Vitamin K prophylaxis revisited

Focus on risk factors

Peter M. van Hasselt

Peter M. van Hasselt

ISBN 9789490122089
Foto cover Christ window by Marc Chagall (1967)

Lay-out Barbara Hagoort, Multimedia, UMC Utrecht
Print Gildeprint drukkerijen, Enschede

Printing of this thesis was financially supported by:
Sigma Tau
Actelion
BioMarin
Swedish Orphan
Shire Human Genetic Therapies
Genzyme
Nutricia Advanced Medical Nutrition
Orphan Europe

Vitamin K prophylaxis revisited
Focus on risk factors

Een nieuwe kijk op Vitamine K profylaxe
Aandacht voor risicofactoren

(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht op gezag van de rector magnificus, prof.dr. J.C. Stoof, ingevolge het besluit van het college voor promoties in het openbaar te verdedigen op dinsdag 21 april 2009 des middags te 12.45 uur

door

Peter Marin van Hasselt
geboren op 10 februari 1970 te Rijswijk

Promotoren	Prof.dr. J.L.L. Kimpen Prof.dr. R. Berger
Co-promotoren	Dr. T.J. de Koning Dr. R.H.J. Houwen

CONTENTS

Chapter 01

Chapter 01

General Introduction

In 1894 Townsend, a Boston physician, described 50 neonates developing a generalized bleeding tendency in the first week of life.[1] While some of these infants died, the bleeding tendency appeared transient in the majority of these infants, in contrast with other causes of bleeding in infancy recognized at that time, mainly hemophilia. He called this clinical entity hemorrhagic disease of the newborn, a term which is still used by some authors until today. In the following years other descriptions of similar clinical cases noted a reduced capability of the blood of these infants to clot[2] as well as reduced levels of prothrombin.[3] The exact cause behind the observed bleeding tendency, however, remained enigmatic until the early 1930s. Around that time Henrik Dam noted a bleeding tendency in chicks fed a diet from which cholesterol (and other similarly lipophilic molecules) had been removed, while studying the endogenous cholesterol production. The bleeding tendency resembled scurvy, but failed to respond to vitamin C administration.[4] He followed up on this serendipitous observation, and performed a series of investigations which finally led to the identification of a fat soluble substance from alphalpha that could reverse the bleeding tendency. This substance was termed vitamin K (for Koagulation, the Danish name for coagulation).

Vitamin K

Molecular structure Vitamin K is one of the four fat soluble vitamins. Like many other vitamins, it is actually a group of molecules (also called "vitamers") with a common function. The substance first extracted by Dam from alphalpha is now known as phylloquinone (or vitamin K1). It consists of a 2-methyl-1,4-naphtoquinone double-ring structure with a phytyl-side chain at the c3-position. Menaquinones (together known as vitamin K2), share the double-ring structure but differ in their side-chain, which consists of a varying number of prenyl-groups. The number of prenyl groups is used to denote menaquinone (MK) subspecies (MK-n). Menadione (vitamin K3) lacks a side chain, which greatly increases its solubility in water.

Function fourty years after Dam's discovery of vitamin K Suttie and Stenflo elucidated its molecular function. Vitamin K serves as a cofactor in the conversion of protein-bound glutamate (Glu) residues to gamma-carboxylglutamate (Gla) residues.[5-7] These Gla-residues are capable of binding calcium-ions, a prerequisite for proper functioning of prothrombin (factor II), as well as the other vitamin K dependent coagulation factors (factor VII, IX and X).[8] A number of proteins containing a glutamate-rich motif have since been discovered (with a function unrelated to coagulation), which are also dependent on vitamin K for the conversion of these Glu-residues into Gla-residues, hence the name Gla-proteins. Two of these, bone-Gla protein (known as osteocalcin) and matrix Gla protein (MGP) play a pivotal role in balancing calcification of bone as well as other tissues.

Recycling In vertebrates, vitamin K has to be reduced to vitamin K-hydroquinone to serve as a co-factor. During glutamyl-carboxlation, vitamin K is converted into vitamin K epoxide. Regeneration of vitamin K-hydroquinone is subsequently established by the vitamin K epoxide reductase complex (VKORC), closing the circle. A single vitamin K molecule is thought to be recycled ~1000 times.[9] Warfarin and its derivatives block the effect of VKORC, explaining their anticoagulative effect. Similarly, polymorphisms in VKORC have recently been associated with indices of poor vitamin K status.[10]

Biochemical indices of vitamin K deficiency

A variety of indices of vitamin K status are available. The properties of these parameters are summarized below.

Prothrombin time (PT) Provides information on the ability of the so called extrinsic pathway and final common pathway of coagulation to form fibrin. This process requires functionally active vitamin K dependent coagulation factors II, VII and X, as well as thromboplastin and factor V. While a prolonged PT itself is aspecific, normalization upon vitamin K administration is considered diagnostic for vitamin K deficiency. A variety of assays are used, with different normal ranges. To facilitate comparison between the variety of available assays (with different normal ranges) a Prothrombin ratio (PR) can be calculated by dividing the measured PT (in seconds) with the control PT.

Prothrombin percentage Requires a very small sample size which is an advantage in neonates. Changes in prothrombin percentage have little effect on coagulation until dropping below ~20%, while changes below this level have a large impact. As a consequence, this measurement is relatively sensitive to changes in the normal range and insensitive to changes in the hypocoagulable state. Conversion tables are available to estimate the IINR based on this measurement.

Des-gamma-carboxyprothrombin (PIVKA II) Measures relatively undercarboxylated prothrombin species, which are specific for vitamin K deficiency. The assays that are now commercially available are very sensitive and also measure subclinical vitamin K deficiency (VKD).[11] A disadvantage of this high sensitivity is that a clear cut-off point to define a clinically relevant degree of deficiency is difficult to determine. The delayed disappearance of these species after administration of vitamin K is exploited to confirm that a bleeding was due to VKD up to days after administration of vitamin K and normalization of prothrombin time. PIVKA-II levels are not routinely available in the Netherlands and most other countries.

Vitamin K plasma levels Vitamin K is present at picomolar(10^{-12} mol/L) levels in healthy controls, close to the limit of quantification of presently available assays, which involve the use of high pressure liquid chromatography and/or mass spectrometry.[12] A deficiency of vitamin K may result in unquantifiable plasma levels, precluding differentiation between varying degrees of deficiency. Furthermore, plasma levels fluctuate with recent intake and, as vitamin K traffics primarily via VLDL,[13,14] (depend on the fed state), it should ideally be measured after an 8 hour fast.[15] Vitamin K plasma levels are useful to quantify the absorption of vitamin K.

Vitamin K deficiency bleeding (VKDB)

Case definition To allow comparison between countries an international case definition of VKDB has been agreed upon. According to this definition, VKDB is certain if: 1) a PT is at least 4 times higher than the control value (PR>4) and 2) at least one of the following statements is true: a) normal or raised platelet count, normal fibrinogen and absent fibrin degradation products; b) PT returning to normal after vitamin K administration; c) PIVKA-II level exceeding normal controls.[16] Three types of VKDB are recognized.[17] Although this subdivision is based solely on the timing of the bleeding, each type is associated with a different pathogenic mechanism.[17]

Classical VKDB The bleeding tendency first described by Townsend is now known as classical VKDB. Bleedings develop in the first week of life, usually on day 2-5. Most bleedings involve the gastrointestinal tract, the skin, the nose or the umbilicus. Intracranial haemorrhages may also occur, but are rarer. The timing of these bleedings is well understood. Newborns are born with negligible vitamin K stores.[11] As a consequence, they depend on vitamin K intake. The fact that breastfeeding contains little vitamin K,[18] combined with the low milk intake in the first days of life induces a physiological dip in functional prothrombin from day 2-5.[19] Once breastfeeding is built up the higher vitamin K intake allows formation of functional vitamin K dependent proteins, and by the end of the first week prothrombin activity is normal.[20,21] Mild deviations, such as a somewhat delayed initiation or a slower build up of breastfeeding, are known to exaggerate the physiological dip in prothrombin levels to a degree at which spontaneous haemorrhages may occur.[20] Conversely, timely initiation of breastfeeding can decrease the physiological dip and the occurrence of classical VKDB.[8] Although this relationship between milk intake and VKDB was already noted in Townsend first description,[1] (he noted that "…wet nurse feeding, the mother's supply proving a failure, was what saved the baby"), the awareness of this relationship has waxed and waned. The relatively low vitamin K content of human milk is an additional explanation.[19]

Early VKDB VKDB in the first day of life (early VKDB) indicates that vitamin K was already deficient at birth, which implies deficient vitamin K levels in the mother. Indeed, early VKDB is strongly associated with the maternal use of drugs that interfere with vitamin K metabolism, mostly antiepileptic drugs.[8]

Late VKDB. In 1939 Dam et al. reported in the Lancet on a 26 days old boy with "jaundice, a haemorrhagic state and anaemia".[21] He suspected a vitamin K deficiency and checked plasma prothrombin status, which was indeed greatly decreased. Subsequent treatment with vitamin K led to the disappearance of the haemorrhagic state. To our knowledge this is the first report of biochemically confirmed late VKDB. The case history is illustrative of the clinical presentation of late VKDB, even to date. Dam described a boy that was jaundiced from birth onwards, but had looked otherwise healthy up to 3 days before admission. At that time he bled from a small scratch near the nose for about 12 hours. This was followed by vomiting of fresh blood on the day before admission. Stools were greenish yellow, urine was dark. On examination an ill looking child was seen 'prostrated, very jaundiced, and anemic". Petechiae and ecchymoses were present on oral mucosa and back, respectively. Bile pigments were found in urine The following days there were "repeated small muscle spasms in the face and limbs", compatible with an intracranial bleeding. Visible bleeding seized after vitamin K administration. Prothrombin had normalized when it was checked two days afterward. His clinical condition was described as 'satisfactory' later, and cholestatic jaundice disappeared.[21] The clinical description can be summarized by 1) the presence of cholestasis; 2) the appearance of mucosal or skin bleedings; and 3) if the relevance of these bleedings is not appreciated, the development of an intracranial haemorrhage. Late VKDB takes place at a time when breastfeeding has been established. It is relatively rare, but the bleedings are usually severe. Intracranial haemorrhages are present in 50-80 percent in most case series.[22,23] As a result these bleedings are associated with significant mortalilty and morbidity.[24,25] The importance of "warning bleeds" –apparently harmless bleedings preceding a devastating intracranial haemorrhage - was first addressed by McNinch.[22] In this first description, these bleedings were present in 7 out of 10 cases in whom an ICH due to VKDB had developed. Although late VKDB may be due to causes similar to those found in classical VKDB (low milk intake, low vitamin K content of milk), underlying resorptive disorders are often found.[22,23,26,27]

Vitamin K prophylaxis

Within years of Dam's discovery of vitamin K, Waddell and Guerry reported that vitamin K administration directly after birth could prevent the 'physiological' drop in prothrombin levels in the first week of life.[20] Results of administrating vitamin K to the mothers just prior to birth had a similar, although somewhat more variable, effect.[28] Together, these results suggested that vitamin K, when

given prophylactically, could help prevent (classical) VKDB. The first trials investigating prophylactic administration of vitamin K were published in the early 1940s. In these trials, vitamin K was administered to the mother prior to birth, again with variable success.[29] In 1944, Lehmann published the first clinical trial of administering vitamin K to newborns shortly after birth.[29] This trial was made possible by the commercial availability of a synthetic variant of vitamin K, now known as menadione. A dose of 1 mg was shown to significantly reduce the number of bleedings after the first day, but within the first week of life, compared with a historical cohort.[29] A methodological weakness of this study, apart from the use of a historical cohort, was the lack of biochemical confirmation that the remaining cases of bleeding were in fact due to vitamin K deficiency. Based on the hypothesis that the dose used was insufficient to protect all infants, massive dosages (as high as 50 mg!) were used. In the 1950s it became clear that menadione, especially when such high dosages were used, could provoke haemolytic anemia, especially in preterm infants.[30-32] This effect of menadione, but not other vitamin K species, induced a more restricted use of vitamin K, which was followed by an increase in the incidence of VKDB.[33] It also evoked a switch to the use of naturally occurring vitamin K (phylloquinone), which is used in most industrialized countries until today. In the USA, routine administration of 1 mg intramuscular vitamin K shortly after birth is the standard of care since its introduction in 1961. Most other countries lack a uniform national prophylactic regimen: until today local policies may differ in respect with the vitamin K species used, the vitamin K formulation, the route of administration and the dosage. The use of these prophylactic vitamin K regimens were effective in preventing classical VKDB , but seemed to differ in their ability to prevent late VKDB.

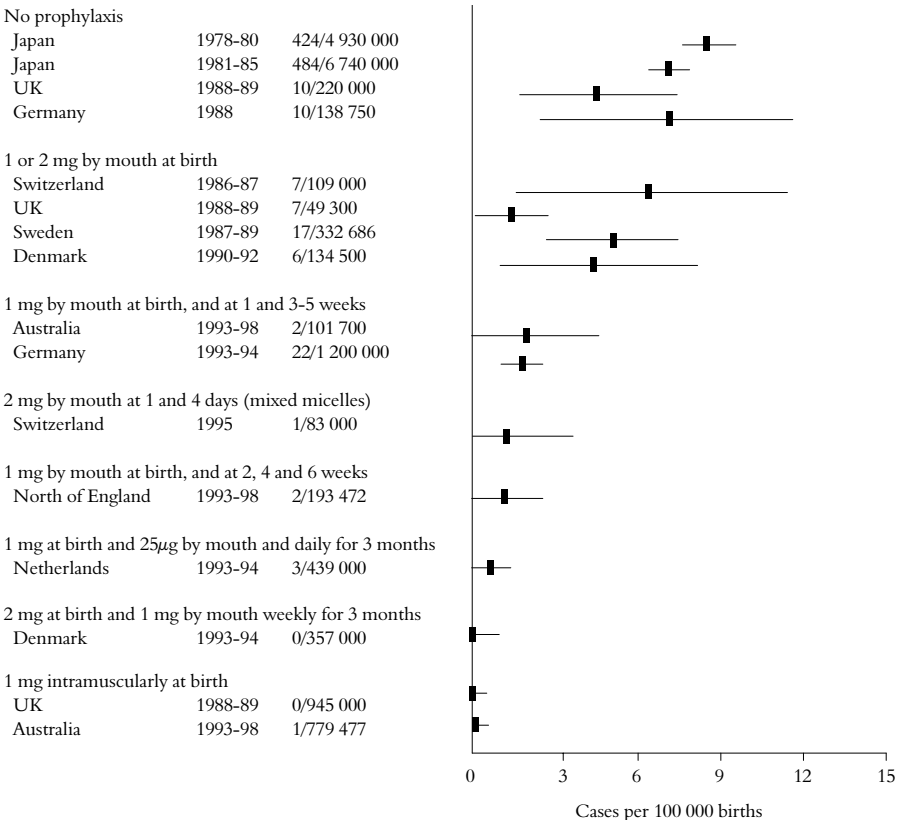
Monitoring the efficacy of vitamin K prophylaxis

Ideally, the efficacy of a prophylactic regimen should be confirmed in a randomized controlled trial. However, the low incidence of late VKDB, even without prophylaxis, combined with the lack of commercial gain, makes it unlikely that such a trial will ever be performed. Thus far, data on the efficacy of various regimens are most commonly deduced from surveillance studies.

Surveillance studies These studies estimate the incidence of a (rare) clinical entity by asking doctors (usually one for each hospital) to report possible cases. A "nothing to report"-option is included and the reporting rate is based on the percentage of cards returned.[22] The patient files of possible cases are reviewed for confirmation/exclusion and to obtain additional clinical information. The case definition for VKDB (see above) is used for confirmation. The number of confirmed cases is divided by the number exposed (for VKDB usually the number of live borns) to estimate the incidence under a certain regimen. Surveillance studies are based on the assumption that there is a direct and inverse relation between the incidence of VKDB and the efficacy of a prophylactic regimen. Moreover, they assume that other factors, such as the rate of reporting/recognition, as

well as risk factors for VKDB, e.g. breastfeeding, dietary deficiency and adherence to the regimen, are also present in comparable proportions. Wariyar compared the incidences of VKDB under various regimens (figure 1).[34] The different reported incidences without prophylaxis indicate that the above assumptions may not be valid. The reported incidence of late VKDB in East-Asian countries is significantly higher than those in Northern European countries.[8,34] This is supported by more recent data from Vietnam, in which the estimated incidence of late VKDB without prophylaxis was as high as 116/100,000.[25] Nevertheless, these data clearly indicate that 1) intramuscular prophylaxis appears to prevent almost all cases of late VKDB; 2) a single oral administration does not prevent late VKDB, regardless of the dose; 3) the efficacy of oral regimen can be improved by increasing the number of dosages. Recent data from Germany indicate that, given a certain multiple dosing regime, a higher dosage may also increase the efficacy.[35] Changing the formulation did not have a significant effect.[27]

Figure 1
Incidence of late VKDB without prophylaxis and under various prophylactic regimens (Wariyar et al.,2000).[34]



Clinical studies Clinical studies have mostly focused on biochemical indices of vitamin K deficiency in healthy subjects. These studies have cast light on the efficacy of a single dose of IM prophylaxis. As first suggested by Loughnan, this is most likely due to a depot mechanism.[36] In support, PIVKA values in 1 month old infants are still undetectable upon IM administration at birth, while the proportion of infants with detectable PIVKA levels in infants receiving oral prophylaxis did not differ from a group in which no prophylaxis was given.[37] The observation that IV administration, a route associated with similarly high vitamin K concentrations, does not offer long term protection against VKDB offers further support.[38] Determination of vitamin K levels in the first months of life in healthy infants revealed that formula feeding (which protects against VKDB) was associated with vitamin K levels well above the normal adult range, and significantly higher than found in breastfed infants, even when supplemented.[39] This is thought to be due to the supplementation of infants formulas with vitamin K, so that they contain approximately 50mcg/L.

Prophylactic failures

Surveillance studies indicate that, despite a much larger cumulative dose of vitamin K compared with IM prophylaxis, most, if not all, oral prophylactic regimens offer less protection against VKDB.[8] Clinical information from these prophylactic failures suggest that VKDB despite prophylaxis is associated with (partial) disadherence, and exclusive breastfeeding.[27,35,40,41] The majority of failures, however, occur in infants with an (unidentified) underlying resorptive disorders.[26,27,34,35,41] Of these, infants with cholestatic liver disease appear to form the largest group. These data suggest that the poorer protection offered by oral prophylaxis may be explained by its inability to protect infants with a (relative) inability to absorb vitamin K.

Absorption of vitamin K

Physiology Vitamin K1 is extremely hydrophobic. Absorption of vitamin K and similarly hydrophobic compounds is attained through the following steps. First, the low pH in the stomach aids in separating hydrophobic compounds from hydrophilic compounds. The amount of lipids subsequently passed on to the intestine is tightly controlled to accommodate rapid elevation of the pH to around 6 by pancreatic bicarbonate as well as solubilisation by bile constituents. These simultaneous processes allow easy access for pancreatic lipase enzymes to the hydrophobic components of the chymus and optimalize pH for hydrolysis. In this environment the different lipase species (triglyceride lipase, phospholipase A, bile acid dependent lipase), in conjunction with ancillary proteins (such as co-lipase), will hydrolyse ester bonds. In general, the degradation products are more hydrophilic and some of these (mainly fatty acids and monoacylglycerides) further aid in solubilisation. During this process hydrophobic compounds can be found in a continuum of structures ranging from large aggregates, which are in equilibrium with smaller particles, such as vesi-

cles (size range 20-60nm) and (mixed) micelles with a size of 2-8 nm).[42,43] Mixed micelles are composed of bile acids, (lyso)phospholipids, fatty acid degradation products and may contain triglycerides, cholesterol and fat soluble vitamins.[44] In contrast with more hydrophilic compounds (such as shorter chain fatty acids) which can, albeit at a low speed, independently move to the brush border, absorption of vitamin K and other extremely hydrophobic compounds critically depends on micelle formation.[45] Incorporation in micelles enables traffic over the unstirred water layer, overlaying the epithelial lining of the intestine.[46] The acidic microclimate, together with the ongoing absorption of micellar components, presumably induces micellar decomposition,[47,48] thereby allowing vitamin K absorption. For vitamin K1 this process primarily takes place in the proximal intestine.[49-51] In adults, oral phylloquinone appears in plasma in about 20 minutes, peaking at 2 hours and then declining exponentially up to 48 to 72 hours.[49]

Pathology Absorption of vitamin K requires bile acids and pancreatic secretions for absorption.[52] Limited data are available regarding the absorption of vitamin K in conditions causing fat malabsorption. Blomstrand and Forsgren investigated the absorption of vitamin K in 5 adults with obstructed bile flow.[53] They observed an uptake of 0-3%, compared with an absorption of 37-62 % in adults with intact biliary flow.[53,54] Shearer investigated the absorption of vitamin K in patients with various forms of fat malabsorption[55] and confirmed that a lack of bile had a more profound effect than pancreatic insufficiency.

Effects of pharmaceutical formulation on vitamin K absorption The molecular structure of vitamin K does not allow esterification, a method successfully exploited in the design of d-alpha-tocopheryl polyethylene glycol 1000 succinate (TPGS), which allows vitamin E absorption under cholestatic conditions.[56,57] Interestingly, administration of vitamin D together with TPGS under cholestatic conditions induced a substantial increase in? absorption of this fat soluble vitamin.[58] To our knowledge, the effect of co-administration has not been studied for vitamin K. In view of the pivotal role of micelle formation in the absorption of vitamin K there were high hopes that a formulation in which vitamin K was dissolved in mixed micelles (Konakion MM®) would lead to a much better protection of infants with an underlying resorptive disorder. Initial studies indeed suggested a higher degree of protection, compared with the former vitamin K formulation in which vitamin K was emulsified using cremophor (Konakion EL®).[59] However, introduction of Konakion MM in oral vitamin K prophylactic schemes had no measurable effect on the incidence of VKDB.[27] In addition the majority of patients who had a failure of the prophylactic regimen turned out to have an underlying cholestatic disorder.[26,27] A more recent study revealed that the absorption of vitamin K in infants with conjugated bilirubinemia was indeed low, and erratic.[60]

The Dutch vitamin K prophylaxis

In 1990, a revised national vitamin K prophylactic regimen was introduced in the Netherlands.[61] This regimen was based on an entirely different approach than other oral prophylactic regimens. While most oral prophylactic regimens consisted of a relatively few large doses of vitamin K, usually administered by health professionals, the Dutch approach also started with a dose of 1mg at birth by a health professional but thereafter advised parents of breastfed infants to administer relatively small amounts (25 mcg) of vitamin K each day, from the second week of life until the infant was either 3 months old or received more than half of its feedings as formula.[61] A food supplement was introduced for this purpose, in which vitamin K was dissolved in arachid oil. The concept of this regimen was to mimic the protection offered by formula. In the Netherlands, as in most Western countries (Germany being an exception) infant formula is fortified and contains approximately 50 mcg of vitamin K per liter. This means that an average newborn, weighing 3 kg and consuming 150 ml/kg/day would receive ~25 mcg shortly after birth, gradually increasing to around 40mcg at the age of 3 months. A surveillance study performed shortly after the introduction of the Dutch regimen appeared to support its efficacy:[41] The risk of late VKDB (idiopathic and secondary causes) in infants receiving prophylaxis was 0.2 (95% CI 0-1.3)/100,000. The total incidence of VKDB was somewhat higher: 1.1/100,000, but one infant did not receive any prophylaxis while 3 others were labelled as treatment failures,[16] meaning that the underlying disease was recognized, but not acted upon. It was concluded that the protection offered by this regimen appeared to be comparable to the protection offered by IM prophylaxis.[41] The introduction of the Dutch prophylaxis coincided with a debate on a putatively increased risk of childhood malignancies in newborns that had received vitamin K intramuscularly.[62,63] As a result, in reviews and commentaries that appeared on the subject in the following years, the Dutch approach was praised and compared to solving the Gordian knot:[64] while the low dosage circumvented the high peak vitamin K levels found upon IM administration (one of the prime suspects of the putative increased risk of childhood malignancies in infants receiving IM vitamin K), the high dosing frequency –appreciative of the short half life of vitamin K - appeared to offer protection against VKDB.[64] However, in subsequent years a number of case reports were published that described infants developing life threatening haemorrhages under the Dutch prophylactic regimen.[65,66] Unrecognized underlying resorptive disorders were found in all these infants.

Vitamin K prophylaxis revisited: focus on risk factors

The protection offered to infants with unrecognized cholestasis appeared to be the Achilles' tendon of oral prophylactic regimens. This is biological plausible, in view of the extreme dependency of vitamin K on micellar formation for its absorption. This thesis is therefore based on the idea that, in view of the well established risk factors for late VKDB (particularly breastfeeding and unrecognized resorptive disorders) a focussed investigation in patients with these risk factors may improve our understanding of the role of each of these factors, and hopefully, to find ways to improve vitamin K prophylaxis.

Aims of this thesis

- To compare the protection against VKDB offered to infants with underlying resorptive disorders by the Dutch prophylactic regimen with other prophylactic regimens.
- To investigate the role of different feeding types on the risk of VKDB at initial presentation of cholestasis.
- To investigate interventions to improve the absorption of vitamin K in the absence of bile in an animal model.

Outline of this thesis

Biliary atresia (BA) is the most frequent cause of cholestatic liver disease in infancy. In **chapter 2**, we used the Dutch and Danish national registries for BA to compare the risk of VKDB in exclusively breastfed infants with BA under the present Dutch prophylaxis with the risk under the former and present Danish prophylaxis (consisting of an oral dose of 2 mg at birth followed by weekly doses of 1 mg and a single IM dose of 2 mg at birth, respectively). The risk of each of these regimens was compared with the risk of VKDB in formula fed infants.
In **chapter 3** we focused on infants with alpha-1-antitrypsin deficiency (A1ATD). A1ATD is the second most frequent cause of cholestasis in infancy. It is associated with a variable, and generally milder, degree of cholestasis compared with BA. We compared the risk of VKDB in exclusively breastfed infants and (partially) formula-fed infants presenting with cholestasis due to A1ATD.
In **chapter 4** we combined data from the BA and A1ATD registry from January 1991 to December 2006 to determine whether a rise in breastfeeding rates in these populations at risk, could help explain an observed rise of VKDB in the Netherlands. Furthermore, we performed a national survey to establish the adherence to the Dutch prophylactic regimen.
In **chapter 5** we focused on (partially) formula fed infants with BA or A1ATD to investigate whether certain types of formula, particularly hypoallergenic formulas, were associated with a higher risk of biochemical VKD
In **chapter 6** we investigated whether reliable absorption of vitamin K in the absence of bile could be attained by encapsulation of vitamin K in polymeric micelles. For this study we compared the

absorption of vitamin K from this formulation in sham operated rats with the absorption in bile duct ligated rats, a model for cholestatic liver disease.
In **chapter 7** we combined in vitro and in vivo studies to investigate whether the poor and erratic absorption of orally delivered vitamin K formulated as a mixed micelle could be explained by the conditions in the gastric environment.
In **chapter 8** the results of these studies are summarised and discussed.
Chapter 9 provides a Dutch summary.

REFERENCES

1. Townsend CW. The haemorrhagic disease of the newborn. Arch Pediatr 1894;11:559-65.

2. Schwartz H, Ottenberg R. Amer J Med Sci 1910;140:17.

3. Whipple GH. Arch intern Med 1912;9:365.

4. Dam H, Schonheyder F. A deficiency disease in chicks resembling scurvy. Biochem J 1934;28:1355-9.

5. Shah DV, Suttie JW. Mechanism of action of vitamin K: evidence for the conversion of a precursor protein to prothrombin in the rat. Proc Natl Acad Sci USA 1971;68:1653-7.

6. Suttie JW. Mechanism of action of vitamin K: demonstration of a liver precursor of prothrombin. Science 1973;179:192-4.

7. Stenflo J, Fernlund P, Egan W, Roepstorff P. Vitamin K dependent modifications of glutamic acid residues in prothrombin. Proc Natl Acad Sci USA 1974;71:2730-3.

8. Shearer MJ. Vitamin K deficiency bleeding (VKDB) in early infancy. Blood Rev 2009;23:49-59.

9. Suttie JW, Preusch PC, McTigue JJ. In: Johnson BC, editor. Posttranslational Covalent Modifications of Proteins.New York: Acadamic Press; 1983;253-79.

10. Nimptsch K, Nieters A, Hailer S, Wolfram G, Linseisen J. The association between dietary vitamin K intake and serum undercarboxylated osteocalcin is modulated by vitamin K epoxide reductase genotype. Br J Nutr 2008; doi:10.1017/S0007114508131750.

11. Shearer MJ. Vitamin K metabolism and nutriture. Blood Rev 1992;6:92-104.

12. Suhara Y, Kamao M, Tsugawa N, Okano T. Method for the determination of vitamin k homologues in human plasma using high-performance liquid chromatography-tandem mass spectrometry. Anal Chem 2005;77:757-63.

13. Sadowski JA, Hood SJ, Dallal GE, Garry PJ. Phylloquinone in plasma from elderly and young adults: factors influencing its concentration. Am J Clin Nutr 1989;50:100-8.

14. Lamon-Fava S, Sadowski JA, Davidson KW, O'Brien ME, McNamara JR, Schaefer EJ. Plasma lipoproteins as carriers of phylloquinone (vitamin K1) in humans. Am J Clin Nutr 1998;67:1226-31.

15. Saupe J, Shearer MJ, Kohlmeier M. Phylloquinone transport and its influence on gamma-carboxyglutamate residues of osteocalcin in patients on maintenance hemodialysis. Am J Clin Nutr 1993;58:204-8.

16. Sutor AH, von Kries R, Cornelissen EA, McNinch AW, Andrew M. Vitamin K deficiency bleeding (VKDB) in infancy. ISTH Pediatric/Perinatal Subcommittee. International Society on Thrombosis and Haemostasis. Thromb Haemost 1999;81:456-61.

17. Lane PA, Hathaway WE. Vitamin K in infancy. J Pediatr 1985;106:351-9.

18. von Kries R, Shearer M, McCarthy PT, Haug M, Harzer G, Gobel U. Vitamin K1 content of maternal milk: influence of the stage of lactation, lipid composition, and vitamin K1 supplements given to the mother. Pediatr Res 1987;22:513-7.

19. Motohara K, Matsukane I, Endo F, Kiyota Y, Matsuda I. Relationship of milk intake and vitamin K supplementation to vitamin K status in newborns. Pediatrics 1989;84:90-3.

20. Waddell WW, Dupont Guerry BWE. JAMA 1939;112:2259.

21. Dam H, Tage-Hansen E, Plum P. Vitamin-K lack in normal and sick infants. Lancet 1939;234:1157-61.

22. McNinch AW, Tripp JH. Haemorrhagic disease of the newborn in the British Isles: two year prospective study. BMJ 1991;303:1105-9.

23. Sutor AH, Dagres N, Niederhoff H. Late form of vitamin K deficiency bleeding in Germany. Klin Padiatr 1995;207:89-97.

24. Cekinmez M, Cemil T, Cekinmez EK, Altinors N. Intracranial hemorrhages due to late-type vitamin K deficiency bleeding. Childs Nerv Syst 2008;24:821-5.

25. Danielsson N, Hoa DP, Thang NV, Vos T, Loughnan PM. Intracranial haemorrhage due to late onset vitamin K deficiency bleeding in Hanoi province, Vietnam. Arch Dis Child Fetal Neonatal Ed 2004;89:F546-F550.

26. Schubiger G, Berger TM, Weber R, Banziger O, Laubscher B. Prevention of vitamin K deficiency bleeding with oral mixed micellar phylloquinone: results of a 6-year surveillance in Switzerland. Eur J Pediatr 2003;162:885-8.

27. von Kries R, Hachmeister A, Gobel U. Oral mixed micellar vitamin K for prevention of late vitamin K deficiency bleeding. Arch Dis Child Fetal Neonatal Ed 2003;88:F109-F112.

28. The Lancet. Haemorrhage in the newborn. Lancet 1944;243:506-8.

29. Lehmann J. Vitamin K as a prophylactic in 13,000 infants. Lancet 1944;243:493-4.

30. Beamish RE, Storrie VM. Severe haemolytic reaction following the intravenous administration of emulsified vitamin K (mephyton). Can Med Assoc J 1956;74:149-52.

31. Allison AC, Moore T, Sharman IM. Haemolysis and haemoglobinuria in vitamin-E deficient rats after injections of vitamin-K substitutes. Br J Haematol 1956;2:197-204.

32. Allison AC. Danger of vitamin K to newborn. Lancet 2008;265:669.

33. McNinch AW, Orme RL, Tripp JH. Haemorrhagic disease of the newborn returns. Lancet 1983;1:1089-90.

34. Wariyar U, Hilton S, Pagan J, Tin W, Hey E. Six years' experience of prophylactic oral vitamin K. Arch Dis Child Fetal Neonatal Ed 2000;82:F64-F68.

35. von Kries R, Hachmeister A, Gobel U. Can 3 oral 2 mg doses of vitamin K effectively prevent late vitamin K deficiency bleeding? Eur J Pediatr 1999;158:S183-6.

36. Loughnan PM, McDougall PN. Does intramuscular vitamin K1 act as an unintended depot preparation? J Paediatr Child Health 1996;32:251-4.

37. Motohara K, Endo F, Matsuda I. Vitamin K deficiency in breast-fed infants at one month of age. J Pediatr Gastroenterol Nutr 1986;5:931-3.

38. Loughnan PM, McDougall PN, Balvin H, Doyle LW, Smith AL. Late onset haemorrhagic disease in premature infants who received intravenous vitamin K1. J Paediatr Child Health 1996;32:268-9.

39. Cornelissen EA, Kollee LA, van Lith TG, Motohara K, Monnens LA. Evaluation of a daily dose of 25 micrograms vitamin K1 to prevent vitamin K deficiency in breast-fed infants. J Pediatr Gastroenterol Nutr 1993;16:301-5.

40. McNinch A, Busfield A, Tripp J. Vitamin K deficiency bleeding in Great Britain and Ireland: British Paediatric Surveillance Unit Surveys, 1993 94 and 2001-02. Arch Dis Child 2007;92:759-66.

41. Cornelissen M, von Kries R, Loughnan P, Schubiger G. Prevention of vitamin K deficiency bleeding: efficacy of different multiple oral dose schedules of vitamin K. Eur J Pediatr 1997;156:126-30.

42. Hernell O, Staggers JE, Carey MC. Physical-chemical behavior of dietary and biliary lipids during intestinal digestion and absorption. 2. Phase analysis and aggregation states of luminal lipids during duodenal fat digestion in healthy adult human beings. Biochemistry 1990;29:2041-56.

43. Staggers JE, Hernell O, Stafford RJ, Carey MC. Physical-chemical behavior of dietary and biliary lipids during intestinal digestion and absorption. 1. Phase behavior and aggregation states of model lipid systems patterned after aqueous duodenal contents of healthy adult human beings. Biochemistry 1990;29:2028-40.

44. Hofmann AF. Micellar solubilization of fatty acids and monoglycerides by bile salt solutions. Nature 1961;190:1106-7.

45. Woollett LA, Wang Y, Buckley DD, Yao L, Chin S, Granholm N et al. Micellar solubilisation of cholesterol is essential for absorption in humans. Gut 2006;55:197-204.

46. Thomson AB, Schoeller C, Keelan M, Smith L, Clandinin MT. Lipid absorption: passing through the unstirred layers, brush-border membrane, and beyond. Can J Physiol Pharmacol 1993;71:531-55.

47. Shiau YF. Mechanism of intestinal fatty acid uptake in the rat: the role of an acidic microclimate. J Physiol 1990;421:463-74.

48. Shiau YF, Levine GM. pH dependence of micellar diffusion and dissociation. Am J Physiol 1980;239:G177-G182.

49. Hollander D, Rim E. Effect of luminal constituents on vitamin K1 absorption into thoracic duct lymph. Am J Physiol 1978;234:E54-E59.

50. Hollander D, Rim E, Muralidhara KS. Vitamin K1 intestinal absorption in vivo: influence of luminal contents on transport. Am J Physiol 1977;232:E69-E74.

51. Hollander D. Vitamin K1 absorption by everted intestinal sacs of the rat. Am J Physiol 1973;225:360-4.

52. Mann JD, Mann FD, Bollman JL. Hypoprothrombinemia due to loss of lymph. Am J Physiol 1949;158:311-4.

53. Forsgren L. Studies on the intestinal absorption of labelled fat-soluble vitamins (A, D, E, and K) via the thoracic-duct lymph in the absence of bile in man. Acta Chir Scand Suppl 1969;399:1-29.

54. Blomstrand R, Forsgren L. Vitamin K1-3H in man. Its intestinal absorption and transport in the thoracic duct lymph. Int Z Vitaminforsch 1968;38:45-64.

55. Shearer MJ, Mallinson CN, Webster GR, Barkhan P. Absorption of tritiated vitamin K1 in patients with fat malabsorption. Gut 1970;11:1063-4.

56. Traber MG, Kayden HJ, Green JB, Green MH. Absorption of water-miscible forms of vitamin E in a patient with cholestasis and in thoracic duct-cannulated rats. Am J Clin Nutr 1986;44:914-23.

57. Sokol RJ, Butler-Simon N, Conner C, Heubi JE, Sinatra FR, Suchy FJ et al. Multicenter trial of d-alpha-tocopheryl polyethylene glycol 1000 succinate for treatment of vitamin E deficiency in children with chronic cholestasis. Gastroenterology 1993;104:1727-35.

58. Argao EA, Heubi JE, Hollis BW, Tsang RC. d-Alpha-tocopheryl polyethylene glycol-1000 succinate enhances the absorption of vitamin D in chronic cholestatic liver disease of infancy and childhood. Pediatr Res 1992;31:146-50.

59. Amedee-Manesme O, Lambert WE, Alagille D, De Leenheer AP. Pharmacokinetics and safety of a new solution of vitamin K1(20) in children with cholestasis. J Pediatr Gastroenterol Nutr 1992;14:160-5.

60. Pereira SP, Shearer MJ, Williams R, Mieli-Vergani G. Intestinal absorption of mixed micellar phylloquinone (vitamin K1) is unreliable in infants with conjugated hyperbilirubinaemia: implications for oral prophylaxis of vitamin K deficiency bleeding. Arch Dis Child Fetal Neonatal Ed 2003;88:F113-F118.

61. Uitentuis J. [Administration of vitamin K to neonates and infants]. Ned Tijdschr Geneeskd 1990;134:1642-6.

62. Golding J, Greenwood R, Birmingham K, Mott M. Childhood cancer, intramuscular vitamin K, and pethidine given during labour. BMJ 1992;305:341-6.

63. Golding J, Paterson M, Kinlen LJ. Factors associated with childhood cancer in a national cohort study. Br J Cancer 1990;62:304-8.

64. Tripp JH, McNinch AW. The vitamin K debacle: cut the Gordian knot but first do no harm. Arch Dis Child 1998;79:295-7.

65. Merelle ME, Griffioen RW, nkert-Roelse JE. Cystic fibrosis presenting with intracerebral haemorrhage. Lancet 2001;358:1960.

66. van Hasselt PM, Houwen RH, van Dijk AT, de Koning TJ. [Vitamin K deficiency bleeding in an infant despite adequate prophylaxis]. Ned Tijdschr Geneeskd 2003;147:737-40.

Chapter 01
Introduction

Chapter 02

Chapter 02

Prevention of Vitamin K Deficiency Bleeding in Breastfed Infants: *Lessons from the Dutch and Danish Biliary Atresia Registries*

Peter M van Hasselt[a], Tom J de Koning[a], Nina Kvist[b], Elsemieke de Vries[c], Christina Rydahl Lundin[d], NeSBAR[e], Ruud Berger[f], Jan LL Kimpen[a], Roderick HJ Houwen[a], Marianne Horby Jorgensen[d], Henkjan J Verkade[c]

[a]Department of Pediatrics, Wilhelmina Children's Hospital, University Medical Center Utrecht, the Netherlands; [b]Department of Pediatric Surgery, University Hospital of Copenhagen, Copenhagen, Denmark; [c]Department of Pediatrics, University Medical Center Groningen, Groningen, the Netherlands; [d]Department of Pediatrics, University Hospital of Copenhagen, Copenhagen, Denmark; [e]Netherlands Study group for Biliary Atresia Registry: DC Aronson, A Kindermann, CMF Kneepkens, LWE van Heurn, AM van den Neucker, ZJ de Langen, PMJG Peeters, G Madern, JH Escher, DC van der Zee, PNMA Rieu, JJM Tolboom; [f]Laboratory for Metabolic Diseases, University Medical Center Utrecht, Utrecht, The Netherlands.

Pediatrics 2008;121;e857-e863

ABSTRACT

Objective

Newborns routinely receive vitamin K to prevent Vitamin K deficiency bleeding. The efficacy of oral vitamin K administration may be compromised in infants with unrecognized cholestasis. We aimed to compare the risk of VKDB under different propylactic regimens in infants with biliary atresia.

Patients and Methods

From Dutch and Danish national biliary atresia registries we retrieved infants that were either breastfed and received 1 mg oral vitamin K at birth followed by 25 μg daily oral vitamin K prophylaxis (Netherlands, 1991-2003), 2 mg oral vitamin K at birth followed by 1 mg weekly oral prophylaxis (Denmark, 1994-May 2000), or 2 mg of intramuscular prophylaxis at birth (Denmark, June 2000-2005), or were fed by formula. We excluded infants in whom the type of prophylaxis was ambiguous. We determined the absolute and relative risk of severe vitamin K deficiency and vitamin K deficiency bleeding on diagnosis in breastfed infants on each prophylactic regimen and in formula-fed infants.

Results

Vitamin K deficiency bleeding was noted in 25/30 of breastfed infants on 25 μg daily oral prophylaxis, in 1 of 13 on 1 mg of weekly oral prophylaxis, in 1 of 10 receiving 2 mg of intramuscular prophylaxis at birth, and in 1 of 98 formula fed infants ($p < 0.001$). The relative risk of a bleeding in breastfed compared with formula-fed infants was 77.5 for 25 μg of daily oral prophylaxis; 7.2 for 1 mg of weekly oral prophylaxis and 9.3 for 2 mg intramuscular prophylaxis at birth.

Conclusions

A daily dose of 25 μg vitamin K fails to prevent bleedings in apparently healthy infants with unrecognized cholestasis because of biliary atresia. One milligram of weekly oral prophylaxis offers significantly higher protection to these infants and is of similar efficacy as 2 mg of intramuscular prophylaxis at birth. Our data underline the fact that event analysis in specific populations at risk can help to evaluate and improve nationwide prophylactic regimens.

Vitamin K is essential for effective coagulation, and a deficiency may result in spontaneous life-threatening hemorrhages.[1] As a consequence of the short half-life of vitamin K compared with other vitamins, newborns can become deficient within days in case of inadequate intake. A bleeding because of vitamin K deficiency (VKD) shortly after birth is known as hemorrhagic disease of the newborn, or classical VKD bleeding (VKDB). A bleeding after the first week of life is called "late VKDB".[2] Approximately 50% of infants with late VKDB present with an intracranial hemorrhage.[3–5]

Breastfeeding is recognized as a major risk factor for both forms of VKD bleedings.[4,6,7] This is presumably because of a lower vitamin K intake in breastfed infants: whereas human milk contains 1 to 2 μg/L, most infant formulas are artificially fortified and contain ~50 μg/L. A variety of prophylactic regimens have been introduced to prevent VKDB in breastfed infants.[7–10] A single intramuscular dose of vitamin K at birth is considered most efficacious, reducing the incidence of classical, as well as late, VKDB to <0.2 per 100 000, although recent evidence suggests that effectiveness may be hampered by a higher risk of omission of prophylaxis.[10–12] Oral administration of vitamin K at birth prevents classical VKDB but fails to prevent late VKDB, even when administered in very high dosages.[13] Based on these observations, breastfed infants on oral prophylaxis receive additional doses of vitamin K in the first months of life.[12] This strategy has substantially reduced the incidence of late VKDB.[12–14] However, prophylactic failures have continued to occur, mostly in infants who later proved to have a cholestatic liver disease.[3,9,15–18]

Cholestatic infants are especially sensitive to suboptimal vitamin K availability, because the absence of intestinal bile greatly reduces the absorption of vitamin K and other fat-soluble vitamins.[19,20] The repeated occurrence of prophylactic failures in infants with unrecognized cholestatic liver disease warrants careful evaluation of the efficacy of vitamin K prophylactic regimens in these infants. National registries for biliary atresia provide a unique opportunity to quantitate the efficacy of vitamin K prophylaxis in a well-defined and homogeneous population of cholestatic infants. These registries allow us to determine the absolute risk of VKDB in breastfed infants under different prophylactic regimens. Moreover, these risks can be weighed against the risk in formula-fed infants. We performed a retrospective cohort study in Dutch and Danish infants with biliary atresia to compare the efficacy of frequent (daily or weekly) oral vitamin K prophylaxis with intramuscular (IM) vitamin K prophylaxis at birth.

METHODS

Study Population

Data from Dutch biliary atresia patients born between 1991 and 2003 were obtained from the Netherlands Study Group for Biliary Atresia Registry, a joint effort of the Dutch Society for Pediatrics and the Dutch Society for Pediatric Surgery. Danish biliary atresia patients born between 1994 and 2005 were retrieved from the University Hospital of Copenhagen (Rigs Hospital) Department of Pediatric Surgery. The Rigs Hospital is the Danish national referral center for patients with suspected biliary atresia and for Kasai hepatoportoenterostomy. Routine vitamin K prophylaxis aims to prevent VKDB in apparently healthy term infants. Infants with a gestational age of <37 weeks or a birth weight of <2000 g were excluded, because these infants routinely receive additional vitamin K prophylaxis. Patients hospitalized from birth until diagnosis were also excluded, because they may have received additional vitamin supplements, as well as other diets. Late VKDB has been defined to occur after the first week of life and before the age of 6 months.[2] Accordingly, infants were excluded if cholestasis was diagnosed before the first week of life or after the age of 6 months. Patient files were reviewed to obtain relevant demographic data and clinical characteristics. Age at diagnosis was defined as the age that cholestasis was first diagnosed. Cholestasis was defined as a total serum bilirubin concentration of >50 μmol/L with a direct fraction of >20%. Clinical or biochemical indications for a VKD at diagnosis were recorded, as well as information on (adherence to) vitamin K prophylaxis. Infants were categorized as "breastfed" if they had received exclusively breastfeeding from birth onward. All of the other infants were categorized as "formula fed."

Vitamin K Prophylaxis

Since 1990, all of the infants born in the Netherlands receive an oral dose of 1 mg of vitamin K directly after birth. On breastfeeding, parents are advised to give their child a daily oral dose of 25 μg of vitamin K from the second week of life until the end of the 13th week. For daily dosing of vitamin K, a dietary supplement is used in which vitamin K is solved in arachid oil. The vitamin K prophylaxis can be stopped earlier if breastfeeding accounts for <50% of the feedings.[16] In Denmark, 2 different vitamin K prophylaxis regimens have been used. Between 1994 and June 2000, all of the infants born after an uncomplicated delivery received an oral dose of 2 mg of vitamin K directly after birth. In the case of a complicated delivery (forceps or vacuum extraction, cesarean section, perinatal asphyxia, and prematurity) the vitamin K dose of 2 mg was administered intramuscularly.[13] Subsequently, breastfed infants received a weekly oral dose of 1 mg of vitamin K (Konakion EL, Roche, Basel, Switzerland). Parents were advised to continue vitamin K administra-

tion as long as infants were breastfed for >50% of their daily feedings. Since June 2000, the Danish prophylactic regimen has consisted of a single IM dose of 2 mg of vitamin K (Konakion MM, Roche, Basel, Switzerland) after birth for all of the infants.[13] In both countries, formula-fed infants only receive vitamin K prophylaxis directly after birth. Thereafter, they are expected to receive sufficient amounts of vitamin K, because the formula feedings commercially available in these countries contain ~50 μg of vitamin K per liter.[21] Data from formula-fed infants receiving oral prophylaxis at birth from both countries were used to assess the efficacy of formula feeding.

VKD

We calculated the prothrombin ratio (PR) at initial diagnosis to be able to compare coagulation parameters from different hospitals in both countries and used it to assess the presence of VKD. The PR was determined as follows. If available, the international normalized ratio was used as PR. If a prothrombin percentage had been determined, the international normalized ratio was estimated from the conversion table supplied by the producer (Axis Shield, Oslo, Norway). In case of a prothrombin time (PT) in seconds, the PR was determined as follows: $PR = PT_{patient}:PT_{control}$. If a $PT_{control}$ was not determined by the laboratory, it was defined as the mean of the provided reference range. VKD was defined as a PR of >1.5 in combination with a normal thrombocyte count. A PR above this threshold is rare in healthy infants after the first week of life.[22,23] Significant PR elevations in otherwise healthy biliary atresia infants are unlikely to be because of other causes.[24] A PR of >4 was designated as "severe" VKD. VKDB was defined as bruising, bleeding, or intracranial hemorrhage in combination with a PR of >4 in any infant between 8 days and 6 months of age and normalizing after administration of vitamin K.[2] The number of bleedings and their locations were noted.

Statistical Analysis

We performed a 2-way analysis of variance of clinical and biochemical parameters with a normal distribution pattern to test for statistical differences between groups. Kruskal-Wallis analysis was used for those parameters with a nonnormal distribution. In case statistical significant differences were found, a Bonferroni test for multiple comparisons or a Mann-Whitney U test was used for posthoc analysis, respectively. Fisher's exact test was performed to determine statistical significance between groups in case of dichotomous parameters. The relative risks (RRs) and 95% confidence intervals (CIs) for VKDB and biochemical levels of VKD were calculated. We performed conditional logistic regression analysis to assess potential confounding. First, all of the clinical and biochemical parameters were considered as possible confounders and included, 1 by 1, as covariates. Risk factors that changed the odds ratio (OR) by >10% were added to a model and were maintained in the final model if they induced a change of >10% in that model. SPSS 12.01 (SPSS Inc, Chicago, IL) was used for all of the analyses.

RESULTS

Between January 1991 and December 2003, 139 biliary atresia patients were included into the Netherlands Study Group of Biliary Atresia Registry (Table 1). Seventeen of these infants did not meet the inclusion criteria; 9 had stayed in a hospital from birth until diagnosis, 7 were born outside of the Netherlands, and 1 was excluded because of prematurity. Thirty (25%) of the remaining 122 infants were exclusively breastfed, and 88 (72%) were formula fed. In 4 infants, the type of feeding was not documented. In total, 63% of all of the infants received any amount of breastfeeding, which is similar to the 51% to 63% of infants using breastfeeding at the age of 6 weeks in the Netherlands from 1991 to 2003, as known from epidemiologic data (www.cbs.nl/statline). A total of 46 patients born between January 1994 and December 2005 were reported to the Danish Biliary Atresia Registry. Nine infants were excluded; 6 had stayed in a hospital from birth until diagnosis and 3 were excluded because of prematurity. In addition, 5 infants born before June 2000 were excluded because vitamin K was administered IM after a complicated delivery. Twenty three of the remaining 32 infants (77%) were breastfed, of which 13 were born before June 2000.

Clinical Characteristics

Infants from both countries were categorized according to the type of prophylaxis that they received. Table 2 summarizes the clinical characteristics in breastfed infants on frequent (daily or weekly) oral or IM prophylaxis and the combined group of formula-fed infants. Overall, breastfed infants had a slightly, but significantly, higher birth weight than formula-fed infants. Cholestasis was found ~14 days earlier in Dutch breastfed infants compared with formula-fed infants ($P < .001$). The age at diagnosis correlated significantly with parameters of cholestatic liver disease.

VKD in Breastfed Infants

VKD was evident in all (30 of 30) Dutch breastfed infants with biliary atresia (Table 3). VKD was severe in 29 of 30 (96%), and this was associated with a VKDB in 25 of 30 (83%). Fifteen infants (50%) had multiple bleedings. An intracranial hemorrhage was diagnosed in 13 of 30 (43%) Dutch breastfed infants. In contrast, VKDB occurred in only 1 of the Danish infants after weekly oral prophylaxis (1 of 13 [8%]) and also in 1 after IM prophylaxis (1 of 10 [10%]; each $P < .001$ compared with the Dutch daily oral prophylaxis). No intracranial hemorrhages were documented in Danish breastfed infants on either regimen. Nevertheless, VKD was a relatively frequent finding, present in 5 of 13 (39%) of the Danish breastfed infants on weekly oral prophylaxis and in 3 of 10 (30%) of the infants on IM prophylaxis at birth.

Efficacy of Vitamin K Prophylaxis in Breastfed Infants: Comparison With Formula Feeding

We compared the risk of (severe) VKD and VKDB in breastfed infants receiving frequent oral or IM prophylaxis with the risk in formula-fed infants (Table 4). Only 1 of the 93 formula-fed infants had a VKDB. Dutch breastfed infants were poorly protected against VKDB compared with formula-fed infants, with a relative risk for a VKDB of 77.5 (95% CI: 11.0–548.0). Similar results were obtained when only Dutch formula-fed infants were used for comparison (RR: 73.3; 95% CI: 10.4–518.0).

Table 1 Patients and populations

	Netherlands (1991-2003)	Denmark (1994-June 2000)	Denmark (July 2000-2005)
prophylactic regimen	1mg oral at birth 25 μg daily oral	2mg oral at birth 1 mg weekly oral	2 mg IM at birth
No. of live births ⋆	2 571 602	440 529	356 602
enlisted in biliary atresia registry, n	139	26	20
incidence of biliary atresia	1:18 501	1:16 943	1:17 830
excluded			
born abroad	7	0	0
hospitalised from birth	9	1	5
premature	1	2	1
unknown feeding type [¶]	4	0	0
complicated delivery [#]		5	
formula-fed [$]			4
included, n	**122**	**18**	**14**
breast-fed	30	13	10
formula-fed	88	5	

⋆ Data on Dutch and Danish live births were derived from the Central Bureau of Statistics (http://statline.cbs.nl), and Denmark Statistics (http://www.dst.dk/HomeUK.aspx). [#] received two types of prophylaxis; IM and weekly oral. [$] received two types of propylaxis; IM and formula. [¶] type of prophylaxis could not be determined

Both Danish regimens offered substantially better protection. Nevertheless, the risk of a severe VKD in Danish breastfed infants on either IM or weekly oral prophylaxis was still clearly elevated when compared with formula-fed infants. The risk for VKDB was not significantly different between Danish breastfed and formula-fed infants.

To evaluate whether the occurrence of VKDB could be the result of diagnostic delay, the age of diagnosis in infants with VKDB was compared with infants without VKDB. Instead, as depicted in Figure 1, infants with VKDB presented significantly earlier, suggesting that a VKDB leads to an earlier diagnosis.

Table 2 Comparison of characteristics for each type of prophylaxis

	25 μg daily oral (n=30)	**1 mg weekly oral (n=13)**	**2 mg IM at birth (n=10)**	**Formula-fed (n=93)**	**P**
male gender - no. (%) ¶	10 (33)	7 (54)	6 (60)	49 (53)	.26
birth weight - g ⋆	3351 ± 404	3517 ± 347	3304 ± 525	3115 ± 629	.05
age at diagnosis - days §	35 (28-43)	46 (26-59)	54 (16-70)	49 (38-61)	.001
weight at diagnosis - g ⋆	3959 ± 423	4531 ± 922	4576 ± 1186	4150 ± 845	.08
bilirubin total - μmol/L §	147 (104-202)	153 (97-289)	197 (140-304)	178 (122-257)	.08
bilirubin direct - μmol/L §	98 (71-135)	124 (85-193)	135 (82-182)	127 (93-176)	.13
bilirubin indirect -μmol/L §	41 (27-76)	193 (152-233)	181 (125-237)	154 (125-180)	.09
ASAT - U/L §	144 (94-235)	131 (103-154)	86 (63-150)	104 (84-133)	.42
ALAT - U/L §	88 (66-135)	52 (34-111)	49 (43-88)	37 (25-62)	.08

Infants with biliary atresia. All infants in first three columns are exclusively breast-fed. ⋆ Plus-minus values are means ± SD, p value was determined with ANOVA. § Values are median (interquartile range), p value was determined with Kruskal Wallis.¶ P value was determined with Fisher's Exact.

Theoretically, other clinical characteristics could have served as confounding factors for the higher risk of VKDB in Dutch breastfed versus formula-fed biliary atresia infants. However, conditional logistic regression resulted in an initial OR of 435 (95% CI: 48.6–3897.0) for VKDB in breastfed versus formula-fed infants and an adjusted OR of 933 (95% CI: 28.7–30 371.0). Logistic regression revealed that female gender was a risk factor for VKDB (OR: 4.1; 95% CI: 1.5–11.3). However, female gender was not significantly associated with (severe) VKD.

Table 3 Risk of vitamin K deficiency in breast-fed infants with biliary atresia under different prophylactic regimens

	25 μg daily oral	1 mg weekly oral	2mg IM at birth	P	daily vs weekly oral		daily oral vs IM at birth	
					RR	(95% CI)	RR	(95% CI)
vitamin K deficiency ¶	30/30 (100%)	5/13 (39%)	3/10 (30%)	< .001	2.6	(1.3-5.2)	3.3	(1.3-8.6)
severe vitamin K deficiency ¶	29/30 (97%)	3/13 (23%)	2/10 (20%)	< .001	4.2	(1.5-11.3)	4.8	(1.4-16.7)
VKDB	25/30 (83%)	1/13 (8%)	1/10 (10%)	< .001	10.8	(1.6-71.7)	8.3	(1.3-53.9)
intracranial haemorrhage	13/30 (43%)	0/13 (0%)	0/10 (0%)	.001	*		*	

Data are number/total number (%). The p value was determined with Fisher's exact. ¶ defined by PR>1.5 for VKD and PR>4.0 for severe VKD and normal thrombocyte count. * cannot be computed. P value was 1.0 for all comparisons between weekly oral and IM prophylaxis.

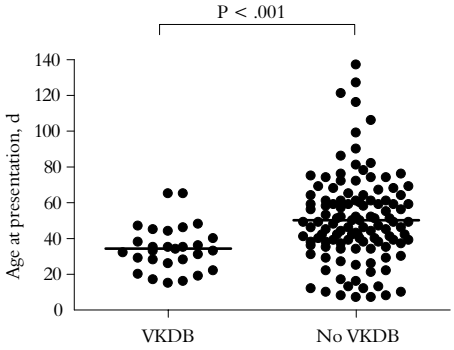


Figure 1
Age at presentation in Dutch and Danish biliary atresia infants. Infants with VKDB presented significantly earlier (34 vs 50 days, p<0.001). There was a significant interaction with feeding type (R=0.609, p<0.001): 27 of the 28 VKDB infants (96%) and 26/118 (14%) of the "no VKDB" infants were breast-fed (p<0.001).

Table 4 Relative risk of vitamin K deficiency in breast-fed infants with biliary atresia despite prophylaxis compared with formula-fed infants

		vitamin K deficiency [¶]		severe vitamin K deficiency [¶]		Vitamin K deficiency Bleeding	
		RR	**(95% CI)**	**RR**	**(95% CI)**	**RR**	**(95% CI)**
formula-fed [$]	(N=93)	1.0		1.0		1.0	
breast-fed + 2mg IM at birth	(N=10)	4.7	(1.4-15.8)	18.6	(1.9-187)	9.3	(0.6-138)
breast-fed + 1mg weekly oral	(N=13)	6.0	(2.1-16.8)	21.5	(2.4-191)	7.2	(0.5-108)
breast-fed + 25 mcg daily oral	(N=30)	15.5	(7.2-33.6)	89.9	(12.7-632)	77.5	(11.0-548)

[$] Dutch and Danish formula-fed infants with biliary atresia receiving oral (not IM) prophylaxis were combined. VKDB was found in 1/93, severe VKD in 1/93 and VKD in 6/93 of these infants. The risk in formula-fed infants was used to compare the risk in breast-fed infants under the three prophylactic regimens. [¶] defined by PR>1.5 for VKD and PR>4.0 for severe VKD. [¶] p values as determined with Fisher's exact were .04 for vitamin K deficiency, .02 for severe vitamin K deficiency and .19 for VKDB. [$] P values were .004 for vitamin K deficiency, .005 for severe vitamin K deficiency and .23 for VKDB. [#] P values were <.001 for all comparisons. Similar results were found when PR>2 was chosen to define VKD; RR for IM prophylaxis 6.2 (1.2-32.8), for weekly oral prophylaxis 9.5 (2.4-37.9), for daily oral prophylaxis 31 (10.2-94.4).

DISCUSSION

Our data show that the Dutch vitamin K prophylactic regimen does not protect breastfed infants with biliary atresia. More than 80% of these infants had developed a VKDB at the time that cholestasis was diagnosed. The clinical significance of this is illustrated by the fact that 43% of breastfed infants with biliary atresia presented with an intracranial hemorrhage. The risk of VKDB in Dutch breastfed infants with biliary atresia was 8 to 10 times higher than in breastfed infants on either weekly oral prophylaxis or IM prophylaxis at birth and ~80 times higher than in formula-fed infants.

The design of the present study was set out to minimize the risk of bias. To reduce selection bias, we focused on infants with biliary atresia, a rarely missed diagnosis for which national registries are available. The incidence of biliary atresia in the Netherlands and Denmark, calculated from our present study, was 1:21 321 and 1:17 329, respectively. These incidences are in close agreement with the earlier reported incidence of 1:20 000.[25] Theoretically, the high overall risk of VKDB in this study could be attributed to a higher detection rate of mild cases. However, the observed fraction of infants with VKDB presenting with an intracranial hemorrhage was in line with previous data,[3,5] which makes this option unlikely. We excluded infants who might have received additional vitamin K, such as preterm infants, to reduce the risk of misclassification. Although some clinical parameters differed significantly between groups, these parameters did not significantly influence the risk of VKDB in breastfed infants on conditional logistic regression analysis.

Our definition of a VKD was based on an elevated PR in the absence of thrombocytopenia, parallel to the case definition of VKDB. Although this definition largely excludes diffuse intravascular coagulation, it does not exclude other causes of PR elevation and may, therefore, give rise to misclassification. However, we do not consider this a major concern in the context of infants with biliary atresia, a condition characterized by poor vitamin K absorption. Moreover, liver dysfunction in biliary atresia is unlikely to cause a PR of >1.5 during the first months of life.[24] Similarly, a "physiologic" PR of >1.5 is rare after the first week of life.[22,23] We also analyzed the data using a higher cutoff value of the PR (>2), but the results were essentially identical (Table 4).

The observed failure of the current Dutch regimen to protect breastfed biliary atresia infants contrasts with surveillance data obtained after introduction of this regimen.[12] These surveillance data indicate that daily oral vitamin K prophylaxis was of similar efficacy as IM prophylaxis. This conclusion was supported by the observation that Dutch breastfed infants had adequate vitamin K levels throughout the first 3 months of life and that their coagulation parameters remained within normal limits.[26] However, these studies focused on healthy infants and did not address the efficacy of vitamin K prophylaxis in conditions with poor absorption of vitamin K, such as that observed in cholestatic infants. This approach may give rise to equivocal results: recent surveillance data from the Netherlands under the same prophylactic regimen seem to contradict the previous findings, with a reported incidence of 3:100 000.[27] Five of 6 reported failures had an unrecognized cholestatic liver disease.

Reliable information on the efficacy of prophylactic regimens in high-risk infants is of increasing importance, because these infants now represent the vast majority of prophylactic failures.[3,9,15] The method presented here, comparison of well-delineated and homogeneous risk populations, may be very helpful in this respect. In contrast to nationwide surveillance studies, which are sensitive to underreporting,[3,12] it seems to be a reliable method to detect prophylactic failures. The presently applied method not only allows us to determine the number of failures but also to calculate the failure rate, because clinical information can be obtained from all of the infants within such a population. It also enables a comparison of failure rates between different subpopulations, such as breastfed infants. Moreover, the availability of biochemical parameters can be used to establish the risk of a "near miss," (eg, a severe VKD diagnosed before a bleeding could develop). Most importantly, as presently shown, this strategy allows the detection of differences between regimens in their ability to protect high-risk populations, which remained undetected using surveillance studies. Comparison of the 2 oral regimens indicates that 1-mg weekly prophylaxis is more effective than 25 μg daily prophylaxis. The most likely explanation for this observation is the dosage; the cumulative dose per week was >5 times higher in the weekly regimen (1.00 mg vs 0.18 mg for weekly and daily oral prophylaxis, respectively). It has been hypothesized that a high dosing frequency may require a relatively low dose of vitamin K to obtain good efficacy.[28] However, our present data do not support the hypothesis that a daily dose of 25 μg is sufficient.

This study firmly establishes the efficacy of formula feeding in preventing VKDB in infants with unrecognized cholestasis. Formula feeding is even more effective than IM prophylaxis, the "gold standard," to prevent VKDB. The mechanism underlying the preventive efficacy of formula feeding remains unclear. The vitamin K content of formula offers an insufficient explanation, because the average estimated daily intake of vitamin K in formula in our cohort (based on a daily

formula intake of 150 mL/kg) is 25 to 50 μg, similar to the dose prescribed in the Dutch prophylactic regimen. A higher production of vitamin K by colonic bacteria in formulafed infants might play a role.[5] However, even a mixed-micellar vitamin K formulation, containing bile acids, is poorly absorbed in infants with biliary atresia.[29] Logistic regression revealed that female gender was a risk factor for VKDB. Although both genders were at a similar risk to present with a (severe) biochemical VKD, female infants were at a significantly higher risk of developing a VKDB. This result has not been reported previously, and the meaning of this finding is presently unknown.

CONCLUSIONS

We quantified the efficacy of frequent oral vitamin K prophylactic regimens in a cohort of Dutch and Danish biliary atresia infants. Our data clearly indicate that the current Dutch prophylaxis of 25 μg of daily oral vitamin K is insufficient to protect exclusively breastfed infants with biliary atresia from VKDB. Effective oral prophylaxis is feasible, as shown by the efficacy of a weekly oral dose of 1 mg of vitamin K in protecting these infants. Based on these data, the current Dutch prophylactic regimen is presently being reevaluated. We feel that nationwide prophylaxis should be tailored to protect those individuals who are at the highest risk to develop an adverse event. An event analysis in high-risk populations, as a means of "postmarketing" surveillance, could help to achieve this objective.

Acknowledgments
We thank the Netherlands Study Group for Biliary Atresia Registry, which includes the following individuals: D. C. Aronson, A. Kindermann, C. M. F. Kneepkens, L. W. E. van Heurn, A. M. van den Neucker, Z. J. de Langen, P. M. J. G. Peeters, G. Madern, J. H. Escher, D. C. van der Zee, P. N. M. A. Rieu, and J. J. M. Tolboom.

REFERENCES

1. Dam H, Dyggve H, Larsen H, Plum P. The relation of vitamin K deficiency to hemorrhagic disease of the newborn. Adv Pediatr. 1952;5(1):129–153

2. Sutor AH, von Kries R, Cornelissen EA, McNinch AW, Andrew M. Vitamin K deficiency bleeding (VKDB) in infancy. ISTH Pediatric/Perinatal Subcommittee. International Society on Thrombosis and Haemostasis. Thromb Haemost. 1999;81(3):456–461

3. von Kries R, Hachmeister A, Gobel U. Oral mixed micellar vitamin K for prevention of late vitamin K deficiency bleeding. Arch Dis Child Fetal Neonatal Ed. 2003;88(2):F109–F112

4. Sutherland JM, Glueck HI, Gleser G. Hemorrhagic disease of the newborn: breast feeding as a necessary factor in the pathogenesis. Am J Dis Child. 1967;113(5):524–533

5. Lane PA, Hathaway WE. Vitamin K in infancy. J Pediatr. 1985; 106(3):351–359

6. Chaou WT, Chou ML, Eitzman DV. Intracranial hemorrhage and vitamin K deficiency in early infancy. J Pediatr. 1984; 105(6):880–884

7. McNinch AW, Orme RL, Tripp JH. Haemorrhagic disease of the newborn returns. Lancet. 1983;1(8333):1089–1090

8. Hey E. Vitamin K–what, why, and when. Arch Dis Child Fetal Neonatal Ed. 2003;88(2):F80–F83

9. Wariyar U, Hilton S, Pagan J, Tin W, Hey E. Six years' experience of prophylactic oral vitamin K. Arch Dis Child Fetal Neonatal Ed. 2000;82(1):F64–F68

10. Busfield A, McNinch AW, Tripp JH. Neonatal vitamin K prophylaxis in Great Britain and Ireland: the impact of perceived risk and product licensing on effectiveness. Arch Dis Child. 2007;92(9):754–758

11. McNinch AW, Tripp JH. Haemorrhagic disease of the newborn in the British Isles: two year prospective study. BMJ. 1991; 303(6810):1105–1109

12. Cornelissen M, von Kries R, Loughnan P, Schubiger G. Prevention of vitamin K deficiency bleeding: efficacy of different multiple oral dose schedules of vitamin K. Eur J Pediatr. 1997; 156(2):126–130

13. Hansen KN, Minousis M, Ebbesen F. Weekly oral vitamin K prophylaxis in Denmark. Acta Paediatr. 2003;92(7):802–805

14. Tripp JH, McNinch AW. The vitamin K debacle: cut the Gordian knot but first do no harm. Arch Dis Child. 1998;79(4): 295–297

15. Schubiger G, Berger TM, Weber R, Banziger O, Laubscher B.Prevention of vitamin K deficiency bleeding with oral mixed micellar phylloquinone: results of a 6-year surveillance in Switzerland. Eur J Pediatr. 2003;162(12):885–888

16. van Hasselt PM, Houwen RH, van Dijk AT, de Koning TJ. Vitamin K deficiency bleeding in an infant despite adequate prophylaxis [in Dutch]. Ned Tijdschr Geneeskd. 2003;147(16): 737–740

17. Loughnan PM, McDougall PN. Epidemiology of late onset haemorrhagic disease: a pooled data analysis. J Paediatr Child Health. 1993;29(3):177–181

18. McNinch A, Busfield A, Tripp JH. Vitamin K deficiency bleeding in the Great Britain and Ireland; British Paediatric Surveillance Unit Surveys, 1993–94 and 2001–02. Arch Dis Child. 2007;92(9):759–766

19. Forsgren L. Studies on the intestinal absorption of labelled fat-soluble vitamins (A, D, E, and K) via the thoracic-duct lymph in the absence of bile in man. Acta Chir Scand Suppl. 1969;399(1):1–29

20. Shearer MJ, Mallinson CN, Webster GR, Barkhan P. Absorption of tritiated vitamin K1 in patients with fat malabsorption. Gut. 1970;11(12):1063–1064

21. Greer FR, Marshall SP, Foley AL, Suttie JW. Improving the vitamin K status of breastfeeding infants with maternal vitamin K supplements. Pediatrics. 1997;99(1):88–92

22. Andrew M. The relevance of developmental hemostasis to hemorrhagic disorders of newborns. Semin Perinatol. 1997;21(1): 70–85

23. Clarke P, Mitchell SJ, Sundaram S, Sharma V, Wynn R, Shearer MJ. Vitamin K status of preterm infants with a prolonged prothrombin time. Acta Paediatr. 2005;94(12): 1822–1824

24. Yanofsky RA, Jackson VG, Lilly JR, Stellin G, Klingensmith WC III, Hathaway WE. The multiple coagulopathies of biliary atresia. Am J Hematol. 1984;16(2):171–180

25. Houwen RH, Zwierstra RP, Severijnen RS, et al. Prognosis of extrahepatic biliary atresia. Arch Dis Child. 1989;64(2):214–218

26. Cornelissen EA, Kollee LA, van Lith TG, Motohara K, Monnens LA. Evaluation of a daily dose of 25 micrograms vitamin K1 to prevent vitamin K deficiency in breast-fed infants. J Pediatr Gastroenterol Nutr. 1993;16(3):301–305

27. Ijland MM, Pereira RR, Cornelissen EA. Incidence of late vitamin K deficiency bleeding in newborns in the Netherlands in 2005: evaluation of the current guideline. Eur J Pediatr. 2008; 167(2):165–169

28. Tripp JH, McNinch AW. The vitamin K debacle: cut the Gordian knot but first do no harm. Arch Dis Child. 1998;79(4): 295–297

29. Pereira SP, Shearer MJ, Williams R, Mieli-Vergani G. Intestinalabsorption of mixed micellar phylloquinone (vitamin K1) is unreliable in infants with conjugated hyperbilirubinaemia: implications for oral prophylaxis of vitamin K deficiency bleeding. Arch Dis Child Fetal Neonatal Ed. 2003;88(2):F113–F118

Chapter 02

Preventing vitamin K deficiency bleeding in breastfed infants

Chapter 03

Chapter 03

Vitamin K deficiency bleeding in cholestatic infants with alpha-1-antitrypsin deficiency

Peter M van Hasselt[a], Karin Kok[b,c], Adriane DM Vorselaars[a], Lotte van Vlerken[a], Richard de Vries[c], Ed Nieuwenhuys[d], Tom J de Koning[a], Roderick HJ Houwen[a]

[a]Department of Pediatrics, Wilhelmina Children's Hospital, University Medical Center Utrecht, the Netherlands; [b]Department of Medicine, Division of Gastroenterology and Hepatology, Radboud University Medical Center Nijmegen, the Netherlands; [c]Department of Gastroenterology and Hepatology, Rijnstate Hospital, Arnhem, the Netherlands; [d]Department of Immunochemistry, Sanquin Diagnostics, Amsterdam, the Netherlands.

Accepted: Archives of Diseases in Childhood

ABSTRACT

Objective
Exclusively breast-fed infants with unrecognized cholestatic jaundice are at risk of a vitamin K deficiency bleeding (VKDB). It is presently unknown whether (the size of) this risk depends on the degree of cholestasis. Since α-1-antitrypsin deficiency (A1ATD) induces a variable degree of cholestasis, we assessed the risk of VKDB in infants with cholestatic jaundice due to A1ATD.

Patients and methods
Infants with a ZZ or SZ phenotype born in the Netherlands between January 1991 and December 2006 were retrieved from the databases of the 5 Dutch diagnostic centres for α-1-antitrypsin phenotyping and/or genotyping. We determined the risk of VKDB upon diagnosis in breastfed and formula-fed infants and searched for correlations between serum levels of conjugated bilirubin and the risk of bleeding.

Results
A total of 40 infants with A1ATD were studied. VKDB was noted in 15/20 (75%) of breast-fed infants, compared with 0/20 of formula-fed infants with A1ATD. The relative risk for VKDB in breast-fed versus formula-fed infants was at least 15.8 (95% confidence interval 2.3 to 108). Conjugated bilirubin levels did not correlate with the risk of VKD(B).

Conclusions
The risk of VKDB in breast-fed infants with A1ATD was high and did not correlate with serum level of conjugated bilirubin. A similar absolute risk was previously reported in breast-fed infants with biliary atresia under the same prophylactic regimen. This suggests that - without adequate prophylaxis - the risk of VKDB is uniformly high in exclusively breast-fed infants with cholestatic jaundice, irrespective of underlying etiology.

Vitamin K prophylaxis aims to protect infants against the potentially life threatening coagulation disorder that results from severe vitamin K deficiency. In breast-fed infants a vitamin K deficiency bleeding (VKDB) may occur within days from birth due to the low vitamin K stores at birth[1], the short half life of vitamin K[2,3] and the low vitamin K content of human milk[4]. Vitamin K prophylaxis at birth effectively prevents VKDB in the first week of life. However, to prevent a vitamin K dependent coagulation disorder after the first week of life (late VKDB) ongoing oral vitamin K prophylaxis is necessary in breast-fed infants. Even with these regimens some infants do develop a late VKDB, usually due to unrecognised cholestasis causing vitamin K malabsorption.[5-8] As late VKDB is associated with intracranial haemorrhage (ICH) – resulting in significant mortality and long term morbidity – in approximately 50% of cases,[9-11] it is especially important to prevent this complication.

It is unknown whether the high risk of VKDB and ICH recently described in breast-fed Dutch infants with biliary atresia[11] can also be found in other forms of neonatal cholestasis. Jaundice is generally less profound in these cases and consequently the cholestasis induced vitamin K malabsorption might also be less severe. Interestingly, Alpha-1-antitrypsin deficiency (A1ATD) - the next most frequent cause of cholestatic jaundice in infancy after biliary atresia – is associated with varying degrees of liver involvement.[12,13] Cholestatic jaundice is present in only approximately 1 in 10 infants with a ZZ phenotype[12] and is even less frequent in patients with an SZ phenotype. Moreover, the degree of cholestasis in symptomatic infants can vary from mild to severe.[12] Thus, A1ATD induced cholestatic liver disease represents an attractive model to study the influence of varying degrees of cholestasis on the development of VKDB at initial presentation.

In this study we retrieved infants with a ZZ or SZ phenotype born in the Netherlands from January 1991 until December 2006 by searching the databases of the 5 Dutch diagnostic centers involved in A1AT phenotyping and/or genotyping. The clinical data of these infants were used 1) to determine the absolute and relative risk of VKBD at initial presentation of cholestatic jaundice induced by A1ATD in exclusively breast-fed versus formula fed infants and 2) to investigate whether varying degrees of jaundice are associated with a different risk of bleeding.

METHODS

Study population

Dutch patients born from January 1991 until December 2006 presenting with neonatal cholestasis due to A1ATD were retrieved from the databases of the 5 centres in the Netherlands involved in A1AT genotyping or phenotyping. Patients were provisionally included if a ZZ or SZ genotype or phenotype was generated within the first 6 months after birth. Subsequently, patient files were reviewed at the hospital where the patients originated to obtain relevant demographic data and clinical characteristics. The same exclusion criteria were used as described previously for biliary atresia.[11] Briefly, exclusion criteria were: 1) a gestational age less than 37 weeks 2) a birth weight below 2000 grams, 3) absence of cholestasis (with cholestasis defined as a total serum bilirubin concentration above 50 μmol/L, with a direct fraction of at least 20 percent), 4) a diagnosis of cholestasis before age 8 days. In addition, the presence of an older sib with cholestatic jaundice due to A1ATD was used as exclusion criteria, as this should affect the advised prophylactic regimen. Infants were categorized as "breast-fed" if they had received exclusively breast feeding from birth onwards. All other infants were categorized as "formula-fed".

Vitamin K prophylaxis

Since 1990 all infants born in the Netherlands receive an oral dose of 1 mg vitamin K directly after birth. Upon breastfeeding, parents are advised to give their child a daily oral dose of 25 μg vitamin K from the second week of life until the end of the 13th week. For daily dosing of vitamin K, a dietary supplement is used in which vitamin K is solved in arachid oil. The vitamin K prophylaxis can be stopped earlier if breastfeeding accounts for less than fifty percent of the daily intake.[14] Formula-fed infants only receive vitamin K prophylaxis directly after birth. Thereafter, they are expected to receive sufficient amounts of vitamin K, since the formula feedings commercially available in the Netherlands contain approximately 50 μg vitamin K per litre.[15]

Vitamin K deficiency

We calculated the prothrombin ratio (PR) at initial diagnosis to be able to compare coagulation parameters from different hospitals, and used it to assess the presence of vitamin K deficiency. If available, the International Normalized Ratio (INR) was used as PR. In case of a prothrombin time (PT) in seconds the PR was determined as follows: PR = $PT_{patient}$: $PT_{control}$. If a $PT_{control}$ was not determined by the laboratory, it was defined as the mean of the provided reference range. Vitamin K deficiency (VKD) was labelled "mild" when PR was between 1.5 and 4 in line with our previous study.[11] A PR of more than 4 was designated as "severe" VKD. VKDB was defined as bruising,

bleeding or intracranial haemorrhage in combination with a PR of more than 4 in any infant between eight days and six months of age and normalising after administration of vitamin K.[9] ICH was determined based on radiological confirmation. Clinical signs suggestive of ICH (high pitched cry, irritable, convulsions) but without radiological confirmation were also listed, but not included as such. Life threatening VKDB was defined as either an ICH or isolated respiratory and /or circulatory insufficiency.

Statistical analysis

We performed a student t-test of clinical and biochemical parameters with a normal distribution pattern to test for statistical differences between groups. Kruskal-Wallis analysis was used for parameters with a non-normal distribution. A Chi Square test was performed to determine statistical significance between groups in case of dichotomous parameters. The relative risks and 95% confidence intervals for VKDB and biochemical levels of VKD were calculated. Since computation of a RR requires at least one positive case in each quadrant a dummy case was added if necessary to be able to compute a (minimal) relative risk.

Approval for the study was obtained from the University Medical Center Utrecht ethics committee.

RESULTS

Between January 1991 and December 2006, 56 patients with A1ATD were included into the Dutch A1ATD registry. Fifty five infants were ZZ, one was SZ. Sixteen infants did not meet the inclusion criteria, as described in detail in table 1. Twenty (50%) of the remaining 40 infants were exclusively breast-fed. Infants were categorized according to the type of prophylaxis they received. Table 2 summarizes the clinical characteristics in breast-fed infants on daily oral prophylaxis and formula-fed infants. A trend for earlier presentation of breast-fed infants was found, in line with previous data in infants with biliary atresia.[11]

Table 1 Patients and population

live births*	3,138,576
no. of documented cases	56
incidence of documented cases	1.8/100.000
excluded	**16**
positive family history; no cholestasis	5
positive family history; cholestasis	2
gestational age<37 wks	3
diagnosed before first week	4
unknown feeding type	2
included	**40**
breastfed	20
formulafed	20

* Derived from the central bureau of statics (http://statline.cbs.nl)

Vitamin K deficiency in A1AT infants at initial presentation

Severe VKD was evident in 16 of 20(80%) exclusively breast-fed infants with A1AT and was associated with a VKDB in 15/20 (75%).(table 3) Life threatening consequences were found in 8/20(40%) infants. One infant presented with respiratory insufficiency without documented intracranial haemorrhage. In seven infants an intracranial haemorrhage was documented and one of these infants died as a consequence. Additionally, two infants had symptoms suggestive of an intracranial hemorrhage (convulsions in 1, irratibility in 1), without radiological confirmation. The risk of (severe) vitamin K deficiency was significantly lower in infants receiving formula feeding ($p<0.001$). None of the infants receiving formula presented with a VKDB, and only one had a (mild) VKD. The minimal relative risk for VKDB in breast-fed versus formulafed infants was 15.8 (95% confidence interval 2.3-108). This calculation was performed after adding a positive dummy, based on the assumption that the next included patient would have received formula and presented with a VKDB.

Cholestatic jaundice and the risk of Vitamin K deficiency bleeding

Total and conjugated bilirubin levels in the group of 20 breast-fed infants analyzed were 104 μmol/L and 63 μmol/L, respectively and did not correlate with the risk of vitamin K deficiency or VKDB. Instead, total bilirubin levels in breast-fed infants presenting with an ICH (n=7) were significantly lower than in infants without ICH (n=13), (80 vs 116 μmol/l, p=0.02).

Table 2 Patient characteristics

	Total	Breastfed +25 μg daily oral (n=20)	Formula-fed (n=20)	P value
		(n=20)	(n=20)	
male sex - no. (%) [¤]	28(70)	16(80)	12(60)	0.30
birthweight - g ⋆	3285±548	3388±523	3193±554	0.27
age at diagnosis - days [§]	35(28-47)	31(27-42)	39(30-60)	0.10
weight at diagnosis - g ⋆	3907±660	4134±736	3660±483	0.09
bilirubin total - μmol/L ⋆	107±41	104±34	117±55	0.36
bilirubin direct - μmol/L ⋆	69±32	63±25	74±38	0.35
ASAT - U/L [§]	98(78-141)	95(83-138)	100(82-141)	0.70
ALAT - U/L [§]	57(39-83)	56(31-72)	61(43-108)	0.22
GGT - U/L	556(315-874)	567(230-875)	584(335-918)	0.81

⋆ Plus-minus values are means ± SD, p value was determined with independent sample t- test. [§] Values are median (interquartile range), p value was determined with Mann Whitney U.[¤] P value was determined with Fisher's Exact.

Table 3 Risk of vitamin K deficiency in breast-fed and formula-fed infants with a-1-antitrypsin deficiency

	Breastfed +25 μg daily oral	Formula-fed	Relative Risk (95% CI)	P value
vitamin K deficiency ¶	16/20 (80%)	1/20 (5%)	16.0 (2.3-109)	< .001
severe vitamin K deficiency ¶	16/20 (80%)	0/20(0%)	16.8* (2.5-115)*	< .001
VKDB	15/20 (75%)	0/20 (0%)	15.8* (2.3-108)*	< .001
intracranial haemorrhage	7/20 (35%)	0/20 (0%)	7.4* (1.0-54.5)*	.008

Data are number/total number (%). The p value was determined with Fisher exact. ¶ defined by PR>1.5 for VKD and PR>4.0 for severe VKD and normal thrombocyte count. *a positive dummy was added for formula to compute a (minimal) RR.

DISCUSSION

The results of this study indicate that exclusively breast-fed infants with A1ATD have a high risk of developing a VKDB prior to recognition of cholestatic jaundice under the present Dutch vitamin K prophylaxis. Of breast-fed infants, 75% presented with a VKDB and 35% presented with an ICH. No VKDB was documented in (partially) formula fed infants. These findings extend our previous observations in infants with biliary atresia[11] to a disease entity with a milder and more variable degree of conjugated hyperbilirubinemia.

To our knowledge this is the first nation-wide study examining the risk of VKDB in infants with A1ATD. Based on the premise that A1AT phenotyping is a regular part of the workup of cholestatic jaundice in infancy we investigated the databases from the diagnostic centers performing A1AT phenotyping in the Netherlands. Out of a birth cohort of 3,530,583 infants 55 cases of PiZZ (and one with PiSZ) were retrieved. Fifty of the PiZZ infants had documented cholestatic jaundice, amounting to an incidence of 1.4/100,000. This incidence is very similar to the expected incidence of 1.3/100,000, which is based on the estimated incidence of PIZZ in the Netherlands (1:9536)[16] and the previously documented risk of developing cholestatic jaundice in infancy in case of PI ZZ phenotype of 12%[12]. Since we aimed to estimate the risk of late VKDB under the current Dutch prophylaxis we carefully excluded infants who may have received additional vitamin K, such as premature infants. Another source of misclassification may originate from the assumption that all breast-fed infants indeed received the prophylaxis according to national guidelines, unless otherwise stated in patient files. However, data from other European countries indicate that adherence to a prophylactic regimen executed by parents is approximately 90%.[8 17 18]

In our group of breast-fed infants with A1ATD total and conjugated bilirubin levels (104/63 μmol/L) were significantly lower than the levels encountered in biliary atresia patients in the Netherlands (147/98 μmol/L, $p<0.001$). Nevertheless, the risk to develop VKDB was similar in both groups (75 and 83%, respectively, $p=0.49$), as was the age at presentation (35 and 36 days, respectively). No association was found in the present study between the degree of (conjugated) hyperbilirubinemia and the risk of VKDB. These findings suggest that all infants with cholestatic jaundice are at a similarly high risk of VKDB, regardless of its degree. This notion is supported by other lines of evidence. First, VKDB has been repeatedly reported in infants with minimal cholestatic jaundice (total bilirubin 21-62 μmol/L).[19-22] Second, in a Japanese study investigating the incidence of subclinical VKD, strongly elevated PIVKA-II levels were found in 8/22.000 infants

at age 4 weeks, all of whom were breast-fed and had moderately elevated conjugated bilirubin levels (range 17-45 μmol/l).[23] The incidence of cholestatic jaundice found in this study was 1/2750, similar to the reported incidence of (transitory) cholestasis in infancy[24], suggesting that nearly all breast-fed infants with some degree of cholestatic jaundice developed coagulation abnormalities.

It could be argued that VKDB in infants with an underlying cholestatic liver disease should not be prevented by routine prophylaxis, but rather by a timely diagnosis and treatment of cholestatic jaundice. However, our data in infants with A1ATD and biliary atresia[11] indicate that the age at diagnosis would have to be reduced by approximately 3 weeks to prevent all bleedings. Thus far, various attempts aimed at increasing awareness and early recognition by parents[25] and health professionals[26,27] only had a modest effect on the age at diagnosis. Theoretically, newborn screening would be an attractive option, but quantification of serum bile acids failed to separate infants with cholestatic jaundice from healthy infants.[28] Therefore, under the present circumstances adequate routine vitamin K prophylaxis seems to be the best way to protect infants with unrecognized cholestasis.

In previous studies vitamin K prophylaxis was shown not only to reduce the risk of VKDB but also to postpone its occurrence.[5,10] Interestingly, the age at presentation in the cohort described here is similar to the age reported in infants who only received a single oral dose at birth. Therefore, it is doubtful whether the additional daily dosages of vitamin K – requiring a significant effort from all parents of breast-fed infants - had any effect at all in infants at the highest risk of VKDB. Although this lack of effect may in part be explained by the comparatively low dose of vitamin K provided,[11] the vitamin K formulation is likely to play a significant role as well. Vitamin K is an extremely hydrophobic compound and emulsification and mixed micelle formation are essential for its uptake. Most commercially available formulations are emulsified or in fact administered as mixed micelles. The present Dutch formulation, in which vitamin K is solved in arachid oil, will remain unemulsified without bile, representing a tremendous hurdle for the absorption of vitamin K under cholestatic conditions.

If oral prophylaxis is to be continued in the Netherlands it seems prudent to choose a formulation with proven efficacy. However, the oral vitamin K formulation which significantly,[11] albeit still imperfectly, protected cholestatic infants is no longer commercially available. As surveillance data indicate that a mixed micellar formulation is equally effective,[5,7] we suggest using this, when implementing a new oral prophylaxis scheme in the Netherlands. Hopefully, strategies to specifically improve the uptake of orally administered vitamin K in cholestatic jaundice will enable

an oral prophylactic regimen in the future that offers breast-fed cholestatic infants the same level of protection as is currently provided to formula fed infants.

In conclusion, the absolute risk of VKDB in exclusively breast-fed infants with cholestatic jaundice due to A1ATD is similar to the risk in infants with biliary atresia and is not affected by the degree of conjugated hyperbilirubinemia. These findings suggest that, regardless of etiology, under the current Dutch regimen infants with cholestatic jaundice are at a high risk of VKDB and support the need to change.

Acknowledgements
We thank Petra van Embden, trial nurse, the participant paediatricians and the centers involved in diagnosing A1ATD in the Netherlands for their contribution to the study.

REFERENCES

1. Widdershoven J, Lambert W, Motohara K, Monnens L, de Leenheer A, Matsuda I, et al. Plasma concentrations of vitamin K1 and PIVKA-II in bottle-fed and breast-fed infants with and without vitamin K prophylaxis at birth. Eur J Pediatr 1988;148:139-42.

2. Shearer MJ, Barkhan P, Webster GR. Absorption and excretion of an oral dose of tritiated vitamin K1 in man. Br J Haematol 1970;18:297-308.

3. McNinch AW, Upton C, Samuels M, Shearer MJ, McCarthy P, Tripp JH, et al. Plasma concentrations after oral or intramuscular vitamin K1 in neonates. Arch Dis Child 1985;60:814-8.

4. Haroon Y, Shearer MJ, Rahim S, Gunn WG, McEnery G, Barkhan P. The content of phylloquinone (vitamin K1) in human milk, cows' milk and infant formula foods determined by high-performance liquid chromatography. J Nutr 1982;112:1105-17.

5. von Kries R, Hachmeister A, Gobel U. Oral mixed micellar vitamin K for prevention of late vitamin K deficiency bleeding. Arch Dis Child Fetal Neonatal Ed 2003;88:F109-F112.

6. von Kries R, Hachmeister A, Gobel U. Can 3 oral 2 mg doses of vitamin K effectively prevent late vitamin K deficiency bleeding? Eur J Pediatr 1999;158 Suppl 3:S183-6.

7. Schubiger G, Berger TM, Weber R, Banziger O, Laubscher B. Prevention of vitamin K deficiency bleeding with oral mixed micellar phylloquinone: results of a 6-year surveillance in Switzerland. Eur J Pediatr 2003;162:885-8.

8. Wariyar U, Hilton S, Pagan J, Tin W, Hey E. Six years' experience of prophylactic oral vitamin K. Arch Dis Child Fetal Neonatal Ed 2000;82:F64-F68.

9. Sutor AH, von Kries R, Cornelissen EA, McNinch AW, Andrew M. Vitamin K deficiency bleeding (VKDB) in infancy. ISTH Pediatric/Perinatal Subcommittee. International Society on Thrombosis and Haemostasis. Thromb Haemost 1999;81:456-61.

10. McNinch AW, Tripp JH. Haemorrhagic disease of the newborn in the British Isles: two year prospective study. BMJ 1991;303:1105-9.

11. van Hasselt PM, de Koning TJ, Kvist N, de VE, Lundin CR, Berger R, et al. Prevention of vitamin K deficiency bleeding in breastfed infants: lessons from the Dutch and Danish biliary atresia registries. Pediatrics 2008;121:e857-e863.
12. Sveger T. Liver disease in alpha1-antitrypsin deficiency detected by screening of 200,000 infants. N Engl J Med 1976;294:1316-21.

13. Odievre M, Martin JP, Hadchouel M, Alagille D. Alpha1-antitrypsin deficiency and liver disease in children: phenotypes, manifestations, and prognosis. Pediatrics 1976;57:226-31.

14. Uitentuis J. [Administration of vitamin K to neonates and infants]. Ned Tijdschr Geneeskd 1990;134:1642-6.

15. Greer FR, Marshall SP, Foley AL, Suttie JW. Improving the vitamin K status of breastfeeding infants with maternal vitamin K supplements. Pediatrics 1997;99:88-92.

16. Blanco I, de Serres FJ, Fernandez-Bustillo E, Lara B, Miravitlles M. Estimated numbers and prevalence of PI*S and PI*Z alleles of alpha1-antitrypsin deficiency in European countries. Eur Respir J 2006;27:77-84.

17. Hansen KN, Minousis M, Ebbesen F. Weekly oral vitamin K prophylaxis in Denmark. Acta Paediatr 2003;92:802-5.

18. Croucher C, Azzopardi D. Compliance with recommendations for giving vitamin K to newborn infants. BMJ 1994;308:894-5.

19. Sutor AH, Dagres N, Niederhoff H. Late form of vitamin K deficiency bleeding in Germany. Klin Padiatr 1995;207:89-97.

20. Ono S, Tokiwa K, Aoi S, Iwai N, Nakanoin H. A bleeding tendency as the first symptom of a choledochal cyst. Pediatr Surg Int 2000;16:111-2.

21. von Kries R, Kordass U, Shearer M, Gobel U. Idiopathic late hemorrhagic disease of newborn and conjugated hyperbilirubinemia. J Pediatr Gastroenterol Nutr 1993;16:328-30.

22. Humpl T, Bruhl K, Brzezinska R, Hafner G, Coerdt W, Shearer MJ. Fatal late vitamin K-deficiency bleeding after oral vitamin K prophylaxis secondary to unrecognized bile duct paucity. J Pediatr Gastroenterol Nutr 1999;29:594-7.

23. Matsuda I, Nishiyama S, Motohara K, Endo F, Ogata T, Futagoishi Y. Late neonatal vitamin K deficiency associated with subclinical liver dysfunction in human milk-fed infants. J Pediatr 1989;114:602-5.
24. Dick MC, Mowat AP. Hepatitis syndrome in infancy--an epidemiological survey with 10 year follow up. Arch Dis Child 1985;60:512-6.
25. Matsui A, Ishikawa T. Identification of infants with biliary atresia in Japan. Lancet 1994;343:925.
26. Hussein M, Howard ER, Mieli-Vergani G, Mowat AP. Jaundice at 14 days of age: exclude biliary atresia. Arch Dis Child 1991;66:1177-9.
27. Hsiao CH, Chang MH, Chen HL, Lee HC, Wu TC, Lin CC, et al. Universal screening for biliary atresia using an infant stool color card in Taiwan. Hepatology 2008;47:1233-40.
28. Mushtaq I, Logan S, Morris M, Johnson AW, Wade AM, Kelly D, et al. Screening of newborn infants for cholestatic hepatobiliary disease with tandem mass spectrometry. BMJ 1999;319:471-7.

Chapter 04

Chapter 04

Changing attitude towards breastfeeding in infants with jaundice unveils the inefficacy of Dutch vitamin K prophylaxis

Peter M van Hasselt[a], Roderick HJ Houwen[a], Elsemieke de Vries[b], Rozemarijn de Rooij[a], Willemien de Vries[b], Ruud Berger[c], Jan LL Kimpen[a], Jacobus P van Wouwe[d], Caren Lanting[d], Henkjan J Verkade[b], Tom J de Koning[a]; Netherlands Studygroup for Biliary Atresia Registry

[a]Department of Pediatrics, Wilhelmina Children's Hospital, University Medical Center Utrecht, the Netherlands; [b]Department of Pediatrics, University Medical Center Groningen, Groningen, the Netherlands; [c]Laboratory for Metabolic Diseases, University Medical Center Utrecht, Utrecht, The Netherlands; [d]Netherlands Organization for Applied Scientific Research TNO Quality of Life, Leiden, The Netherlands; Netherlands Study group for Biliary Atresia Registry: DC Aronson, A Kindermann, CMF Kneepkens, LWE van Heurn, AM van den Neucker, ZJ de Langen, PMJG Peeters, G Madern, JH Escher, DC van der Zee, PNMA Rieu, JJM Tolboom.

Submitted

ABSTRACT

Background
A recent surveillance study documented a substantial rise over the last decade in the incidence of late vitamin K deficiency bleeding (VKDB) in the Netherlands. As we reported previously that especially breastfed -as opposed to formula fed- infants with biliary atresia (BA) or alpha-1-antitrypsin deficiency (A1ATD) have an increased risk to develop VKDB, we hypothesized that changes in breastfeeding rates in these groups might explain this rise.

Methods
We assessed changes in breastfeeding rate and the risk of VKDB in Dutch infants presenting with BA and A1ATD between January 1991 and December 2006. In addition, we performed a survey amongst caretakers of healthy infants to establish the degree of adherence to the prophylactic regimen.

Results
A total of 184 infants with BA and A1ATD were included for analysis. While the overall incidence of breastfeeding in Dutch newborns changed only marginally, the breastfeeding rate in infants with BA or A1ATD increased from 6/45 (13%) infants in 1991-1994 to 21/44 (47%) in 2003-2006. This increase was paralleled by an increase in the incidence of VKDB in this cohort from 11 to 41% and a dramatic increase of intracranial haemorrhage (from 0 to 20%). The reported adherence to vitamin K prophylaxis was 85%, indicating that the uniformly high risk of VKDB in exclusively breastfed infants in this time frame could not be explained by a lack of adherence to prophylaxis.

Conclusions
A significant rise in the breastfeeding rate in infants with unidentified cholestasis unveiled the ineffectiveness of the Dutch vitamin K prophylaxis

Vitamin K deficiency bleeding after the first week of life (late VKDB) is a life threatening condition associated with a high risk of intracranial haemorrhage (ICH),[1,2] resulting in significant mortality and long term morbidity.[2-4] The resurgence of this clinical entity was first described by McNinch et al, who published a number of illustrative cases of breastfed infants with VKDB who had not received vitamin K prophylaxis.[5] More importantly these authors subsequently used and advocated surveillance data to determine the efficacy of prophylactic regimens that were introduced to prevent these bleedings.[1]

Similar surveillance studies in the Netherlands initially showed a very low incidence of VKDB (0-0.7/100,000) during the 1993-1994 period.[6] However the incidence of VKDB in 2005 had risen to 3/100,0000 live borns,[7] which is close to the incidence of VKDB without vitamin K prophylaxis.[8] Yet the prophylactic regimen had remained unchanged during this period.

The difference in the risk of VKDB between breastfed and formula-fed infants with unrecognized cholestasis in the Netherlands[10,11] suggests that changes in breastfeeding patterns would significantly affect the incidence of VKDB. We therefore hypothesized that an increase in breastfeeding rates could explain the observed increase in VKDB in the Netherlands. To test this hypothesis we combined data from the Dutch registries for the major causes for cholestatic liver disease in infancy (biliary atresia (BA) and alpha-1-antitrypsin deficiency (A1ATD)) from1991-2006 and assessed time dependent changes in breastfeeding rate and its effect on the risk of VKDB. In addition to this we assessed parental compliance to vitamin K prophylaxis regimen, because compliance is always an issue of concern in oral prophylaxis.[12]

Here we provide evidence that a specific increase of breastfeeding rates in high risk populations has unveiled the ineffectiveness of the Dutch vitamin K prophylaxis.

METHODS

Patient

Infants with biliary atresia (BA) and alpha-1-antitrypsin deficiency (A1ATD) born in the Netherlands between January 1991 and December 2006 were identified from the Netherlands Studygroup for Biliary Atresia Registry (NeSBAR) and the Dutch centers for A1AT phenotyping and genotyping, respectively. These registries have national coverage and a demonstrated a high retrieval rate.[10,11] Briefly, inclusion was based on the following criteria: documented conjugated hyperbilirubinemia, known feeding type, born in the Netherlands, gestational age above 37 weeks and birthweight higher than 2000 grams, and an age at diagnosis between 8 days and 6 months.

Definitions

VKDB was defined according to the international case definition[13] as documented hemorrhage or prolonged bleeding, a Prothrombin Ratio higher than 4, a normal thrombocyte count and cessation of bleeding after administration of vitamin K. Cholestatic jaundice was defined as a conjugated bilirubin above 30 μmol/L in combination with a direct fraction of at least 20 percent. As described previously,[10] infants were categorized as "breastfed" if they had received exclusively breastfeeding from birth onwards. All other infants were categorized as "formula fed".

Breastfeeding rate

Changes in the breastfeeding rate were assessed by calculating the proportion of infants being exclusively breastfed at presentation of cholestasis in four time frames: 1991-1994; 1995-1999; 1999-2002; 2003-2006. For comparison, breastfeeding rates in healthy infants at 6 weeks of age in these timeframes were kindly provided by the Dutch Center for biostatistics (http://statline.cbs.nl).

Adherence to vitamin K prophylaxis

From February until April 2007 a total of 5380 questionnaires were handed out by primary health care workers in 263 healthy baby clinics across the country to parents of infants under 6 months of age as part of a biannual random sample survey.[14] The questionnaire included items on feeding type and age.[14] Compliance to the vitamin K prophylactic regimen was assessed by asking parents of exclusively breastfed infants younger than 13 weeks of age the following question with the previous week in mind: Was vitamin K administered? a) yes, every day. b) yes 5 or 6 times. c) yes, 3 or 4 times. d) yes, once or twice. e) no, never gave it. Analyses was restricted to those infants who were supposed to have received vitamin K prophylaxis each day of the preceding week. In view of the Dutch vitamin K prophylactic regimen (advising vitamin K from 1-13 weeks of age) only infants between 2 and 13 weeks of age were included.

RESULTS

Between January 1991 and December 2006 a total of 184 infants with BA (n= 143) and A1ATD (n=41) were identified. As depicted in figure 1, the breastfeeding rate in this cohort increased significantly from 6/45 (13 percent) in 1991-1994 to 21/44 (47 percent) in 2003-06. The rise in breastfeeding rate in these cholestatic infants was much more pronounced than the moderate rise in the overall BF rate observed in nationwide surveys (from 54.2 to 62.1%). In parallel, the risk of VKDB rose from 11 to 41 percent. Moreover, the risk in VKDB was accompanied by substantial increase in the risk of ICH. ICH was not observed in VKDB cases in the period from 1991-1994, whereas 20 percent of VKDB infants in 1999-2002 presented with ICH.

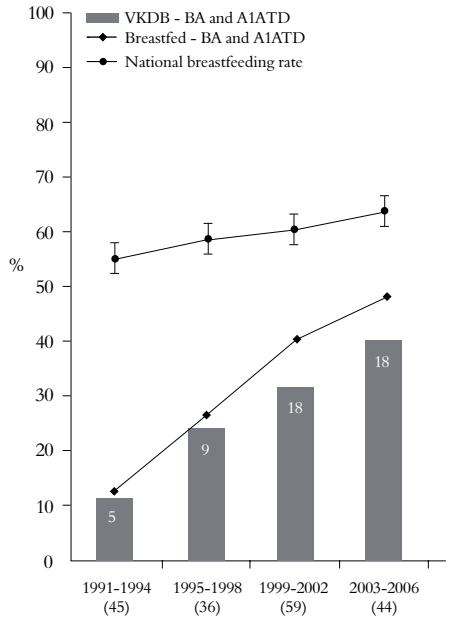


Figure 1
Rising rate of breastfeeding and VKDB in infants with BA and A1ATD. Data on national breastfeeding rate are derived from central bureau for statistics (http://statline.cbs.nl). Numbers between brackets represent the number of patients with BA and A1ATD in each period. Numbers in bars represent absolute numbers of patients with VKDB.

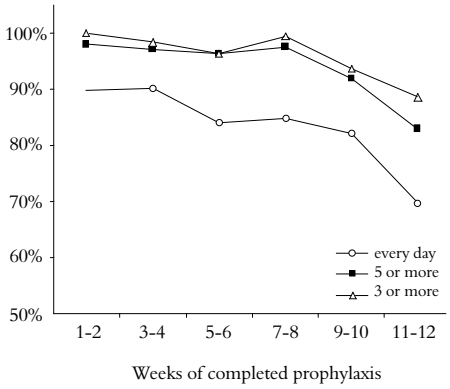


Figure 2
Adherence to vitamin K prophylactic regimen in the Netherlands. Weeks represent number of completed weeks under prophylaxis. Lines depict different degrees of adherence (each day, at least 5 out of 7 days and at least 3 out of 7 days of the preceding week).

Adherence

We have no reason to suspect that the high risk of VKDB in breastfed infants could be attributed to a low adherence to the prophylactic regimen. Of the 5380 questionnaires 2768 (51%) were completed. Of these 911 involved exclusively breastfed infants of which 567 were 2-13 weeks of age. 85% of these infants' parents reported that they administered the vitamin K daily in the preceding week, 95% of the parents gave between 5 and 7 doses per week and more than 97% gave at least 3 doses. There was a trend towards a lower adherence in the last weeks of the first trimester (figure 2). However, since most (84%) of infants with VKDB in this study presented within the first 2 months of life this decrease in adherence in the third month of life is unlikely to have substantially affected the risk of VKDB.

DISCUSSION

We here show that the breastfeeding rate in Dutch cholestatic infants with BA and A1ATD, the two major causes of cholestasis in infancy, has increased significantly in recent years. The rise in breastfeeding rate in these high risk groups was accompanied by a four-fold increase in the risk of VKDB, and a substantial risk of ICH. In our opinion, these findings explain the discrepant data from the two Dutch surveillance studies performed in this time frame: the low breastfeeding rate in the early nineties in children at risk for VKDB may have concealed the lack of protection offered by the Dutch prophylactic regimen, whereas the pronounced rise in breastfeeding rate unveiled its inefficacy.

The rising breastfeeding rate in both cohorts of infants with cholestatic jaundice was unexpected given the marginal increase of the overall Dutch breastfeeding rate in this period. It is tempting to speculate that the raised breastfeeding rate in infants with cholestatic jaundice is due to a different attitude towards breastfeeding in all jaundiced infants. Several reports since the early nineties have stated that jaundice, which is seen more often in breastfed infants, should not be a reason to switch to infant formula, as was common practice in the past.[15,16] These reports were of course aimed at infants with unconjugated hyperbilirubinemia and the rise in breastfeeding rates within populations of infants with cholestatic jaundice may have been an unforeseen and unwanted side effect of this legitimate appeal.

The excellent adherence in cholestatic infants to the vitamin K prophylactic regimen we described previously[10,11] was based solely on parental reporting. The reliability of such information gathered at a time when parents are confronted with a potentially life threatening disorder can be questioned. We therefore investigated the degree of adherence to the prophylactic regimen in healthy babies. The reported adherence rate of approximately 85% is comparable with others.[17] Assuming that adherence in infants with unrecognized cholestasis is similar, lack of compliance is not a good explanation for the increasing risk of VKBD in breastfed infants in the Netherlands.

In conclusion, we show that the incidence of VKDB in the Netherlands has changed over time, likely because breastfeeding rates specifically increased in infants with neonatal cholestasis. Our data underline that caution is in place when deducing the efficacy of a prophylactic regimen from general surveillance data. In our opinion, targeted surveillance in cohorts of patients particularly at risk should be used to complement "untargeted" surveillance data.

1 McNinch AW, Tripp JH. Haemorrhagic disease of the newborn in the British Isles: two year prospective study. BMJ 1991;303:1105-9.

REFERENCES

2. Sutor AH, Dagres N, Niederhoff H. Late form of vitamin K deficiency bleeding in Germany. Klin Padiatr 1995;207:89-97.

3. Cekinmez M, Cemil T, Cekinmez EK, Altinors N. Intracranial hemorrhages due to late-type vitamin K deficiency bleeding. Childs Nerv Syst 2008;24:821-5.

4. Danielsson N, Hoa DP, Thang NV, Vos T, Loughnan PM. Intracranial haemorrhage due to late onset vitamin K deficiency bleeding in Hanoi province, Vietnam. Arch Dis Child Fetal Neonatal Ed 2004;89:F546-F550.

5. McNinch AW, Orme RL, Tripp JH. Haemorrhagic disease of the newborn returns. Lancet 1983;8333:1089-90.

6. Cornelissen M, von Kries R, Loughnan P, Schubiger G. Prevention of vitamin K deficiency bleeding: efficacy of different multiple oral dose schedules of vitamin K. Eur J Pediatr 1997;156:126-30.

7. Ijland MM, Pereira RR, Cornelissen EA. Incidence of late vitamin K deficiency bleeding in newborns in the Netherlands in 2005: evaluation of the current guideline. Eur J Pediatr 2008;167:165-9.

8. Wariyar U, Hilton S, Pagan J, Tin W, Hey E. Six years' experience of prophylactic oral vitamin K. Arch Dis Child Fetal Neonatal Ed 2000;82:F64-F68.

9. Shearer MJ. Vitamin K deficiency bleeding (VKDB) in early infancy. Blood Rev 2008 Epub September 18.

10. van Hasselt PM, de Koning TJ, Kvist N, de Vries E, Rydahl Lundin C, Berger R et al. Prevention of vitamin K deficiency bleeding in breastfed infants: lessons from the Dutch and Danish biliary atresia registries. Pediatrics 2008;121:e857-e863.

11. van Hasselt PM, Kok K, Vorselaars ADM, van Vlerken L, de Vries R, Nieuwehuys E et al. Vitamin K deficiency bleeding in cholestatic infants with Alpha-1-Antitrypsin deficiency. submitted 2009.

12. Clarke P, Shearer MJ. Vitamin K deficiency bleeding: the readiness is all. Arch Dis Child 2007;92:741-3.

13. Sutor AH, von Kries R, Cornelissen EA, McNinch AW, Andrew M. Vitamin K deficiency bleeding (VKDB) in infancy. ISTH Pediatric/Perinatal Subcommittee. International Society on Thrombosis and Haemostasis. Thromb Haemost 1999;81:456-61.

14. Van Wouwe JP, Lanting CI, van DP, Treffers PE, van BS. Breastfeeding duration related to practised contraception in the Netherlands. Acta Paediatr 2008 E pub September 2.

15. Maisels MJ. What's in a name? Physiologic and pathologic jaundice: the conundrum of defining normal bilirubin levels in the newborn. Pediatrics 2006;118:805-7.

16. Martinez JC, Maisels MJ, Otheguy L, Garcia H, Savorani M, Mogni B et al. Hyperbilirubinemia in the breast-fed newborn: a controlled trial of four interventions. Pediatrics 1993;91:470-3.

17. Hansen KN, Minousis M, Ebbesen F. Weekly oral vitamin K prophylaxis in Denmark. Acta Paediatr 2003;92:802-5.

Chapter 05

Chapter 05

Hydrolysed formula is a risk factor for vitamin K deficiency in infants with unrecognised cholestasis

P.M. van Hasselt[a], W. de Vries[b], E. de Vries[b], K. Kok[c], E.C.M. Cranenburg[d], T.J. de Koning[a], L.J. Schurgers[d], H.J. Verkade[b], R.H.J. Houwen[a]

[a]Department of Pediatrics, Wilhelmina Children's Hospital, University Medical Center Utrecht, the Netherlands; [b]Department of Pediatrics, University Medical Center Groningen, Groningen, the Netherlands; [c]Department of Medicine, Division of Gastroenterology and Hepatology, Radboud University Medical Center Nijmegen, the Netherlands; [d]Cardiovascular Research Institute and VitaK, Maastricht University, the Netherlands.

Submitted

ABSTRACT

Background
Vitamin K deficiency may cause life threatening haemorrhages in infancy. In contrast with breastfed infants, who receive multiple doses of vitamin K to prevent VKDB after the first week of life (late VKDB), formula fed infants rely on the protection offered by formula. In this study we assessed whether the risk of VKD(B) in formula fed cholestatic infants is associated with certain formula types, in particular hypoallergenic formula.

Methods
Infants born in the Netherlands between January 1991 and December 2006 with cholestatic jaundice due to biliary atresia (BA) and alpha-1-antitrypsin deficiency (A1ATD) were identified through the Netherlands Studygroup for Biliary Atresia Registry (NeSBAR) and the Dutch A1ATD registry, respectively. The relative risk of VKD(B) for each type of formula used was calculated. The influence of partial breastfeeding was also assessed.

Results
Of 118 formula fed infants, 8 infants presented with VKD. Six (75%) of these infants received hypoallergenic formula (whey based hydrolysate in 4, casein based hydrolysate in 1, soy based in 1). Only 2 received regular feeding. One infant on whey based hydrolysed formula presented with VKDB. Risk factor analysis revealed that infants on hydrolysed, in particular whey based, formula, had a strongly increased risk of VKD (Relative Risk 25.0 (6.4-97.2), $p<0.001$)) compared with infants receiving regular formula.

Conclusion
Cholestatic infants receiving (whey based) hydrolysed formula are at risk of developing VKD, which may lead to overt bleeding. Therefore, infants on whey based hydrolysed formulas might need additional vitamin K prophylaxis, similar to breastfed infants.

Vitamin K deficiency (VKD) causes life threatening hemorrhages in infancy, known as vitamin K deficiency bleeding (VKDB). To prevent VKDB, all newborns receive vitamin K directly after birth. Formula fed infants, in contrast with breastfed infants, do not receive additional doses of vitamin K in the first months of life and thus depend on the protection offered by formula feeding.

Although the degree of protection offered by formula feeding is generally very high,[1] this may not be true for all types of formula.[2,3] The majority of formula fed infants that developed a VKDB received hypoallergenic –soy or hydrolysed- formula.[3-6] These observations suggest a suboptimal protection against VKDB by these types of formula, but do not warrant more definite conclusions. Insight into this putative risk factor has gained importance in view of the increased use of hydrolysed formulas to reduce the risk of allergic disease.[7]

We recently reported the use of national registries for the two most prevalent causes of cholestatic jaundice in infancy (BA and A1ATD) to delineate the risk of VKDB in cholestatic infants under various prophylactic regimens.[1,8] While more than 80 percent of exclusively breastfed infants presented with VKDB, biochemical VKD was absent in the vast majority of (partially) formula fed infants. However, VKD did occur in formula fed infants. Interestingly, the degree of deficiency in those formula fed infants presenting with VKD was significant and even resulted in VKDB.[1]

We hypothesized that the occurrence of VKD in formula fed cholestatic infants could be explained by the use of hypoallergenic formula. Here, we combined data from the Dutch registries for BA and A1ATD to compare the risk of VKD(B) in infants on hypoallergenic formula with those on regular infant formula. Furthermore, we evaluated whether the vitamin K content of various commercially available formulas could serve as an explanation for such an increased risk of VKD(B).

METHODS

Patients

Infants born in the Netherlands between January 1991 and December 2006 with cholestatic jaundice due to BA and A1ATD were retrieved from the Netherlands Studygroup for Biliary Atresia Registry (NeSBAR) and the databases from the 5 laboratories in the Netherlands which perform alpha-1-antitrypsin phenotyping/genotyping, respectively.[1,8] Inclusion criteria were 1) cholestatic jaundice (defined as a conjugated bilirubin>30 mcmol/l; conjugated fraction>20%), 2) gestational age above 37 weeks, birthweight above 2000 grams, 3) born in the Netherlands, 4) documented feeding type, 5) age at diagnosis of cholestasis after the age of 8 days and before 6 months of age. Infants with A1ATD who had an older sib with A1ATD were excluded because this knowledge should lead to an intensified vitamin K prophylaxis. Patient files were reviewed to obtain relevant clinical and biochemical characteristics. In addition, the type of feeding and clinical and biochemical indications for a VKD were documented at the time cholestatic jaundice was first recognized, as described previously.[8]

Type of feeding

Types of formula were defined based on the feeding received at the time cholestasis was first diagnosed and were divided as follows. Regular formula - all cow milk based formulas, including those for hungry babies; hydrolysed formula - all formulas containing partially or extensively hydrolysed milk proteins (subdivided in whey and casein based) and soy based formula. Since partial breastfeeding may increase the risk of VKD additional risk factor analysis was performed on the use of breastfeeding; any combination of breastfeeding and formula, a recent switch from breastfeeding (<2 weeks before presentation), and formula for at least 2 weeks.

Vitamin K deficiency

The Prothrombin ratio (PR) was used to assess the presence of a VKD, as described previously.[8] When prothrombin time (PT) expressed in seconds was available the PR was determined as follows: PR = $PT_{patient}$: $PT_{control}$. If a $PT_{control}$ was not determined by the laboratory, it was defined as the mean of the provided reference range. Otherwise, the International Normalized Ratio (INR) was used as PR. In line with our previous study VKD was defined as a PR above 1.5, while a PR of more than 4 was designated as severe VKD.[1] VKDB was defined in line with an established case definition as bruising, bleeding or intracranial hemorrhage in combination with a PR more than 4 in any infant between 8 days and six months of age not due to an inherited coagulopathy or disseminated intravascular coagulation.[9]

Vitamin K determination in commercially available formulas

The amount of vitamin K species (vitamin K1, MK4, MK5, MK7) in the 9 different formulas (2 regular, 4 whey based (2 partially hydrolysed, 2 intensively hydrolysed), 2 casein based, 1 soy) was measured using reversed phase HPLC as described previously.[10] All samples were measured in duplicate.

Statistical analysis

SPSS 15.01 was used for statistical analysis. Risk factor analysis in the combined population of cholestatic infants was performed by calculating relative risks and 95% confidence intervals and corresponding p-values.

RESULTS

Patients

A total of 222 infants with BA(166) and A1ATD (56) were identified. Forty three infants were excluded because of a place of birth outside the Netherlands and hence exposure to a different Vitamin K profylactic regimen (8), a positive family history for A1ATD (7), prematurity (6), a diagnosis of cholestasis before age 7 days (15), and incomplete documentation on type of feeding given (7). Sixty-one infants were exclusively breastfed and were also excluded. Of the remaining 118 infants that were (partially) formula-fed VKD was documented in 8 infants (6.8%) and manifested as a VKDB in one (0.8%).

Risk of VKD in infants on hypoallergenic formula compared with regular formula

To assess the risk of VKD in infants on hypoallergenic formulas we calculated the relative risk (RR) of each type of formula compared with regular formula. Risk factor analysis was restricted to the 59 infants in whom detailed information on the formula type used could be retrieved. As shown in table 1, the absolute risk of VKD in infants on hydrolysed formula was 5/6 (83%), significantly higher than found in infants on regular formula (2/50: 4%), RR 20.8 (95% CI 5.1-84.8). Whey based hydrolysate was associated with the highest risk of VKD as well as the highest absolute PR values (figure 1). VKDB was found in 1/4 of these infants (p=0.07, compared with regular formula). Soy based formula was associated with VKD in 1/3 infants. To our surprise, partial breastfeeding was not significantly associated with VKD(B) (table 2). However, a significant association with VKDB was found when breastfeeding was combined with a (whey based) hydrolysate (p=0.02).

Table 1 Risk of vitamin K deficiency in infants with BA and A1ATD on hypoallergenic formulas compared with infants on regular formula

	Vitamin K deficiency number (percent)	Relative Risk (95% CI)	P Value	Severe VKD number (percent)	P Value	VKDB number (percent)	P Value
regular (n=50)	2 (4%)	1.0		0 (0%)			
hydrolysed (n=6)	5 (83%)	20.8 (5.1-84.8)	**<0.001**	2 (33%)	**0.01**	1 (17%)	0.11
whey based (n=4)	4 (100%)	25.0 (6.4-97.2)	**<0.001**	0 (0%)	**0.004**	1 (25%)	0.07
casein based (n=2)	1 (50%)	12.5 (1.8-87.0)	0.11	0 (0%)		0 (0%)	
soy-based (n=3)	1 (33%)	8.3 (1.0-67.9)	0.16	0 (0%)		0 (0%)	
Total (n=59)	**8 (13%)**			**2 (3.3%)**		**1 (1.7%)**	

The risk in infants on regular formula was used to determine the relative risk in infants on different types of hydrolysed formula. P value was determined with Fisher exact (2-sided).

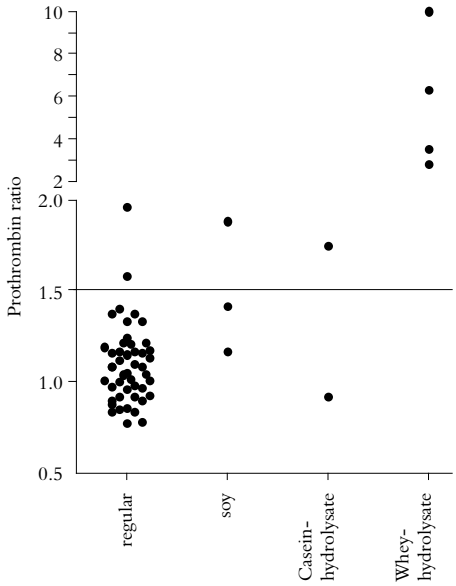


Figure 1
Prothrombin ratio in infants with BA/A1ATD at initial presentation by formula type.

Comparison of the Vitamin K content of regular and hypoallergenic formulas

It has been suggested that the association between hypo-allergenic formulas and VKDB is related to a lower vitamin K content of these formulas.[5] Therefore, we analysed the vitamin K content in 9 different commercially available formulas. All but one formula contained 50μg/L of vitamin K1 or more, arguing against this explanation (Table 3). In addition, the other analysed vitamin K species, with similar biological properties, were found to be present in comparable quantities, and therefore can not explain the differences observed between the relative risk of different fomulas.

Table 2 Risk of vitamin K deficiency in infants with BA and A1ATD with a positve breastfeeding history

	Vitamin K deficiency number (percent)	Relative Risk (95% CI)	P Value	severe VKD	P Value	VKDB number (percent)	P Value
negative breastfeeding history (n=28)	4 (14%)	1		0 (0%)			
positive breastfeeding history (n=31)	4 (13%)	0.9 (0.3-3.3)	1.00	2 (6%)	0.49	1 (17%)	0.11
partial breastfeeding (n=9)	1 (11%)	0.8 (0.09-10.1)	1.00	1 (11%)	0.24	1 (11%)	0.24
switch from breast-feeding< 2 wks (n=7)	2 (29%)	2.0 (0.5-8.8)	0.58	0 (0%)	1.00	0 (0%)	
switch from breast-feeding> 2 wks (n=15)	1 (7%)	0.5 (0.3-3.8)	0.64	1 (7%)	0.35	0 (0%)	

The risk in infants with a negative breastfeeding history was used to determine the relative risk in infants with a positive breastfeeding history. P value was determined with Fisher exact (2-sided).

Table 3 Vitamin K content in commercially available regular and hypoallergenic formulas

	whey	casein	vitamin K1 [µg/l]	MK4 [µg/l]	MK5 [µg/l]	MK7 [µg/l]
regular formula A			65.4	1.5	9.3	0.0
regular formula B			68.0	1.7	7.3	3.6
partially hydrolysed A	+	+	128.2	57.0	1.7	0.0
partially hydrolysed B	+		59.3	2.2	6.7	0.0
intensively hydrolysed A	+		66.1	13.1	9.6	0.0
intensively hydrolysed B	+		101.0	3.3	12.1	0.0
very intensively hydrolysed A		+	73.3	2.9	5.7	4.2
very intensively hydrolysed B		+	38.3	0.3	0.9	0.0
soy			72.4	1.8	12.5	1.5

DISCUSSION

The present data indicate that the use of hydrolysed formula, in particular whey based hydrolysed formula, is a significant risk factor for VKD in infants with unrecognized cholestatic jaundice. More than 80 percent of infants on hydrolysed formula presented with VKD, contrasting with only 4% of infants fed regular formula. The discrepant risk of VKD could not be explained by a lower vitamin K content of commercially available hypoallergenic formulas as compared with regular formulas.

For this study we combined data from national registries for the two most prevalent causes of cholestatic jaundice in infancy in the Netherlands (BA and A1ATD), from a birth cohort of more than 3 million live borns. The lack of efficacy of the Dutch prophylactic regimen (which remained unchanged since its introduction in 1990) in cholestatic infants,[1,8] enabled us to study the protection offered by hypoallergenic formulas.

The exact formula type was unknown in a significant proportion of infants, a problem associated with the retrospective design of our study. We conservatively excluded infants in whom the formula type was not clearly stated from risk factor analysis. Since none of the infants on 'unknown formula' had VKD this may have led to an underestimation of the true risk posed by hydrolysed formula. Although we cannot exclude the possibility that hypoallergenic formula served as a confounder for allergic enteritis, it seems more likely that the poor growth commonly associated with fat malabsorption due to cholestasis induced changes in type of feeding and formula.

Previous, mostly casuistic, reports have suggested an association between hypoallergenic formula and VKDB.[2-5] The rarity of these events, however, precluded more definite conclusions. In this study we hypothesized that biochemical VKD, although harmless in itself, could be viewed as a marker for an increased risk of VKDB, which may potentially be life threatening. We used a prothrombin ratio above 1.5 as an index for probable VKD. As described previously, a PR above 1.5 after the first week of life is unlikely to stem from other causes than VKD.[11] By using this strategy in a large birth cohort, we were able to underpin hydrolysed formula as a risk factor for VKD in infants with unidentified cholestasis.

In view of the high risk of VKDB in exclusively breastfed infants the lack of association between VKD and the use of (partial) breastfeeding is surprising. Most likely, this highlights the protection offered by regular formula, even when used for short periods of time or in combination with breastfeeding. Our data suggest that when such a protective effect is absent - as appears to be the case for hydrolysed formula - coagulation abnormalities associated with the use of breastfeeding are not resolved, may get worse and may even result in a VKDB.

The explanation for the higher risk of VKD in infants on hypoallergenic formula remains elusive. We did not find support for a previous suggestion that this can be explained by a lower vitamin K content.[6] We suspect that hydrolysed formulas decrease the absorption of vitamin K. First, the shortened transit time observed in infants on hydrolysed formula[12] has been associated with decreased vitamin K absorption in experimental studies.[13] Secondly, hydrolysation of the protein content of formula might reduce absorption of vitamin K by impeding emulsification.[14]

In conclusion, cholestatic infants receiving hydrolysed, especially whey based, formula are at a substantial risk of developing VKD(B). These data suggest that infants fed with hydrolysed formula, especially when combined with breastfeeding, should receive additional doses of vitamin K.

Acknowledgements
We would like to thank the members of NeSBAR (Netherlands Study group for Biliary Atresia Registry: DC Aronson, A Kindermann, CMF Kneepkens, LWE van Heurn, AM van den Neucker, ZJ de Langen, PMJG Peeters, G Madern, JH Escher, DC van der Zee, PNMA Rieu, JJM Tolboom) as well as the participant paediatricians and the centers involved in diagnosing A1ATD in the Netherlands for their contribution to the study.

REFERENCES

1. van Hasselt PM, de Koning TJ, Kvist N, de VE, Lundin CR, Berger R et al. Prevention of vitamin K deficiency bleeding in breastfed infants: lessons from the Dutch and Danish biliary atresia registries. Pediatrics 2008 April;121(4):e857-e863.

2. McNinch AW, Tripp JH. Haemorrhagic disease of the newborn in the British Isles: two year prospective study. BMJ 1991 November 2;303(6810):1105-9.

3. Sutor AH, Dagres N, Niederhoff H. Late form of vitamin K deficiency bleeding in Germany. Klin Padiatr 1995 May;207(3):89-97.

4. Moss MH. Hypoprothrombinemic bleeding in a young infant. Association with a soy protein formula. Am J Dis Child 1969 May;117(5):540-2.

5. McNinch A, Busfield A, Tripp J. Vitamin K deficiency bleeding in Great Britain and Ireland: British Paediatric Surveillance Unit Surveys, 1993 94 and 2001-02. Arch Dis Child 2007 September;92(9):759-66.

6. Pellegrino M, Sacco M, D'Altilia MR, Meleleo D, De Anseris AG. Hemorrhagic disease in an infant fed on a vitamin K-deficient soya formula. J Pediatr Gastroenterol Nutr 1996 November;23(4):413-4.

7. Host A, Halken S. Primary prevention of food allergy in infants who are at risk. Curr Opin Allergy Clin Immunol 2005 June;5(3):255-9.

8. van Hasselt PM, Kok K, Vorselaars ADM, van Vlerken L, de Vries R, Nieuwehuys E et al. Vitamin K deficiency bleeding in cholestatic infants with Alpha-1-Antitrypsin deficiency. submitted.

9. Sutor AH, von Kries R, Cornelissen EA, McNinch AW, Andrew M. Vitamin K deficiency bleeding (VKDB) in infancy. ISTH Pediatric/Perinatal Subcommittee. International Society on Thrombosis and Haemostasis. Thromb Haemost 1999 March;81(3):456-61.

10. Schurgers LJ, Vermeer C. Determination of phylloquinone and menaquinones in food. Effect of food matrix on circulating vitamin K concentrations. Haemostasis 2000 November;30(6):298-307.

11. Andrew M. The relevance of developmental hemostasis to hemorrhagic disorders of newborns. Semin Perinatol 1997 February;21(1):70-85.

12. Milhatsch WA, Hogel J, Pohlandt F. Hydrolysed protein accelerates the gastrointestinal transport of formula in preterm infants. Acta Paediatr 2001;90(2):196-8.

13. Hollander D, Rim E. Factors affecting the absorption of vitamin K-1 in vitro. Gut 1976 June;17(6):450-5.

14. Singh AM, Dalgleish DG. The emulsifying properties of hydrolysates of whey proteins. J. Dairy Sci 1998;81:919-924

Hydrolysed formula is a risk factor for vitamin K deficiency in infants with unrecognised cholestasis

Chapter 06

Chapter 06

The influence of bile acids on the oral bio-availability of vitamin K encapsulated in polymeric micelles

P.M. van Hasselt[a], G.E.P.J. Janssens[b], T.K. Slot[b], M. van der Ham[c], T.C. Minderhoud[a], M. Talelli[b], L.M. Akkermans[d], C.J.F. Rijcken[b], C.F. van Nostrum[b]

[a]Wilhelmina Children's Hospital, University Medical Center Utrecht, Lundlaan 6, 3584 EA Utrecht, The Netherlands; [b]Department of Pharmaceutics, Utrecht Institute of Pharmaceutical Sciences, Utrecht University, P.O. Box 80082, 3508 TB, Utrecht, The Netherlands; [c]Laboratory for metabolic and endocrine diseases, University Medical Center Utrecht, Lundlaan 6, 3584 EA Utrecht, The Netherlands; [d]Department of Surgery, University Medical Center Utrecht, Lundlaan 6, 3584 EA Utrecht, The Netherlands.

J Control Release. 2009;133:161-8

ABSTRACT

The purpose of this study was assess the ability of polymeric micelles to enable gastrointestinal absorption of the extremely hydrophobic compound vitamin K, by comparison of its absorption in bile duct ligated and sham operated rats. Hereto, Vitamin K was encapsulated in micelles composed of $mPEG_{5000}$-*b*-p(HPMAm-lac_2), a thermosensitive block copolymer. Vitamin K plasma levels rose significantly upon gastric administration of 1 mg vitamin K encapsulated in polymeric micelles in sham operated rats, but not after bile duct ligation (AUC 4543 and 1.64 ng/mL/hr respectively, $p<0.01$). Duodenal administration of polymeric micelles together with bile acids in bile duct ligated rats fully restored absorption. Dynamic light scattering time series showed a significant and dose dependent rise in micellar size in the presence of bile acids in vitro, indicating the gradual formation of mixed micelles during the first 3 hrs of incubation. The highest bile acid amounts (11 mM deoxycholic acid and 41 mM taurocholic acid) eventually caused aggregation of the loaded micelles after the formation of mixed micelles. These data suggest that the gastrointestinal absorption of encapsulated vitamin K from polymeric micelles is mediated by free bile and that uptake of intact micelles through pinocytosis is insignificant.

Through evolution the mammalian gastrointestinal tract has come up with an intricate system for optimizing the uptake of certain extremely hydrophobic compounds which are essential for life, e.g. fat-soluble vitamins and cholesterol. The bulk of dietary fat is effectively processed through hydrolysis by lipases, resulting in less hydrophobic degradation products, such as fatty acids and monoacylglycerides. Bile constituents, mainly phospholipids and bile acids, then aid in solubilization and, together with the fatty degradation products, form mixed micelles which are essential for trafficking extrewmely hydrophobic compounds through the unstirred water layer to the enterocytes for further uptake.[1]

The crucial function of intraluminal constituents for the adequate absorption of extremely hydrophobic compounds is illustrated by the consequences of bile deficiency, as occurs in cholestatic liver disease. The lack of bile virtually annihilates the absorption of fat soluble vitamins.[2,3] The resulting deficiencies of these vitamins impede a wide array of biological functions including immunocompetence, hemostasis, bone health and cognition.[4-6] Effective oral suppletion is available for some, but not all, lipophilic vitamins. Specifically, the presently available oral vitamin K formulations are ineffective in case of cholestatic liver disease[7], which results in spontaneous haemorrhages[8].

Polymeric micelles, constituted of amphiphilic block copolymers, represent a promising delivery system for hydrophobic drugs.[9,10] The unimers have an amphiphilic chemical structure and can form micelles above the (relatively low) critical micelle concentration (CMC). While the hydrophilic blocks serve as a shell and enhance the degree of water solubility, the hydrophobic blocks form a core in which hydrophobic drugs can be stored.[10,11] Compared with other nanoparticles, polymeric micelles are highly versatile, have a high storage capacity, are generally stable, and have been shown to prolong the half-life of drugs upon parenteral administration.[12] There is a growing body of evidence which suggests that polymeric micelles can also be used to enhance the oral uptake of a variety of hydrophobic drugs.[13-16] Specifically, absorption of unimers as well as compounds incorporated in polymeric micelles have been shown, both in vitro and in vivo.[17,18]

The mechanism through which oral absorption of polymeric micelles would occur remains elusive. Two different hypotheses have been suggested. The main stream hypothesis states that polymeric micelles can enhance the absorption of hydrophobic compounds by serving as a carrier/shuttle to the intestinal mucosa, followed by *in toto* absorption of drug loaded micelles through pinocytosis. Alternatively, the (enhanced) absorption of hydrophobic compounds using polymeric micelles is attained through interplay with physiological constituents of the gastrointestinal tract. Theoretically, the degradation properties of lipases as well as the detergent capacity of bile may cause

destabilization of polymeric micelles. The released hydrophobic compounds could subsequently be shuttled through the unstirred water layer, towards the intestinal mucosa by means of the normal physiological machinery. The documented increased uptake using polymeric micelles may then be merely the result of an increase in membrane permeability[19] and/or inhibition of P-glycoprotein (P-gp) efflux, which has been established for several types of micelles (or its unimers) [20], including TPGS[21] and pluronic P85 [22].

The poor absorption of lipophilic vitamins in the absence of bile offers a unique opportunity to test the validity of the above stated hypotheses. An adequate uptake of an extremely hydrophobic compound entrapped in a polymeric micelle under both physiological and bile deficient conditions would strongly support an independent effect of the polymeric micelle on absorption, whereas a strongly reduced uptake under bile deficient conditions would lend support to the 'interplay' hypothesis.

In this study, we used an *in vivo* rat model to compare the absorption of vitamin K loaded in polymeric micelles under physiological and bile deficient conditions. For the choice of the type of polymeric micelles we considered that the micelles should preferably be loaded in an efficient and convenient way, and that the polymers should be biodegradable and/or bioresorbable. Therefore, micellar loading of vitamin K was accomplished using $mPEG_{5000}$-*b*-p(HPMAm-lac_2), a bioresorbable and thermosensitive block copolymer which has been used by us previously for the very efficient encapsulation of drugs with a large structural variety, i.e. paclitaxel[23] and a hydrophobic photosensitiser[24]. The thermosensitivity of the block copolymers enables easy micelle formation and drug loading by mild heating of an aqueous mixture of polymer and active compound. The present work includes *in vitro* experiments to determine the micellar stability in the presence of bile and other luminal constituents.

METHODS

General

Acetonitrile (ACN), dichloromethane (DCM), ethanol, and methanol were HPLC grade quality and purchased from Biosolve Ltd. (Valkenswaard, The Netherlands). 4,4-azobis-(4-cyanopentanoic acid) (ABCPA), monomethylether of poly(ethyleneglycol) with Mw 5000 g/mol ($mPEG_{5000}$), and vitamin K1 were obtained from Fluka Chemie AG (Buchs, Switzerland). *N*-(2-hydroxypropyl)-methacrylamide dilactate (HPMAm-Lac_2) was synthesized as described by Soga *et al*.[25] Ammoniumacetate, hydrochloric acid 25 %, and ethanol and hexane for plasma analyses were obtained from Merck KGaA (Darmstadt, Germany). Vitamin K in arachid oil (10 mg/g) is a product of Bipharma (Weesp, the Netherlands). Porcine pancreatic lipase and Taurocholic acid (TA) were obtained from Sigma-Aldrich GmbH (Steinheim, Germany). Deoxycholic acid (DA) Sodium Salt Monohydrate was purchased from MP Biomedicals, Inc. (Eschwege, Germany). Phosphate buffered saline (PBS, pH 7.4) was obtained from Braun Melsungen AG (Melsungen, Germany). Vitamin K1-D4 was obtained from Buchem BV (Apeldoorn, The Netherlands). Buprenorfine HCl was purchased from Schering-Plough BV (Utrecht, The Netherlands). Isoflurane is a product of Abbott Animal Health (Abbott Laboratories, Chicago, IL, USA). Silicone cannulae were purchased from Degania Silicone, Inc. (Cumberland, RI, USA). Heparin was obtained from Leo Pharma BV (Breda, The Netherlands).

All buffers were filtered through 0.2 μm cellulose acetate filter (Schleicher & Schuell MicroScience GmbH, Dassel, Germany) or through 0.02 or 0.2 μm filters 'Anotop' (Whatman International Ltd., Maidstone, England) prior to use. Micellar solutions were filtered through 0.45 μm cellulose syringe filter (Alltech Associates Inc., Deerfield, IL, USA). Milli-Q water refers to demineralized water filtered through a 0.22 μm Millipak® 100 filter (Millipore Corporation, Billerica, MA, USA). Vivaspin 10 and 20 mL centrifugal concentrators with a 5000 MWCO filter were obtained from Sartorius AG (Goettingen, Germany). For MS measurements, a Quattro Ultima triple quadrupole mass spectrometer (tandem MS) (Waters, Manchester, UK) interfaced with an atmospheric pressure chemical ionisation (APCI) source and equipped with an Alliance 2795 HPLC (Waters, Etten-Leur, The Netherlands) was used. Masslynx software (Version 4.0, SP 4, Waters, Manchester, UK) was used for instrument control, data acquisition and data processing.

Synthesis of block copolymer, PEG_{5000}-b-p(HPMAm-lac_2)

PEG_{5000}-*b*-p(HPMAm-lac_2) was synthesized as described in detail by Soga *et al*.[25] In brief, HPMAm-lac_2 was dissolved in ACN (concentration 300 mg/mL) together with $(methoxy\text{-}PEG)_2$-ABCPA, which served as radical macro-initiator. The polymerization process was carried out in a nitrogen atmosphere for 24 hours, at a temperature of 70 °C. The synthesized polymer was precipitated in diethyl ether, the precipitate was collected by centrifugation and dried in a vacuum oven and then dissolved in water (21 °C), filtered (0.22 μm pore size) and freeze-dried. The resulting polymer had a molecular weight of 32000 g/mol as measured by gel permeation chromatography (using polyethylene glycol standards) and a critical micelle temperature of 4 °C, which are similar to the values described before.[25]

Vitamin K incorporation in PEG5000-b-p(HPMAm-Lac2) micelles

PEG_{5000}-*b*-p(HPMAm-lac_2) (2 to 10 mg/mL) was dissolved in 120 mM ammonium acetate buffer pH 5, and stored overnight at 4 °C. Vitamin K stock solutions of 0.1 to 10 mg/mL in ethanol were prepared and kept in the dark. The vitamin K loaded micelles were produced by the rapid heating procedure as previously described.[25] Briefly, the polymer solution was cooled in a glass vial for at least 1 minute at 0 °C. Subsequently, vitamin K solution was added to the vial in a ratio 9:1 v/v (polymer: vitamin K) and immediately transferred into a 50 °C water bath for 1 minute while vigorously stirring. The resulting micellar solution was cooled to room temperature and filtered through a 0.45 μm cellulose syringe filter. Micellar solutions were concentrated by centrifugation at a specific speed and for the indicated time, using a Sigma 4K10 centrifuge (Salm en Kipp B.V., Breukelen, the Netherlands) and Vivaspin 20 mL centrifugal concentrators with a 5000 MWCO filter. The temperature of the centrifuge was set to 30 °C. During the entire procedure, vitamin K containing solutions were protected from daylight to prevent photo-oxidation of vitamin K.

Dynamic light scattering (DLS)

Micelle diameter and polydispersity indices (PDI) were measured by Dynamic Light Scattering (DLS), using an ALV/CGS-3 Compact Goniometer System (ALV GmbH, Langen, Germany) in combination with a Photon Counting Module (PerkinElmer Optoelectronics, Vaudreuil, Canada). The scattering angle was 90° and temperature was set to 25 °C, unless stated otherwise. Analysis was performed using ALV-Correlator Software Version 3.0.2.1 (ALV-GmbH, Langen, Germany). Results were corrected for the viscosity of 10 % aqueous ethanol mixtures (1.45 mPa·s) where appropriate.

Cryogenic Transmission Electron Microscopy (Cryo-TEM)

Thin aqueous films of empty and loaded micelles, obtained from 10mg/mL polymer and 1mg/mL vitamin K (9:1 v/v) followed by 150 min centrifugation at 3000xG, were formed by placing sample

drops on a 200 mesh glow discharged copper grid covered with Quantifoil holey carbon foil (Quantifoil Micro Tools GmbH, Jena, Germany) at 22 ºC and at 100 % relative humidity followed by dry blotting during 0.5 sec. The thin film was vitrified by quickly plunging the grid into liquid ethane. The grids with vitrified thin films were transferred into the microscope chamber using a GATAN 626 cryo-holder system. A Tecnai12 transmission electron microscope (Koninklijke Philips Electronics NV, Eindhoven, the Netherlands) operating at 120 kV was used with the specimen at -180 ºC and using low dose imaging conditions to avoid melting of the vitrified film. Images were recorded on SIS-CCD camera and processed with AnalySIS software.

Animals and experimental procedure

Male Wistar rats (*Rattus norvegicus* Cpb:WU) were purchased from Harlan (Horst, the Netherlands). They were kept under constant housing conditions (temperature 22 °C, relative humidity 60 %, and a 12 hour light/dark cycle) with free access to water and food upon arrival and were allowed to adjust to these conditions for at least one week prior to surgery. The rats were randomly assigned to undergo either bile duct ligation or a sham operation. All surgical procedures were performed with sterile instruments under aseptic conditions on a heated operating table under general anesthesia using a combination of 2 % Isoflurane gas through a snout mask and intramuscular 0.15 mL 5 % buprenorphin. Through a midline incision the abdomen was opened and the liver gently retracted, exposing the common bile duct. Using a 6-0 silk ligature the common bile duct was ligated. The sham operation consisted of the same procedure, however without the ligature being placed. Subsequently the muscular part of the stomach was exposed, and a gastric cannula was inserted in the stomach, and fixed in place using a purse-string suture. Alternatively, for duodenal administration, the cannula was placed 10 – 15 mm distal from the pylorus in the duodenum. The abdominal incision was closed in layers using a running suture and 4-0 (Vicryl; Ethicon) for the abdominal wall and the skin. The right jugular vein was cannulated as follows. Through a small, v-shaped incision the silicone cannula was inserted 4.2 cm in the direction of the heart. After checking for intact flow in the cannula, the system was flushed using heparin in physiological saline (100 Units/mL) and subsequently filled with a locking solution containing 50 % dextrose and heparin 500 IU/mL. Both cannulas were tunneled subcutaneously to the back, puncturing the skin between the shoulder blades and were kept in place using a rodent infusion jacket (Uno Zevenaar BV, Zevenaar, The Netherlands). After the surgical procedure animals were placed in separate cages with free access to water and regular rodent chow. The following day, the first blood sample (0.5 mL) was taken. Subsequently, rats received either 1 mg (0.1 mL) vitamin K in arachid oil and 0.9 mL water or 1 mL vitamin K-loaded polymeric micelle solution intragastrically. When bile was added to the micelle solution it was administered intraduodenally to mimic the physiological situation. After administration of vitamin K, blood samples were taken at one hour intervals for 5 hours. The exact concentration of the polymeric micelle solutions (range 0.85-1.29 mg/mL) were

determined by UV spectrometry (see next section) and were used to adjust the plasma values compared with baseline per 1 mg of vitamin K. Rats were euthanized by blood loss under anesthesia. The protocol was approved by the Animal Experiment Committee of the University Medical Center, Utrecht, the Netherlands. SPSS 15.01 was used for statistical analysis. Paired t-tests were performed on the plasma vitamin K levels upon gastric administration of the loaded polymeric micelles. T-tests were performed for comparison between groups of the vitamin K concentration at each time point, as well as for the peak level (defined as the highest concentration in an individual animal compared with baseline) and the area under the curve (AUC).

Vitamin K determination

The amount of vitamin K in the micellar solution was measured using UV-spectrometry (UV-Vis Spectrometer Lambda 2; PerkinElmer Instruments, Oosterhout, the Netherlands) at a detection wavelength of 248nm. Prior to measurement, the micellar samples were diluted at least 10 fold in ethanol. Ethanol induces micellar destabilization by solvation of the individual polymers and is a solvent for vitamin K. A calibration curve was made with vitamin K in pure ethanol. The detection limits were 6.25 μg/mL as lower limit and 50 μg/mL as upper limit, and R^2 was 0.9986. The polymers were shown not to affect the measurement of vitamin K (data not shown). Rat plasma samples were prepared as follows. 100 μl internal standard (vitamin K-D4) was added to 200 μl rat plasma followed by addition of 500 μl ethanol. After vortexing for 30 seconds, vitamin K was extracted twice with 1.5 mL hexane by vortexing and centrifugation at 2000 rpm during 1 minute. The hexane fractions were collected and evaporated followed by dissolving the residue in 120 μl ethanol. After filtration with Sirocco protein precipitation plate (Waters, Massachusetts, USA) aliquots of 50 μl were injected to the LC-MS/MS system. Samples were analyzed using LC-MS as described by Suhara *et al*. with few modifications.[26] Chromatographic separation was performed, using an Atlantis dC18 analytical column (Waters, Massachusetts, USA) by 40 °C. A linear gradient of 2 minutes between solvent A (methanol 0.1 % acetic acid 95:5 v/v) and solvent B (ethanol 100 %) by a flow of 1 mL/min was used. The MS operated in $APCI^+$ with the following settings: corona discharge needle voltage, 3 kV; cone voltage, 35 V; source temperature, 130 °C; desolvation temperature, 550 °C; collision energy, 20 eV. For multiple reaction monitoring, the transitions *m/z* 445.4 ® 186.9 (Vitamin K) and *m/z* 451.4 ® 186.9 (Vitamin K-D4) were measured. For quantification of vitamin K, a calibration curve in high range (1 - 542 ng/mL, $r^2 = 0.999$) with high internal standard (70 ng/mL Vitamin K-D4) and a calibration curve in lower range (0.3 - 2.2 ng/mL, $r^2 = 0.993$) with lower internal standard (7 ng/mL Vitamin K-D4), both dissolved in ethanol, were used. Linearity was checked with high internal standard till 6775 ng/mL ($r^2 = 0.995$) and with lower internal standard till 1084 ng/mL ($r^2 = 0.999$). For HPLC measurements of vitamin K a Waters 2695 System (Waters Associates Inc., Milford, MA, USA) was used with a LiChrospher®

100 RP-18 column (Merck KGaA, Darmstadt, Germany). ACN/DCM/MeOH 60:20:20 was used as eluent as described before.[27] The flow was 1 mL/min. Compounds were detected with a UV detector at 210 and 248 nm. A calibration curve ($r^2 = 1.000$) was established using Empower 2 Pro Software as analysing software.

Micellar characteristics in the presence of luminal bowel constituents

To investigate the effect of luminal constituents on micellar stability we assessed changes in particle size (Z_{ave}) and polydispersity index (PDI) over time. Dynamic light scattering (DLS) measurements were taken every 10 minutes for 12 hours at 37 °C. For these experiments a polymer concentration of 2mg/mL was used, which induces a 20 percent larger particle size compared with the 10mg/mL polymer concentration, without affecting polydispersity and encapsulation efficiency. The effect of the acidic gastric pH was assessed by comparing the stability of vitamin K loaded micelles at pH 2 and pH 7.4. Micelles were formed as described above in PBS buffer pH 7.4 or ammonium acetate buffer pH 5 (2 mg/mL polymer, 1 mg/mL vitamin K (9:1 v/v)). The latter solution was adjusted to pH 2 by addition of 4 M HCl. The influence of lipase on micellar stability was determined by adding 100 uL lipase solution (porcine pancreatic lipase, 30 mFIP-U/mL or 6000 FIP-U/mL, in 5 mM $CaCl_2$) to 1 mL micellar solution (pH 7.4). The former concentration was previously shown to induce degradation of a polymeric micelle[28], the latter concentration (20-fold higher than found in humans[29]), was chosen to rule out an effect of lipase, even at peak concentrations. Micellar stability in the presence of bile acids was determined as follows. 250 mg taurocholic acid (TA) and 49.5 mg deoxycholic acid (DA) were separately dissolved in 1 mL 100 mM PBS buffer pH 7.4. Thereafter, 5-100 μl TA solution and 5-100 μl DA solutions were added to 1 mL micellar solution, with maximal final bile acid concentrations of 20.8 mg/mL (41 mM) and 4.1 mg/mL (11 mM), respectively. These concentrations reflect the physiological range of intraduodenal bile acid concentrations.[30]

Effect of bile acids on vitamin K retention

To investigate the effect of bile acids on vitamin K retention in the micelles, we performed an ultrafiltration experiment. Micelles (2 mg/mL polymer, 1 mg/mL vitamin K in ethanol, 9:1 v/v) were subjected to the above described range of bile acid concentrations. Subsequently, the samples were immediately placed in membrane tubes (5000 MWCO filter) and centrifuged for approximately 25 minutes at 3000 x *g* at 40 °C until a 2-fold volume reduction was reached. The filtrates and the residues were collected and Z_{ave} and PDI of both fractions were measured by DLS. The filter was rinsed with 2mL ethanol to dissolve any precipitated vitamin K. The amount of vitamin K in each fraction was measured using HPLC as described above. The same procedure was performed for samples which had been pre-incubated with bile acids for three hours.

RESULTS

Vitamin K encapsulation in mPEG$_{5000}$-b-p(HPMAm-Lac$_2$) micelles

Previous reports from our laboratory showed that hydrophobic compounds can be stably and efficiently entrapped into mPEG$_{5000}$-*b*-p(HPMAm-Lac$_2$) micelles by simply heating an aqueous polymer-drug mixture.[23-24] A polymer concentration of 10 mg/mL was chosen since it resulted in the lowest diameter at a constant vitamin K concentration of 0.1 mg/mL in the final dispersion (from DLS experiments, data not shown). At this polymer concentration empty micelles had a diameter of 45 nm. Encapsulation of vitamin K at concentrations up to 0.1 mg/mL resulted in a moderate rise of Z_{ave} (to ~65 nm), with an encapsulation efficiency, defined as the ratio of vitamin K loaded in micelles to the initial amount of vitamin K added, of ~90 %. Higher vitamin K concentrations resulted in a rapid increase of size and polydispersity (figure 1A).

Also, the encapsulation efficiency decreased, resulting in a maximum micellar vitamin K concentration of ~0.3 mg/mL. Thus, the loading capacity, defined as the maximum weight of vitamin K with respect of the weight of polymer, was 3%. For the in vivo studies, a vitamin K concentration of approximately 1 mg/mL is required, and therefore micellar solutions with a vitamin K concentration of 0.1 mg/mL were concentrated by centrifugal membrane filtration. Figure 1B shows that

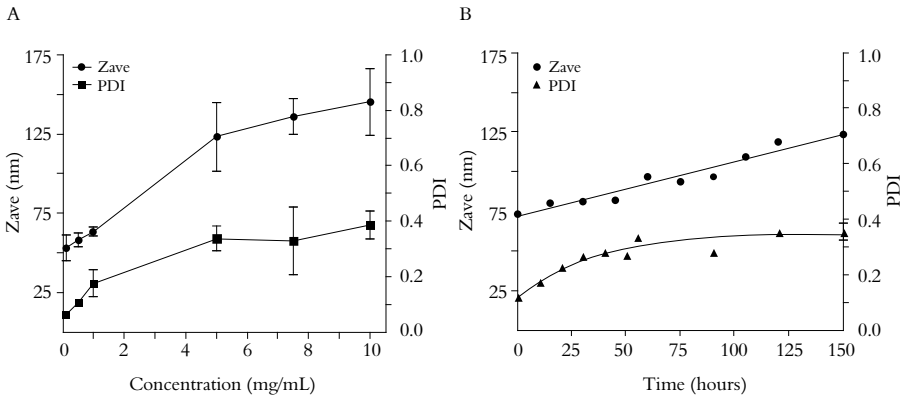


Figure 1

Effect of increasing vitamin K concentration in the stock solutions (A) and increasing centrifugation times (B, using a vitamin K stock solution of 1 mg/mL) on Z_{ave} diameter and polydispersity index (PDI) of the formed micelles. One part of vitamin K stock solutions was added to nine parts of polymer stock solutions of 10 mg/mL.

the vitamin K concentration increased linearly with increasing centrifuge time. The required 10 × volume reduction was attained after 150 minutes of centrifugation at the expense of a rise in micelar size (~120 nm) and a higher polydispersity (~0.35) as determined by DLS. Particle size and size distribution was confirmed using Cryo TEM (figure 2). The vitamin K concentration in the final samples used for the in vivo experiments was, as expected, 1.0 ± 0.14 mg/mL.

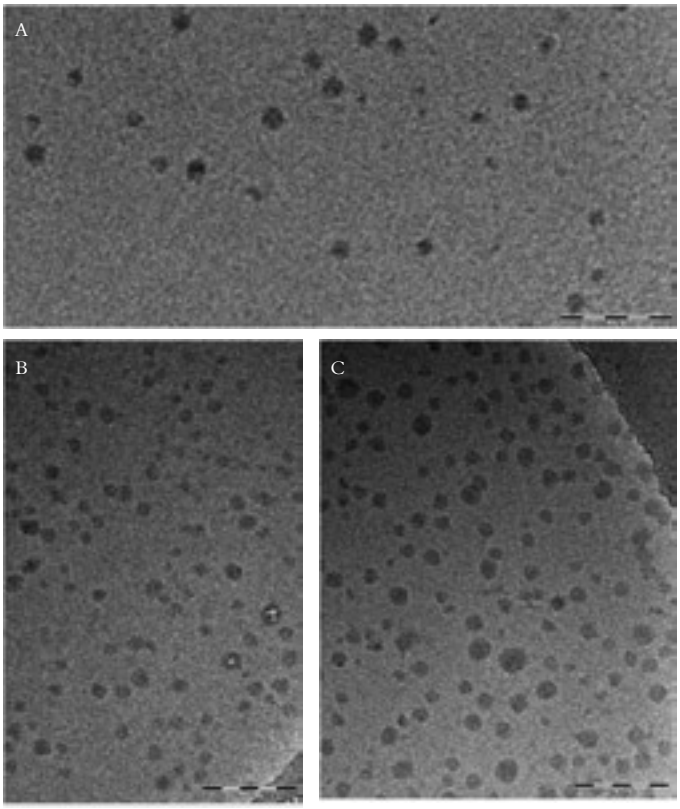


Figure 2

Cryo-TEM pictures of vitamin K loaded micelles before centrifugation (A), and empty (B) and vitamin K loaded micelles (C) after 150 minutes of centrifugation. Initial polymer and vitamin K concentrations were 10 mg/mL and 1 mg/mL, respectively.

Influence of bile acids on the in vivo absorption of vitamin K encapsulated in $mPEG_{5000}$-b-p(HPMAm-Lac_2) micelles

Under physiological conditions (i.e. with sham operated rats), a significant rise in plasma vitamin K concentration (p=0.03) compared with baseline was found after intragastric administration of vitamin K encapsulated in $mPEG_{5000}$-b-p(HPMAm-Lac_2) micelles and formulated in oil (figure 3A). However, at t=3 and t=4 hrs, plasma vitamin K levels were significantly lower when micelles were administered compared with those found upon administration of vitamin K in oil (p=0.02 and p=0.04 respectively). A trend in the same direction was observed for peak plasma vitamin K levels (2304 in micelles vs 3928 ng/mL in oil) and AUC (4534 vs 11460 ng/mL/hr in micelles and oil, respectively), but these differences were not significant (p=0.14 and 0.07, respectively).

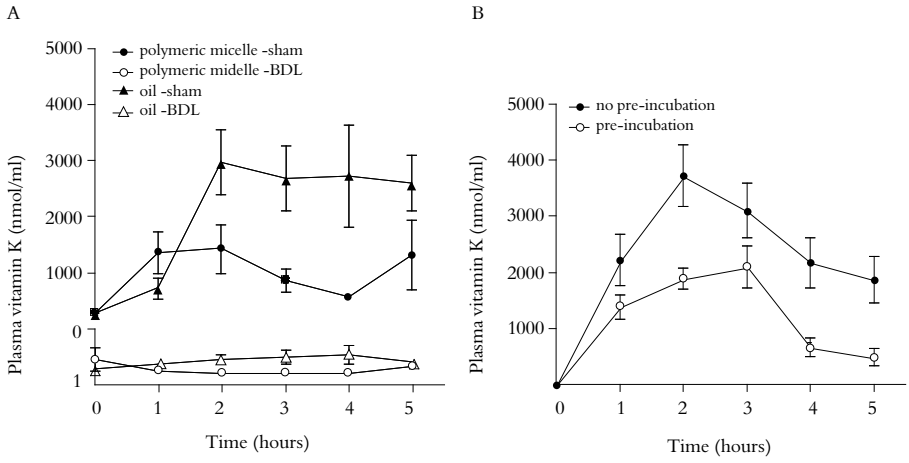


Figure 3

The absorption of vitamin K in sham operated and bile duct ligated (BDL) rats upon administration of vitamin K loaded polymeric micelles (10x concentrated) and vitamin K dissolved in oil.(A) The absorption of vitamin K from polymeric micelles in BDL rats after addition of bile acids (100 μl of saturated TA and DA solutions) with and without pre-incubation (3 hrs) of polymeric micelles with the bile acids.(B)

Bile duct ligation (BDL) almost completely abolished vitamin K absorption from both micellar vitamin K and vitamin K dissolved in oil. AUC values were 1.6 and 2.7 ng/ml/hr, respectively (p=0.08). Interestingly, vitamin K absorption in BDL rats was fully restored by duodenal administration of polymeric micelles together with bile acids (100 μl of saturated TA and DA solutions), resulting in an AUC of 13870 ng/mL/hr (figure 3B). Pre-incubation of polymeric micelles with the same amounts of bile acids for 3 hours partially inhibited this effect, as indicated by significantly lower vitamin K plasma concentrations and AUC (6581 ng/mL/hr, p=0.01).

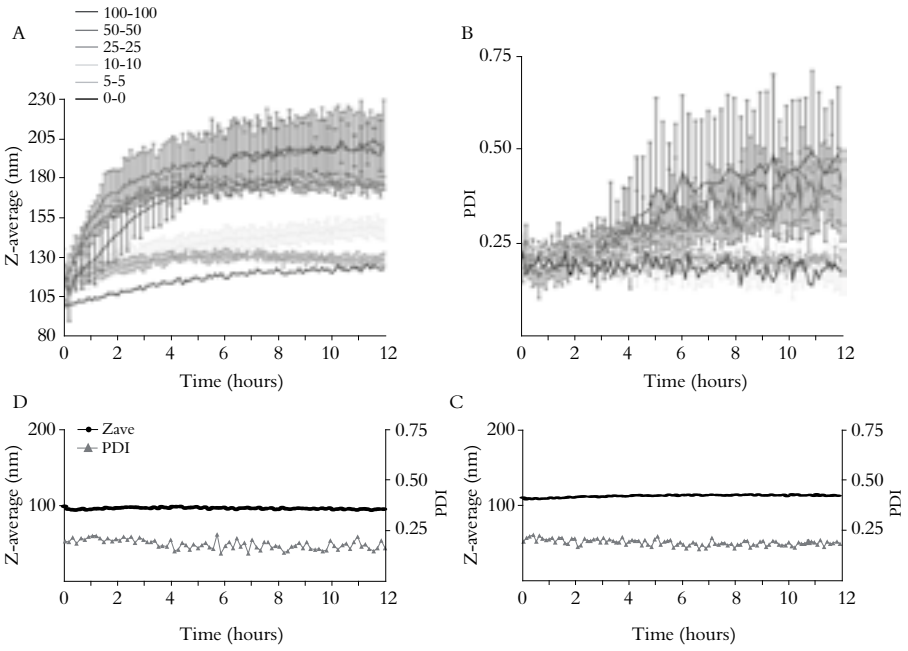


Figure 4
Micellar properties in the presence intraluminal constituents in time.
Z_{ave} diameter (A) and PDI (B) in the presence of different amounts of bile acids (numbers are the amounts of saturated TA and DA solutions given in microliters); Z_{ave} and PDI at pH 2 (C) and in the presence of pancreatic lipase (3 mU/mL) (D). Polymer and vitamin K concentrations were 2 mg/mL and 0.1 mg/mL, respectively.

Stability of vitamin K-encapsulated $mPEG_{5000}$-b-p(HPMAm-Lac_2) micelles in the presence of bile

The difference between the uptake of the micellar vitamin K formulation in the absence and presence of bile strongly suggests that the uptake is bile dependent and that the micelles alone are not able to transfer their contents through the enterocytes into the systemic circulation. Therefore, the influence of varying amounts of bile on the integrity of the loaded micelles was investigated. Indeed, as depicted in figures 4A and 4B, repeated measurements indicated a rise of Z_{ave} diameter and PDI over time, which was more pronounced at higher bile concentrations. Contrarily, empty micelles were not affected by the addition of bile (results not shown). Relatively low amounts of bile acids (< 25 μl TA and DA solutions) gave only a moderate increase in the particle size of the loaded micelles without affecting the size distribution. These 'swollen' micelles were stable during the time of investigation. As shown in figure 5 for the highest bile concentrations (100 μl TA + 100 μl DA), polymeric micelles and bile appeared to fuse gradually into a large monomodal peak during the first 3 hours. The size corresponding to this peak was ~300 nm, much larger than the particle size of bile micelles (~3 nm) and the polymeric micelles alone (65 nm), suggesting the formation of mixed 'micelles'. Larger aggregates appeared after approximately 10 hours, suggesting saturation and consequently instability of the mixed micelles, in contrast to micelles that contained less than 25 μl of the saturated bile acid solutions (figure 5C and supplementary video).

Stability of vitamin K-encapsulated $mPEG_{5000}$-b-p(HPMAm-Lac_2) micelles in the presence of other luminal constituents

Theoretically, micellar stability could also be affected by other luminal constituents, mainly the low gastric pH which may cause hydrolysis of the polymers, and the hydrolyzing capacity of lipase. However, size and size distribution of vitamin K loaded micelles remained stable at pH 2 (figure 4C), and in the presence of lipase at concentrations of 3mU/mL (figure 4D) and 600 U/mL (data not shown).

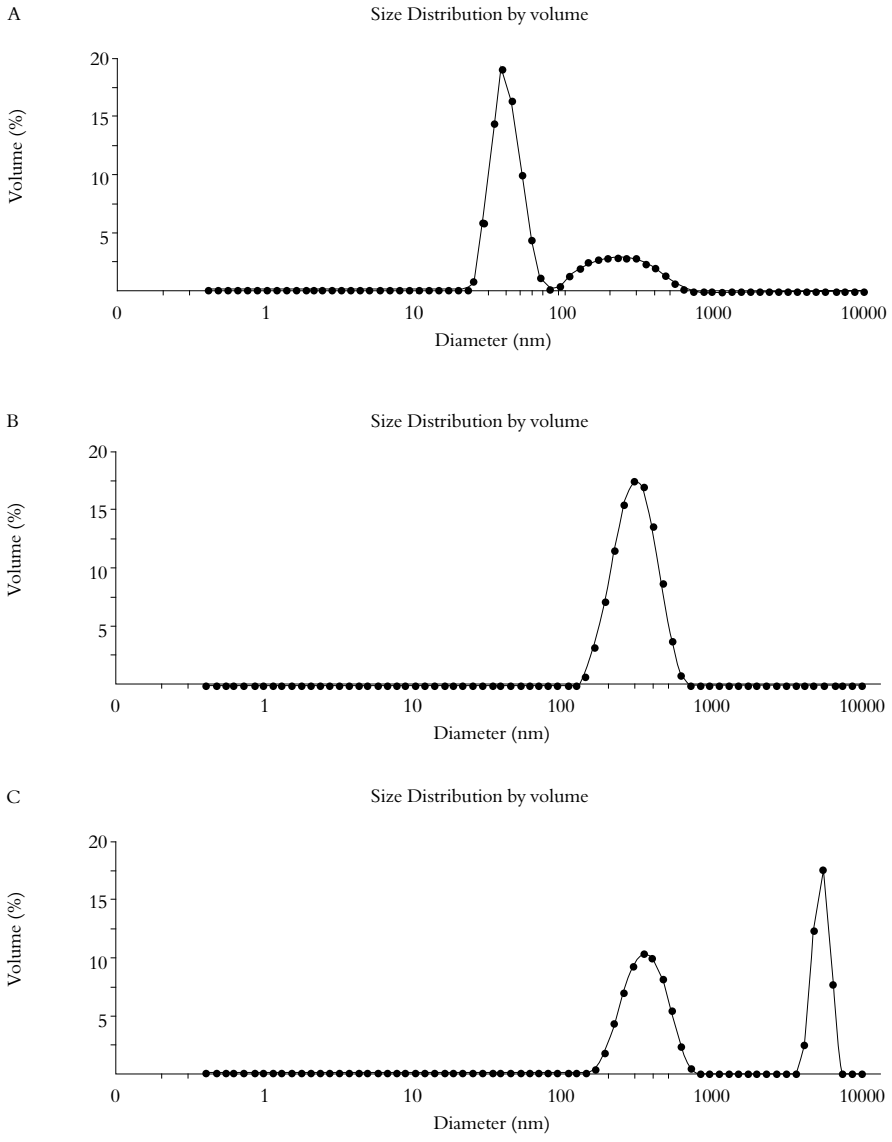


Figure 5
Size distribution by volume following the addition of 100 l of bile acids (saturated TA and DA solutions) to vitamin K loaded polymeric micelles after 1 hour (A), 3 hours (B) and 10 hours (C). Polymer and vitamin K concentrations were 2 mg/mL and 0.1 mg/mL, respectively.

Vitamin K retention in the presence of bile acids: ultrafiltration experiments

To determine whether the observed changes of the vitamin K-loaded micelles in the presence of bile acids had a detrimental effect on the retention of vitamin K in the polymeric micelle, an ultrafiltration experiment was performed. A membrane with MWCO of 5000 was used, which is permeable for vitamin K and bile acids and not for the polymer. However, the amount of vitamin K retrieved from the filtrate was negligible (<1%, Figure 6), while an increasing amount with a maximum of ~20 percent of vitamin K was found to precipitate during the centrifugation with increasing amount of bile acids (Figure 6) No precipitation was observed within the same timeframe (25 min.) when the mixture of micelles and bile acids were kept without centrifugation.

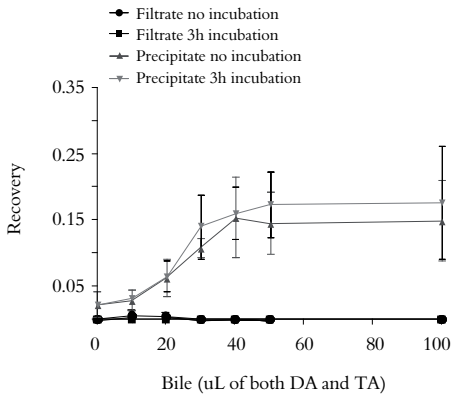


Figure 6

The effect of bile acids on the stability of vitamin K loaded polymeric micelles after ultracentrifugation, expressed as the fractions of vitamin K recovered in the filtrate and after rinsing the emptied centrifuge tubes.

DISCUSSION

Our data indicate that the intestinal absorption of vitamin K encapsulated in $mPEG_{5000}$-b-p(HPMAm-Lac_2) micelles depends on the presence of bile. Plasma levels rose significantly above the background level after a pharmacological dose of vitamin K in sham operated rats but remained unaltered in bile duct ligated rats. Administration of polymeric micelles together with bile acids completely restored absorption in bile duct ligated rats. Taken together, these findings provide strong evidence against the widespread notion that orally administered polymeric micelles can enhance the absorption of hydrophobic drugs by way of *in toto* pinocytosis.[31,32] Our in vitro data show that, whereas vitamin K encapsulation is unaffected by an acidic environment or the presence of lipase, the presence of relatively large amounts of bile acids results in micellar instability and subsequent precipitation of vitamin K.

Since we only examined one type of micelle it remains to be determined whether these findings can be extrapolated to other micellar types. Thus far, evidence for the uptake of other types of intact micelles is indirect. Zastre et al, using MePEG-b-PCL diblock copolymers, observed that the uptake of Rhodamine-123 in Caco-2 cells was negligible below the CMC but increased substantially at polymer concentrations well above the CMC. [19,31] Similarly, Dabholkar described an increase in cell associated fluorescence as well as Rhodamine-123 accumulation after adding paclitaxel loaded Rhodamine labeled PEG-PE/TPGS micelles, compared with free Rhodamine.[33] However, apico-basolateral transport of the drug and the polymer across a Caco-2 monolayer were not determined. Such transport studies in Caco-2 monolayers were recently performed by Mathot, using risperidone encapsulated in a mPEG750-b-P(CL-co-TMC) micelle (diameter 24nm).[17] Although they did observe a dose dependent rise in retrieved unimers on the basolateral side, they also noted a dramatic drop in the apparent permeability coefficient at concentrations above the CMC. In addition, they found a discrepancy between the apparent permeability of the unimers and the encapsulated compound, being 20 times higher for risperidone. In a second study, they showed that the apparent permeability of the encapsulated compound was only determined by its degree of hydrophobicity and was unaffected by encapsulation. Even though they did observe a measurable ATP and clathrin dependent transport above the CMC, they concluded that the free drug seemed to be the major absorbed fraction.[17] Although we cannot exclude some degree of endocytotic uptake for our micelles, it is unlikely to be of a magnitude that is clinically relevant.

Micellar stability in the presence of luminal constituents was investigated previously by Dabholkar et al.[33] They concluded that paclitaxel-loaded PEG-PE/TPGS micelles were stable in the presence of bile acids at a concentration of 5mM. We, however, observed a slow and moderate swelling of the micelles at the same bile acid concentration, probably because of the formation of mixed micelles. At higher bile acid concentrations (in the range of 12.5 mM to 51 mM), a clear dose dependent rise in micellar size and polydispersity was documented in our study. Remarkably, no such effect on micellar stability was found in the empty micelles, suggesting that micellar stability is critically dependent on the presence, and perhaps the properties, of the encapsulated compound.

Although bile acids had a pronounced effect on micellar size and eventually evoked formation of large aggregates, this destabilising effect appeared unessential for vitamin K absorption. First, vitamin K absorption occurred within an hour in the presence of bile, thus before the formation of appreciable amounts of mixed micelles as observed by in vitro DLS measurements. Second, pre-incubation significantly impeded absorption in stead of inducing a rapid and pronounced rise of plasma vitamin K levels. We speculate that two separate processes operate simultaneously after addition of bile. In the first process, bile acids outside the micelle may extract the encapsulated vitamin K and act as a shuttle to transport vitamin K to the endothelial lining. This is supported by the observation that duodenal co-administration of vitamin K and bile acids at a concentration that was 2-3 fold higher than commonly found in vivo[30] (without pre-incubation) resulted in higher plasma vitamin K levels (Figure 3B). Compared with the oil formulation, lower vitamin K levels were found in sham operated rats when vitamin K was administered as a micelle (Figure 3A), which suggests that the extraction process is incomplete under physiological conditions. This may be in part due to the second process, which is the gradually merging of bile acids with the loaded polymeric micelles to form 'swollen' mixed micelles. This process will eventually result in aggregate formation which both decrease the mobility of the particle towards the endothelium as well as impede the release of vitamin K. This hypothesis is supported by the lower vitamin K concentrations found after pre-incubation with bile acids.

The present findings have important implications for tailoring the design of polymeric micelles for oral delivery. In view of the minor role for *in toto* pinocytosis on the absorption of a drug, efforts to enhance oral absorption should not be directed at increasing the stability of micelles. Instead, micelles should be able to release the entrapped drug rather rapidly after passing the stomach, but preferably only after crossing the unstirred water layer, which is one of the main barriers for the absorption of hydrophobic compounds. In this respect, targeting micelles for the mucus layer may be a promising venue to prolong the duration of contact with the endothelial lining thereby

increasing the time for degradation and release.[34-36] Furthermore, P-gp inhibiting properties of the polymeric micelle or its unimers, as have been documented for some Pluronics species as well as for TPGS, may be helpful.[21,22]

In conclusion, the absorption of vitamin K encapsulated in $mPEG_{5000}$-b-p(HPMAm-Lac_2) micelles depends on the availability of bile acids, indicating that the polymeric micelles are incapable of inducing measurable absorption by themselves. We suggest that the bile dependent uptake may well be a more universal theme than suggested so far in the literature, and that measurement of the uptake using a bile deficient animal model could be used as an important index for the *in toto* uptake of drug loaded nanoparticles through the endothelial lining of the gastrointestinal tract.

Acknowledgments
The authors wish to acknowledge André Verheem, Hans Kanbier and Raymond Mouw (UMCU) for technical assistance.

Supplementary file
Video showing the change in particle size distribution upon addition of saturated bile acid solutions to vitamin K loaded micelles is available online.

REFERENCES

1. L.A. Woollett, Y. Wang, D.D. Buckley, L. Yao, S. Chin, N. Granholm et al, Micellar solubilisation of cholesterol is essential for absorption in humans, Gut 55 (2006) 197-204

2. L. Forsgren, Studies on the intestinal absorption of labelled fat-soluble vitamins (A, D, E, and K) via the thoracic-duct lymph in the absence of bile in man, Acta Chir. Scand. Suppl. 399 (1969) 1-29.

3. M.J. Shearer, C.N. Mallinson, G.R. Webster, P. Barkhan, Absorption of tritiated vitamin K1 in patients with fat malabsorption, Gut 11 (1970) 1063-1064.

4. A.T. de Albuquerque Taveira, M.I. Fernandes, L.C. Galvao, R. Sawamura, V. de Mello, F.J. de Paula. Impairment of bone mass development in children with chronic cholestatic liver disease, Clin. Endocrinol. Oxf. 66 (2007) 518-523.

5. R.J. Sokol, Fat-soluble vitamins and their importance in patients with cholestatic liver diseases, Gastroenterol. Clin. North Am. 23 (1994) 673-705.

6. H. Chen, Q. Zhuo, W. Yuan, J. Wang, T. Wu. Vitamin A for preventing acute lower respiratory tract infections in children up to seven years of age, Cochrane Database Syst. Rev. (2008) CD006090.

7. S.P. Pereira, M.J. Shearer, R. Williams, G. Mieli-Vergani, Intestinal absorption of mixed micellar phylloquinone (vitamin K1) is unreliable in infants with conjugated hyperbilirubinaemia: implications for oral prophylaxis of vitamin K deficiency bleeding, Arch. Dis. Child. Fetal Neonatal Ed. 88 (2003) F113-F118.

8. P.M. van Hasselt, T.J. de Koning, N. Kvist , V.E. de, C.Rydahl Lundin, R. Berger, J.L. Kimpen, R.H. Houwen, M.H. Jorgensen, H.J. Verkade, Netherlands Studygroup for Biliary Atresia Registry, Prevention of vitamin K deficiency bleeding in breastfed infants: lessons from the Dutch and Danish biliary atresia registries, Pediatrics 121 (2008) e857-e863.

9. A. Mahmud, X.B. Xiong, H.M. Aliabadi, A. Lavasanifar, Polymeric micelles for drug targeting, J. Drug Target. 15 (2007) 553-584.

10. D.D. Lasic, Mixed micelles in drug delivery, Nature 355 (1992) 279-280.

11. S. Muranishi, Absorption enhancers, Crit. Rev. Ther. Drug Carrier Syst. 7 (1990) 1-33.

12. K. Kawano, M. Watanabe, T. Yamamoto, M. Yokoyama, P. Opanasopit, T. Okano, Y Maitani, Enhanced antitumor effect of camptothecin loaded in long-circulating polymeric micelles, J. Control. Release 112 (2006) 329-332.

13. M.V. Varma, R. Panchagnula, Enhanced oral paclitaxel absorption with vitamin E-TPGS: effect on solubility and permeability in vitro, in situ and in vivo, Eur. J. Pharm. Sci. 25 (2005) 445-453.

14. P.C. de Smidt, M.A. Campanero, I.F. Troconiz. Intestinal absorption of penclomedine from lipid vehicles in the conscious rat: contribution of emulsification versus digestibility, Int. J. Pharm. 270 (2004) 109-118.

15. V.P. Sant, D. Smith, J.C. Leroux, Enhancement of oral bioavailability of poorly water-soluble drugs by poly(ethylene glycol)-block-poly(alkyl acrylate-co-methacrylic acid) self-assemblies, J. Control. Release 104 (2005) 289-300.

16. L. Bromberg, Polymeric micelles in oral chemotherapy, J. Control. Release 128 (2008) 99-112.

17. F. Mathot, A. des Rieux, A. Arien, Y.J. Schneider, M. Brewster, V. Preat, Transport mechanisms of mmePEG750P(CL-co-TMC) polymeric micelles across the intestinal barrier, J. Control. Release 124 (2007) 134-143.

18. F., Mathot, L. van Beijsterveldt, V. Preat, M. Brewster, A. Arien, Intestinal uptake and biodistribution of novel polymeric micelles after oral administration, J. Control. Release 111 (2006) 47-55.

19. J. Zastre, J. Jackson, H. Burt, Evidence for modulation of P-glycoprotein-mediated efflux by methoxypolyethylene glycol-block-Polycaprolactone amphiphilic diblock copolymers, Pharm. Res. 21 (2004) 1489-1497.

20. K. Bogman, F. Erne-Brand, J. Alsenz, J. Drewe, The role of surfactants in the reversal of active transport mediated by multidrug resistance proteins, J. Pharm. Sci. 92 (2003) 1250-1261.

21. J.M. Dintaman, J.A. Silverman. Inhibition of P-glycoprotein by D-alpha-tocopheryl polyethylene glycol 1000 succinate (TPGS), Pharm. Res. 16 (1999) 1550-1556.

22. E.V. Batrakova, H.Y. Han, V.Y. Alakhov, D.W. Miller, A.V. Kabanov, Effects of pluronic block copolymers on drug absorption in Caco-2 cell monolayers, Pharm. Res. 15 (1998) 850-855.

23. O. Soga, C.F. van Nostrum, M. Fens, C.J. Rijcken, R.M. Schiffelers, G. Storm, W.E. Hennink. Thermosensitive and biodegradable polymeric micelles for paclitaxel delivery, J. Control. Release 103 (2005) 341-353.

24. C.J. Rijcken, J.W. Hofman, Z.F. van, W.E. Hennink, C.F. van Nostrum, Photosensitiser-loaded biodegradable polymeric micelles: preparation, characterisation and in vitro PDT efficacy, J. Control. Release 124 (2007) 144-153.

25. O. Soga, C.F. van Nostrum, A. Ramzi, T. Visser, F. Soulimani, P.M. Frederik, P.H. Bomans, W.E. Hennink, Physicochemical characterization of degradable thermosensitive polymeric micelles, Langmuir 20 (2004) 9388-9395.

26. Y. Suhara, M. Kamao, N. Tsugawa, T. Okano, Method for the determination of vitamin k homologues in human plasma using high-performance liquid chromatography-tandem mass spectrometry, Anal. Chem. 77 (2005) 757-763.

27. S. Otles, O. Cagindi, Determination of vitamin K1 content in olive oil, chard and human plasma by RP-HPLC method with UV–Vis detection, Food Chemistry 100 (2007) 1220-1222.

28. M.G. Carstens, C.F. van Nostrum, R. Verrijk, L.G. de Leede, D.J. Crommelin, W.E. Hennink,. A mechanistic study on the chemical and enzymatic degradation of PEG-Oligo(epsilon-caprolactone) micelles, J Pharm Sci 97 (2008) 506-18.

29. W. Schwizer, K. Asal, C. Kreiss, C. Mettraux, J. Borovicka, B. Remy, et al, Role of lipase in the regulation of upper gastrointestinal function in humans, Am J Physiol 273 (1997) G612-G620.

30. Z. Weizman, P.R. Durie, H.R. Kopelman, S.M. Vesely, G.G. Forstner, Bile acid secretion in cystic fibrosis: evidence for a defect unrelated to fat malabsorption, Gut 27 (1986) 1043-8.

31. J. Zastre, J. Jackson, M. Bajwa, R. Liggins, F. Iqbal, H. Burt, Enhanced cellular accumulation of a P-glycoprotein substrate, rhodamine-123, by Caco-2 cells using low molecular weight methoxypoly-ethylene glycol-block-polycaprolactone diblock copolymers, Eur. J. Pharm. Biopharm. 54 (2002) 299-309.

32. A.V. Kabanov, V.Y. Alakhov, Pluronic block copolymers in drug delivery: from micellar nanocontainers to biological response modifiers, Crit. Rev. Ther. Drug Carrier Syst. 19 (2002) 1-72.

33. R.D. Dabholkar, R.M. Sawant, D.A. Mongayt, P.V. Devarajan, V.P. Torchilin. Polyethylene glycol-phos-phatidylethanolamine conjugate (PEG-PE)-based mixed micelles: some properties, loading with paclitaxel, and modulation of P-glycoprotein-mediated efflux, Int. J. Pharm. 315 (2006) 148-157.

34. M.H. Dufresne, M.A. Gauthier, J.C. Leroux, Thiol-functionalized polymeric micelles: from molecular recognition to improved mucoadhesion, Bioconjug. Chem. 16 (2005) 1027-1033.

35. S. Chayed, F.M. Winnik, In vitro evaluation of the mucoadhesive properties of polysaccharide-based nanoparticulate oral drug delivery systems, Eur. J. Pharm. Biopharm. 65 (2007) 363-370.

36. N. Thirawong, J. Thongborisute, H. Takeuchi, P. Sriamornsak, Improved intestinal absorption of calcitonin by mucoadhesive delivery of novel pectin-liposome nanocomplexes, J. Control. Release 125 (2008) 236-245.

Chapter 07

Chapter 07

The influence of the gastric environment on the absorption of vitamin K from orally administered mixed micelles under physiological and bile deficient conditions

P.M. van Hasselt[a], T.C. Minderhoud[a], T.K. Slot[b], M. van der Ham[c], G.E.P.J. Janssens[b], M. de Sain[c], M. Talelli[b], L.M. Akkermans[d], R.H.J. Houwen[a], T.J. de Koning[a], C.F. van Nostrum[b]

[a]Department of Pediatrics, Wilhelmina Children's Hospital, University Medical Center Utrecht, Lundlaan 6, 3584 EA Utrecht, The Netherlands; [b]Department of Pharmaceutics, Utrecht Institute for Pharmaceutical Sciences, Utrecht University, P.O. Box 80082, 3508 TB, Utrecht, The Netherlands; [c]Laboratory for metabolic and endocrine diseases, University Medical Center Utrecht, Lundlaan 6, 3584 EA Utrecht, The Netherlands; [d]Department of Surgery, University Medical Center Utrecht, Lundlaan 6, 3584 EA Utrecht, The Netherlands.

Submitted

ABSTRACT

Background
Absorption of vitamin K depends on its incorporation in mixed micelles. We studied whether the poor absorption of Vitamin K from orally delivered mixed micelles under cholestatic conditions could be explained by exposure to gastric conditions.

Methods
Dynamic Light Scattering (DLS) was used to monitor particle size upon dilution, pH decrease, and its combination. In vivo absorption studies in bile duct ligated rats were used to determine the absorption of mixed micellar vitamin K upon gastric and duodenal administration and the effect of pharmaceutical elevation of the gastric pH.

Results
Dilution of the mixed micelles did not visually affect the dispersions and had minimal effect on particle size. In contrast, pH reduction induced turbidity and phase separation. Although this pH-effect appeared completely reversible visually, DLS analysis upon pH reversal revealed that reformation of micelles was incomplete. Moreover, a pH reduction combined with a 10-fold dilution completely abolished reformation of mixed micelles. In vivo absorption in bile duct ligated rats showed that the poor and highly variable vitamin K absorption compared with sham operated rats upon gastric administration could be rescued by duodenal administration and, to a lesser extent, by elevating gastric pH.

Conclusions
Absorption of orally delivered mixed micellar vitamin K under bile deficient conditions is impeded by micellar decomposition in the stomach. Prevention of micellar decomposition by raising gastric pH, for instance by proton pump inhibition, could lead to a more reliable absorption of vitamin K under cholestatic conditions.

The unstirred water layer – a system of laminar flow overlaying the epithelial lining of the gastrointestinal tract - represents a major hurdle for the absorption of extremely lipophilic compounds. Under physiological conditions, this hurdle is overcome by their incorporation in mixed micelles. Mixed micelles are nano-scaled disc-like structures (with a radius of 2-4 nm) composed of bile acids, phospholipids, and fatty acid degradation products (monoacylglycerides and free fatty acids).[1] The small size and the hydrophilic outer side of these mixed micelles enable trafficking of incorporated hydrophobic compounds over the unstirred water layer.[2] Decomposition of these micelles (aided by the lower pH in the mucous overlaying the endothelial cells) subsequently sets the entrapped compounds free and enables absorption.[3]

Mixed micelle formation depends on the presence of a sufficient concentration of bile. The minimal concentration is known as the critical micelle concentration (CMC). Obstructive jaundice, irrespective of the underlying cause, results in intraluminal bile acid concentrations below the CMC and virtually abolishes the absorption of fat soluble vitamins.[4-6] The clinical consequences of the resultant deficiencies can be dramatic, and include bone disease,[7] impaired immune response,[8-10] neurocognitive decline[10] and a potentially life threatening coagulation disorder.[11]

Theoretically, the pharmaceutical formulation of bioactive hydrophobic compounds in mixed micelles that mimic the physiological situation represents an attractive venue to enable absorption in the absence of endogenous bile. This option has been exploited for vitamin K, which is commercially available as Konakion MM®. In this formula, vitamin K is incorporated in a mixed micelle consisting of a phospholipid (phosphatidylcholine) and a bile acid (glycocholic acid). Although initially promising,[12] a trial in patients with a variety of cholestatic liver diseases showed that the uptake of vitamin K from this formulation was both low and unreliable.[13] Similarly, surveillance data failed to detect a decrease in the incidence of VKDB after switching to this formulation.[14] No satisfactory explanation for this surprisingly poor effect was given.

We hypothesized that the erratic absorption of vitamin K from orally administered mixed micelles under cholestatic conditions may be explained by premature micellar decomposition. We explored this hypothesis by combining *in vitro* analyses of micellar stability with *in vivo* absorption studies in bile duct ligated rats.

METHODS

General

Acetonitrile, dichloromethane, ethanol, and methanol were HPLC grade quality and purchased from Biosolve Ltd. (Valkenswaard, The Netherlands). Konakion MM (vitamin K concentration 10 mg/mL) is a product of Roche (Basel, Switzerland), vitamin K in arachid oil (10 mg/g) is a product of Bipharma (Weesp, the Netherlands). Vitamin K1-D4 was obtained from Buchem BV (Apeldoorn, The Netherlands). Buprenorphine HCl was purchased from Schering-Plough BV (Utrecht, The Netherlands). Isoflurane is a product of Abbott Animal Health (Abbott Laboratories, Chicago, IL, USA). Heparin was obtained from Leo Pharma BV (Breda, The Netherlands). Silicone cannulae were purchased from Degania Silicone, Inc. (Cumberland, RI, USA). Polymethylmethacrylate gastric cannulae for chronic implantation were kindly provided by Astra Zeneca (AstraZeneca R&D, Mölndal, Sweden). For mass spectrometric (MS) measurements, a Quattro Ultima triple quadrupole mass spectrometer (Waters, Manchester, UK) interfaced with an atmospheric pressure chemical ionisation (APCI) source and equipped with an Alliance 2795 HPLC (Waters, Etten-Leur, The Netherlands) was used. Masslynx software (Version 4.0, SP 4, Waters, Manchester, UK) was used for instrument control, data acquisition and data processing.

Animals

Male specific pathogen-free Wistar rats (HsdCpb:Wu), 275-375 grams, were used for this experiment. The animals were kept under constant housing conditions (temperature 22 °C, relative humidity 60 %, and a 12 hour light/dark cycle) and had free access to water and food and were allowed to adjust to these conditions for one week prior to surgery. After the procedure they were housed individually with free access to water and vitamin K deficient chow (diet 4165.00 Arie Blok bv, Woerden.). The protocol was approved by the Animal Experiment Committee of the University Medical Center, Utrecht, the Netherlands. The animals were allocated to 6 experimental groups. Five groups received vitamin K intragastrically: Konakion MM and vitamin K in oil in sham operated and bile duct ligated (BDL) rats (2x2 groups); Konakion MM after proton pump inhibition in BDL rats. One group of BDL rats received Konakion MM intraduodenally.

Surgical procedures

All surgical procedures were performed on a heated operating table under general anesthesia using a combination of 2 % Isoflurane gas through a snout mask and intramuscular 0.15 mL 5% buprenorphin. All surgical procedures were performed with sterile instruments under aseptic condi-

tions. The right jugular vein was cannulated, and the 12 cm cannula was tunneled subcutaneously to the back, puncturing the skin between the shoulder blades. Through a small, v-shaped incision the silicone cannula was inserted 4.2 cm in the direction of the heart. After checking for intact flow in the cannula, the system was flushed using heparin in physiological saline (100 IE/mL). Through a midline incision (2 cm) the abdomen was opened and the liver gently retracted, exposing the common bile duct. Using a 6-0 silk ligature the common bile duct was ligated. Subsequently the muscular part of the stomach was exposed. For intragastric administration, a polymethylmethacrylate cannula was placed in the stomach, and fixed in place using a purse-string suture. The wings of the cannula were sutured to the abdominal wall. A small incision, parallel to the midline incision was used to exteriorize the fistula. For duodenal administration a 15 cm silicon cannula was used, which was inserted in the stomach and positioned 1 – 1,5 cm distal from the pylorus. The proximal end of the cannula was tunneled subcutaneously to the back, and exteriorized using the same puncture hole as used for the jugular vein cannula. The abdominal incision was closed in layers using a running suture and 4-0 (Vicryl, Ethicon) for the abdominal wall and the skin.
The jugular cannula and the duodenal cannula (if placed) were kept in place using a rodent infusion jacket (Uno Zevenaar BV, Zevenaar, The Netherlands). The jugular cannula was filled with a locking solution containing 50% Dextrose and heparin 500 U/mL. The protocol was approved by the Animal Experiment Committee of the University Medical Center, Utrecht, the Netherlands.

Proton pump inhibition and pH measurement
The animals allocated to the proton pump inhibition (PPI) group received 20 mg omeprazole (AstraZeneca BV, Zoetermeer, Netherlands) dissolved in 1 mL sodium hydrogen carbonate intragastrically on day 1 (8 AM and 6 PM) and on day 2 (8 AM).[15] Konakion MM was administered 2 hours after the last omeprazole dose, to ensure full effect of the PPI. Intragastric pH was measured using a pH Electrode Ingold Glass (MMS Orion pH study, Enschede, Netherlands).

Collection of blood samples
Blood was drawn by connecting a 1 mL syringe to the blunt needle inserted in the rodent infusion jacket. Each sample (0.45 mL) was replaced with an equal amount of warm saline (0,9 % NaCl, 25 IU heparin/mL). The first blood sample was taken two days after surgery, just prior to administration of vitamin K. Subsequently, blood samples were taken every hour for 5 hours. The last sample was taken under general anesthesia and was followed by euthanasia by blood loss. Blood samples were collected in Lithium heparin microtainer containers. After centrifugation, plasma was extracted and stored at -20 ^{o}C.

Vitamin K analysis

Rat plasma samples were prepared as follows. 100 μL internal standard (vitamin K-D4) was added to 200 μl rat plasma followed by addition of 500 μL ethanol. After vortexing for 30 seconds, vitamin K was extracted twice with 1.5 mL hexane by vortexing and centrifugation at 2000 rpm during 1 minute. The hexane fractions were collected and evaporated followed by dissolving the residue in 120 μl ethanol. After filtration with Sirocco protein precipitation plate (Waters, Massachusetts, USA), aliquots of 50 μL were injected to the LC-MS/MS system. Samples were analyzed using LC-MS as described by Suhara et al.[16] with few modifications.[17]

Dynamic light scattering (DLS)

Konakion MM micelle diameter and polydispersity index (PDI) were measured by Dynamic Light Scattering (DLS), using an ALV/CGS-3 Compact Goniometer System (ALV GmbH, Langen, Germany) in combination with a Photon Counting Module (PerkinElmer Optoelectronics, Vaudreuil, Canada). The scattering angle was 90° and temperature was set to 25 °C, unless stated otherwise. Analysis of particle size distribution was performed using ALV-Correlator Software Version 3.0.2.1 (ALV-GmbH, Langen, Germany) and Malvern DTS v.4.00 software (Malvern Instruments, Worcestershire, UK). Normalized size distributions were obtained by calculating the first derivative of the volume percentage integral at each particle size. In addition, the volume percentage integral above a given radius (4.2 nm) was computed from the raw DLS data. A radius of 4.2 nm represents the upper limit of the size of physiological mixed micelles.[1]

Titration experiments

To determine the effect of dilution on particle size distribution, Konakion MM was serially diluted by a factor 1.5 with water followed by DLS measurements. The effect of decreasing pH was determined by titrating Konakion MM or 10 fold diluted Konakion MM with 0.5-5 μL HCl (1M), followed by 5s mixing, pH determination (Metrohm/620, accuracy ± 0.1) and DLS measurement (see above) until pH 3. The same procedure was used for rising pH but using NaOH (1M). Titration experiments were performed in triplo.

Statistical analysis

All statistical analyses were performed using SPSS for Windows 14.0 (SPSS Inc. Chicago). T-tests were performed for comparison between groups of the vitamin K concentration at each time point, as well as for the peak level (defined as the highest concentration in an individual animal compared with baseline) and the area under the curve (AUC). Graphpad Prism 5 was used to compute the AUC and to fit one phase exponential decay. For each fit a r^2-value was calculated.

RESULTS

Peak plasma levels were similar in sham operated rats when vitamin K was administered intragastrically as mixed micelle or in oil (4010±991 and 4272 ± 2333 ng/mL respectively, p=0.82). As shown in figure 1A, in sham operated rat, mixed micellar vitamin K resulted in a faster absorption compared with vitamin K in oil as reflected by a higher plasma level at t=1 (p<0.001) and a significantly lower level at t=5 (p=0.01). Bile duct ligation dramatically impeded the absorption of vitamin K in oil, with plasma levels remaining around baseline. The average absorption in BDL rats appeared higher for mixed micellar vitamin K (figure 1A), but this difference was not statistically significant due to a high interindividual variability. Interestingly, a marked rise (above 10 ng/mL) was observed in 3/10 rats, while plasma levels remained at baseline in 7 other rats (figure 1B). Peak

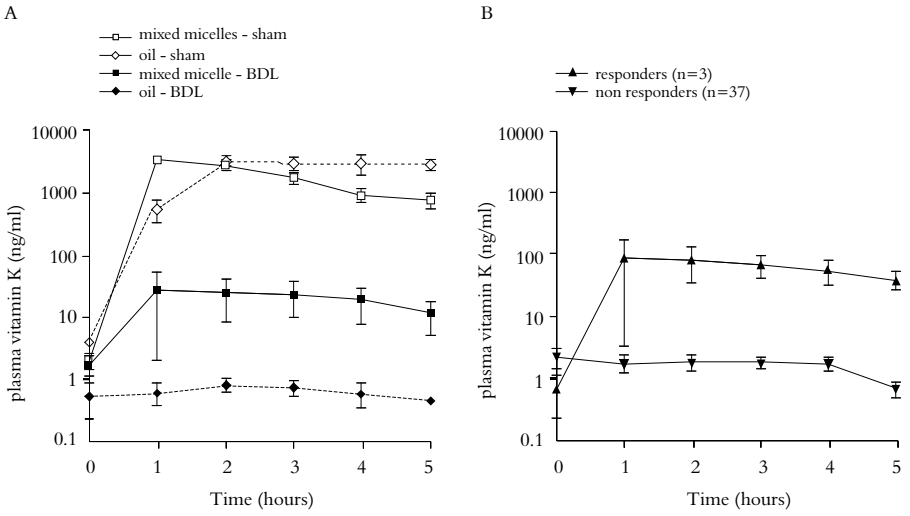


Figure 1

Vitamin K plasma concentrations after a single gastric dose of 1 mg vitamin K. Error bars depict SEM. A: Absorption from vitamin K administered as a mixed micelle or oil-solubilisate in sham operated and bile duct ligated rats. B: Vitamin K absorption in bile duct ligated rats administered as mixed micelle in responders and non-responders.

levels (1.9 ± 1.1 vs 120 ± 120 ng/mL, p=0.02) and AUC (7.9 ± 5.4 vs 303 ± 281 ng/mL, p<0.02) differed significantly between rats who seemed to be able to absorb MM (responders) and those who did not (non responders).

These findings seemed to mimic the erratic absorption of mixed micellar vitamin K previously observed in cholestatic subjects/infants.(13) We hypothesized that orally administered mixed micellar vitamin K is (partly) destabilised by gastrointestinal factors. We further hypothesized that reliable absorption might be attained under cholestatic conditions if these factors could be favourably modified. We therefore set out to delineate these factors.

In *vitro* stability of mixed micellar vitamin K: effect of dilution and pH

The physicochemical properties of the mixed micelles suggested two major causes for micellar destabilisation. First, dilution could lead to a bile acid concentration below the critical micelle concentration (CMC). Second, the low pH in the stomach will lead to protonation of the individual bile acids. To assess the influence of these factors on the micellar stability we monitored their effect on particle size distribution.

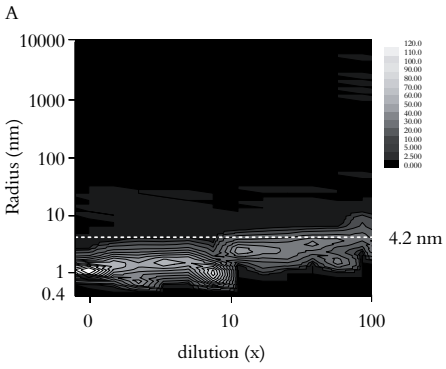


Figure 2

Effect of dilution on particle size distribution of Konakion MM. Colours depict the first derivative of the volume percentage integral at a given particle size (arbitrary units, highest values are depicted in red).

As shown in figure 2, particle size increased only slightly when mixed micellar vitamin K was diluted up to a 100-fold. DLS analysis did not permit reliable quantification at higher degrees of dilution, because of low scattering intensity, but suggested a rise in particle size at dilutions in excess of 1000 (results not shown). Upon decreasing the pH of the undiluted mixed micelles (at a vitamin K concentration of 10 mg/mL) a modest rise in particle size was observed initially (Figure 3A). At a pH of 4.3, close to the pK_a of glycocholic acid ($pK_a \approx 3.95$), the percentage of particles in the micellar size range (<4.2 nm radius) decreased rapidly (figure 3A and 3C). Visual turbidity first appeared around pH 5, increased at lower pH and obscured DLS measurements below pH 3.5-4. Turbidity was completely reversed by increasing the pH back to neutral, but the average particle size remained slightly higher and volume analysis indicated that at pH 6 approximately 25% still had a radius above 4.2 nm, the upper limit of the mixed micellar range (figure 3B and 3C).

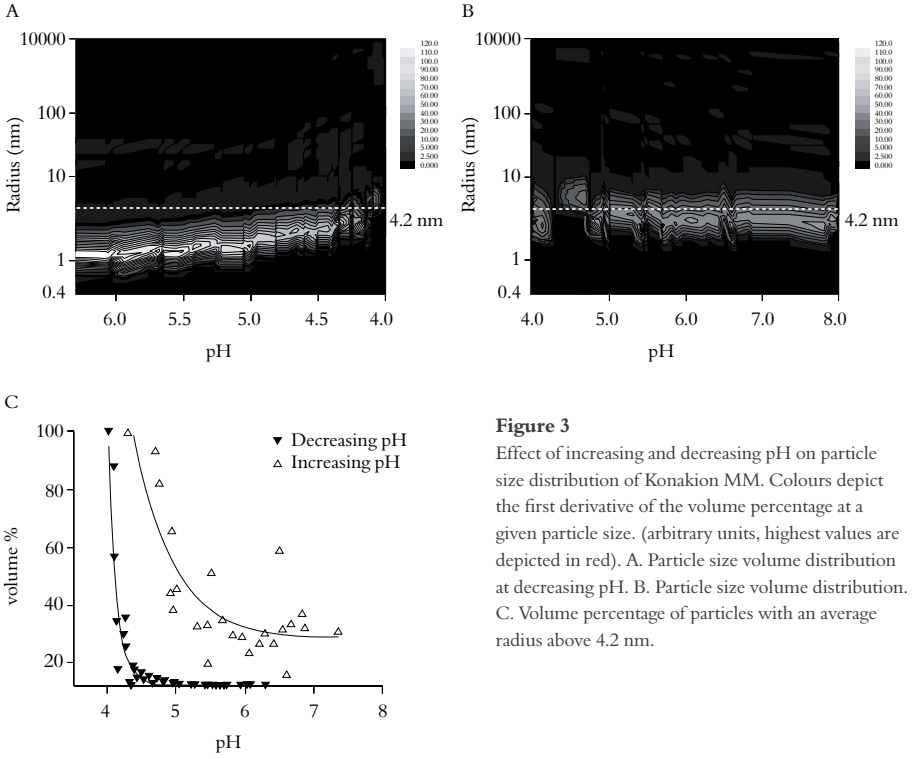


Figure 3
Effect of increasing and decreasing pH on particle size distribution of Konakion MM. Colours depict the first derivative of the volume percentage at a given particle size. (arbitrary units, highest values are depicted in red). A. Particle size volume distribution at decreasing pH. B. Particle size volume distribution. C. Volume percentage of particles with an average radius above 4.2 nm.

Next, we assessed micellar stability under the simultaneous influence of dilution and protonation, a situation which more closely resembles the gastric circumstances. The results are presented in Figure 4. Particle size increased dramatically with decreasing pH in a 10-fold diluted mixed micellar vitamin K solution as compared to the undiluted mixtures. At pH 5, nearly all particles had a size above the micellar range (figure 4A) and micellar size continued to increase thereafter. While turbidity was reversed quickly upon pH increase, the decrease in particle size was much less pronounced than observed in the undiluted mixture (figure 4B). Even at pH 7, average particle radius remained approximately 100 nm, which is far above the size of the initial mixed micelles, and no particles in the micellar range could be detected.

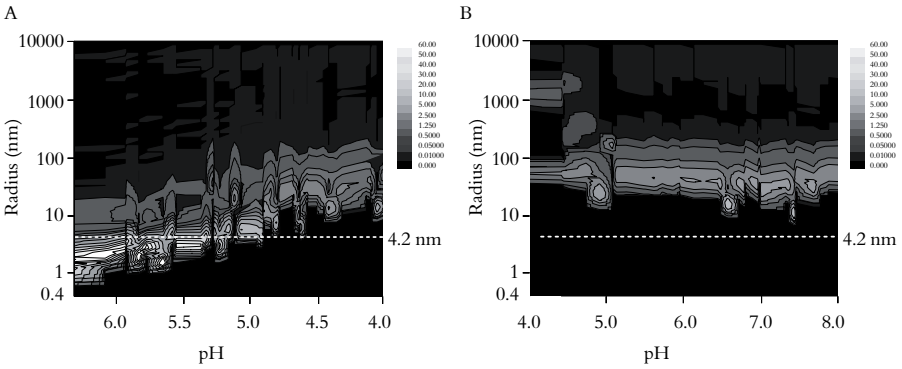


Figure 4

Effect of increasing and decreasing pH on particle size distribution of mixed micellar vitamin K upon 10 time dilution. Colours depict the first derivative of the volume percentage at a given particle size (arbitrary units, highest values are depicted in red). A. Particle size volume distribution at decreasing pH. B. Particle size volume distribution at increasing pH.

Modulation of *in vivo* absorption of mixed micellar vitamin K by pH

The in vitro data provided strong support for destabilisation of micelles at low pH, especially when combined with dilution, and suggested that if a low pH could be prevented this would positively affect vitamin K absorption. Indeed, duodenal administration of mixed micellar vitamin K completely restored vitamin K absorption in bile duct ligated rats, and resulted in significantly higher peak levels (9381 vs 37.3 ng/mL, p=0.007) and AUC levels (15804 vs 96.6, p=0.001) compared with gastric administration. Elevation of the gastric pH using a proton pump inhibitor (gastric pH range 3.6-5.2) had a similar, although less pronounced, effect, with a mean peak level of 1608 ng/mL (range 149-6206 ng/mL). Interestingly, the species with the highest measured gastric pH (5.2) at the time of administration had a peak plasma level (6206 ng/mL) that was similar to those found after duodenal administration (figure 5). When this rat was excluded, vitamin K levels after proton pump inhibition at t = 3, 4 and 5 h were significantly ($p<0.01$) lower compared with those found after duodenal administration (figure 5).

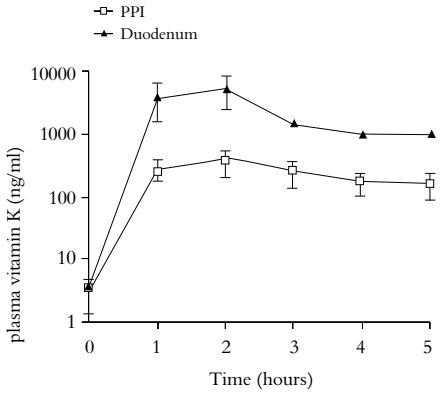


Figure 5
Vitamin K plasma concentrations in bile duct ligated rats after a single dose of 1 mg vitamin K. Error bars depict SEM. Duodenal administration (n=5) prevented exposure to gastric acidity and dilution. PPI (n=4) increased gastric pH thereby limiting exposure to pH level below the pK_a of glycocholic acid.

DISCUSSION

In the present study we provide evidence that the erratic (and generally poor) absorption of mixed micellar vitamin K under cholestatic conditions is caused by gastric decomposition.[13] Using dynamic light scattering analysis we showed that mixed micelles are destabilised by a low pH in vitro. Although the effect of pH reduction alone was mild and reversible, pH reduction combined with dilution had a dramatic effect on particle size and – more importantly – completely prevented the reformation of mixed micelles at the pH found in the proximal intestine (pH 6), the main site of vitamin K absorption.[18] Absorption studies in bile duct ligated rats confirmed that bypassing the stomach – by administering vitamin K directly into the duodenum – fully restored absorption of mixed micellar vitamin K. Of clinical relevance, elevating the gastric pH through proton pump inhibition increased vitamin K absorption at least a hundredfold compared with vitamin K in oil.

The mechanism which impedes absorption could not have been suspected from the reversible turbidity observed in vitro upon exposure to low pH conditions. Turbidity of mixed micellar vitamin K at low pH is explained by protonation of bile acids, which deprives bile acids of their amphiphilic character, inhibits emulsification and results in phase separation of vitamin K from the aqueous phase. The observed reversible turbidity suggested that deprotonation of bile acids resulted in micellar reformation. Use of a pharmaceutical technique (DLS) enabled us to visualise the events at a nano-scale and disproved this assumption.

Interestingly, our results indicate that the particle size distribution at a given pH differs in case of rising or decreasing pH, a phenomenon known as hysteresis. Especially after 10 times dilution, once phase separated at low pH, primary micelles are not reconstituted upon increasing pH. We propose that this hysteresis is a kinetic effect that can be explained as follows. At equilibrium, the concentration of freely dissolved amphiphiles in a micellar dispersion is always at the CMC level. Dilution causes a deviation from the equilibrium situation eventually resulting in dissociation of micelles such that the concentration of free amphiphiles is maintained at the CMC level. However, this dissociation is generally a slow process,[19] and this process may be even further slowed down by the interaction between the bile acids and the payload (vitamin K) of the mixed micelles.[20,21] Thus, initially, only the concentration of freely dissolved bile salts is decreased upon dilution, while the number of micellar associated bile molecules is hardly changed. This is actually the reason why the particle size did not change upon 10 or 100 times dilution (see Figure 2).

In contrast, after phase separation at low pH and reformation of the mixed micelles upon pH reversal, the concentration of freely dissolved bile salts will be at the expected CMC level and, consequently, less bile salts are indeed available for mixed micelle formation in the diluted situation. This explains the larger particle size after pH increase.

Our data underline that formulations that are highly effective in healthy controls may be grossly ineffective under certain pathological conditions. The relative rarity of each of these conditions sustains a 'knowledge deficit' and hinders the development of therapeutics that are specifically adapted to each of these pathological circumstances. The present data exemplify that model systems may be used to disentangle disease specific mechanisms to allow the development of tailored therapeutics.

Although malabsorption of vitamin K under bile deficient conditions was recognized within years of its discovery in 1934,[22] a pharmaceutical answer which solves this biological riddle has not yet been given. Pharmaceutical efforts, including solubilisation with cremophor[12] and incorporation in a polymeric micelle[17] have been unsuccessful. Coupling to a hydrophilic polymer was successful for vitamin E,[23] but is not feasible for vitamin K due to the lack of reactive groups necessary for such coupling through esterification. As a consequence, malabsorption of vitamin K is currently being tackled in patients with cholestatic liver disease either by administering massive oral doses or by parenteral administration. The present data suggest an alternative approach. Our data indicate that an intragastric pH above 4 (preferably 5) at the time of administration of mixed micellar vitamin K suffices to attain peak plasma levels of at least 10 percent of normal in patients with cholestatic liver disease. This would be a major improvement in view of the sheer undetectable absorption in the absence of bile using other pharmaceutical formulations.[17]

The ultimate goal would be a formulation which allows absorption of vitamin K (and other extremely lipophilic compounds) in all patients, including those with an underlying resorptive disorder. Such a formulation would be especially useful for vitamin K prophylaxis, which aims to prevent rare but life threatening bleedings in infancy. These bleedings occur primarily in infants with unidentified cholestasis.[24,25] In order to be efficacious, healthy infants – the vast majority- are exposed to supraphysiological vitamin K levels. A formulation which is absorbed well even in the presence of cholestasis would allow an efficacious prophylaxis at a much lower dose, decreasing the burden on healthy infants.

In conclusion, we here give evidence that the generally poor absorption of mixed micellar vitamin K under cholestatic conditions is caused by micellar dissociation in the stomach and can be alleviated using proton pump inhibition.

Acknowledgments
The authors wish to acknowledge André Verheem, Hans Kanbier and Raymond Mouw (UMCU) for technical assistance.

REFERENCES

1. Wang DQ. Regulation of intestinal cholesterol absorption. Annu Rev Physiol 2007;69:221-48.

2. Thomson AB, Schoeller C, Keelan M, Smith L, Clandinin MT. Lipid absorption: passing through the unstirred layers, brush-border membrane, and beyond. Can J Physiol Pharmacol 1993;71:531-55.

3. Shiau YF. Mechanism of intestinal fatty acid uptake in the rat: the role of an acidic microclimate. J Physiol 1990;421:463-74.

4. Sokol RJ, Farrell MK, Heubi JE, Tsang RC, Balistreri WF. Comparison of vitamin E and 25-hydroxyvitamin D absorption during childhood cholestasis. J Pediatr 1983;103:712-7.

5. Sokol RJ, Heubi JE, Iannaccone S, Bove KE, Balistreri WF. Mechanism causing vitamin E deficiency during chronic childhood cholestasis. Gastroenterology 1983;85:1172-82.

6. Argao EA, Heubi JE. Fat-soluble vitamin deficiency in infants and children. Curr Opin Pediatr 1993;5:562-6.

7. de Albuquerque Taveira AT, Fernandes MI, Galvao LC, Sawamura R, de M, V, de Paula FJ. Impairment of bone mass development in children with chronic cholestatic liver disease. Clin Endocrinol (Oxf) 2007;66:518-23.

8. Chen H, Zhuo Q, Yuan W, Wang J, Wu T. Vitamin A for preventing acute lower respiratory tract infections in children up to seven years of age. Cochrane Database Syst Rev 2008:CD006090.

9. Sokol RJ, Heubi JE, Butler-Simon N, McClung HJ, Lilly JR, Silverman A. Treatment of vitamin E deficiency during chronic childhood cholestasis with oral d-alpha-tocopheryl polyethylene glycol-1000 succinate. Gastroenterology 1987;93:975-85.

10. Sokol RJ, Heubi JE, Iannaccone ST, Bove KE, Balistreri WF. Vitamin E deficiency with normal serum vitamin E concentrations in children with chronic cholestasis. N Engl J Med 1984;310:1209-12.

11. Shearer MJ. Vitamin K deficiency bleeding (VKDB) in early infancy. Blood Rev 2009;23:49-59.

12. Amedee-Manesme O, Lambert WE, Alagille D, De Leenheer AP. Pharmacokinetics and safety of a new solution of vitamin K1(20) in children with cholestasis. J Pediatr Gastroenterol Nutr 1992 February;14:160-5.

13. Pereira SP, Shearer MJ, Williams R, Mieli-Vergani G. Intestinal absorption of mixed micellar phylloquinone (vitamin K1) is unreliable in infants with conjugated hyperbilirubinaemia: implications for oral prophylaxis of vitamin K deficiency bleeding. Arch Dis Child Fetal Neonatal Ed 2003;88:F113-F118.

14. von Kries R, Hachmeister A, Gobel U. Oral mixed micellar vitamin K for prevention of late vitamin K deficiency bleeding. Arch Dis Child Fetal Neonatal Ed 2003;88:F109-F112.

15. Bastaki SM, Chandranath IS, Singh J. The anti-secretory and anti-ulcer activities of esomeprazole in comparison with omeprazole in the stomach of rats and rabbits. Mol Cell Biochem 2008;309:167-75.

16. Suhara Y, Kamao M, Tsugawa N, Okano T. Method for the determination of vitamin k homologues in human plasma using high-performance liquid chromatography-tandem mass spectrometry. Anal Chem 2005;77:757-63.

17. van Hasselt PM, Janssens GE, Slot TK, van der HM, Minderhoud TC, Talelli M et al. The influence of bile acids on the oral bioavailability of vitamin K encapsulated in polymeric micelles. J Control Release 2009;133:161-8.

18. Hollander D, Rim E. Effect of luminal constituents on vitamin K1 absorption into thoracic duct lymph. Am J Physiol 1978;234:E54-E59.

19. Elhasi S, Astaneh R, Lavasanifar A. Solubilization of an amphiphilic drug by poly(ethylene oxide)-block-poly(ester) micelles. Eur J Pharm Biopharm. 2007;65:406-13.

20. Soga O, van Nostrum CF, Fens M, Rijcken CJ, Schiffelers RM, Storm G et al. Thermosensitive and biodegradable polymeric micelles for paclitaxel delivery. J Control Release 2005;103:341-53.

21. Petrov P, Yuan J, Yoncheva K, Müller AH, Tsvetanov CB.Wormlike morphology formation and stabilization of "pluronic p123" micelles by solubilization of pentaerythritol tetraacrylate. J Phys Chem B. 2008;112:8879-83.

22. Dam H. The antihaemorrhagic vitamin of the chick. Biochem J 1935;29:1273-85.

23 Sokol RJ, Butler-Simon N, Conner C, Heubi JE, Sinatra FR, Suchy FJ et al. Multicenter trial of d-alpha-tocopheryl polyethylene glycol 1000 succinate for treatment of vitamin E deficiency in children with chronic cholestasis. Gastroenterology 1993;104:1727-35.

24 van Hasselt PM, de Koning TJ, Kvist N, de VE, Lundin CR, Berger R et al. Prevention of vitamin K deficiency bleeding in breastfed infants: lessons from the Dutch and Danish biliary atresia registries. Pediatrics 2008;121:e857-e863.

25 Sutor AH, Dagres N, Niederhoff H. Late form of vitamin K deficiency bleeding in Germany. Klin Padiatr 1995;207:89-97.

Chapter 08

Chapter 08

Summary and General Discussion

SUMMARY

The main aims of this thesis were: a) to investigate the efficacy of the Dutch vitamin K prophylaxis regimen in protecting infants against Vitamin K Deficiency Bleeding (VKDB), with an emphasis on infants with underlying resorptive disorders; b) to investigate the role of different feeding types on the risk of VKDB; c) to investigate the efficacy of interventions to improve the absorption of vitamin K in the absence of bile.

In **chapter 2** we compared the risk of VKDB under different prophylactic regimens in infants with biliary atresia, the largest cause of cholestatic jaundice in infancy. From the Dutch and Danish national biliary atresia registries we retrieved infants who were either breastfed and received 1 mg of oral vitamin K at birth followed by 25 mcg daily (the Dutch oral vitamin K prophylaxis), 2 mg of oral vitamin K at birth followed by 1 mg of weekly oral prophylaxis (Denmark, 1994 to May 2000) or 2 mg of intramuscular prophylaxis at birth (Denmark, June 2000-2005) or were fed by formula. We determined the absolute and relative risk of severe vitamin K deficiency and VKDB at diagnosis in breastfed infants on each prophylactic regimen and in formula-fed infants. VKDB was found in 25 of 30 of breastfed infants on 25 mcg of daily oral prophylaxis, in 1 of 13 on 1 mg of weekly oral prophylaxis, in 1 of 10 receiving 2 mg of intramuscular prophylaxis at birth, and in 1 of 98 formula-fed infants. The relative risk of a bleeding in breastfed compared with formula-fed infants was 77.5 for 25 mcg of daily oral prophylaxis, 7.2 for 1 mg of weekly oral prophylaxis, and 9.3 for 2 mg of intramuscular prophylaxis at birth. Based on these data it was concluded that the Dutch regimen fails to prevent bleedings in apparently healthy infants with unrecognized cholestasis due to biliary atresia. In addition it was shown that oral prophylaxis can provide similar protection against VKDB as intramuscular prophylaxis at birth when given at a 6x times higher cumulative dose.

In biliary atresia, by definition, efflux of bile is absent. Theoretically, residual bile excretion, as found in several other causes of cholestasis in infancy, may allow some vitamin K absorption and thus reduce the risk of VKDB. Therefore, in **chapter 3**, we assessed the risk of VKDB in infants with cholestatic jaundice due to alpha-1-antitrypsin deficiency (A1ATD), the second largest cause of cholestasis in infancy, in which the degree of cholestasis is known to vary. For this purpose we identified infants with a ZZ or SZ phenotype born in the Netherlands between January 1991 and December 2006 from the databases of the 5 Dutch diagnostic centres for alpha-1-antitrypsin phenotyping and/or genotyping. We determined the risk of VKDB upon diagnosis of A1ATD in breastfed and formula-fed infants and searched for correlations between serum levels of (conju-

gated) bilirubin and the risk of bleeding. VKDB was noted in 15/20 (75%) of breast-fed infants, compared with 0/20 of formula-fed infants with A1ATD.
Conjugated bilirubin levels at diagnosis did not correlate with the risk of vitamin K deficiency nor with VKDB. These data showed that the risk of VKDB in breastfed infants with A1ATD was similar to the risk in BA, despite lower and variable degrees of conjugated bilirubinemia. It was concluded that - without adequate prophylaxis - the risk of VKDB is uniformly high in exclusively breast-fed infants with cholestatic jaundice, irrespective of underlying etiology.

Intriguingly, despite the uniformly high risk of VKDB in exclusively breastfed infants with unidentified cholestasis, the first surveillance (1992-94) indicated that the risk of VKDB in the Netherlands under this regimen was low, comparable even to the incidence found in countries on IM prophylaxis. In contrast, the second surveillance study reported a risk of 3/100.000, close to the incidence found in countries in which only a single oral dose of vitamin K at birth is given. As VKDB is almost exclusively seen in cholestatic infants who have exclusive breastfeeding, we hypothesized in **chapter 4** that changes in the breastfeeding rate in these infants could explain the observed overall rise in the incidence of VKDB in the Netherlands under an unchanged prophylactic regimen. To investigate this, we assessed changes in breastfeeding rate and the risk of VKDB in Dutch infants presenting with biliary atresia (BA) and alpha-1-antitrypsin deficiency (A1ATD) between January 1991 and December 2006 that were identified through their respective national registries. In addition, we performed a survey amongst caretakers of healthy infants to establish the degree of adherence to the prophylactic regimen. While the overall incidence of breastfeeding in Dutch newborns changed only marginally, the breastfeeding rate in infants presenting with BA/A1ATD increased from 6/45 (13%) infants in 1991-1994 to 21/44 (47%) in 2003-2006. This increase was paralleled by a quadrupling of the incidence of VKDB in this cohort from 11 to 41%. Reported adherence was 85%, indicating that the uniformly high risk of VKDB in exclusively breastfed infants (~80%) in this time frame could not be explained by a lack of adherence to prophylaxis. These data strongly suggest that the lack of protection offered by the Dutch vitamin K prophylactic regimen was unveiled by a dramatic rise in the breastfeeding rate in infants with unidentified cholestasis.

In **chapter 5** we used the same combined cohort of infants with BA and A1ATD to assess whether the risk of vitamin K deficiency in bottle fed cholestatic infants is associated with certain formula types, particulary hypoallergenic formulas. In contrast with breastfed infants, who receive multiple doses of vitamin K to prevent VKDB after the first week of life, formula fed infants rely on the protection offered by formula. Formula fed infants with cholestasis due to BA or A1ATD and in whom the formula type was documented, were used to calculate the relative risk to develop vita-

min K deficiency under each formula type. The influence of prior or ongoing use of breastfeeding was also assessed. Of 118 formula fed infants 8 (7%) presented with vitamin K deficiency; 6 (75%) of these infants received hypoallergenic formula (whey based hydrolysate in 4, casein based hydrolysate in 1, soy based in 1). Risk factor analysis in 59 infants with known formula type revealed that infants on hypoallergenic formula had a 20 times higher risk of vitamin K deficiency as compared with infants receiving regular formula. The higher risk of vitamin K deficiency could not be explained by a lower vitamin K content of these formulas. Although partial breastfeeding was not significantly associated with a higher risk of vitamin K deficiency, the only case of VKDB was found in the combined presence of hypoallergenic formula and partial breastfeeding. These data suggest that cholestatic infants receiving hydrolysed formula, and especially whey based hydrolysate, are at risk of presenting with biochemical vitamin K deficiency. This may lead to VKDB, especially in the presence of additional risk factors for VKDB, such as partial breastfeeding.

VKDB has also been described in infants with resorptive disorders other than cholestasis, particu-

larly pancreatic insufficiency. In **chapter 6** we therefore assessed the risk of vitamin K deficiency and VKDB in infants presenting with Cystic Fibrosis (CF) in a retrospective cohort study. Simultaneously the impact of several factors known to influence vitamin K status was assessed in this cohort. Of 74 patients presenting with CF in the first 6 months of life only one developed a VKDB. An additional two infants presented with vitamin K deficiency but did not develop a bleeding. In each of these 3 infants multiple known risk factors for vitamin K deficiency were present. Of these, only conjugated hyperbilirubinemia was associated with an increased relative risk of vitamin K deficiency (RR 27.6 (95% CI 3.0-255), $p=0.01$). This risk was even higher when patients were pancreatic insufficient or were fed by hypoallergenic formula. Thus, under the present Dutch regimen the risk of VKDB in infants with CF is low compared with other populations at risk. Impediments of bile flow, not pancreatic insufficiency, may be primarily responsible for the risk of vitamin K deficiency.

The high risk of VKDB in cholestatic infants is thought to be due to the poor absorption of vitamin K in the absence of bile. Due to its hydrophobicity, trafficking of Vitamin K to the enterocytes critically depends on micellar incorporation. In **chapter 7** we assessed the ability of polymeric micelles to enable gastrointestinal absorption of vitamin K, by comparison of its absorption in bile duct ligated and sham operated rats. To that end, vitamin K was encapsulated in micelles composed of mPEG5000-b-p(HPMAm-lac2), a thermosensitive block copolymer. Vitamin K plasma levels rose significantly upon gastric administration of 1 mg vitamin K encapsulated in polymeric micelles in sham operated rats, but not after bile duct ligation (AUC 4543 and 1.64 ng/mL/h respectively, $p<0.01$). Duodenal administration of polymeric micelles together with bile acids in bile duct ligat-

ed rats fully restored absorption. Dynamic light scattering (DLS) time series showed a significant and dose dependent rise in micellar size in the presence of bile acids in vitro, indicating the gradual formation of mixed micelles during the first 3 h of incubation. These data suggest that the gastrointestinal absorption of encapsulated vitamin K from polymeric micelles is mediated by free bile and that uptake of intact polymeric micelles through pinocytosis is insignificant. Since encapsulation in polymeric micelles could not mimic the role of natural bile we focused on the use of mixed micelles, a pharmaceutical formulation in which vitamin K is entrapped in a micelle consisting of a bile acid and phosphatidylcholine. In **chapter 8** we investigated whether the poor absorption of vitamin K from orally delivered mixed micelles under cholestatic conditions could be explained by gastric conditions. DLS was used to monitor particle size upon dilution, pH decrease, and its combination. In vivo absorption studies in bile duct ligated (BDL) rats were used to determine the absorption of mixed micellar vitamin K upon gastric and duodenal administration and the effect of pharmaceutical elevation of the gastric pH. Dilution of the mixed micelles did not visually affect dispersion and had minimal effect on particle size. In contrast, pH reduction induced turbidity and phase separation, pointing at desintegration of mixed micelles. Although this pH-effect appeared completely reversible visually, DLS analysis upon pH reversal revealed that reformation of micelles was incomplete. Moreover, a pH reduction combined with a 10-fold dilution completely abolished reformation of mixed micelles. In vivo absorption in BDL rats showed that the poor and highly variable vitamin K absorption compared with sham operated rats upon gastric administration could be rescued by duodenal administration and, to a lesser extent, by elevating gastric pH. Based on these data, we concluded that the absorption of orally delivered mixed micellar vitamin K under bile deficient conditions is impeded by micellar decomposition in the stomach. Prevention of micellar decomposition by raising gastric pH, for instance by proton pump inhibition, could lead to a more reliable absorption of vitamin K under cholestatic conditions.

GENERAL DISCUSSION

In view of the ease with which prophylactic strategies are implemented, the number of studies performed to determine their efficacy is remarkably low. This may in part be related to the fact that determining the efficacy of a regimen is actually difficult. The results in this thesis underline an important theme that complicates determining the efficacy of a regimen: *prophylaxis is given to all, because of the few who really need it.* These few differ from the vast majority, but cannot be recognized beforehand. As a consequence, prospective studies need to be extremely large to have sufficient power to determine the efficacy of a regimen in at risk infants. Smaller studies using surrogate parameters can be (and have been) performed, but it is questionable whether biochemical indices of subclinical vitamin K deficiency in healthy infants can be taken as a marker for vitamin K deficiency bleeding (VKDB) in those at risk.

Most data on the efficacy of vitamin K prophylaxis have been obtained from surveillance studies. **Chapter 4** confirms that such studies are capable of providing a reasonable estimate of the incidence of VKDB (albeit with some degree of underreporting), but also illustrate that determining the efficacy of a regimen based solely on incidence figures can be hazardous. To circumvent the shortcomings of nationwide surveillance, we used a different strategy in this thesis, termed targeted surveillance by others.[1] Targeted surveillance enabled us to compare the efficacy of regimens in protecting important risk populations. This strategy was based on the insight that while infants at risk cannot be detected beforehand, they can be identified retrospectively. We therefore performed retrospective cohort studies that focused on infants at risk, mainly cholestatic liver disease.

In a commentary on **chapter 1** entitled "Making life safe for canaries" this approach was compared with the use of canaries in British mines.[2] As stated by Hey "miners once used canaries because, if the bird stopped singing, that was a sure sign that miner's damp (either carbon monoxide or methane) was starting to accumulate". The canaries thus allowed miners to leave the mine in time. Similarly, infants with biliary atresia alarmed us that the Dutch prophylaxis fails. But does it fail for all patients? Likely, not. It seems that prophylaxis is only insufficient for a selected group: "the canaries". This idea is supported by several lines of evidence. First, recent Dutch surveillance data reported that 5/6 infants with documented VKDB had an underlying cholestatic liver disease.[3] Secondly, the combined incidence of VKDB in patients with biliary atresia and alpha-1-antitrypsin deficiency in the Netherlands in 2005 (**chapter 4**) was close to the overall incidence of VKDB.

Thirdly, a study performed in Japan, measuring prothrombin percentage in healthy babies at 1 month of age, elevated conjugated bilirubin levels were found in all infants with grossly abnormal values.[4] We therefore suspect that infants without a resorptive disorder (the miners) are very unlikely to develop late VKDB.
The strong association between late VKDB and breastfeeding observed in this thesis has been recognized from its earliest descriptions[5] and it is widely acknowledged that the incidence of VKDB varies with breastfeeding rates.[1,6] Data presented in **chapter 4** illustrate that more subtle changes in a society can affect the risk of VKDB as well. The increased breastfeeding rate in infants with cholestatic jaundice - perhaps due to a more lenient attitude towards jaundice in breastfed infants – may well have been responsible for the rise in overall incidence of late VKDB in the Netherlands. Previously, McNinch et al. suggested that the resurgence of VKDB in the 80s was associated with a reduced tendency to give supplemental feedings in the first days of life.[7] Borderline vitamin K deficiency in mothers may also help explain the observed differences in the baseline incidence of late VKDB from country to country. In support, VKDB is seen more frequently in countries were a high breastfeeding rate is combined with the use of rice as staple food.[8-10] The observation that a rice based diet induces vitamin K deficiency in rats is suggestive of a causal link.[11] We hypothesize that one or more of the above mentioned risk factors were present in Boston around 1894 explaining why Townsend could report a "new" clinical entity in as many as 50 newborns.[5]

In this thesis (**chapter 2, 3 and 5)**, we did not only determine the risk of VKDB, but also looked at biochemical vitamin K deficiency. The general assumption behind this approach was that VKDB likely represents the "tip of the iceberg". This view under the surface helped us to further delineate the role of several factors that had previously been associated with VKDB in case reports and case series.[12-16] First, as expected, our data confirm that VKDB is associated with very high prothrombin ratios, mostly even above the upper limit of detection. Second, the appreciation that a grossly prolonged prothrombin time may remain asymptomatic is also supported by our data. Third, the observation in **chapter 2** that most cholestatic breastfed infants that did not develop a VKDB still presented with biochemical vitamin K deficiency underlines the universality of the risk in exclusively breastfed infants with unidentified cholestasis without adequate prophylaxis. Fourth, we had expected to find biochemical vitamin K deficiency in a significant proportion of formula fed infants as well. This expectation was based on the assumption that – in view of the similar amount of vitamin K given to breastfed and formula fed infants – the risk of developing vitamin K deficiency would only be mildly lower. In theory, a small increase in the degree of protection could delay the development of a severe vitamin K deficiency sufficiently to allow recognition of cholestatic jaundice and thus significantly reduce the risk of VKDB. The finding in **chapter 2 and 3** that the vast majority of formula fed cholestatic infants had a normal prothrombin ratio highlights the degree of protection offered by formula. This protective effect was further supported by the

normalization of prothrombin ratio upon switching to formula.(data not shown) Fifth, the data on biochemical vitamin K deficiency enabled us to perform risk factor analysis, which led to the suggestion that hydrolysed formula is a risk factor for developing vitamin K deficiency in cholestatic infants (**chapter 5**).

The importance of giving adequate vitamin K prophylaxis to all infants is to a large extent related to the devastating consequences of intracranial haemorrhages due to VKDB. The ability to prevent these life threatening haemorrhages is therefore an important index of the efficacy of a prophylactic regimen. We propose that national Pediatric Intensive Care Unit (PICU) diagnosis registries, as recently organised in the Netherlands, may be an additional way to assess the incidence of intracranial haemorrhage due to VKDB. Data from these registries, when combined with data from "untargeted surveillance" would allow capture-recapture analysis. This would not only afford a more precise estimate of the true incidence of intracranial haemorrhage due to VKDB but also provide valuable information on the degree of (under)ascertainment of general surveillance.

Although vitamin K prophylaxis should be aimed at protecting all apparently healthy infants, including those with an underlying resorptive disorder, the design of oral prophylaxis is still aimed at protecting healthy infants. Such a design may be appropriate for classical VKDB (which occurs primarily in healthy infants in whom the initiation of breastfeeding is delayed) but is inappropriate for preventing late VKDB. **Chapter 2 and 3** provide strong evidence that the Dutch vitamin K prophylaxis fails to protect exclusively breastfed cholestatic infants against VKDB. However, the reason(s) for this lack of efficacy cannot be inferred from these data. It is likely that the poorer efficacy is related to the six-fold lower cumulative weekly dose, compared with the Danish regimen. Additionally, the vitamin K formulation presently used in the Netherlands - drops of vitamin K dissolved in oil - may contribute to its poor efficacy. While administration of vitamin K together with oil induces excellent absorption under physiological conditions,[17] this formulation may be grossly insufficient in patients with fat malabsorption due to cholestasis. Experimental data in **chapter 6 and 7** confirm that absorption from vitamin K administered in oil is indeed extremely poor if bile is absent. Since we did not study the absorption of vitamin K emulsified in cremophor, a direct comparison with the formulation used in the former Danish regimen cannot be made. The sparse published data indicate that vitamin K is absorption is poor from this formulation as well.[18] However, even small increments in the degree of absorption may affect the risk of VKDB.

In **chapter 6** we studied whether vitamin K absorption can be enhanced when encapsulated in polymeric micelles. Since previous data suggested that pinocytosis of intact micelles with a diameter up to 100 nm would be feasible,[20,21] we chose a polymeric micelle within this size limit that also afforded stable encapsulation of vitamin K. However, we found that absorption of vitamin K by

pinocytosis was insignificant. Bile acids remained necessary for absorption of vitamin K. Future studies should therefore be directed at the design of micelles that "offload their luggage" at the cellular membrane of the epithelial cell without bile. This strategy may be combined with strategies which increase the available time for the micelles to deliver their payload by attaching to the mucous layer covering the epithelial cells in the gastrointestinal tract.[22] If successful, these strategies are likely to be relevant for other extremely hydrophobic compounds as well. The results presented in **chapter 7** suggest another attractive strategy to increase the absorption of vitamin K under cholestatic conditions. Combining commercially available vitamin K wrapped in mixed micelles (Konakion MM) with proton pump inhibition (in rats) allows substantial absorption of vitamin K, as this stabilizes the mixed micelles in an acidic environment. This combination treatment, however, is not suitable to be used in a prophylactic scheme for all (breastfed) infants.

The data presented in this thesis suggest that it is possible to improve the efficacy of vitamin K prophylaxis by tailoring it to protect infants with a high risk of VKDB, particularly infants with unidentified cholestatic liver disease. However, the fact that none of the presently available oral formulations induces reliable absorption in the absence of bile at the usual dose also means that a substantial dosage increment would be needed to achieve adequate protection for all breastfed infants. The decision that needs to be made is if, and to what extent, we want to "make life safe for canaries".[2] This requires balancing the benefits in infants with unrecognized cholestasis with the risks in healthy babies exposed to higher doses of vitamin K. Available data indicate that the risk of this higher exposure, if any, is extremely low. First, formula fed infants are already exposed to high vitamin K levels for several months,[23] without any known harmful consequences. Secondly, the suggestion by Golding of an increased risk of childhood malignancies when vitamin K is administered intramuscularly[24] has not been confirmed in subsequent studies, and may have been due to methodological flaws.[25] Thirdly, within the Danish regimen, almost half a million infants received a weekly oral dose of 1 mg vitamin K for 3 months, without any ill effects.[26] Nevertheless, the large cumulative number of healthy infants that would be exposed if the Dutch prophylaxis is adjusted upward warrants prudence.

The data in **chapter 2** indicate that both Danish regimens (a single dose of 2 mg vitamin K IM at birth as well as an oral dose of 2 mg, followed by weekly oral doses of 1 mg) will give an adequate level of protection. Theoretically, an equivalent dose administered daily may have a more favourable pharmacodynamic profile, by reducing peak levels as well as the depth and duration of a dip in vitamin K levels. In view of the short biological half life of vitamin K,[27] it may also reduce the consequences of accidentally missed doses of vitamin K. The few patients still at risk of VKDB under this improved regimen may be recognized in time if efforts for an improved detection and treat-

ment of cholestatic jaundice in infancy have effect at last.[28] Furthermore, professionals involved in the care of newborns should be alerted by minor "sentinel" bleedings indicative of vitamin K deficiency. We can only repeat earlier warnings that a bleeding due to vitamin K deficiency is far from a trivial condition; it may prove fatal if not recognized and treated promptly.[7]

The ultimate goal would be an oral prophylactic regimen which protects those at risk, without exposing healthy infants to supraphysiological vitamin K levels. Data from **chapter 7** suggest that this goal may come within reach if a formulation can be designed which affords safe delivery of mixed micelles in the duodenum.

REFERENCES

1. Shearer MJ. Vitamin K deficiency bleeding (VKDB) in early infancy. Blood Rev 2009;23:49-59.

2. Hey E. Making life safe for canaries. Pediatrics 2008;121:1048-9.

3. Ijland MM, Pereira RR, Cornelissen EA. Incidence of late vitamin K deficiency bleeding in newborns in the Netherlands in 2005: evaluation of the current guideline. Eur J Pediatr 2008;167:165-9.

4. Matsuda I, Nishiyama S, Motohara K, Endo F, Ogata T, Futagoishi Y. Late neonatal vitamin K deficiency associated with subclinical liver dysfunction in human milk-fed infants. J Pediatr 1989;114:602-5.

5. Townsend CW. The haemorrhagic disease of the newborn. Arch Pediatr 1894;11:559-65.

6. Cornelissen M, von Kries R, Loughnan P, Schubiger G. Prevention of vitamin K deficiency bleeding: efficacy of different multiple oral dose schedules of vitamin K. Eur J Pediatr 1997;156:126-30.

7. McNinch AW, Orme RL, Tripp JH. Haemorrhagic disease of the newborn returns. Lancet 1983;1:1089-90.

8. Danielsson N, Hoa DP, Thang NV, Vos T, Loughnan PM. Intracranial haemorrhage due to late onset vitamin K deficiency bleeding in Hanoi province, Vietnam. Arch Dis Child Fetal Neonatal Ed 2004;89:F546-F550.

9. Hanawa Y, Maki M, Matsuyama E, Tada H, Urayama T, Yamada K et al. The third nationwide survey in Jpan of vitamin K deficiency in infancy. Acta Paediatr Jpn 1990;32:51-9.

10. Chuansumrit A, Isarangkura P, Hathirat P. Vitamin K deficiency bleeding in Thailand: a 32-year history. Southeast Asian J Trop Med Public Health 1998;29:649-54.

11. Mathers JC, Fernandez F, Hill MJ, McCarthy PT, Shearer MJ, Oxley A. Dietary modification of potential vitamin K supply from enteric bacterial menaquinones in rats. Br J Nutr 1990;63:639-52.

12. Sutor AH, Dagres N, Niederhoff H. Late form of vitamin K deficiency bleeding in Germany. Klin Padiatr 1995;207:89-97.

13. Pellegrino M, Sacco M, D'Altilia MR, Meleleo D, De Anseris AG. Hemorrhagic disease in an infant fed on a vitamin K-deficient soya formula. J Pediatr Gastroenterol Nutr 1996;23:413-4.

14. Moss MH. Hypoprothrombinemic bleeding in a young infant. Association with a soy protein formula. Am J Dis Child 1969;117:540-2.

15. McNinch A, Busfield A, Tripp J. Vitamin K deficiency bleeding in Great Britain and Ireland: British Paediatric Surveillance Unit Surveys, 1993 94 and 2001-02. Arch Dis Child 2007;92:759-66.

16. McNinch AW, Tripp JH. Haemorrhagic disease of the newborn in the British Isles: two year prospective study. BMJ 1991;303:1105-9.

17. Cornelissen EA, Kollee LA, De Abreu RA, Motohara K, Monnens LA. Prevention of vitamin K deficiency in infancy by weekly administration of vitamin K. Acta Paediatr 1993;82:656-9.

18. Amedee-Manesme O, Lambert WE, Alagille D, De Leenheer AP. Pharmacokinetics and safety of a new solution of vitamin K1(20) in children with cholestasis. J Pediatr Gastroenterol Nutr 1992;14:160-5.

19. Argao EA, Heubi JE, Hollis BW, Tsang RC. d-Alpha-tocopheryl polyethylene glycol-1000 succinate enhances the absorption of vitamin D in chronic cholestatic liver disease of infancy and childhood. Pediatr Res 1992;31:146-50.

20. Zastre J, Jackson J, Bajwa M, Liggins R, Iqbal F, Burt H. Enhanced cellular accumulation of a P-glycoprotein substrate, rhodamine-123, by Caco-2 cells using low molecular weight methoxypoly-ethylene glycol-block-polycaprolactone diblock copolymers. Eur J Pharm Biopharm 2002;54:299-309.

21. Kabanov AV, Alakhov VY. Pluronic block copolymers in drug delivery: from micellar nanocontainers to biological response modifiers. Crit Rev Ther Drug Carrier Syst 2002;19:1-72.

22. Thirawong N, Thongborisute J, Takeuchi H, Sriamornsak P. Improved intestinal absorption of calcitonin by mucoadhesive delivery of novel pectin-liposome nanocomplexes. J Control Release 2008;125:236-45.

23. Cornelissen EA, Kollee LA, van Lith TG, Motohara K, Monnens LA. Evaluation of a daily dose of 25 micrograms vitamin K1 to prevent vitamin K deficiency in breast-fed infants. J Pediatr Gastroenterol Nutr 1993;16:301-5.

24. Golding J, Greenwood R, Birmingham K, Mott M. Childhood cancer, intramuscular vitamin K, and pethidine given during labour. BMJ 1992;305:341-6.

25. Passmore SJ, Draper G, Brownbill P, Kroll M.Case-control studies of relation between childhood cancer and neonatal vitamin K administration.BMJ. 1998;316:178-84.

26. Hansen KN, Minousis M, Ebbesen F. Weekly oral vitamin K prophylaxis in Denmark. Acta Paediatr. 2003;92:802-805.

27. Greer FR, Marshall SP, Foley AL, Suttie JW. Improving the vitamin K status of breastfeeding infants with maternal vitamin K supplements. Pediatrics 1997;99:88-92.

28. Bekhof J, de Langen ZJ, Verkade HJ. [Icterus prolongatus is a reason for laboratory investigation, also in breastfed neonates] Ned Tijdschr Geneeskd. 2005;149:613-7.

Chapter 09

Chapter 09

Nederlandse samenvatting

De belangrijkste doelen van dit proefschrift waren: a) het onderzoeken van de effectiviteit van de Nederlandse vitamine K profylaxe bij het voorkomen van vitamine K deficiëntie bloedingen (VKDB), met name bij zuigelingen met een onderliggende resorptiestoornis; b) het in kaart brengen van de invloed van verschillende soorten zuigelingenvoeding op het risico op VKDB; c) een aanzet te geven tot het verbeteren van de opname van oraal toegediend vitamine K wanneer er geen gal in de darm is.

Achtergrond

Vitamine K deficientie bloedingen (VKDB) op de zuigelingenleeftijd zijn zeldzaam maar levensbedreigend. Meest gevreesd zijn spontaan optredende hersenbloedingen, met een hoge kans op overlijden en op neurologische schade in geval van overleven. Aangezien bij de geboorte niet kan worden voorspeld bij welke zuigelingen een bloeding zal optreden, wordt in de meeste Westerse landen aan alle pasgeborenen kort na de bevalling vitamine K gegeven: de zogenaamde vitamine K profylaxe. In Nederland wordt daartoe 1 mg vitamine K oraal (= via de mond) gegeven. Dit beschermt goed tegen een bloeding in de eerste levensweek maar biedt, in tegenstelling tot een injectie in de spier (intramusculaire toediening), nauwelijks bescherming tegen het optreden van een bloeding ná de eerste levensweek. Om bloedingen na de eerste week te voorkomen wordt in Nederland sinds 1990 geadviseerd om borstgevoede zuigelingen (waarvan bekend is dat ze een duidelijk hoger risico lopen op een vitamine K tekort) dagelijks een kleine dosis vitamine K te geven (25 microgram). Voor flesgevoede zuigelingen is dit niet nodig, omdat vitamine K al is toegevoegd aan de flesvoeding.

De effectiviteit van profylaxe maatregelen in het algemeen, en dus ook van vitamine K profylaxe, wordt meestal bepaald met behulp van zogenaamde surveillance studies. Een surveillance studie werkt als volgt: aan alle kinderartsen wordt (meestal via een contactpersoon per ziekenhuis) gedurende een periode van 1-2 jaar maandelijks gevraagd of ze willen doorgeven of ze in de afgelopen maand een patiënt met VKDB hebben gezien. Op deze manier kan de incidentie (= de frequentie van voorkomen) worden bepaald, welke een maat is voor de effectiviteit van de profylaxe. Kort na het advies in 1990 om borstgevoede zuigelingen dagelijks 25 microgram vitamine K te geven leek de incidentie van VKDB in Nederland laag, lager dan in andere landen, vergelijkbaar zelfs met landen waarin vitamine K intramusculair wordt gegeven. Daarop werd geconcludeerd dat de profylaxe maatregel effectief was. In de daaropvolgende jaren werden echter verschillende gevallen van VKDB beschreven in de medische literatuur, zodat twijfel ontstond over de eerder gerapporteerde effectiviteit. Opvallend was dat de zuigelingen bij wie zich ondanks de profylaxe toch een bloeding voordeed, in vrijwel alle gevallen een onderliggende stoornis bleken te hebben, die de opname van vitamine K in negatieve zin beïnvloedde. In de meeste gevallen ging het hierbij

om een belemmerde afvloed van gal naar de darm (cholestase). Dat een falende profylaxe vooral voorkwam bij zuigelingen met niet onderkende cholestase werd ook in andere landen, met andere orale profylaxe maatregelen, geobserveerd.

Een nieuwe kijk op vitamine K profylaxe: aandacht voor risicofactoren
In dit proefschrift richtten we ons op factoren die sterk geassocieerd zijn met VKDB. In **hoofdstuk 2 en 3** onderzochten we het risico op VKDB bij zuigelingen die zich presenteerden met een ziekte die de opname van vitamine K bemoeilijkte.

In **hoofdstuk 2** werd dit bij zuigelingen met galgangatresie onderzocht. Galgangatresie is de meest voorkomende oorzaak van cholestase op de zuigelingenleeftijd. Bij deze aandoening is geen galafvloed mogelijk. Naast het risico op VKDB werd in deze groep patiënten tevens de bescherming geboden door het Nederlandse vitamine K profylaxe regime vergeleken met twee andere regimes: het voormalige Deense regime, waarbij vitamine K - net als in Nederland - oraal wordt gegeven, maar met een hogere dosering bij de geboorte (2 mg) en een hogere wekelijkse dosis daarna (1 mg per week) en daarnaast ook het huidige Deense regime (eenmalig 2 mg intramusculair bij de geboorte). We maakten daarbij gebruik van de Nederlandse en Deense landelijke registraties voor kinderen met galgangatresie. Aangezien borstvoeding een belangrijke risicofactor is voor het ontwikkelen van een VKDB, vergeleken we vooral het risico op VKDB bij borstgevoede zuigelingen onder de drie bovengenoemde regimes. Tachtig procent van de Nederlandse exclusief borstgevoede zuigelingen met galgangatresie bleek zich met een VKDB te presenteren. Bij ongeveer de helft van hen trad een hersenbloeding op. Dit risico was bijna 80 maal hoger dan het risico bij flesgevoede zuigelingen. De kans op een VKDB bij borstgevoede zuigelingen onder beide Deense regimes was aanzienlijk lager (ongeveer 10 procent).

De resultaten van **hoofdstuk 2** laten dus een zeer hoog risico zien bij borstgevoede zuigelingen met galgangatresie, ondanks de Nederlandse profylaxe. Dit suggereert dat het risico op VKDB ook bij andere oorzaken van cholestase verhoogd is. Echter, bij veel andere oorzaken van cholestase is (in tegenstelling tot galgangatresie) enige galafvloed mogelijk. In theorie zou de mate waarin de galafvloed is belemmerd van invloed kunnen zijn op het vermogen vitamine K op te nemen, en daarmee het risico op VKDB. In **hoofdstuk 3** onderzochten we daarom het risico op VKDB bij Nederlandse zuigelingen met alpha-1-antitrypsine deficientie (A1ATD), een aandoening waarin de mate van cholestase sterk varieert. De kans op VKDB onder exclusief borstgevoede zuigelingen met A1ATD was 75%, vergelijkbaar met het risico bij zuigelingen met galgangatresie. Er werd geen verband gevonden tussen de mate van geelheid (als maat voor de ernst van de galafvloed belemmering) en de kans op een bloeding. Deze uitslagen doen vermoeden dat het risico op VKDB hoog

is bij alle borstgevoede zuigelingen met galafvloedbelemmering, onafhankelijk van de onderliggende oorzaak. Dit hoge risico op VKDB in Nederlandse cholestatische zuigelingen werd bevestigd door een in 2005 uitgevoerde landelijke surveillance studie, waarbij 5 van de 6 zuigelingen met een VKDB een onderliggende cholestatische ziekte bleken te hebben, die op het moment van presentatie nog niet ontdekt was. De incidentie in deze studie was veel hoger dan die in de eerdere surveillance studie.

In **hoofdstuk 4** onderzochten we vervolgens of het verschil tussen de twee surveillance studies kon worden verklaard door veranderende borstvoedingsgewoontes. We combineerden daarvoor de data uit de landelijke registraties voor galgangatresie en A1ATD van 1991 t/m 2006 en keken naar het percentage zuigelingen binnen deze risicogroep die uitsluitend borstvoeding kregen. Het landelijke borstvoedingspercentage bij zuigelingen van 6 weken (~ de leeftijd waarop de bloedingen zich voordoen), steeg in deze periode van 54 naar 62%. Het percentage exclusief borstgevoede zuigelingen met galgangatresie of A1ATD steeg echter van 13 procent in de periode 1991-1994 tot 47 procent in de periode 2003-2006. Parallel hieraan steeg het risico op VKDB van 11 naar 41 procent. Deze data maken aannemelijk dat de lage borstvoedingsfrequentie bij zuigelingen met een hoog risico op VKDB begin jaren 90 de matige bescherming van het Nederlandse regime verdoezelde. Het onvermogen van de Nederlandse vitamine K prophylaxe om zuigelingen met een niet onderkende cholestatische leverziekten afdoende te beschermen werd pas duidelijk na een sterke stijging in het borstvoedingpercentage in deze risico populaties.

Hoewel het risico op VKDB onder flesgevoede zuigelingen veel kleiner was dan bij borstgevoede zuigelingen met een galafvloed belemmering was het risico ook onder hen nog steeds hoger dan bij gezonde zuigelingen. In **hoofdstuk 5** onderzochten we of dit risico samenhing met het soort flesvoeding. We maakten daarbij opnieuw gebruik van de gecombineerde database van zuigelingen met galgangatresie en A1ATD en vergeleken het risico op vitamine K deficientie bij zuigelingen die reguliere flesvoeding kregen met het risico bij zuigelingen die andere soorten flesvoeding kregen Het risico op vitamine K deficientie bij zuigelingen die gehydrolyseerde voeding kregen (met name voeding waarbij gebruik werd gemaakt van wei-eiwit) bleek ruim twintig keer hoger te liggen dan bij zuigelingen die reguliere flesvoeding kregen.

In **hoofdstuk 6 en 7** onderzochten we manieren om de opname van oraal toegediend vitamine K te verbeteren als er sprake is van cholestase. Hiervoor is het van belang kort stil te staan bij de reden dat vitamine K niet goed kan worden opgenomen als de galafvloed belemmerd is. Vitamine K is erg hydrofoob,"water vrezend", hetgeen betekent dat het in water nauwelijks oplost. Om toch te worden opgenomen uit de darm heeft het hulp nodig. In normale omstandigheden wordt deze

hulp geboden door de alvleesklier en de gal. De alvleesklier scheidt enzymen uit waarvan een aantal betrokken is bij de afbraak van verschillende soorten vetten. Bij dit proces ontstaan deeltjes die iets beter in water opgelost kunnen worden, zoals vetzuren en monoglyceriden. Deze deeltjes mengen zich met de watervrezende stoffen, waaronder vitamine K; een proces, dat emulsificatie wordt genoemd. Gal, dat door de lever wordt gemaakt, en daarna tijdelijk wordt opgeslagen in de galblaas bestaat o.a. uit galzouten en fosfolipiden. Eenmaal in de darm aangekomen helpen deze stoffen de gevormde vetemulsie te stabiliseren. Galzouten hebben verder de eigenschap dat ze micellen kunnen vormen; dit zijn extreem kleine bolletjes, of schijfjes (met een doorsnede van een paar nanometer, dat wil zeggen een paar miljardste meter) waarvan de buitenkant waterlievend is (hydrofiel) en de binnenkant hydrofoob. De hydrofobe binnenkant van zo'n micel maakt het een geschikt transport middel om hydrofobe stoffen - zoals vitamine K- te vervoeren naar de darmwand. Het 'opslagvermogen' wordt sterk verbeterd als gemengde micellen worden gevormd: deze bevatten naast galzouten de eerder beschreven afbraakproducten van vet alsmede de meegeleverde fosfolipiden. Gezien de cruciale rol van micellen bij de opname van vitamine K vroegen we ons af in hoeverre door een apotheker gemaakte micellen de rol van de natuurlijke gevormde micellen zouden kunnen overnemen. We maakten hierbij gebruik van een diermodel voor cholestase (ratten waarbij de gemeenschappelijke galweg werd afgebonden, zogenaamde BDL ratten) en vergeleken de vitamine K opname bij deze ratten met de opname bij ratten waarbij de galweg niet werd afgebonden.

In **hoofdstuk 6** onderzochten we het vermogen van door een apotheker gemaakte, zogenaamde polymere micellen (geladen met vitamine K) om de functie van natuurlijk gevormde gemengde micellen over te nemen. In plaats van galzouten en fosfolipiden bestaan deze micellen uit polymeren. Terwijl de opname van vitamine K uit deze polymere micellen bij ratten met een intacte galafvloed redelijk was, kon bij BDL ratten geen opname worden aangetoond. Om het verschil tussen beide groepen te verklaren onderzochten we *in vitro*, dat wil zeggen in een laboratoriumschaaltje, het effect van galzouten op de polymere micellen. Galzouten destabiliseerden de met vitamine K geladen polymere micel, waardoor vitamine K vrij kon komen en beschikbaar kwam voor opname. Inderdaad kon bij de proefdieren de vitamine K opname worden teruggebracht naar het niveau van ratten met intacte galafvloed als de polymere micel gelijktijdig met gal werd toegediend. Omdat gal dus nodig blijft voor een goede vitamine K opname concludeerden we dat deze polymere micellen niet in staat zijn om de rol van natuurlijk gevormde micellen over te nemen.

In **hoofdstuk 7** richtten we ons op een van de op dit moment commercieel beschikbare vitamine K preparaten: Konakion MM. Dit preparaat bestaat uit een apotheek vervaardigde gemende micel,

die net als de natuurlijke gemengde micel bestaat uit galzouten en fosfolipiden waaraan vitamine K is toegevoegd. Bij introductie van dit preparaat werd gehoopt dat het ook goed opgenomen zou kunnen worden door patiënten met cholestase. Echter, recent bleek bij zuigelingen met cholestase de opname onvoorspelbaar te zijn. In de meeste gevallen was de opname laag, er waren echter uitschieters richting normaal. Een duidelijke verklaring voor deze observatie werd niet gegeven. Op zoek naar een verklaring onderzochten we daarom de opname van vitamine K uit Konakion MM bij BDL ratten. De opname bij deze ratten leek sterk op de opname bij cholestatische zuigelingen. Bij 7 van de 10 ratten werd geen meetbare opname gevonden, bij de overige 3 was de opname beter, hoewel nog steeds beduidend lager dan bij ratten met intacte galwegen. We vroegen ons vervolgens af of de omstandigheden in de maag deze grote verschillen zouden kunnen verklaren. In de maag treedt verdunning op, alsmede blootstelling aan zuur. Om dit verder uit te zoeken bestudeerden we *in vitro*, dat wil zeggen in een laboratoriumschaaltje, het effect van blootstelling aan verdunning en zuur op de deeltjesgrootte van het preparaat. Verdunning had een relatief beperkte invloed op de deeltjesgrootte. Het effect van verlaging van de zuurgraad op de deeltjes grootte was zeer groot (passend bij uiteenvallen van de micellen), maar als de zuurgraad werd teruggebracht naar de oorspronkelijke waarde vormden de meerderheid van de micellen zich opnieuw. Echter, als verlaging van de zuurgraag werd gecombineerd met verdunning verdwenen de micellen sneller, en was dit proces bij het terugbrengen van de zuurgraag niet langer omkeerbaar. Het voorkomen van blootstelling aan een lage zuurgraad, door de Konakion MM micellen direct in de twaalfvingerige darm in te brengen, of door het toedienen van een maagzuur remmer, leidde bij BDL ratten tot een veel betere opname. Dit onderzoek suggereert dat de vitamine K opname uit Konakion MM micellen bij patiënten met cholestase sterk kan worden verbeterd door het te combineren met het gebruik van maagzuur remmers.

Toekomst

De gegevens uit dit proefschrift geven aan dat de huidige Nederlandse vitamine K profylaxe exclusief borstgevoede zuigelingen met de twee meest voorkomende oorzaken van cholestase op de zuigelingen leeftijd nauwelijks beschermt tegen het ontstaan van een VKDB. Vermoedelijk geldt dit ook voor andere vormen van cholestase. De observatie dat andere vitamine K profylaxe regimes (zowel IM als oraal) een veel betere bescherming bieden, ondersteunt de noodzaak de Nederlandse profylaxe aan te passen en geeft richting aan de wijze waarop de effectiviteit kan worden verbeterd. Vermoedelijk zou een wekelijkse orale dosering van 1 mg vitamine K het risico op VKDB bij borstgevoede zuigelingen aanzienlijk kunnen beperken. Een alternatief zou zijn om de huidige dagelijkse dosering bij borstgevoede zuigelingen van 25 microgram op te hogen naar 100 microgram. Ook dan zal gerichte surveillance in risicogroepen, zoals patiënten met galgangatresie en A1ATD, moeten blijven plaatsvinden om zo de effectiviteit van dit aangepaste regime in de

Nederlandse situatie te bevestigen. Voor de verdere toekomst is de hoop een vitamine K preparaat te ontwikkelen dat net zo goed opgenomen wordt in de darm van zuigelingen met cholestase als door gezonde zuigelingen.

Chapter 10

Chapter 10

Nawoord
Curriculum Vitae
List of publications

NAWOORD

Dit proefschrift markeert het (voorlopig) eindpunt van een zoektocht die het gevolg was van een toevallige klinische observatie.

Tijdens mijn opleiding tot kinderarts was ik gedurende een van de afdelingsstages (op afdeling "Dolfijn") in korte tijd betrokken bij twee zuigelingen die zich presenteerde met intracraniële bloedingen als gevolg van een ernstig vitamine K tekort. Aanvankelijk werd aangenomen dat dit tekort was ontstaan door ontrouw aan de profylaxe. Echter, anamnestisch werd deze aanname niet ondersteund. Ouders gaven juist aan zeer trouw te zijn geweest met het toedienen van de vitamine K druppeltjes. Omdat bij beide zuigelingen aanwijzingen gevonden werden voor een verstoorde opname van vitamine K, concludeerden we dat dit een veel aannemelijker verklaring was en, samen met Tom de Koning, Atty van Dijk en Roderick Houwen, beschreef ik deze klinische les in het NTVG.

Mijn nieuwsgierigheid was geprikkeld. Twee gevallen tijdens een stage van 3 maanden leek in tegenspraak met de beschikbare literatuur die suggereerde dat VKDB onder de huidige profylaxe eigenlijk niet voorkwam. Puur toeval? Of was er sprake van een stijging van de incidentie? En: hoe groot was eigenlijk de kans op een VKDB bij zuigelingen met een resorptiestoornis, en kwam het ook voor zonder? Ik nam contact op met Leslie Beks, medewerker van de diagnose registratie afdeling van het UMC Utrecht. Het bleek mogelijk om binnen een leeftijdscategorie te zoeken naar specifieke diagnoses. Om zoveel mogelijk gevallen op te sporen namen we ook diagnoses mee waarbij het risico op een vitamine K tekort verhoogd zou kunnen zijn. Er bleken - alleen al in het UMC Utrecht - ongeveer 2 gevallen van VKDB per jaar terug te vinden, veel meer dan op basis van de eerder gerapporteerde incidentie zou worden verwacht. Opvallend was dat de bloedingen vrijwel zonder uitzondering werden aangetroffen bij zuigelingen waarbij zowel sprake was van een resorptiestoornis (meestal cholestase) als van borstvoeding. Deze eerste zoektocht vormde de opmaat voor dit promotie traject.

Chapter 10
Nawoord

De volgende mensen zou ik graag danken voor hun bijdrage aan de totstandkoming van dit proefschrift.

Hooggeleerde Kimpen, beste Jan. Als een echte bestuurder heb je dit promotie traject grotendeels van afstand 'gemanaged'. Bij de genese van dit traject heb je daarnaast een cruciale rol gespeeld door - bij het refereren van een artikel over de ontdekking van het humaan metapneumovirus - het belang van het opvolgen van opvallende klinische observaties te beklemtonen. Ik ben blij dat ik van jou de kans heb gekregen dit in de praktijk te brengen.

Hooggeleerde Berger, beste Ruud. Je Don Corleone-achtige stemgeluid dragen er aan bij dat er naar jou geluisterd wordt. Jij zorgde ervoor dat er in het metabole lab plaats kwam voor 'vitamine K'. Niet alleen werd er dankzij jou door Maria van de Ham een meetmethode opgezet voor het meten van vitamine K, ook het meten van de grote aantallen monsters werd, onder auspiciën van Monique de Sain, mogelijk gemaakt.

Weledelzeergeleerde de Koning, beste Tom. Jij was vanaf het eerste moment betrokken bij dit vitamine K project. Ik bewonder je oog voor "nieuwe" dingen en je vermogen om over de grenzen van het metabole vakgebied heen te kijken. Voor mij was je een ideaal klankbord bij het zoeken naar oplossingen voor de obstakels die een promotietraject kenmerken. Waar nodig nam je obstakels weg door aan de noodzakelijke touwtjes te trekken. Ik hoop als collega nog lang te genieten van je vermogen er onder alle omstandigheden 'de humor van in te zien.'

Wedelzeergeleerde Houwen, beste Roderick. Ook jij was vanaf het eerste begin betrokken. We zijn in de loop van de tijd steeds beter op elkaar ingespeeld geraakt. Ik bewonder je kijk op het hele wetenschappelijke palet – grote lijnen, details (de eenheid van bilirubine..), planning, politiek. Jij legde de eerste contacten met Denemarken en maakte later het onderzoek binnen het cohort alfa-1-antitrypsine patiënten mogelijk.

Hooggeleerde Verkade, beste Henkjan. Zonder jou had dit proefschrift er vermoedelijk anders uitgezien. Dankzij je scherpe analyse van de eerste Utrechtse data ontstond het plan om deze bevindingen in een strak gedefinieerd landelijk cohort van galgangatresie patiënten te repliceren. Elsemieke de Vries en, later, Willemien de Vries werden door jou enthousiast gemaakt om de NeSBAR database te updaten, zodat dit plan ook gestalte kon krijgen. Mijn dank aan hen gaat verder dan hun "rondje Nederland".

Weledelzeergeleerde van Nostrum, beste René. In 2005 nam ik contact met je op met de vraag of er niet een farmacologische manier was te bedenken om vitamine K zo aan de darm aan te bieden dat het - ook zonder gal - zou kunnen worden opgenomen. We vatten toen het plan op om hiervoor je temperatuursgevoelige polymere micellen te gebruiken. Met dank aan Christianne en Gwylim lukte het om vitamine K in de micellen te krijgen. Ik waardeer je volharding, ook toen onze pogingen om de opname van vitamine K te bestudeerden in Caco-2 cellen tegen nogal wat methodologische obstakels opliepen. Je stond open voor mijn soms ongebruikelijke oplossingen en hielp deze, waar zinvol, te realiseren. Daarnaast bewonder ik je vermogen om met betrekkelijk kleine ingrepen een manuscript een beter aanzien te geven. Hopelijk ontwerpen we in de toekomst nog eens een 'magic vitamin K bullet'.

Dr. Hørby Jørgensen, dear Marianne. My two visits to the Rigs hospital in Copenhagen (where Nobel laureate Henrik Dam made his first observations on vitamin K deficiency in humans!) are among the most memorable parts of this PhD project. It was intriguing to see the different ways of an academic hospital in a country that is so often mistaken for ours. I must confess I was a bit optimistic regarding my ability to grasp the written Danish language and would like to thank you, Nina Kvist and Christina Rydahl Lundin for your aid in retrieving and translating the case records of Danish biliary atresia patients.

Weledelzeergeleerde Lanting, beste Caren. Ik ben erg blij dat je mogelijkheden zag om een aantal vragen over vitamine K toe te voegen aan de landelijke twee-jaarlijkse vragenlijst vanuit TNO aan ouders van jonge zuigelingen. Dank voor je hulp bij het opstellen van methodologisch verantwoorde vragen en je betrokkenheid bij het verwerken van de antwoorden. Wellicht kunnen we deze exercitie nog eens herhalen als het profylactisch regime in Nederland eenmaal gewijzigd is!

Weledelzeergeleerde Schurgers en van Summeren, beste Leon en Marieke. Het is een genoegen om met jullie te kunnen praten over onze gezamenlijke hobby, vitamine K. Misschien wel vooral dankzij de verschillende invalshoeken. Leon, jou dank ik daarnaast voor het meten van vitamine K in verschillende soorten flesvoeding, samen met Ellen Cranenburg.

Hooggeleerde Akkermans, beste Louis. De dierexperimentele studies in dit proefschrift waren niet mogelijk geweest zonder te kunnen beschikken over goed microchirurgisch instrumentarium. Ik ben blij dat je het belang van het onderzoek zag en me de mogelijkheid hebt gegeven mijn onderzoek in jouw lab uit te voeren.

Chapter 10

Nawoord

Beste André. De alias van je werkplek (chez André) is goedgekozen, en contrasteert met de strakke organisatie in veel andere laboratoria. Ondanks het ogenschijnlijke gebrek aan structuur, slaagde je er met je aanleg voor McGiverism altijd in dat te vinden wat nodig was, of anders wat daarvoor dienst kon doen. Samen doorstonden we een hele serie mislukte pogingen om microchirurgisch de hoofdlymfe permanent te canuleren om zo lymfe te verkrijgen in een vrij bewegende rat, om tot de conclusie te komen dat hetzelfde onderwerp wellicht beter in bloed kon worden bestudeerd.

Beste Maria. Dank voor al je inspanningen die er voor zorgden dat het mogelijk werd om 'in house' vitamine K spiegels te bepalen. Jij wist de massaspectrometer zo in te stellen dat de soms lage spiegels toch quantificeerbaar waren. De logistieke uitdaging die gepaard gaat met het meten van grote hoeveelheden vitamine K monsters werd mede dankzij jou tot een goed einde gebracht. Raymond en Hans dank ik voor het - eindeloos pipetterend - doorstaan van ettelijke lange, donkere dagen.

Beste Tanca. Nadat bovenstaande conclusie getrokken was kwam jij in beeld en tijdens je wetenschappelijke stage ging je energiek aan de slag met de nieuwe strategie. Ik bewonder het ogenschijnlijk gemak waarmee je je de microchirurgische vaardigheden eigen maakte. De manier waarop je anderen benadert maakt dat mensen bereid zijn wat voor je te doen. De door je stage gegeneerde data vroegen om een vervolg en ik prijs me nog steeds erg gelukkig dat je, dit keer als arts-onderzoeker, bereid was dit zelf ter hand te nemen.

Beste Thierry. Jij wist als whizzkid data op een manier in beeld te helpen brengen die ik wel kan bedenken, maar niet zelf kan verwezenlijken. Veel dank hiervoor.

Beste Gepke. Niet alleen een goede buur is beter dan een verre vriend, zeker geldt dat voor een kamergenoot. Je hebt het vermogen echt mee te denken en je gedachten (die soms net zo springerig zijn als je haar) zetten me meer dan eens weer op het juiste spoor. Ik ben blij dat je me als paranimf ter zijde zal staan.

Beste Robert. Alhoewel je professioneel een andere tak van sport beoefent, slaan we beiden graag een bal. Als oud-huisgenoten zijn we, onafhankelijk van de lengte van de tussenpoze, ook buiten de baan altijd zo weer op elkaar ingespeeld. Ook al treed je het adagium 'see one, do one, teach one' met voeten, toch heb ik alle vertrouwen in jou als paranimf.

Beste Casper, Annemarie, Margriet, Cuno, Titia, Louis, Yves, Carin en Leo. Af en toe liep ik tegen een vraag op waarbij ik behoefte had aan een hulplijn. Dank voor het ruimhartig delen van jullie ervaring en expertise.

Beste Floris. Ooit hielp ik je, door een oefensessie, bij je sollicitatie voor de opleiding tot kinderarts. Dat heb je de afgelopen periode meer dan goed gemaakt. Mede dankzij jouw komst als fellow metabole ziekten kwam er voor mij meer tijd om mijn promotietraject af te ronden. Ik ben benieuwd of je mijn voorbeeld gaat volgen!

Beste Debbie en Ellen. Jullie vormen een onmisbaar onderdeel van ons metabole team en ik prijs me daarmee gelukkig. Dankzij jullie bleef ik de afgelopen jaren nog enigszins op de hoogte van andere belangrijke nieuwe ontwikkelingen (zoals 'scrappen' en 'wii-en').
Beste Johanna. Je secretariële ondersteuning in de afrondingsfase van dit proefschrift was van onschatbare waarde. Je denkt mee, gaat rustig aan de slag (behalve als ik je erg op de vingers kijk..) en rondt een verzoek altijd weer sneller af dan ik verwacht.

Beste Dorine en Ems. Het subspecialisme metabole ziekten is niet compleet zonder de mogelijkheid tot een goede dieetbehandeling. Dankzij de combinatie van kennis, ervaring en betrokkenheid maken jullie voor veel ouders het verschil. Ik hoop na een vergelijkbaar aantal ervaringsjaren nog net zoveel liefde voor het vak uit te stralen.

Beste Monique, Nanda, Inge en Berthil. Een van de leukste aspecten van de metabole ziekten is de korte lijn tussen kliniek en lab. Beide onderdelen zijn, vergelijkbaar met Yin-Yan, weliswaar verschillend maar toch onlosmakelijk met elkaar verbonden. Dank voor de wijze waarop jullie hier als onze 'counterparts' invulling aan geven.

Beste collega's van het Sylvia Toth Centrum. Het is enorm verrijkend om samen met specialisten uit andere disciplines naar een patiënt met een ontwikkelingsachterstand te kijken, om zo gezamenlijk te proberen de onderliggende oorzaak te doorgronden. Zeker ook dankzij het 'eigen huiskamer'-concept, de secretariële en verpleegkundige ondersteuning wordt er een niveau van patiëntenzorg geboden waarvan ik hoop dat deze nog lang mogelijk zal blijven.

Beste WKZ-collega's. Een prettige werkplek is een niet te onderschatten bron van levensvreugde. Het gebouw speelt daarin een rol (architectonisch is er geen mooiere kinderkliniek), de rol van mensen is echter oneindig veel groter. Ik beschouw het als een groot voorrecht om te werken in een ziekenhuis zo vol met betrokken en inspirerende professionals op alle niveaus.

Beste Barbara. Het gaat in het leven niet alleen om de inhoud, de 'buitenkant' speelt een grote rol. Als vormgever wist je niet alleen mijn globale gedachte over de vormgeving ogenschijnlijk moeiteloos te vertalen, je vergat niet het je eigen 'touch' te geven.

Chapter 10

Nawoord

Lieve dierbaren. Promoveren is een wonderlijke, ietwat anachronistische bezigheid. Het is een academische proeve van bekwaamheid, maar ook een tijd en aandacht slurpend fenomeen om te komen tot een boekje met een beperkte lezersschare. Aangezien zowel tijd als aandacht eindig bleken, heeft deze professionele uitdaging ook zijn weerslag gehad op jullie leven. Ik ben blij dat jullie mij, op allerlei mogelijke manieren, terzijde hebben gestaan.

CURRICULUM VITAE

Peter Marin van Hasselt werd op 10 februari 1970 geboren te Rijswijk als zoon van Rolf Jan van Hasselt en Tienke Korthals-Altes. Hij genoot zijn middelbare school opleiding op het Eemland College Zuid, te Amersfoort waar hij in 1988 zijn VWO diploma behaalde. Dankzij een beurs verkregen via het Netherlands America Committee for Educational Exchange (NACEE) studeerde hij het navolgende jaar aan het Wittenburg College, Springfield, Ohio in de Verenigde Staten. In 1989 begon hij aan zijn studie Geneeskunde aan de Rijksuniversiteit Groningen. Hij was tijdens deze periode o.a. actief als lid van de Almanakcommissie en de Galacommissie. Daarnaast was hij bestuurslid van Panacea, de geneeskunde studievereniging en secretaris van het bestuur van WINGS (Werkgroep Internationalisering Groninger Studenten). In 1994 behaalde hij zijn doctoraal examen. Na het behalen van zijn artsexamen in 1997 was hij een half jaar werkzaam als AGNIO op de afdeling Neonatologie in het (oude) Wilhelmina Kinderziekenhuis (WKZ). Hierna deed hij een jaar onderzoek aan het Nederlands Herseninstituut te Amsterdam begeleid door Ruud Buis, Coby Heinen en Frank van Bel. Per 1 januari 1999 startte hij met de opleiding tot kinderarts, eerst in de Isala klinieken locatie Sophia te Zwolle (opleider Wim Baerts), later in het Wilhelmina Kinderziekenhuis te Utrecht (opleider Jan Kimpen). Sinds de afronding van zijn opleiding tot kinderarts is hij verbonden aan de afdeling metabole ziekten van het WKZ. Eerst als fellow, vervolgens, vanaf medio 2007, als kinderarts metabole ziekten. In 2005 werd een aanvang gemaakt met het promotie-onderzoek naar vitamine K prophylaxe, wat uiteindelijk resulteerde in dit proefschrift. Peter is getrouwd met Annika Braak. Samen hebben ze twee dochters: Madelief (2004) en Famke (2006).

Chapter 10

Curriculum Vitae

LIST OF PUBLICATIONS

van Hasselt PM, Janssens GE, Slot TK, van der Ham M, Minderhoud TC, Talelli M, Akkermans LM, Rijcken CJ, van Nostrum CF. The influence of bile acids on the oral bioavailability of vitamin K encapsulated in polymeric micelles. J Control Release. 2009 Jan 19;133(2):161-8.

Engbers HM, Berger R, van Hasselt P, de Koning T, de Sain-van der Velden MG, Kroes HY, Visser G. Yield of additional metabolic studies in neurodevelopmental disorders. Ann Neurol. 2008 Aug;64(2):212-7.

van Hasselt PM, de Koning TJ, Kvist N, de Vries E, Lundin CR, Berger R, Kimpen JL, Houwen RH, Jorgensen MH, Verkade HJ; Netherlands Study Group for Biliary Atresia Registry. Prevention of vitamin K deficiency bleeding in breastfed infants: lessons from the Dutch and Danish biliary atresia registries. Pediatrics. 2008 Apr;121(4):e857-63.

Aldenhoven M, Klomp LW, van Hasselt PM, de Koning TJ, Visser G. [From gene to disease; Menkes disease: copper deficiency due to an ATP7A-gene defect] Ned Tijdschr Geneeskd. 2007 Oct 13;151(41):2266-70.

Flipsen-ten Berg K, van Hasselt PM, Eleveld MJ, van der Wijst SE, Hol FA, de Vroede MA, Beemer FA, Hochstenbach PF, Poot M. Unmasking of a hemizygous WFS1 gene mutation by a chromosome 4p deletion of 8.3 Mb in a patient with Wolf-Hirschhorn syndrome. Eur J Hum Genet. 2007 Nov;15(11):1132-8.

Koelink CJ, van Hasselt P, van der Ploeg A, van den Heuvel-Eibrink MM, Wijburg FA, Bijleveld CM, van Spronsen FJ. Tyrosinemia type I treated by NTBC: how does AFP predict liver cancer? Mol Genet Metab. 2006 Dec;89(4):310-5.

de Vries MC, Rodenburg RJ, Morava E, van Kaauwen EP, ter Laak H, Mullaart RA, Snoeck IN, van Hasselt PM, Harding P, van den Heuvel LP, Smeitink JA. Multiple oxidative phosphorylation deficiencies in severe childhood multi-system disorders due to polymerase gamma (POLG1) mutations. Eur J Pediatr. 2007 Mar;166(3):229-34.

van Hasselt PM, Houwen RH, van Dijk AT, de Koning TJ. [Vitamin K deficiency bleeding in an infant despite adequate prophylaxis]Ned Tijdschr Geneeskd. 2003 Apr 19;147(16):737-40.

Kavelaars A, van der Pompe G, Bakker JM, van Hasselt PM, Cats B, Visser GH, Heijnen CJ. Altered immune function in human newborns after prenatal administration of betamethasone: enhanced natural killer cell activity and decreased T cell proliferation in cord blood. Pediatr Res. 1999 Mar;45(3):306-12.