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ORIGINAL ARTICLE

Exclusively breastmilk-fed preterm infants are at high risk of developing subclinical vitamin K deficiency despite intramuscular prophylaxis at birth

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Abstract

Background: There is near-global consensus that all newborns be given parenteral vitamin K_1 (VK_1) at birth as prophylaxis against VK deficiency bleeding (VKDB). Breastmilk has a low VK content and cases of late VKDB are reported in exclusively breastmilk-fed preterm infants despite VK prophylaxis at birth.

Objectives: To assess the prevalence of functional VK insufficiency in preterm infants based on elevated under-γ-carboxylated (Glu) species of Gla proteins, factor II (PIVKA-II), and osteocalcin (GluOC), synthesized by liver and bone, respectively.

Patients/Methods: Prospective, multicenter, observational study in preterm infants born <33 weeks' gestation. Blood samples and dietary history were collected before hospital discharge, and after discharge at 2–3 months' corrected age. Outcome measures were serum VK_1, PIVKA-II, and %GluOC (GluOC as a percentage of the sum of GluOC plus GlaOC) compared between exclusively breastmilk-fed and formula/mixed-fed infants after discharge.

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Results: After discharge, breastmilk-fed babies had significantly lower serum VK_1 (0.15 vs. 1.81 μg/L), higher PIVKA-II (0.10 vs. 0.02 AU/ml) and higher %GluOC (63.6% vs. 8.1%) than those receiving a formula/mixed-feed diet. Pre-discharge (based on elevated PIVKA-II), only one (2%) of 45 breastmilk-fed infants was VK insufficient. After discharge, eight (67%) of 12 exclusively breastmilk-fed babies were VK insufficient versus only one (4%) of 25 formula/mixed-fed babies.

Conclusions: Preterm infants who remain exclusively or predominantly human breastmilk-fed after neonatal unit discharge are at high risk of developing subclinical VK deficiency in early infancy. Routine postdischarge VK_1 supplementation of breastfed infants to provide intakes comparable to those from formula milks should prevent this deficiency.

KEYWORDS
hemorrhage, infant, nutrition, vitamin K deficiency, vitamin K1

1 | INTRODUCTION

Vitamin K (VK) is an essential fat-soluble micronutrient that functions as a cofactor for the microsomal enzyme γ-glutamyl carboxylase (GGCX). This enzyme catalyzes the conversion of specific peptide-bound glutamate (Glu) to γ-carboxyglutamate (Gla), a modification that imparts biological activity to all VK-dependent proteins.[1] The only potentially life-threatening syndrome associated with acute VK insufficiency is the inability to synthesize sufficient numbers of biologically active γ-carboxylated molecules of the four VK-dependent procoagulants (factors II, VII, IX, and X), resulting in a hypocoagulable state. The hemostatic system has considerable capacity to function adequately at low-factor concentrations of Gla proteins but, as insufficiency progresses to deficiency, a point will be reached when the procoagulatory mechanisms fail and bleeding occurs.

Besides the classical VK-dependent procoagulants synthesized in the liver, other VK-dependent proteins are synthesized in extrahepatic tissues. They include osteocalcin (OC) and matrix Gla protein, both originally isolated from bone. OC, the most abundant noncollagenous bone protein, is synthesized only by osteoblasts and odontoblasts, whereas matrix Gla protein has a broad tissue distribution and is synthesized by several cell types.[2,3] Analogous to the role that Gla residues play in facilitating the calcium ion-dependent binding of VK-dependent coagulation proteins to phospholipid membranes, the three Gla residues of OC facilitate its binding to free calcium ions and hydroxyapatite crystals.[4,5] Circulating OC correlates with the rate of bone formation and osteoblast numbers and originates from new bone synthesis rather than bone breakdown.[2] For these reasons, OC is one of the few specific biomarkers of bone formation.[2] Although the precise molecular function of OC is unclear, recent evidence points to roles in the biomolecular regulation of bone mineral,[4–6] primarily by regulating hydroxyapatite crystal growth,[5] and ensuring the parallel alignment of hydroxyapatite crystallites with collagen fibrils.[6] In all species studied, the osteoblastic synthesis of OC coincides with the onset of mineralization in utero with OC levels in bone and the circulation increasing in concert with the deposition of hydroxyapatite during skeletal growth.[2,4] Bone is continually replenished throughout life by a remodeling process that involves osteoclastic-mediated resorption and osteoblastic-mediated formation. Based on current evidence that carboxylated OC acts to promote the quality and strength of bone, ensuring optimal VK intakes in early infancy could be considered a desirable aim.

The only sector of ostensibly healthy human populations at increased risk of VK insufficiency and overt deficiency are infants in the first 6 months of life, with the exclusively breastfed being at greatest risk.[7,8] The resultant hemostatic syndrome is now known as VK deficiency bleeding (VKDB) of early infancy.[7–9] The progression from a hypocoagulable state to VKDB is highly individual and unpredictable. Worryingly, data from global population surveys of late-onset VKDB (which typically occurs at age 1–2 months), show that 40%–80% of cases first present as intracranial bleeding that

Essentials

- Exclusively breastfed preterm infants may be at risk of vitamin K deficiency bleeding after discharge from the neonatal intensive care unit (NICU).
- We undertook a multi-center study to assess the prevalence of subclinical VK deficiency in preterm infants pre- and post-NICU discharge.
- PIVKA-II and %GluOC data demonstrate that exclusively human breastmilk-fed preterm infants have a high risk of developing subclinical vitamin K deficiency post-NICU discharge.
- Bone carboxylation efficiency was particularly sensitive to VK_1 intakes with mean %GluOC values of 63.6% in breastmilk-fed babies and 8.1% in formula/mixed-fed babies

may be lethal or neurodevelopmentally devastating.[7,10] There is now near-global consensus that all infants, whether born term or preterm, and whether or not subsequently breastfed, should receive VK prophylaxis at birth to prevent VKDB. Even so, occasional cases of late-onset VKDB occur in ex-preterm infants despite prior prophylactic VK at birth.[11–15] For example, the most recent British Paediatric Surveillance Unit survey included a probable case in a 24-week gestation infant who received 0.4 mg/kg vitamin K_1 (VK_1) intramuscularly at birth, had no liver disease, was primarily breastmilk fed, and bled on postnatal day 91.[14] We reported another extremely preterm-born breastfeeding infant with proven VKDB before neonatal intensive care unit (NICU) discharge despite prophylaxis at birth and no other risk factors.[15]

Naturally occurring K vitamins are phylloquinone, VK_1, and menaquinones (vitamin K_2).[7,8] The major VK source for preterm babies during the neonatal period is VK_1 derived from exogenous and nutritional sources (prophylactic dose given at birth and subsequent parenteral and/or enteral feeding).[16] Of the menaquinone (MK) family, only the nonbacterial form MK-4 has dietary significance for breastmilk-fed infants, being synthesized from VK_1 in the mammary gland,[8,17] and probably also from dietary VK during intestinal absorption.[18] Longer side-chain MK, synthesized by human gut bacteria, may make some contribution to hepatic stores of VK in the later neonatal period,[8,19] but little is known of their bioavailability for hepatic Gla synthesis.

Human breastmilk is the best food for babies. Multiple expert international committees recommend breastmilk for pre- and post-discharge nutrition of babies born preterm (<37 weeks' gestation) because of benefits on reducing early neonatal morbidities associated with prematurity (such as necrotizing enterocolitis and sepsis), and for optimizing growth and neurodevelopmental outcomes.[20–22] However, for feeding to preterm infants, breastmilk usually requires additional fortification because it does not provide adequate intakes of most nutrients to meet their exceptionally rapid growth requirements. Commercial multinutrient fortifiers containing macro- and micro-nutrients (including VK_1) are therefore also widely recommended to supplement the human milk feeds of hospitalized preterm and very low birth weight (<1500 g) infants.[20,21,23]

Human breastmilk has a low VK content: average VK_1 concentration is 1–2 μg/L,[7,8,24] and MK-4 concentrations are lower still.[7,8,25] Exclusive breastmilk feeding is often the only risk factor identifiable in idiopathic late-onset VKDB.[7,8,12,14] A significant proportion of breastfed term infants showed evidence of subclinical VK deficiency at age 2–5 months related to breastfeeding duration.[26] Although all preterm infants receive prophylactic VK_1 at birth, and those exclusively fed human breastmilk may receive extra VK_1 from multinutrient milk fortifiers during the NICU stay, scientific committees in North America[27,28] and Europe[29] have not yet provided any recommendations for further VK supplementation after discharge. Exclusively breastfed preterm infants therefore currently remain entirely dependent on the endogenous VK content of human milk for their ongoing VK requirements in early infancy.

Some preterm infants develop undetectable serum VK_1 levels by as early as 3 weeks after birth despite parenteral prophylaxis at birth and early enteral milk feeding.[30] Yet the subsequent VK status of preterm infants has hitherto not been studied in early infancy following discharge home from NICU. We therefore aimed to assess VK status of breastmilk-fed preterm infants before and after discharge by measuring the undercarboxylated species of two different VK-dependent proteins: (1) undercarboxylated factor II (proteins induced by VK absence or antagonism [PIVKA-II]), reflecting functional hepatic insufficiency; and (2) undercarboxylated OC (GluOC), reflecting functional bone insufficiency.[1] Our hypothesis was that, without additional dietary VK supplements, preterm infants who remain exclusively breastmilk-fed develop a high prevalence of subclinical VK deficiency in early infancy in comparison with those fed VK_1-fortified formula milks.

2 | METHODS

2.1 | Design and participating centers

This was a prospective, observational, multicenter study involving four UK neonatal units. In the absence of any prior data, a power calculation was not possible. We therefore set a pragmatic recruitment target of 45 infants.

2.2 | Study population

Eligible infants were inborn at <33 weeks' gestation and had received prophylactic VK_1 parenterally following birth. We targeted for inclusion only those still exclusively or predominantly (arbitrarily defined as >80% breastmilk intake by volume) human breastmilk-fed when approaching discharge (at ~34–36 weeks' postmenstrual age), and whose mothers intended to continue or establish full breastfeeding after discharge. We excluded infants with significant cholestatic jaundice (defined as conjugated serum bilirubin fraction >20% of an elevated total serum bilirubin or conjugated serum bilirubin >20 μmol/L if total serum bilirubin was <100 μmol/L) because, being at increased risk for VKDB, these routinely receive large daily prophylactic oral doses of VK_1.

All participating centers gave 0.4 mg/kg VK_1 prophylactic dose (Konakion MM Paediatric, Neon Healthcare Ltd, Herts, UK) parenterally soon after birth. All centers also routinely supplemented human breastmilk until discharge using a commercial bovine milk-derived fortifier (Cow & Gate Nutriprem Human Milk Fortifier) that provided an extra 6.4 μg of VK_1 per two sachets added to 100 ml breastmilk; centers otherwise gave no other additional VK supplementation routinely pre- or post-discharge.

2.3 | Blood samples

We obtained ~3.5 ml blood samples at two time-points: (1) pre-discharge (baseline), at ~36 weeks postmenstrual age; and (2)

postdischarge, at ~2 months' corrected gestational age (CGA), i.e. at ~2 months after expected delivery date. Dietary enteral feeding histories were obtained from the medical records and from the mother/guardian at each visit.

2.4 | Biomarkers used for assessment of vitamin K status

The main markers of VK status used were serum concentrations of PIVKA-II and GluOC, the latter adjusted for total OC. We define both these biomarkers as measuring "functional VK insufficiency" (i.e., subclinical deficiency). This is because their concentrations reflect the efficiency of VK function as a cofactor for the GGCX enzyme during the synthesis of the relevant Gla protein, which in turn reflects the local availability of total bioactive VK molecules to the GGCX. Both biomarkers are based on the principle that functionally defective VK-dependent proteins are released into the bloodstream when the delivery of the VK cofactor to the GGCX is insufficient. These predominately Glu-containing molecules are part of a heterogeneous spectrum of inactive molecules termed PIVKAs. Because the sites of synthesis of factor II and OC are tissue specific, measurement of their γ-carboxylation status reflects the functional VK status in liver and bone, respectively.[31] In addition, we measured serum concentrations of VK_1 as a recognized surrogate marker of tissue stores of this vitamer.[32]

Serum VK_1 and PIVKA-II were measured by liquid chromatography–tandem mass spectrometry and chemiluminescence immunoassay (Abbott Architect), respectively.[32] For VK_1, the lower limit of detection in serum was 0.1 μg/L and the adult nonfasting reference range was 0.15–1.55 μg/L.[30,32] For PIVKA-II, the heterogeneity of circulating molecular species together with variability in their affinities to antibodies used in different assays has led to the convention of reporting values as Arbitrary Units (AU). This allows assays with differing sensitivities to be calibrated against each other. This is the first neonatal study in which we have used the Abbott Architect PIVKA-II assay, having previously used a manual in-house assay deploying the C4B6 antibody.[30,33,34] Work-up studies showed that the two assays had a high degree of correlation in the measurement range such that a PIVKA-II value of 1 AU is equivalent to 1 μg of electrophoretically pure PIVKA-II.[33] Thus, a PIVKA-II concentration of 0.05 AU/ml, representing the upper limit of the adult reference range for the Abbott method, is equivalent to a gravimetric serum PIVKA-II concentration of 50 ng/ml. This compares to a lower limit of quantification with our C4B6 antibody assay of 0.2 AU/ml (200 ng/ml). Note that the Abbott method reports results in milli (m) AU/ml, with adult reference range of 17.4–50.9 mAU/ml,[35] instead of AU/ml, which was the convention used for most previous studies.[30,33,34] Originally, Abbott developed this assay on its platform to meet a demand for PIVKA-II as a diagnostic marker of hepatocellular carcinoma in adults. The probable rationale behind the unit change from AU/ml to mAU/L is that predictive PIVKA-II concentrations for hepatocellular carcinoma are much higher than for nutritional VK insufficiency. Nevertheless, to provide historical consistency with previous studies, we continue to use the traditional PIVKA-II units of AU/ml.

Undercarboxylated and carboxylated serum osteocalcin (GluOC and GlaOC) were measured by separate immunoassays using their respective enzyme-linked immunosorbent assay kits from Takara Shuzo (Otsu, Shiga, Japan). The sum of GluOC and GlaOC concentrations was used as a measure of total OC, and the VK status of bone was evaluated by expressing the GluOC fraction as a percentage of total OC (%GluOC) as previously evaluated.[4,36,37]

2.5 | Primary outcome and definition used for functional vitamin K insufficiency

The primary outcome was the proportion of infants at ~2 months' CGA who had functional VK insufficiency, defined as a PIVKA-II concentration above the adult reference range upper limit for the Abbott method (i.e., >0.05 AU/ml).[35] Serum VK_1 concentrations were a secondary outcome. The main analysis of interest was comparison of VK status between infants subgrouped according to mode of feeding at the time of the second visit: exclusive breastfed versus exclusive formula and/or mixed fed. Any formerly exclusively breastfed infant who had received ≥1 week of complete formula milk feeds immediately preceding the final visit was categorized into the formula/mixed-fed subgroup for analysis. Our centers' previous data on postdischarge breastfeeding rates indicated that <50% of enrolled babies would likely remain exclusively breastmilk feeding by 2–3 months' CGA. Because subsequently formula/mixed-fed babies would be receiving currently recommended daily VK intakes through adequately supplemented formula milks, their ongoing inclusion despite breastfeeding attrition would by design allow a useful natural comparator group for the still-exclusively breastfeeding infants.

2.6 | Statistical analysis

For the purposes of analysis, undetectable VK_1 concentrations were imputed a value of 0.05 μg/L, equal to half the minimum detectable limit. All concentrations were nonnormally distributed and so were $\log_e$-transformed toward normality then compared using the parametric unpaired *t* test. Proportions were compared using the χ^2 and Fisher exact tests. A two-tailed *p* value <.05 was considered statistically significant.

2.7 | Ethics, consent, and sponsorship

This study had prior ethics review board approval (REC ref. 15/LO/1808). Prior written parental consent was obtained for all infants enrolled. The study was sponsored by Norfolk and Norwich University Hospitals NHS Foundation Trust.

3 | RESULTS

3.1 | Baseline characteristics

Between January 2016 and April 2018, 45 babies were enrolled and underwent a first blood sampling visit; 37 completed the study with a second blood sampling visit. Figure 1 shows study flow and Table 1 presents baseline characteristics.

3.2 | Vitamin K status predischarge and in early infancy

Table 2 shows the results of the biomarkers of VK status, age at blood sampling, and feeding mode at the time of the two study visits for the whole study cohort. Overall VK_1 concentrations were similar between the study visits (Table 2). Two babies had undetectably low VK concentrations before NICU discharge. A higher proportion of babies (16% vs. 4%) had a subnormal VK_1 concentration at the later follow-up visit at ~2 months' CGA (Table 2). Before discharge from the NICU, only one baby was VK insufficient as defined by an elevated PIVKA-II: this was a 660-g baby born at 23^{+6} gestation who had received the standard 0.4 mg/kg of VK_1 intravenously at birth, 11 days' parenteral nutrition, then 10 weeks of VK_1-fortified breastmilk feeds. At the time of sampling at 39^{+4} weeks CGA she remained exclusively fed by expressed breastmilk that had been unfortified in the previous 4 weeks; her serum PIVKA-II was slightly raised (0.08 AU/ml) and serum VK_1 was undetectable (<0.10 μg/L). One other exclusively breastmilk-fed infant had an undetectable VK_1 concentration predischarge, but normal PIVKA-II.

For the group as a whole, the prevalence of a raised PIVKA-II was significantly higher at ~2 months CGA than before discharge (24% vs. 2%, $p = .003$), but overall %GluOC values were similar between visits (Table 2).

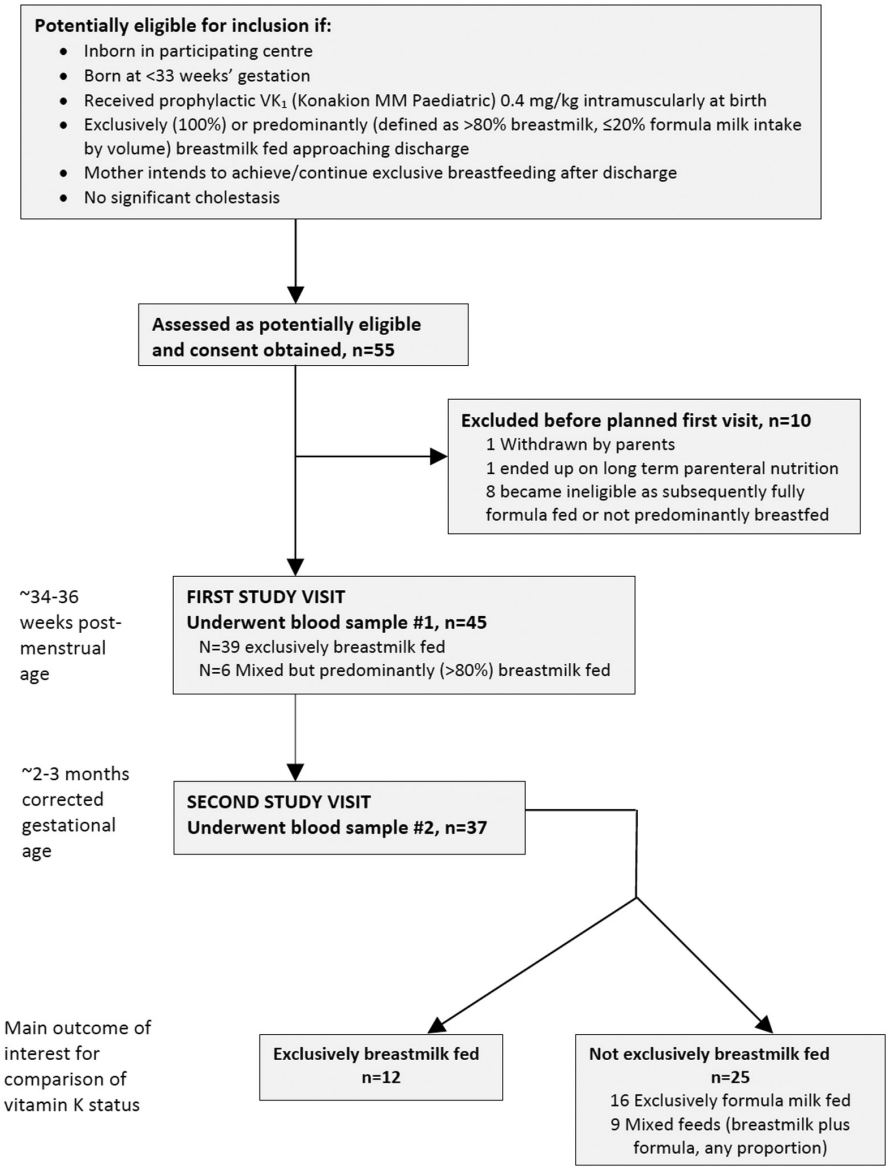

FIGURE 1 Study flow diagram

TABLE 1 Baseline characteristics of the study group, $n = 45$

Characteristic	
Birth gestational age, wk	30.0 (23.9–32.6)
Birth weight, g	1337 (549–2058)
Singleton:twin:triplet, *n*:*n*:*n*	30:13:2
Fetal growth restriction, *n*	4 (9%)
Gender male:female, *n*:*n*	20:25
Prophylactic vitamin K_1 dose at birth, mg/kg	0.40 (0.27–0.75)
Intramuscular:intravenous route, *n*:*n*	42:3
Received parenteral nutrition, *n*	32 (71%)
Days parenteral nutrition[a], *n*	10 (5–31)
Postnatal age when fully enterally fed, days	10 (2–35)

Note: Data are median (range) unless indicated.

[a]Reported for the 32 infants who received any parenteral nutrition.

3.3 | Exclusive breastmilk feeding and risk of developing vitamin K insufficiency

There was a high attrition rate for exclusive breastmilk feeding between study visits, as anticipated, falling from 87% when in the NICU down to only 32% by the time of the final visit at median ~2 months CGA (Figure 1 and Table 2). Table 3 presents the measures of VK status at the final study visit according to mode of feeding.

The primary outcome, prevalence of an elevated PIVKA-II, was much higher in babies still exclusively breastmilk fed at median CGA 2 months, present in 8 (67%) of the 12 exclusively breastfed babies compared with in only one (4%) of the 25 formula/mixed fed infants (p value <.001; Table 3). This sole formula-fed infant with evidence of VK insufficiency was an ex-29 weeks' gestation infant who, aged 5 months corrected, had a slightly raised PIVKA-II (0.13 AU/ml) but normal serum VK_1 concentration. This infant had been feeding exclusively on maternal breastmilk (providing ~0.3 μg/kg/day of VK_1) up until 2 weeks before the study visit when maternal breastfeeding had ceased and she had commenced on a (VK_1-fortified) commercial formula milk feed which then provided a daily VK_1 supplementation of ~40 μg (~6 μg/kg/day based on recent weight and milk intake).

Serum concentrations of VK_1 (commonly used as a surrogate indicator of tissue stores) were much lower in the 12 still-exclusively breastfeeding infants compared with the 25 formula/mixed-fed infants, with mean concentrations some 12-fold lower (0.15 vs. 1.81 μg/L, p <.001). Half of the exclusively breastfed babies had a serum VK_1 concentration below the normal nonfasting adult reference range versus none of the formula/mixed-fed babies (Table 3).

Total OC concentrations were similar in both breastfed and formula/mixed-fed babies (Table 3). Notably, mean total OC concentrations of these rapidly growing preterm infants were markedly higher compared with those that have been reported in older children[38] (by approximately four-fold) and adults[38] (by approximately 20-fold). Exclusively breastmilk-fed babies had higher GluOC concentrations and lower GlaOC concentrations compared with formula/mixed-fed babies (p <.001). The ratio GluOC:GlaOC was 1.86 for exclusively breastmilk-feeding infants and 0.09 for the formula/mixed-fed infants. Their large comparative disparity in OC carboxylation is best represented by %GluOC, the most accepted indicator of bone VK status, with mean values of 63.6% and 8.1% in exclusively breastmilk-fed and formula/mixed-fed babies, respectively (p <.001).

4 | DISCUSSION

4.1 | Prevalence of subclinical VK deficiency based on PIVKA-II concentrations

Our study shows that exclusively breastfeeding preterm infants develop a high prevalence of subclinical VK deficiency after hospital discharge; by age 1–3 months' CGA, the majority (67%) had a raised PIVKA-II compared with only 4% of formula/mixed-fed infants. Our findings were not unexpected, considering the low VK_1 content of mature human milk (1–2 μg/L).[7,8,24,39] An exclusively breastfed preterm infant consuming an average of 150 ml/kg/day of VK_1-unsupplemented/unfortified breastmilk receives only ~0.3 μg/kg/day of VK_1. This intake is approximately 30 times lower than current guidelines of 8–10 μg/kg/day for preterm infants,[40] including those on parenteral nutrition.[41] These recommended intakes are consistent with the estimated mean VK_1 intakes of 8–9 μg/kg/day in a cohort of term infants at 6 and 12 weeks' postnatal age, all of whom had satisfactory or elevated VK_1 concentrations when fed a formula milk containing 55 μg/L VK_1.[39] Preterm babies fed breastmilk containing the commercial bovine-derived breastmilk fortifier product added in our NICUs receive an additional 64 μg VK_1 content per liter of breastmilk and therefore met recommendations with the usual average milk intake of ~150 ml/kg/day. Most infants receive breastmilk fortifier routinely in our NICUs when fully enterally fed until discharge, explaining why VK insufficiency was rare before discharge. However, because fortifier was universally stopped at discharge, all exclusively breastfed babies subsequently relied solely on maternal milk for their ongoing VK_1 requirements. Clearly, exclusively breastfed babies failed to meet their nutritional VK requirements, evidenced by the high prevalence of biomarkers indicating subclinical VK deficiency after discharge (Table 3). In contrast, babies who received a formula/mixed-feed diet were protected against subclinical deficiency, evidenced by their supraphysiological serum VK_1 and normal PIVKA-II levels (Table 3).

4.2 | Does the high prevalence of subclinical VK deficiency matter?

4.2.1 | Interpretation of PIVKA-II values with respect to coagulation function

Although the modestly raised PIVKA-II concentrations (range: >0.05–0.19 AU/ml) do indicate early VK insufficiency, routine

TABLE 2 Biomarkers of vitamin K status with age and feeding characteristics at study visits in the complete study group

	Study visit 1, $n = 45$	Study visit 2, $n = 37$
Visit characteristics		
Corrected gestational age, completed weeks	35 [34–36]	48 [45–54]
Chronological age postnatal, days	41 (15–110) [25–61]	150 (69–238) [102–193]
Weight at visit, kg	2.040 [1.751–2.365]	4.200 [1.500–5.260]
Feeding mode at visit		
Exclusively human breastmilk	39 (87%)	12 (32%)**
Mixed breast and formula milk	6 (13%)	9 (24%)
Formula milk only	0 (0%)	16 (43%)
Started weaning diet	0 (0%)	4 (11%)
Breastmilk fortifier[a], prior days	25 [12–52]	N/A
Markers of vitamin K status		
Vitamin K_1, μg/L	1.04 (0.76, 1.41)	0.81 (0.51, 1.28)
Sub-normal K_1 concentration[b], *n*	2[c] (4%)	6 (16%)
PIVKA-II, AU/ml	0.01 (0.01, 0.02)	0.04 (0.03, 0.05)**
Raised PIVKA-II[d], *n*	1 (2%)	9 (24%)*
GluOC, ng/ml	33.0 (23.6, 46.2)[e]	28.6 (15.7, 51.9)[f]
GlaOC, ng/ml	123.5 (111.9, 136.3)[e]	107.4 (89.7, 128.7)[f]
Total OC[g], ng/ml	175.9 (164.6, 188.1)[e]	171.9 (158.0, 187.0)[f]
%GluOC of total OC	18.8 (13.8, 25.5)[e]	16.6 (9.6, 28.7)[f]

Note: Baseline data are median (range) [interquartile range] or *n* (%); concentrations are geometric means (95% confidence interval) for (log-transformed) skewed distributions.

Abbreviations: AU, arbitrary units; GlaOC, carboxylated osteocalcin; GluOC, undercarboxylated osteocalcin; OC, osteocalcin; PIVKA-II, undercarboxylated factor II.

*$p = .003$; **$p < .001$.

[a]Reported for days before visit 1 only because no baby received postdischarge human milk fortifier.

[b]Vitamin K_1 <0.15 μg/L based on K_1 reference range in healthy nonfasting adults of 0.15–1.55 μg/L.[32]

[c]Both had undetectable K_1 (<0.10 μg/L).

[d]PIVKA-II concentration >0.05 AU/ml.

[e]Data available for $n = 39$ infants.

[f]Data available for $n = 23$ infants.

[g]Total osteocalcin is the sum of GluOC and GlaOC.

coagulation indices such as the prothrombin time (PT) would not have been affected. Based on our previous studies,[30,34] PIVKA-II concentrations up to 1.0 AU/ml are considered insignificant to coagulation, whereas a value of 5.0 AU/ml is approaching the range (6.9–99.5 AU/ml) found in patients stable on warfarin therapy.[34] Thus, unlike the PIVKA-II assay, the PT is too insensitive and nonspecific for diagnosis of VK insufficiency.[42] Suttie showed that PT values remain unchanged until concentrations of plasma prothrombin fall below 50% of normal, after which the PT rises rapidly.[42] In contrast, modern PIVKA-II immunoassays can readily detect abnormal prothrombin well before any change in the PT.[42] Although a prolonged PT is a requisite diagnostic finding in VK-deficient coagulopathy, a raised PIVKA-II (up to ~5.0 AU/ml) indicates a VK-insufficient state that may progress to clinical deficiency. In overt deficiency, by which time PT has become prolonged, PIVKA-II values are invariably very high. Our previously mentioned reported case of late VKDB had a PIVKA-II concentration of 13.4 AU/ml 3 days after VK_1 treatment (concomitant with complete correction of PT), and fell to normal (0.03 AU/ml) within 3 weeks.[15] Another infant with fatal late VKDB had a PIVKA-II of 67.9 AU/ml.[43]

4.2.2 | Risk factors influencing the progression of subclinical deficiency to VKDB

It is not possible to predict whether the developmental subclinical VK deficiency we identified following hospital discharge will remain subclinical over the whole risk period for late-onset VKDB, generally considered to be up until age 6 months. Countries with intramuscular prophylaxis and active surveillance programs still report spontaneous cases of VKDB with incidence rates ~1 per 100 000 births[13,14] and higher rates for oral rather than

TABLE 3 Measures of vitamin K status according to mode of feeding at median 8 weeks' corrected age

	Exclusively breastmilk fed, $n = 12$	Formula/mixed fed, $n = 25$	p value
Vitamin K_1, μg/L	0.15 (0.09, 0.24)	1.81 (1.35, 2.44)	<.001
Subnormal VK_1 concentration[a], *n*	6 (50%)	0 (0%)	<.001
PIVKA-II, AU/ml	0.10 (0.05, 0.19)	0.02 (0.02, 0.03)	<.001
Raised PIVKA-II[b], *n*	8 (67%)	1 (4%)	.001
GluOC, ng/ml	118.5 (83.6, 168.0)[c]	13.4 (7.4, 24.1)[d]	<.001
GlaOC, ng/ml	63.7 (57.7, 70.3)[c]	142.0 (129.6, 155.5)[d]	<.001
Total OC[e], ng/ml	186.3 (155.5, 223.3)[c]	164.7 (149.5, 181.4)[d]	.15
%GluOC of total OC	63.6 (53.1, 76.1)[c]	8.1 (4.8, 13.7)[d]	<.001

Note: Data are geometric means (95% CI) for (log-transformed) skewed distributions, or *n* (%).

Abbreviations: AU, arbitrary units; GlaOC, carboxylated osteocalcin; GluOC, undercarboxylated osteocalcin; OC, osteocalcin; PIVKA-II, undercarboxylated factor II.

[a]Defined as VK_1 <0.15 μg/L based on VK_1 reference range in healthy nonfasting adults of 0.15–1.55 μg/L.[32]

[b]Primary outcome measure, defined as PIVKA-II concentration >0.05 AU/ml.

[c]Data available for $n = 8$ infants.

[d]Data available for $n = 15$ infants.

[e]Total osteocalcin is the sum of GluOC and GlaOC.

intramuscular prophylaxis.[44] Such cases raise the question of the identity of risk factors that act as triggers for VKDB. Some well-known risk factors are associated with the intestinal absorption pathway common to all four fat-soluble vitamins, specifically their dependence on bile salt-mediated luminal solubilization. The main risk factor affecting this pathway is cholestasis-induced malabsorption and, indeed, undiagnosed cholestasis is the single most reported cause of late VKDB.[7,10,12,13] Other nonspecific contributory risk factors affecting VK absorption are chronic diarrhea and persistent emesis.

Given that the time window for VKDB is fairly narrow, we also need to consider whether there are VK-specific risk factors that can explain the vulnerability of neonates to developing VKDB. One such factor is that hepatic stores of VK in early infancy are low and moreover lack the large pool of long-chain MK forms that compose the majority of liver stores in adults.[8,19,45–47] Bacterially derived MK are absent in the liver at birth and only build up slowly over weeks or months.[8,19,45–47] The implication of these findings is that the needs of infants during early life are met largely by dietary VK_1. The impact of reliance on dietary VK_1 becomes significant in circumstances where enteral intakes of VK_1 are severely restricted or where infants have any underlying pathology that may reduce the efficiency of intestinal absorption or tissue utilization of VK.[7,10] Besides low hepatic VK_1 stores, an exacerbating risk factor not found for other fat-soluble vitamins is its high turnover rate and loss through catabolism. Thus, a seminal Japanese study in adult patients fed a diet very low in VK before surgery showed that hepatic VK_1 stores fell precipitously such that two thirds of the original stores were lost within 3 days.[48] In contrast, the larger hepatic pool of long-chain MK in adults turns over at a much lower rate.[48] In neonates, a combination of the reliance on VK_1, its small body pool and high turnover rate explain how, under conditions of limited nutritional supply, a subclinical VK deficiency can progress to VKDB in a relatively short time, even in infants who received VK prophylaxis at birth.

4.3 | Value of %GluOC as a biomarker for VK status

Osteocalcin is the only skeletal Gla protein that is exclusively synthesized in bone matrix; therefore assessment of its degree of γ-carboxylation constitutes a unique measure of the VK status of bone.[2,36,37,49–51] The biomarker of bone VK status used in our study, %GluOC, has been shown to be highly responsive to dietary VK intakes in adults[51,52] and children.[53,54] Pooled data analysis from several community studies in adults show an almost linear, inverse correlation between dietary VK_1 intakes and %GluOC that was not found for GluOC alone.[37] This finding illustrates the need to correct GluOC for total OC when assessing VK status to avoid confounding effects of bone turnover independent of VK intakes.[2] Furthermore, in the same pooled analysis study, there was no correlation between dietary intakes and PIVKA-II showing that %GlucOC is a more sensitive biomarker than PIVKA-II for assessing the effects of diet on VK status.[37]

One difference between the efficiency of post-translational Gla modification during OC synthesis by bone osteoblasts compared to that during factor II synthesis by liver hepatocytes is that OC never becomes fully γ-carboxylated, even at abnormally high dietary intakes.[50] A likely explanation for this incongruity between liver and bone VK status lies with known differences in blood and tissue transport mechanisms to these organs such that the uptake and storage of VK by the liver takes precedence over extrahepatic organs.[37]

GluOC with or without GlaOC has previously been measured in third-trimester fetuses,[55] in newborns,[55,56] and in children aged 3 years and older. Shimizu et al.'s study in 18 full-term infants showed that OC in cord blood serum circulates predominately as the GluOC form.[56] However, after oral prophylaxis at birth (2 mg MK-4), by postnatal day 5 there was a dramatic replacement of GluOC by GlaOC that was entirely attributable to increased VK intakes rather than to any change in bone turnover.[56]

4.3.1 | Interpretation of %GluOC values with respect to bone health

Preterm infants are at particular risk of suboptimal early nutrition at a time of rapid bone turnover and mineral accretion.[57] However, the implications of early nutritional intakes and deficiencies for their longer term bone health are unclear.[58] To date, most studies have concentrated on nutritional intakes of minerals and vitamin D rather than on VK intakes. The high proportion of total OC as GluOC in our cohort of breastfed infants implies that exclusively breastfed preterm infants have a poor bone VK status in early infancy, and demonstrates that their bone needs are not being met by the dietary supply of VK from human milk alone during this important period of rapid growth.

Whether long-term VK insufficiency has a clinical impact on bone health is an ongoing question. Earlier epidemiological studies in adults had suggested that an elevated proportion of GluOC of total OC was an independent risk predictor of bone fracture[59–61] and of low bone mineral density (BMD).[62,63] However, an updated systematic review and meta-analysis of randomized controlled trials of VK supplementation in adults concluded that VK has no significant effect on BMD, although it may reduce clinical fractures.[64] Interestingly, this lack of effect on BMD chimes with new evidence that the mechanism of action of OC is to optimize quality and strength of bone, but not quantity.[6,65,66] This suggests that future studies should focus on effects of VK supplementation on bone strength and quality, as in fact some studies have previously reported.[67–69]

Measurements of the relative circulating concentrations of GluOC and GlaOC reported in the present study also need to be considered in the context of a hypothesis that GluOC (but not GlaOC) is a skeletal hormone that regulates glucose metabolism.[70,71] In brief, this hypothesis, based on findings from the original OC knockout mouse model, suggested that GluOC enhances β-cell proliferation, insulin secretion, and insulin sensitivity.[70,71] However, evidence for this hypothesis is lacking in the majority of human studies,[2,4] including in a recent prospective case-cohort study.[72] Importantly, no influence of GluOC on glucose control was found in two recent OC-deficient mouse models,[65,66,73] raising questions about the reproducibility of OC gene knockout technologies.

4.4 | Is routine postdischarge VK supplementation indicated for preterm infants?

All preterm infants require adequate dietary VK intakes during early infancy to prevent deficiency. The commercially manufactured formula milks contain sufficient supplementary VK (typically 60–70 μg/L of VK_1)[16] to meet currently recommended requirements; these amounts achieve adequate serum VK concentrations[39] and are known to protect against VKDB.[9,10] In stark contrast, exclusively breastmilk-fed preterm babies do not meet these recommended dietary requirements, and major North American and European scientific bodies do not currently provide specific recommendations for ongoing VK_1 supplementation during early infancy.[27–29] Although preterm babies are routinely prescribed daily multivitamin drops throughout infancy, incongruously the multivitamin preparations commonly used in United Kingdom and most other countries do not contain VK. Our data show that preterm babies who remain breastfed have a high risk of developing VK insufficiency in the months following discharge. Therefore, we recommend that preterm babies who are solely breastmilk fed and those whose mothers intend to establish full breastfeeding, should all be provided with additional daily oral VK_1 or VK_2 supplements routinely at hospital discharge. Supplementation should match the VK content of formula milks known to protect against VKDB and should continue for the duration of breastfeeding or at least until weaning. Although not all breastfed babies develop subclinical VK deficiency in early infancy, our data suggest that the majority will do so without provision of supplementary VK. Therefore, an approach of targeting the whole at-risk group at discharge would seem most logical and safest.

4.5 | Strengths and limitations

This is the first study to report on the VK status of preterm infants following NICU discharge. We achieved a high rate of study completion and showed that the risk of developing VK insufficiency was strongly associated with ongoing exclusive breastmilk feeding in early infancy. To the best of our knowledge, these are the first data reporting %GluOC assessment in preterm infants. Our data show that, in infants born preterm, both PIVKA-II and %GluOC biomarkers were highly predictive of VK status and feeding mode in early infancy (Table 3). Measurement of the efficiency of γ-carboxylation of Gla proteins provides an important way of assessing VK insufficiency well before traditional coagulation tests are ever able to detect clinically relevant VK deficiency.

Limitations include that this was a relatively small observational study using proxy biochemical markers of VK deficiency rather than clinical outcomes. With a UK VKDB incidence of ~1 in 100 000 live births,[14] only very large prospective epidemiological studies can detect the rare but important clinical outcomes of VK deficiency. We did not include measurement of PT in study babies because it is an insensitive marker for diagnosing subclinical VK deficiency, and a

prolonged PT also lacks specificity for routine VK deficiency screening in preterm infants.[74]

4.6 | Conclusion and future study

Without additional supplementation, preterm infants who remain exclusively breastmilk fed are at a high risk of developing VK insufficiency in early infancy. Routine postdischarge VK_1 supplementation is safe and should prevent subclinical VK_1 deficiency and risk of deficiency bleeding. The possible role of VK insufficiency in early infancy affecting the future bone quality and strength of breastmilk-fed preterm infants now merits investigation: the clear elevations of %GluOC that we have shown indicate that bone OC in exclusively breastfed infants is grossly undercarboxylated compared with that of formula-fed infants and healthy children and adults.

AUTHOR CONTRIBUTIONS

P. Clarke conceptualized and designed the study, wrote the protocol, obtained ethics review board approvals and funding, designed the data collection instruments, enrolled babies, provided overall research oversight, collected, analyzed, and interpreted the data, wrote the initial and final manuscript drafts, and is guarantor. M. J. Shearer contributed to protocol development, analyzed and interpreted the data, critically reviewed manuscript drafts for important intellectual content, and cowrote the final manuscript draft. D. Card contributed to protocol development and was responsible for vitamin K_1 and PIVKA-II sample analysis, validation, and quality assurance, and critically reviewed manuscript drafts for important intellectual content. A. Nichols, N. Holland, and K. Dockery undertook patient enrollment, blood sampling, and data collection. V. Ponnusamy undertook patient enrollment, blood sampling and data collection, provided research oversight at her site, and critically reviewed manuscript drafts for important intellectual content. A. Mahaveer undertook patient enrollment, blood sampling and data collection, and provided research oversight at his site. K. Voong was responsible for vitamin K_1 and PIVKA-II sample analysis and validation. S. Mulla contributed to the design of the data collection instruments and assisted with patient enrollment, blood sampling, and data collection. L. J. Hall contributed to protocol development and critically reviewed manuscript drafts for important intellectual content. C. Maassen and P. Lux were responsible for GlaOC and GluOC immunoassays, validation, and quality assurance. L. J. Schurgers contributed to protocol development, had overall responsibility for GlaOC and GluOC immunoassays, validation, and quality assurance, and critically reviewed manuscript drafts for important intellectual content. D. J. Harrington contributed to protocol development, had overall responsibility for vitamin K_1 and PIVKA-II sample analysis, validation, and quality assurance, and critically reviewed manuscript drafts for important intellectual content. All authors had access to the complete dataset, contributed to manuscript revisions, approved the final manuscript as submitted, and agree to be accountable for all aspects of the work.

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CONFLICT OF INTEREST

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