		7044	7885	6040
	(10)	7		2890		
	(22)	8		4450		
	(9)	11				5580
BREAST, no vit K	(10)	12		707		
	(22)	10		490		
BREAST, 1 mg oral	(ch 4)	74	815	391		268
1 mg i.m.	(ch 4)	64	1608	615		329
1 mg i.m.	(10)	13		698		
1 mg i.m.	(9)	23				200
1 mg weekly[a]	(ch 5)	25		1223	927	748
25 μg daily[b]	(ch 6)	32		1972	1371	1068

[a] Vitamin K_1 concentrations 6 to 7 days after the latest administration.
[b] In addition to 1 mg vitamin K_1 oral or i.m. after birth. Vitamin K_1 concentrations 20 to 28 hours after the latest administration.

Oral *versus* intramuscular vitamin K prophylaxis at birth

In chapter 4 two groups of about 165 breast-fed infants were randomized to receive, either orally or intramuscularly, 1 mg vitamin K_1 on the first or second day of life. Although vitamin K_1 plasma concentrations were significantly higher in the intramuscular group, Thrombotests, activities of clotting factors VII and X, and PIVKA-II concentrations did not reveal any difference between the two groups. At the age of 2 weeks vitamin K_1 concentrations were still elevated in comparison with reported levels in unsupplemented infants and PIVKA-II was not detectable, indicating that both prophylactic regimens were still effective at that age. At the age of one month vitamin K_1 concentrations had declined to unsupplemented levels, while PIVKA-II was detectable in few infants. Comparison of our results with those of Widdershoven *et al.* (10) revealed that intramuscular vitamin K still has an effect at the age of one month, while oral vitamin K does not. However, at 3 months of age 11.5% of infants had PIVKA-II in their blood, demonstrating that neither

prophylactic regimen was completely effective at that age. Other reports confirm the reappearance of PIVKA-II after a single administration at birth (10, 23). Taking both these results and epidemiological evidence into account, it can be concluded that for a complete protection of breast-fed infants against the risk of late vitamin K deficiency, the administration should be repeated. In chapters 5 and 6, weekly and daily repeated administrations were evaluated.

The infants in whose blood PIVKA-II was detectable did not differ from the infants without PIVKA-II for Thrombotest results and activities of clotting factors VII and X (Table 4.3). However, these conventional coagulation parameters are not sensitive to a biochemical vitamin K deficiency (1). Haemostasis is not yet impaired in persons with low concentrations of PIVKA-II. Vitamin K_1 concentrations were not different in PIVKA-II positive infants as compared with PIVKA-II negative ones. This might be explained as follows. First, because PIVKA-II has a long half-life, it can still be present after the deficiency has been corrected (paragraph 2.2). Secondly, plasma concentrations of vitamin K_1 vary widely, dependent on the time elapsed since the latest vitamin K_1 intake. The plasma half-life of tritiated vitamin K_1 has been reported to be about 2.5 hours (24, 25). Further, besides vitamin K_1 vitamin K_2 is present in the human liver after the second week of life (26). Vitamin K_2 consists of different menaquinones (MK's) produced by certain intestinal bacteria. *In vitro*, MK-2 to MK-6 have been shown to be as active as vitamin K_1 as cofactor for the carboxylase enzyme (27). Maybe a deficiency of vitamin K_2 might cause a biochemical vitamin K deficiency regardless of vitamin K_1 status.
Determination of vitamin K_2 status is complicated since many MK's must be accounted for. To our knowledge, vitamin K_2 plasma concentrations in infancy have not been reported so far. We succeeded in determining MK-3 to MK-7 by HPLC procedure and applied this to 30 blood samples of formula-fed infants and 134 samples of breast-fed infants. The preliminary results indicate that more different MK's and higher concentrations of MK's are present in formula-fed infants. For example, mean concentrations of MK-5, MK-6, and MK-7 amounted to 659 ± 433, 1854 ± 1032, and 2994 ± 2557 pg/ml, respectively in the formula-fed group, while in the breast-fed group MK-5 and MK-7 were not even detectable. MK-6 amounted to 668 ± 630 pg/ml in this latter group. Differences in intestinal flora between human milk-fed and artificial milk-fed infants have been reported, resulting in a lower vitamin K_2 production in the former group (28). Our results indicate that the vitamin K_2 supply may be an important additional factor in the aetiology of HDN. This offers an extra explanation for the fact that vitamin K_1 and PIVKA-II concentrations are not correlated. On the contrary, PIVKA-II is a direct reflection of the availability of *all* vitamin K for the carboxylation of prothrombin. As will be shown in the next paragraphs, supplementation of vitamin K_1 prevented the appearance of PIVKA-II. We conclude that determination of PIVKA-II is more suitable

for assessing vitamin K status than vitamin K_1 plasma concentrations. Altogether, determination of MK's has opened a new field of research.

Weekly vitamin K prophylaxis in breast-fed infants

In chapter 5, 48 breast-fed infants weekly received an oral dose of 1 mg vitamin K_1. No symptoms of vitamin K deficiency occurred; all infants had normal Thrombotest results and PIVKA-II was not detectable. At the age of 12 weeks, the detection rate of PIVKA-II was significantly lower than in infants who had received a single dose of vitamin K_1 postnatally. So, weekly administration of 1 mg vitamin K_1 seems reliable to prevent late signs of vitamin K deficiency. As was to be expected, vitamin K_1 concentrations showed a negative correlation with the number of days elapsed since the latest vitamin K administration. After 6 to 7 days, concentrations ranged from 278 to 2530 pg/ml. Mean values were significantly higher than in infants who received a single dose at birth. A weekly dose of 1 mg corresponds to a daily dose of approximately 150 μg. This exceeds by far the RDI of 5 μg/day and is 3 times the presumed dosage ingested by formula-fed infants. However, vitamin K_1 plasma concentrations in formula-fed infants were higher (Table 9.2). But the levels in weekly supplemented infants probably fluctuate more than those in formula-fed infants, who continually receive small doses of vitamin K_1. No harmful effects of temporarily high vitamin K_1 plasma concentrations have been reported so far, nor have they been discovered in our cytogenetic study reported in chapter 3. Since 1985 weekly oral administration of 1-2 mg vitamin K_1 to breast-fed infants has been recommended in France and no case of late HDN has been reported so far (29). In 1986 the German Paediatric Society recommended to administer 1 mg vitamin K_1 twice weekly when parenteral application was rejected by the parents (30). In these children concentrations were very high when measured 24 hours after the administration (>10 ng/ml), and were in the range of formula-fed infants after 72 hours (31). Our results demonstrate that administration once per week is equally effective. Perhaps still lower doses are adequate.

Daily vitamin K prophylaxis in breast-fed infants

Formula-feeding seems effective to prevent late vitamin K deficiency, as case-reports of late HDN in formula-fed infants are rare, PIVKA-II is never detectable and vitamin K_1 plasma concentrations are high (10). Supplementation of breast-fed infants to an amount comparable to the dose ingested by formula-fed infants would be a logical approach. No studies evaluating such low doses have been performed. In chapter 6, 58 breast-fed infants who were supplemented daily with 25 μg vitamin K_1, as well as 10 infants who were fed with a formula containing at least 50 μg/l vitamin K_1, are reported. The daily supplements prevented the appearance of PIVKA-II (Table 9.1). Vitamin K_1 concentrations in these infants were not correlated with the number of days of vitamin K_1 treatment, but indeed were negatively correlated

with the number of hours elapsed since the latest vitamin K administration. After 20 to 28 hours concentrations ranged from 112 to 9023 pg/ml. Again, concentrations varied widely between individuals. Mean values were significantly higher than in unsupplemented infants, but were significantly lower than in formula-fed infants whose values ranged from 3028 to 10,884 pg/ml. This is in accordance with the fact that a daily intake of 25 μg vitamin K_1 exceeds by far the presumed intake of about 1 μg/day for unsupplemented breast-fed infants and is about half the dose ingested by formula-fed infants. The RDI of 5 μg/day is generously met. It can be concluded that the suggested maintenance dose is reliable for the prevention of late vitamin K deficiency in breast-fed infants. Whether lower doses are also suitable is unknown at present. Again, no accumulation of vitamin K_1 was discovered. On the contrary, concentrations declined during follow-up, probably because the volume of distribution increases with age.

Whether a daily or a weekly regimen should be preferred is subjective. Mean concentrations prior to the next administration were slightly higher in the daily supplemented group than in the weekly group, although not statistically significant. In the daily supplemented group the range of vitamin K_1 concentrations was wider, but evidently all infants had enough vitamin K available for the carboxylation process, as no PIVKA-II was present. In practice, daily administration of vitamin K soon will be routine for the parents, while a weekly administration is likely to be easily forgotten. When an administration is forgotten or the drug is spat out unnoticed, this has fewer lasting consequences for the daily regimen. The total dose administered in the daily regimen amounts to 2.1 mg *versus* 12 mg in the weekly regimen. We suggest to recommend 1 mg vitamin K_1 either orally or intramuscularly after birth in all newborn babies, followed by a daily administration of 25 μg vitamin K_1 from 2 weeks until 3 months of age in breast-fed infants. Current Dutch recommendations start at the age of one week (32), but it is doubtful whether this is necessary. As reported in chapter 4, a single administration of 1 mg vitamin K_1 at birth is still effective after 2 weeks. The limit of 3 months is also arbitrary. We did not study our subjects beyond this period. Late HDN, however, is rarely reported beyond the age of 3 months (3). The low rate of exclusive breast-feeding as well as availability of vitamin K_2 may partly account for this.

Vitamin K prophylaxis in infants exposed to coumarins

A group of infants especially at risk of vitamin K deficiency-haemorrhage, is that exposed to 4-hydroxy-coumarins. When a lactating mother takes these anticoagulants, her baby is exposed via the milk. Extra vitamin K supplementation to the child has been recommended to overcome the vitamin K antagonism. We studied vitamin K_1 concentrations in 4 infants who were exposed to acenocoumarol (Sin-

trom^R) and were prescribed 1 mg vitamin K_1 per day. Vitamin K_1 concentrations were extremely high: 67.4 ± 46.0 ng/ml. Lower doses are to be recommended.

9.4 PREVENTION OF EARLY HAEMORRHAGIC DISEASE OF THE NEWBORN

Another group of infants at risk of vitamin K deficiency-haemorrhage is that exposed to anticonvulsants (AC). More than 40 case-reports have been published on haemorrhages in neonates born to mothers treated with AC (33). These bleedings occur within 24 hours after birth and are often life-threatening. Vitamin K prophylaxis *after* birth cannot always prevent these intrapartum or early haemorrhages (33, 34). Antenatal vitamin K supplementation via the mother has been suggested as an alternative. Before studying the effects of daily supplementation of 10 mg vitamin K_1 during the last month of pregnancy (chapter 8), an observational study was conducted to establish the incidence of vitamin K deficiency in mothers and neonates exposed to AC (chapter 7).

Incidence of vitamin K deficiency in neonates exposed to anticonvulsants

In a multicenter case-control study, 25 mother-infant pairs on AC therapy and 25 control pairs were studied for PIVKA-II and vitamin K_1 concentrations at 32 weeks of pregnancy and at delivery (chapter 7). Vitamin K_1 plasma concentrations during normal pregnancy are reported to be normal (35) or slightly decreased (22). Vitamin K_1 concentrations in pregnant women using AC were not reported before. They were found to be significantly lower than in control women, especially at delivery. This supports the previously mentioned hypothesis of an increased turnover of vitamin K due to induction of hepatic enzymes by AC (36, 37). Despite the decreased vitamin K_1 concentrations, the women were not vitamin K deficient, since PIVKA-II was rarely detectable. Additional stores of vitamin K_2 may be responsible for this. Neonates, on the contrary, lack hepatic vitamin K_2 in the first 2 weeks of life (26). Because AC readily cross the placenta, they also induce fetal liver enzymes. Together with a placental barrier for vitamin K_1 (paragraph 1.5.2), it is imaginable that fetuses exposed to AC are more likely to develop a vitamin K deficiency. In accordance with the literature, vitamin K_1 concentrations in cord blood of both groups were predominantly below the limit of detection (10,22,35). PIVKA-II was present in low concentrations in 20% of control cord samples, which is in agreement with other reports (10, 38). This again illustrates the marginal vitamin K status of normal infants at birth. In cord blood of neonates with antenatal exposure to AC, PIVKA-II was detected more frequently (54%) and in higher concentrations. Remarkably, valproic acid did not induce PIVKA-II in cord blood of exposed neonates, which is in accordance with the fact that valproic acid is not enzyme-inducive (39). In conclusion, symptoms of vitamin K deficiency are more frequent

and more serious in neonates intra-uterinely exposed to enzyme-inducive AC than in controls. The mothers themselves have decreased vitamin K_1 plasma concentrations, but usually are not deficient in vitamin K.

Antenatal vitamin K prophylaxis

In a consecutive multicenter study, 16 epileptic women using enzyme-inducive AC were treated with 10 mg vitamin K_1 per day from the 36th week of gestation onwards (chapter 8). PIVKA-II was not detectable in any maternal or cord sample. The supplementation raised the median maternal vitamin K_1 level about 60-fold and the median cord level at least 15-fold. The median cord level was 530 pg/ml, which is approximately the normal adult level. A lower dose of vitamin K_1 might be considered. Median maternal-cord ratio was 44, which is exactly the same ratio as reported by Mandelbrot *et al.* (40) in non-epileptic mother-infant pairs. There was no correlation between the cord vitamin K_1 concentrations and the duration of treatment, as confirmed by others (40, 41). In other words, supplementation during just a few days before delivery would be sufficient. To raise cord vitamin K_1 concentrations, the vitamin must be administered at least 4 hours before delivery (18, 41). For correction of haemostasis more time might be necessary. Because the moment of delivery is unpredictable, it may be prudent to start at the 36th or 37th week of gestation. Some reports confirm that prenatal oral administration of vitamin K_1 or K_2 prevents hypoprothrombinaemia in the newborn (42,43,44), others however, do not (45).

In conclusion, antenatal supplementation of vitamin K_1 reverses the increased incidence of (biochemical) neonatal vitamin K deficiency caused by maternal enzyme-inducive AC therapy. This intervention may reduce the frequency of early haemorrhagic disease in newborns of epileptic mothers.

9.5 SUGGESTIONS FOR FUTURE INVESTIGATIONS

To prove efficacy of vitamin K prophylaxis, prospective randomized studies would be most appropriate. Such studies should involve a very large number of babies, since the incidence of HDN is low. For this, as well as for ethical reasons these studies are still lacking.

Currently, research for vitamin K deficiency relies on the assessment of vitamin K status, which is based on either assessment of serum or tissue vitamin K concentrations or on detection of non-carboxylated proteins. Until recently, most data available on tissue and plasma concentrations of vitamin K only regarded vitamin K_1 and not vitamin K_2. Now that determination of menaquinones is possible, much has to be learned about the impact of vitamin K_2 in the development of vitamin K deficiency and HDN. The alternative method to assess vitamin K status is based on the

detection of non or partially carboxylated proteins. In this thesis we used the detection of PIVKA-II and demonstrated that administration of extra vitamin K prevented the appearance of PIVKA-II. It has been questioned, however, whether hepatic proteins, such as the coagulation factors, are the most sensitive markers for a vitamin K deficiency (46). Because it was shown in rats that at low concentrations of vitamin K_1, the liver preferentially absorbs the circulating vitamin K_1 (47), it is conceivable that extrahepatic tissues are depleted first. Consequently, the carboxylation of osteocalcin, a non-hepatic vitamin K-dependent protein (paragraph 1.6.2), might be affected in an earlier phase of vitamin K deficiency than prothrombin. In cord blood of healthy neonates the degree of carboxylation of osteocalcin was reported to be 30-40% of maternal carboxylation (48). It is unknown at present whether vitamin K supplementation increases the degree of carboxylation of osteocalcin. Whether osteocalcin may serve as an additional marker for studying vitamin K requirements, needs further confirmation. Therefore, a study has been initiated in co-operation with the research-group of Vermeer *et al.* (Maastricht) to investigate simultaneously the carboxylation of osteocalcin and prothrombin in cord blood of healthy, term neonates.

Another promising area of research is the development of mixed-micelles solutions of vitamin K_1, which contain glycocholic acid and lecithine as solubilizers in stead of Cremofor EL. It was demonstrated that absorption of vitamin K_1 from mixed-micelles solutions is much better than from conventional solutions, especially in infants with cholestasis (49). The use of such a preparation might result in fewer failures of oral prophylaxis and fewer anaphylactoid reactions after intramuscular and intravenous administration, since preservatives are also omitted. Unfortunately, a suitable product for daily administration of low doses of mixed-micelles vitamin K_1 is not yet available in the Netherlands.

9.6 SUMMARY OF RECOMMENDATIONS

Based on the results of this thesis, we recommend to adapt the recommendations of the Dutch Paediatric Association (32) as follows:

- All healthy, term infants receive an oral dose of 1 mg vitamin K_1 (1 drop of Konakion^R 20 mg/ml, Roche) within a few hours after birth. In other cases, for example perinatal asphyxia, complicated delivery or intra-uterine exposure to anticonvulsants, anticoagulants and rifampicine, an intramuscular injection of 1 mg vitamin K_1 is preferred. When birth weight is less than 1500 grams 0.5 mg should be injected.

- Thereafter breast-fed infants are supplemented with an oral dose of 25 μg vitamin K_1 daily (5 drops of Davitamon K^R, Chefaro) from 2 weeks until 3 months of age. Exclusively formula-fed infants do not need vitamin K supplementation after having recieved 1 mg at birth.

- Pregnant women on enzyme-inducive anticonvulsant medication are prescribed an oral dose of 10 mg vitamin K_1 per day (1 tablet of Konakion^R) from the 36th or 37th week of gestation until delivery.

We hope these recommendations will help to prevent all cases of HDN. In co-operation with the Dutch Surveillance Center of Paediatrics (NSCK) a prospective survey was started to determine the incidence of HDN in the Netherlands and to examine the effect of current methods of vitamin K prophylaxis, using the system of monthly notification cards developed by the British Paediatric Surveillance Unit (50).

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SAMENVATTING

In **hoofdstuk 1** worden de werking en pharmacokinetiek van vitamine K, de verschijnselen bij vitamine K deficiëntie en de controversen met betrekking tot vitamine K profylaxe beschreven.
Vitamine K is een co-factor voor het vitamine K-afhankelijke carboxylase enzym, dat betrokken is bij de produktie van de stollingsfactoren II, VII, IX en X en de stollings-inhibitoren proteine C en S. Door de carboxylering van bepaalde glutaminezuur residuen tot *γ-carboxy*-glutaminezuur residuen, zijn de eiwitten in staat met calcium te binden> Dit is nodig voor een goede haemostatische funktie. Indien de carboxylering gestoord wordt door een tekort aan vitamine K, verschijnen er onvolledig gecarboxyleerde en daardoor a-functionele stollingsfactoren in het bloed. Deze worden "proteins induced by vitamin K absence" (PIVKA) genoemd. PIVKA-II (onvolledig gecarboxyleerd prothrombine) werd in dit proefschrift gebruikt als sensitieve maat voor het bestaan van een vitamine K deficiëntie, naast de vitamine K_1 plasma concentratie (**hoofdstuk 2**).
Een vitamine K deficiëntie leidt tot een gegeneraliseerde bloedingsneiging. Op de zuigelingen-leeftijd worden 3 bloedingspatronen onderscheiden; vroege bloedingen binnen 24 uur na de geboorte, klassieke bloedingen op de 2^e tot 7^e dag, en late bloedingen na de eerste week (Tabel 1.5). De vroege en late bloedingen zijn vaak intracranieel gelocaliseerd, met frequent de dood of neurologische handicaps tot gevolg. Om deze bloedingen te voorkomen wordt op steeds grotere schaal vitamine K profylaxe toegepast. Desalnietemin bestaan er nog vele controversen omtrent de beste wijze, frequentie en dosis van toedienen, resulterend in een niet consistent beleid. **Het doel van dit proefschrift was het vaststellen van adviezen ten aanzien van de preventie van vitamine K deficiëntie bij gezonde jonge zuigelingen.**

Omdat bij het foetale schaap chromosoom schade na intraveneuze injectie van vitamine K_1 is beschreven, werd allereerst een studie verricht naar de genotoxiciteit van vitamine K (**hoofdstuk 3**). Er werd geen toename gezien in zuster-chromatide uitwisselingen, noch in chromosoom breuken in perifere bloed lymfocyten 24 uur na de toediening van 1 mg vitamine K_1 intramusculair aan de pasgeborene. Deze kortetermijn mutageniciteits toetsen gaven aldus geen aanwijzingen voor een genotoxisch effect van de toediening van vitamine K_1 aan de humane neonaat. Echter, langetermijn studies zijn gewenst.

In de **hoofdstukken 4, 5 en 6** werden verschillende vormen van vitamine K profylaxe geëvalueerd op hun betrouwbaarheid in de preventie van láte vitamine K deficienties in gezonde, volledig borstgevoede zuigelingen. Het is onomstreden dat een éénmalige toediening van vitamine K beschermt tegen klassieke bloedingen. Late bloedingen kunnen echter optreden ondanks orale of intramusculaire vitamine K toediening post partum (Tabel 1.6).

In **hoofdstuk 4** worden twee groepen van ongeveer 165 borstgevoede zuigelingen beschreven, die na de geboorte at random ofwel oraal ofwel intramusculair 1 mg vitamine K_1 hadden ontvangen. Ondanks de significant hogere vitamine K_1 plasma concentraties na intramusculaire toediening, was er geen verschil in Thrombotests, activiteiten van de stollingsfactoren VII en X, en PIVKA-II concentraties tussen de groepen. Op de leeftijd van 2 weken waren de vitamine K_1 concentraties in beide groepen hoger dan in ongesuppleerde kinderen en was er geen PIVKA-II aantoonbaar. Dit geeft aan dat beide vormen van vitamine K profylaxe nog effectief zijn na 2 weken. Op de leeftijd van 1 maand waren de vitamine K_1 concentraties overeenkomstig waarden in ongesuppleerde zuigelingen en was bij enkele kinderen PIVKA-II detecteerbaar. Op de leeftijd van 3 maanden was in beide groepen in meer dan 10% van de zuigelingen PIVKA-II aanwezig, wat laat zien dat géén van beide éénmalige toedieningswijzen complete bescherming biedt tegen het optreden van late vitamine K deficiënties.
In **hoofdstuk 5** werden 48 borstgevoede zuigelingen bestudeerd die wekelijks 1 mg vitamine K_1 per os kregen toegediend. Er traden geen tekenen van vitamine K deficiëntie op; alle zuigelingen hadden normale Thrombotest waarden en er was geen PIVKA-II aantoonbaar op de leeftijd van 1, 2 en 3 maanden. Op de leeftijd van 3 maanden was het vóórkomen van PIVKA-II significant minder dan na een éénmalige orale of intramusculaire toediening. De gemiddelde vitamine K_1 concentraties vlak vóór de volgende gift, waren significant hoger dan na een éénmalige vitamine K profylaxe na de geboorte. Er trad geen accumulatie op. Het wekelijks toedienen van 1 mg vitamin K_1 lijkt betrouwbaar in de preventie van late symptomen van vitamine K deficiëntie.
In **hoofdstuk 6** werden 58 zuigelingen met borstvoeding bestudeerd die na de toediening van 1 mg vitamine K_1 per os of intramusculair na de geboorte, vanaf de leeftijd van één week dagelijks 25 μg vitamine K_1 per os kregen toegediend. Eveneens worden hier 10 zuigelingen beschreven die werden gevoed met een kunstvoeding die tenminste 50 μg/l vitamine K_1 bevat. Ook de toediening van 25 μg vitamine K_1 per dag voorkwam het ontstaan van PIVKA-II. Wederom varieerden de vitamine K_1 concentraties sterk interindividueel. Ze waren hoger dan na een éénmalige vitamine K profylaxe, maar lager dan in de flesgevoede zuigelingen. Er trad wederom geen accumulatie op. Of volstaan kan worden met nog kleinere hoeveelheden is vooralsnog onbekend. Of de voorkeur gegeven moet worden aan de dagelijkse of wekelijkse profylaxe toont dit onderzoek niet aan. Enkele overwegingen ten voordele van de dagelijkse toediening zijn:

- minder tijdsfluctuatie in plasma concentratie vitamine K_1
- de totale dosis die gegeven wordt in 3 maanden is beduidend minder (2.1 *versus* 12 mg).
- dagelijkse toediening wordt eerder routine en daardoor minder gemakkelijk vergeten

- indien een gift vergeten wordt of onopgemerkt uitgespuugd wordt, heeft dit minder langdurige consequenties.

Hoofdstukken 7 en 8 hebben betrekking op de preventie van vróege bloedingen. Neonaten waarvan de moeder tijdens de zwangerschap anti-epileptica heeft gebruikt, hebben een risico voor bloedingen die reeds durante partum of binnen 24 uur optreden. Vitamine K toediening na de geboorte kan een ernstige afloop niet altijd meer voorkomen. Voordat het effect van prenatale vitamine K suppletie via de moeder werd bestudeerd, werd middels een observationele studie vastgesteld of de incidentie van vitamine K deficiëntie in deze groep neonaten inderdaad verhoogd is.
In een multicentrum case-control studie werden 25 moeder-kind paren met anti-epileptica en 25 controle paren bestudeerd voor wat betreft PIVKA-II en vitamine K_1 concentraties bij 32 weken amenorrhoe en na de bevalling (**hoofdstuk 7**). Ondanks de significant lagere vitamine K_1 concentraties bij de vrouwen met anti-epileptica, waren zij niet frequent vitamine K deficiënt daar zelden PIVKA-II aantoonbaar was. De beschikbaarheid van vitamine K_2 reserves zou hieraan ten grondslag kunnen liggen. Neonaten daarentegen beschikken de eerste weken nog niet over vitamine K_2 in hun lever en zijn geheel afhankelijk van vitamine K_1. De vitamine K_1 concentratie in het navelstrengbloed was in beide groepen meestal lager dan de detectielimiet, wat overeenkomt met de literatuur. PIVKA-II was aanwezig in 20% van de controle navelstreng-monsters, doch in lage concentraties. In het navelstrengbloed van de neonaten met intra-uteriene blootstelling aan anti-epileptica was PIVKA-II significant vaker (54%) en in hogere concentraties aanwezig. Valproinezuur gaf geen induktie van PIVKA-II. Dit komt overeen met de hypothese dat de verhoogde kans op vitamine K deficiëntie door anticonvulsiva, veroorzaakt wordt door enzym-induktie in de foetale lever. Valproinezuur is immers niet enzym-inducerend. Geconcludeerd werd dat verschijnselen van vitamine K deficiëntie significant vaker en in ernstiger mate optreden bij pasgeborenen die intra-uterien blootgesteld worden aan enzym-inducerende anti-epileptica, dan bij controle kinderen.
Vervolgens werden 16 vrouwen die enzym-inducerende anti-epileptica gebuikten, behandeld met 10 mg vitamine K_1 per os per dag vanaf 36 weken amenorrhoe tot aan de partus (**hoofdstuk 8**). In geen enkel moederlijk of navelstreng monster was PIVKA-II aantoonbaar. De vitamine K_1 concentraties in de navelstreng waren significant gestegen. Geconcludeerd werd dat antenatale vitamine K suppletie de verhoogde kans op neonatale vitamine K deficiëntie - veroorzaakt door maternaal enzym-inducerende anti-epileptica - te niet doet. Deze interventie kan mogelijk bijdragen in de preventie van vroege bloedingen bij pasgeborenen van moeders met anti-epileptica.

In **hoofdstuk 9** worden de resultaten besproken en samengevat. Eveneens worden suggesties voor verder onderzoek gegeven.

Het volgende beleid t.a.v. vitamine K profylaxe wordt voorgesteld:

- Alle gezonde voldragen pasgeborenen ontvangen 1 mg vitamine K_1 per os (1 druppel Konakion^R 20 mg/ml, La Roche) binnen enkele uren na de geboorte. In overige situaties, bv. bij kunstverlossing, asfyxie, aspiratie, expositie aan anticonvulsiva, anticoagulantia of rifampicine, wordt 1 mg vitamine K_1 intramusculair toegediend. Bij een geboortegewicht minder dan 1500 gram wordt 0.5 mg gegeven.
- Zuigelingen met uitsluitend borstvoeding krijgen vervolgens vanaf de leeftijd van 2 weken tot en met 3 maanden, dagelijks 25 μg vitamine K_1 per os toegediend (5 druppels Davitamon K^R, Chefaro). Voor iedere flesvoeding die geintroduceerd wordt vóór de leeftijd van 3 maanden, wordt dagelijks 5 μg (1 druppel) minder gegeven. Zuigelingen gevoed met een gehumaniseerde flesvoeding behoeven geen vitamine K suppletie naast de toediening op de eerste levensdag.
- Aan zwangere vrouwen die enzym-inducerende anticonvulsiva gebruiken, wordt geadviseerd 10 mg vitamine K_1 per dag in te nemen vanaf 36 of 37 weken zwangerschap tot aan de partus.

In samenwerking met het Nederlands Signalerings Centrum Kindergeneeskunde is inmiddels een studie gestart om de incidentie van bloedingen t.g.v. vitamine K deficiëntie in Nederland vast te stellen en het effect van de voorgestelde vitamine K profylaxe te evalueren.

WOORDEN VAN DANK

Hoewel ik tijdens de verdediging van dit proefschrift alleen zal staan, is de tot stand koming van dit proefschrift het werk van velen.
De meeste dank ben ik verschuldigd aan de 459 in dit proefschrift beschreven zuigelingen en hun ouders; voor het in mij gestelde vertrouwen en de gastvrije ontvangst. In totaal werden 20.194 km afgelegd voor 1144 huisbezoeken.
De afdeling Obstetrie (hoofd: Prof. Dr. T.K.A.B. Eskes) en met name 'de kraamafdeling en babykamer' ben ik erkentelijk voor de prettige samenwerking bij de werving van neonaten. Bijna drie jaar lang was er koffie met gebak. Heel plezierig verliep de samenwerking met de verloskundigen Karen Ter Steege, Fenny van der Laan, Jacqueline Gasse en Alie van den Pol, die een deel van de werving op zich namen.

Voor wat betreft de studies omtrent het effect van anti-epileptica ben ik naast dank aan de ruim 66 vrouwen, veel dank verschuldigd aan Prof. Tom Eskes, Régine Steegers-Theunissen en de research-verpleegkundigen Nelleke Hamel, Anneke van de Hurk, Engelien van de Laak en Willie Matthijs. Alleen door aansluiting bij hun project "Primaire Preventie van Aangeboren Afwijkingen" was het mogelijk deze bijzondere patientengroep te bestuderen. Ook dank ik alle (assistent-)gynaecologen en het verloskamer-personeel van de deelnemende centra voor het afnemen van het moederlijk - en navelstrengbloed na de bevalling. Met name wilde ik noemen Dr. H. Bruinse, A.Z.U.; Dr. J. Merkus, Tilburg; en Dhr. J. van der Meer, Groningen. Ook het laboratorium-personeel ben ik dank verschuldigd. Het was de verdienste van de ouders, de portiersdienst van het Radboud Ziekenhuis en de PTT (semafoon, telefoon en pakketdienst) dat het niet vaker mis ging. Irene Willems en Jeanne Kervezée, verloskundigen te Groesbeek, dank ik hartelijk voor de samenwerking bij het verzamelen van een controle groep.

De vitamin K_1 concentraties zijn bepaald in het Laboratorium voor Kindergeneeskunde en Neurologie (hoofd: Prof. Dr. J.M.F. Trijbels). Na voorbereidend werk van Wilma Kruidenberg en Sietske van der Honing (stagiaires L.U. Wageningen), kostte het nog heel wat inspanning van John van Baal, Trude Vogels en Ronney de Abreu om deze bewerkelijke bepaling in eigen huis mogelijk te maken. Later slaagden zij er zelfs in vitamin K_2 te meten. De PIVKA-II concentraties werden met grote nauwkeurigheid door Theo van Lith gemeten. I am very grateful to Kunihiko Motohara and Eisai Co Ltd. for their co-operation on the project and for sending us the indispensable PIVKA-II assay kits.
Het Centraal Haematologisch Laboratorium (hoofd: Dr. J.H.C. Wessels) was eveneens nauw bij dit onderzoek betrokken. Allereerst dank ik Bert Verbruggen, die ondanks de dagelijkse druk van de routine, tijd vrij maakte voor dit onderzoek. Zo

kon Kitty de vele, vele factor VII en X bepalingen uitvoeren. Dank ook aan alle analisten die nog om vijf minuten voor vijf vriendelijk de Thrombotesten uitvoerden. Uitermate leerzaam was de introductie in enkele cytogenetische technieken door Dominique Smeets en Gerard Merkx van de Afdeling Antropogenetica (hoofd: Prof. Dr. H.H. Ropers). Ik voelde mij altijd welkom. Dominique, bedankt voor het corrigeren en bediscussiëren van mijn eerste artikel.

Eveneens wil ik bedanken:
Louis Kollée en Cor van Oostrom voor de enthousiaste begeleiding en steun gedurende de uitvoering van dit project. Louis, door jouw goede contacten in den lande, ben ik steeds met de juiste mensen in contact gekomen.
Dank aan Jan Widdershoven, mijn voorganger in het vitamine K onderzoek. Door zijn medewerking en die van Annemarie Bosman was een snelle start van het onderzoek mogelijk.
Mevr. A. Pietersma-de Bruyn, P. Kuijpers, P. van Haard (R. de Graaf Gasthuis te Delft) en Prof. Dr. J. Ruys; juist omdat we van mening verschillen zijn de discussies zo levendig en vruchtbaar.
Mevr. Benneker, apotheker, ben ik erkentelijk voor de adviezen en de bereidingen van de vitamin K_1 oplossingen.
Ad Theeuwes (voorheen MSA) en Anja Koster (MSA) zorgden voor de statistische ondersteuning. Jan Giesen en Rachel Agbeko, bedankt voor het doorlezen van delen van het manuscript.
Leo Berrevoets en Marian Scholten verzorgden het financiële beheer van dit project op aangename wijze.
Graag wil ik ook het gehele Instituut Kindergeneeskunde (hoofd: Prof. Dr. R.C.A. Sengers) met alle collega-onderzoekers, arts-assistenten, kinderartsen en secretaressen, met name Kitty, José en Jeanne, bedanken voor de geboden faciliteiten en de belangstelling die zij toonden tijdens het onderzoek. Zeker wil ik noemen Conny van Ravenswaay-Arts en Arie van Dijk, want samenwerking met andere "jonge onderzoekers" is onmisbaar én gezellig. Samen stonden we sterk. Achteraf waardeer ik dat de Opleidingscommissie mij gedwongen heeft het manuscript te voltooien vóór het starten van de opleiding tot kinderarts. Anders was dit boekje nog lang niet verschenen. Frans Zegers dank ik voor de vele hand- en spandiensten.
Eveneens dank aan de collegae arts-assistenten en kinderartsen van het St. Joseph Ziekenhuis te Veldhoven voor de steun tijdens de afwerking van het manuscript.

En last but sure not least, wil ik de (pro)motor van dit onderzoek bedanken. Prof. Dr. L.A.H. Monnens, Leo, zonder jouw inzet en steun was dit onderzoek nooit ontstaan, noch uitgevoerd en geslaagd. Jouw altijd durende leer- en nieuwsgierigheid zijn een stimulans voor de wetenschappelijke activiteiten van menig (co-)assistent. Ook ik mocht ervaren dat je steeds weer nieuwe ideeën voor onderzoek hebt. Je begeleiding was perfect; zelden hoefde ik langer dan één week op iets ter correctie

te wachten. Bewonderenswaardig vind ik eveneens dat je naast alle andere literatuur ook de literatuur over vitamine K kon bijhouden, zodat de discussies altijd up to date waren.

Een ieder die ik vergeten ben, wees ervan overtuigd dat ik je zeer dankbaar ben voor alles wat je voor mij of voor het onderzoek betekend hebt. Hierbij denk ik ook aan mijn familie en vrienden die mij met raad en daad bijgestaan hebben. Allen bedankt!

CURRICULUM VITAE

Marlies Cornelissen werd op 8 december 1962 te Terheijden geboren. Nadat zij in 1981 het diploma gymnasium ß behaalde (Newmancollege te Breda), werd begonnen met de studie Geneeskunde aan de Katholieke Universiteit te Nijmegen. In februari 1986 slaagde zij cum laude voor het doctoraal examen, in september 1988 werd het artsexamen afgelegd.
Aansluitend, van oktober 1988 tot en met april 1992, was zij werkzaam als arts-onderzoeker op de afdeling Kindergeneeskunde van het Academisch Ziekenhuis St. Radboud te Nijmegen (hoofd: Prof. Dr. R.C.A. Sengers). Gedurende deze periode werd met subsidie van het Praeventiefonds (project 28-1563) het in dit proefschrift beschreven onderzoek uitgevoerd. In mei 1992 ontving zij de 7^{e} prijs van de European Association of Perinatal Medicine voor een voordracht tijdens het $XIII^{th}$ European Congress of Perinatal Medicine.
Sedert juni 1992 is zij in verband met de opleiding tot kinderarts binnen het cluster Nijmegen, werkzaam als arts-assistent Kindergeneeskunde in het St. Joseph Ziekenhuis te Veldhoven (opleider: Dr. E.J.P. Lommen).

STELLINGEN

behorend bij het proefschrift

PREVENTION OF

VITAMIN K DEFICIENCY

IN INFANCY

E.A.M. Cornelissen

1. Een éénmalige toediening van vitamine K aan borstgevoede zuigelingen is onvoldoende ter preventie van late vitamine K deficiënties (dit proefschrift).

2. De dagelijkse toediening van 25 μg vitamine K_1 is adequaat ter preventie van vitamine K deficiëntie in gezonde jonge borstgevoede zuigelingen (dit proefschrift).

3. De meeste flesvoedingen zijn (te) ruim gesuppleerd met vitamine K_1 (dit proefschrift).

4. Behalve een vitamine K_1 tekort, hebben zuigelingen met borstvoeding ook een relatief tekort aan vitamine K_2 vergeleken met kinderen met flesvoeding (voorlopige resultaten eigen onderzoek).

5. Toediening van vitamine K aan een zwangere vrouw die enzyme-induceren-de anti-epileptica gebruikt, voorkomt een vitamine K tekort bij het kind (dit proefschrift).

6. Routine suppletie van vitamine K aan patiënten met cystic fibrosis is niet nodig (Cornelissen EAM, Lieburg AF van, Motohara K, Oostrom CG van. Acta Paediatr 1992;81:658-61).

7. Kinderen met onbekend spierlijden lopen het gevaar eenzaam op te groeien (Ekeren GJ van, Cornelissen EAM, Stadhouders AM, Sengers RCA. Eur J Pediatr 1991;150:744-50).

8. De Landelijke Neonatale Registratie en het Nederlands Signalerings Centrum Kindergeneeskunde kunnen een belangrijke basis vormen voor beleidsbepaling op het gebied van de kindergeneeskunde.

9. Dat in vitro fertilisatie (IVF) frequent leidt tot drie- en meerlingen is een uiting van onvolwassen technologie met mogelijk kwalijke gevolgen voor ouders en kinderen.

10. Het middels echografie vaststellen van een toegenomen wanddikte van de blaas van een pasgeborene is een belangrijke indikatie voor de diagnose urethrakleppen.

11. Degenen die moedermelk aanraden omdat het het intellect zou bevorderen (Lucas *et al.* Lancet 1992;339:8788), dienen zich te realiseren dat slimmer niet gelijk is aan gelukkiger.

12. Het probleem van de mestoverschotten kan niet alleen op de boeren worden afgewenteld, maar is een verantwoordelijkheid van de hele maatschappij.

13. Het bewerken van een proefschrift tijdens de opleiding kindergeneeskunde is in lichte mate ongunstig voor de opleiding en uitzonderingen daargelaten in grote mate ongunstig voor het onderzoek.

14. Het feit dat babykamers meestal in pastelkleuren blijven ondanks het veranderen van de mode, getuigt van conservatisme in deze periode.

Marlies Cornelissen
Nijmegen, 8 december 1992

ISBN 90-9005406-5