Clarke Paul (Orcid ID: 0000-0001-6203-7632)
Shearer Martin (Orcid ID: 0000-0003-2509-0818)
Card David John (Orcid ID: 0000-0002-4967-4343)
Nichols Amy (Orcid ID: 0000-0002-7657-2588)
Ponnusamy Vennila (Orcid ID: 0000-0002-1987-5531)
Mahaveer Ajit (Orcid ID: 0000-0001-9786-6860)
Voong Kieran (Orcid ID: 0000-0002-2082-0781)
Dockery Karen (Orcid ID: 0000-0002-9781-3176)
Holland Nicky (Orcid ID: 0000-0001-5553-1958)
Mulla Shaveta (Orcid ID: 0000-0002-1008-0767)
Hall Lindsay J (Orcid ID: 0000-0001-8938-5709)
Maassen Cecile (Orcid ID: 0000-0002-7071-4231)
Lux Petra (Orcid ID: 0000-0001-7269-4011)
Schurgers Leon (Orcid ID: 0000-0001-7867-6957)
Harrington Dominic (Orcid ID: 0000-0003-4786-9240)

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

Authors & Affiliations:
Paul Clarke[*,†] MD MRCP(UK) FRCPCH
Martin J Shearer[‡] PhD FRCPath
David J Card[§] MSc
Amy Nichols[*] RN(Child)
Vennila Ponnusamy[¶] PhD MBBS FRCPCH
Ajit Mahaveer[**] MD, MRCPCH
Kieran Voong[‡] MSc
Karen Dockery[**]
Nicky Holland[¶] BN(Child)
Shaveta Mulla[*] MD MRCPCH
Lindsay J Hall[††,‡‡] PhD
Cecile Maassen[§§] BSc
Petra Lux[§§] BSc
Leon J Schurgers[§§] PhD
Dominic J Harrington[§] PhD FRCPath

* Neonatal Intensive Care Unit, Norfolk & Norwich University Hospitals NHS Foundation Trust, Norwich, NR4 7UY, UK
† Norwich Medical School, University of East Anglia, Norwich, UK
‡ Centre for Haemostasis and Thrombosis, Guy's and St Thomas's NHS Foundation Trust, London, UK
§ Nutristasis Unit, Viapath, Guy's and St Thomas's NHS Foundation Trust, London, UK
¶ Neonatal Intensive Care Unit, Ashford and St Peter's Hospitals NHS Foundation Trust, Chertsey, UK.
** Neonatal Intensive Care Unit, St Mary's Hospital, Manchester University NHS Foundation Trust, Manchester, UK.
†† Gut Microbes & Health, Quadram Institute Bioscience, Norwich, UK
‡‡ ZIEL – Institute for Food & Health, Technical University of Munich, Freising, Germany
§§ Department of Biochemistry, Cardiovascular Research Institute Maastricht, Maastricht University, Maastricht, The Netherlands

Drs. Clarke and Shearer contributed equally as co-first authors

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/jth.15874

Drs. Schurgers and Harrington contributed equally as co-senior authors

Corresponding Author:
Prof. P Clarke, Consultant Neonatologist, Neonatal Intensive Care Unit, Norfolk & Norwich University Hospital NHS Foundation Trust, Norwich, NR4 7UY, UK.
ORCID ID: 0000-0001-6203-7632 Email: paul.clarke@nnuh.nhs.uk Tel: +44 1603 286342
Twitter: Paul Clarke @drpaulclarke

Short Title: Vitamin K status of breastmilk-fed preterm infants

Abbreviations
AU/mL, arbitrary units per milliliter
BMD, bone mineral density
CGA, corrected gestational age
GGCX, γ-glutamyl carboxylase
Gla, γ-carboxyglutamate
GlaOC, γ-carboxylated osteocalcin
Glu, glutamate
GluOC, undercarboxylated osteocalcin
MK-n, menaquinones; vitamin K_2
NICU, neonatal intensive care unit
OC, osteocalcin
PIVKA-II, undercarboxylated factor II
PT, prothrombin time
VK, vitamin K
VK_1, phylloquinone; vitamin K_1
VKDB, vitamin K deficiency bleeding

Word count: 4,988

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

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, multi-center, observational study in preterm infants born <33 weeks' gestation. Blood samples and dietary history were collected before hospital discharge, and post 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 post-discharge.

Results: Post 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 1 (2%) of 45 breastmilk-fed infants was VK insufficient. Post-discharge, 8 (67%) of 12 exclusively breastmilk-fed babies were VK insufficient versus only 1 (4%) of 25 formula/mixed-fed babies.

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

Key Words: Vitamin K Deficiency; Infant; Nutrition; Vitamin K1; Hemorrhage

1 INTRODUCTION

Vitamin K (VK) is an essential fat-soluble micronutrient which 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 non-collagenous 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 which 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 defciency bleeding (VKDB) of early infancy.[7-9] The progression from a hypocoagulable state to VKDB is highly individual and unpredictable. Worringly, 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 which 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 subsquently 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 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 prior to Neonatal Intensive Care Unit (NICU) discharge despite prophylaxis at birth and no other risk factors.[15]

Naturally-occurring K vitamins are phylloquinone (vitamin K_1; VK_1) and menaquinones (vitamin K_2; MK-n).[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 MK family, only the non-bacterial 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] While 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. 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: i) undercarboxylated factor II (PIVKA-II), reflecting functional hepatic insufficiency; ii) 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, multi-center study involving four U.K. 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' post-menstrual age), and whose mothers intended to continue or establish full breastfeeding post 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: i) baseline (pre-discharge), at ~36 weeks post-menstrual age; ii) at ~2 months corrected gestational age (CGA), i.e. at ~2 months post 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 historically termed 'Proteins Induced by VK Absence or Antagonism' (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 adult non-fasting 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 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 their 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 ELISA 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 sub-grouped 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 post-discharge breastfeeding rates indicated that <50% of enrolled babies would likely remain exclusively breastmilk feeding by 2-3 months CGA. As 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 non-normally distributed and so were $\log_e$-transformed towards normality then compared using the parametric unpaired t test. Proportions were compared using the χ^2 and Fisher's exact tests. A 2-tailed P-value <0.05 was considered statistically significant.

2.7 Ethics and Consent

This study had prior ethics review board approval (REC ref. 15/LO/1808). Prior written parental consent was obtained for all infants enrolled.

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 pre-discharge 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 prior to NICU discharge. A higher proportion of babies (16% vs. 4%) had a sub-normal VK_1 concentration at the later follow-up visit at ~2 months CGA (Table 2). Prior to 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 pre-discharge, 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-value=0.003), but overall %GluOC values were similar between visits (Table 2).

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 1 (4%) of the 25 formula/mixed fed infants (P-value <0.001; Table 3). This sole formula-fed infant with evidence of VK insufficiency was an ex-29 week 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 prior to the study visit when maternal breastfeeding had ceased and she commenced 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 in the 25 formula/mixed-fed infants, with mean concentrations some 12-fold lower (0.15 vs. 1.81 μg/L, p-value <0.001). Half of the exclusively-breastfed babies had a serum VK_1 concentration below the normal non-fasting 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 to formula/mixed-fed babies (p-values <0.001). 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-value <0.001).

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 to 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 *thirty 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 prior to 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 post 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 While the modestly raised PIVKA-II concentrations (range: >0.05-0.19 AU/mL) do indicate early VK insufficiency, routine 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] While 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 which may progress to clinical deficiency. In overt deficiency, by which time PT has become prolonged, PIVKA-II values are invariably very high. Our aforementioned reported case of late VKDB, had a PIVKA-II concentration of 13.4 AU/mL 3 days post 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 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 which comprise 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

OC is the only skeletal Gla protein that is exclusively synthesized in bone matrix, and 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 which 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 above.[38] 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 which 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 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 of the reproducibility of OC gene knockout technologies.

4.4 Is routine post-discharge 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, 74] 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] While preterm babies are routinely prescribed daily multivitamin drops throughout infancy, incongruously the multivitamin preparations commonly used in U.K. 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 content of formula milks known to protect against VKDB and should continue for the duration of breastfeeding or at least until weaning. While 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 U.K. 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.[75]

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 post-discharge 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 to that of formula-fed infants and healthy children and adults.

Author Contribution

P. Clarke conceptualized and designed the study, wrote the protocol, obtained ethics review board approvals, 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 co-wrote 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 enrolment, blood sampling and data collection.
V. Ponnusamy undertook patient enrolment, blood sampling and data collection, provided research oversight at her site, and critically reviewed manuscript drafts for important intellectual content.
A. Mahaveer undertook patient enrolment, 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 enrolment, 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.

Drs. P. Clarke and M.J. Shearer contributed equally as co-first authors
Drs. L. Schurgers and D.J. Harrington contributed equally as co-senior authors

Acknowledgements:
The authors thank all the parents who generously allowed their babies to be studied; also, all doctors, advanced neonatal nurse practitioners, and research/clinical nurses who kindly assisted sample collection. We acknowledge the kind help of Dr Priyadarshan Ambadkar for supporting the study at James Paget Hospital and Nutricia (U.K.) Ltd for supporting some study costs. Prof. Clarke sincerely thanks Prof. Mark A Turner for valuable guidance which helped conceive this study, Prof. Simon J Mitchell for his first conception of preterm vitamin K studies, and Rebecca and Xavier Clarke for manuscript proofing. The authors thank the anonymous reviewers for helpful comments on an earlier version of our manuscript.

Conflict of Interest: The authors have no conflicts of interest relevant to this article to disclose.

Funding/Support. Danone Nutricia Early Life Nutrition provided a small study grant which supported the travel expenses of parents and the courier costs of transport of specimens to the laboratories. Nutricia Ltd had no other role or involvement in this project and had no input into study design, data interpretation or publication of these results. Other study costs were met by the Norfolk and Norwich University Hospital Neonatal Unit Research Fund.

REFERENCES

1 Berkner KL, Runge KW. The physiology of vitamin K nutriture and vitamin K-dependent protein functions in atherosclerosis. *J Thromb Haemost*. 2004; **2**: 2118-32. doi:10.1111/j.1538-7836.2004.00968.x.

2 Gundberg CM, Lian JB, Booth SL. Vitamin K-dependent carboxylation of osteocalcin: friend or foe? *Adv Nutr*. 2012; **3**: 149-57. doi:10.3945/an.112.001834.

3 Schurgers LJ, Uitto J, Reutelingsperger CP. Vitamin K-dependent carboxylation of matrix Gla-protein: a crucial switch to control ectopic mineralization. *Trends Mol Med*. 2013; **19**: 217-26. doi:10.1016/j.molmed.2012.12.008.

4 Booth SL, Centi A, Smith SR, Gundberg C. The role of osteocalcin in human glucose metabolism: marker or mediator? *Nat Rev Endocrinol*. 2013; **9**: 43-55. doi:10.1038/nrendo.2012.201.

5 Poundarik AA, Boskey A, Gundberg C, Vashishth D. Biomolecular regulation, composition and nanoarchitecture of bone mineral. *Sci Rep*. 2018; **8**: 1191. doi:10.1038/s41598-018-19253-w.

6 Moriishi T, Ozasa R, Ishimoto T, Nakano T, Hasegawa T, Miyazaki T, Liu W, Fukuyama R, Wang Y, Komori H, Qin X, Amizuka N, Komori T. Osteocalcin is necessary for the alignment of apatite crystallites, but not glucose metabolism, testosterone synthesis, or muscle mass. *PLoS Genet*. 2020; **16**: e1008586. doi:10.1371/journal.pgen.1008586.

7 Shearer MJ. Vitamin K deficiency bleeding (VKDB) in early infancy. *Blood Rev*. 2009; **23**: 49-59. doi:10.1016/j.blre.2008.06.001.

8 Shearer MJ, Clarke P. Vitamin K Metabolism in the Fetus and Neonate. In: Polin R, Abman S, Rowitch D, Benitz W, eds. *Fetal and Neonatal Physiology* 6th edn: Elsevier, 2021, 303-10.

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 Loughnan PM, McDougall PN. Epidemiology of late onset haemorrhagic disease: a pooled data analysis. *J Paediatr Child Health*. 1993; **29**: 177-81. doi:10.1111/j.1440-1754.1993.tb00480.x.

11 Clarke P. Vitamin K prophylaxis for preterm infants. *Early Hum Dev*. 2010; **86 Suppl 1**: 17-20. doi:10.1016/j.earlhumdev.2010.01.013.

12 Sutor AH, Dagres N, Niederhoff H. Late form of vitamin K deficiency bleeding in Germany. *Klin Padiatr*. 1995; **207**: 89-97. doi:10.1055/s-2008-1046519.

13 Zurynski Y, Grover CJ, Jalaludin B, Elliott EJ. Vitamin K deficiency bleeding in Australian infants 1993-2017: an Australian Paediatric Surveillance Unit study. *Arch Dis Child*. 2020; **105**: 433-8. doi:10.1136/archdischild-2018-316424.

14 Busfield A, Samuel R, McNinch A, Tripp JH. Vitamin K deficiency bleeding after NICE guidance and withdrawal of Konakion Neonatal: British Paediatric Surveillance Unit study, 2006-2008. *Arch Dis Child*. 2013; **98**: 41-7. doi:10.1136/archdischild-2011-301029.

15 Bryson S PA, Mulla S, Card D, Shearer MJ, Clarke P, Vasu V. Late onset vitamin K deficiency bleeding, extreme prematurity and a human milk based diet. [Poster Presentation]. 3rd Congress of joint European Neonatal Societies (jENS 2019); Maastricht (Netherlands). 18-21 September 2019.

16 Clarke P, Mitchell SJ, Shearer MJ. Total and Differential Phylloquinone (Vitamin K1) Intakes of Preterm Infants from All Sources during the Neonatal Period. *Nutrients*. 2015; **7**: 8308-20. doi:10.3390/nu7105393.

17 Thijssen HH, Drittij MJ, Vermeer C, Schoffelen E. Menaquinone-4 in breast milk is derived from dietary phylloquinone. *Br J Nutr*. 2002; **87**: 219-26. doi:10.1079/BJNBJN2001505.
18 Ellis JL, Fu X, Karl JP, Hernandez CJ, Mason JB, DeBose-Boyd RA, Booth SL. Multiple Dietary Vitamin K Forms Are Converted to Tissue Menaquinone-4 in Mice. *J Nutr*. 2021. doi:10.1093/jn/nxab332.
19 Shearer MJ. Vitamin K metabolism and nutriture. *Blood Rev*. 1992; **6**: 92-104.
20 Lapillonne A, Bronsky J, Campoy C, Embleton N, Fewtrell M, Fidler Mis N, Gerasimidis K, Hojsak I, Hulst J, Indrio F, Molgaard C, Moltu SJ, Verduci E, Domellof M, Nutrition ECo. Feeding the Late and Moderately Preterm Infant: A Position Paper of the European Society for Paediatric Gastroenterology, Hepatology and Nutrition Committee on Nutrition. *J Pediatr Gastroenterol Nutr*. 2019; **69**: 259-70. doi:10.1097/MPG.0000000000002397.
21 Parker MG, Stellwagen LM, Noble L, Kim JH, Poindexter BB, Puopolo KM, Section On Breastfeeding CONCOF, Newborn. Promoting Human Milk and Breastfeeding for the Very Low Birth Weight Infant. *Pediatrics*. 2021; **148**. doi:10.1542/peds.2021-054272.
22 Boquien CY. Human Milk: An Ideal Food for Nutrition of Preterm Newborn. *Front Pediatr*. 2018; **6**: 295. doi:10.3389/fped.2018.00295.
23 Arslanoglu S, Boquien CY, King C, Lamireau D, Tonetto P, Barnett D, Bertino E, Gaya A, Gebauer C, Grovslien A, Moro GE, Weaver G, Wesolowska AM, Picaud JC. Fortification of Human Milk for Preterm Infants: Update and Recommendations of the European Milk Bank Association (EMBA) Working Group on Human Milk Fortification. *Front Pediatr*. 2019; **7**: 76. doi:10.3389/fped.2019.00076.
24 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. doi:10.1203/00006450-198711000-00007.
25 Indyk HE, Woollard DC. Vitamin K in milk and infant formulas: determination and distribution of phylloquinone and menaquinone-4. *Analyst*. 1997; **122**: 465-9. doi:10.1039/a608221a.
26 Shearer M, Harvey J, Hodges S, Savidge G. Raised undercarboxylated prothrombin (PIVKA-II) in healthy 2-5 month-old infants shows evidence of subclinical vitamin K deficiency which is related to duration of breast feeding. [Abstract]. *Blood*. 2001; **98**: 530a.
27 Ng E, Loewy AD. Guidelines for vitamin K prophylaxis in newborns. *Paediatr Child Health*. 2018; **23**: 394-402. doi:10.1093/pch/pxy082.
28 Hand I, Noble L, Abrams SA. Vitamin K and the Newborn Infant. *Pediatrics*. 2022; **149**. doi:10.1542/peds.2021-056036.
29 Mihatsch WA, Braegger C, Bronsky J, Campoy C, Domellof M, Fewtrell M, Mis NF, Hojsak I, Hulst J, Indrio F, Lapillonne A, Mlgaard C, Embleton N, van Goudoever J, Nutrition ECo. Prevention of Vitamin K Deficiency Bleeding in Newborn Infants: A Position Paper by the ESPGHAN Committee on Nutrition. *J Pediatr Gastroenterol Nutr*. 2016; **63**: 123-9. doi:10.1097/MPG.0000000000001232.
30 Clarke P, Mitchell SJ, Wynn R, Sundaram S, Speed V, Gardener E, Roeves D, Shearer MJ. Vitamin K prophylaxis for preterm infants: a randomized, controlled trial of 3 regimens. *Pediatrics*. 2006; **118**: e1657-66. doi:10.1542/peds.2005-2742.
31 Vermeer C, Shearer MJ, Zittermann A, Bolton-Smith C, Szulc P, Hodges S, Walter P, Rambeck W, Stocklin E, Weber P. Beyond deficiency: potential benefits of increased intakes of vitamin K for bone and vascular health. *Eur J Nutr*. 2004; **43**: 325-35. doi:10.1007/s00394-004-0480-4.

32 Card DJ, Gorska R, Harrington DJ. Laboratory assessment of vitamin K status. *J Clin Pathol*. 2020; **73**: 70-5. doi:10.1136/jclinpath-2019-205997.
33 Belle M, Brebant R, Guinet R, Leclercq M. Production of a new monoclonal antibody specific to human des-gamma-carboxyprothrombin in the presence of calcium ions. Application to the development of a sensitive ELISA-test. *J Immunoassay*. 1995; **16**: 213-29. doi:10.1080/15321819508013559.
34 Chuansumrit A, Plueksacheeva T, Hanpinitsak S, Sangwarn S, Chatvutinun S, Suthutvoravut U, Herabutya Y, Shearer MJ. Prevalence of subclinical vitamin K deficiency in Thai newborns: relationship to maternal phylloquinone intakes and delivery risk. *Arch Dis Child Fetal Neonatal Ed*. 2010; **95**: F104-8. doi:10.1136/adc.2009.173245.
35 Ko DH, Hyun J, Kim HS, Park MJ, Kim JS, Park JY, Shin DH, Cho HC. Analytical and Clinical Performance Evaluation of the Abbott Architect PIVKA Assay. *Ann Clin Lab Sci*. 2018; **48**: 75-80.
36 Gundberg CM, Nieman SD, Abrams S, Rosen H. Vitamin K status and bone health: an analysis of methods for determination of undercarboxylated osteocalcin. *J Clin Endocrinol Metab*. 1998; **83**: 3258-66. doi:10.1210/jcem.83.9.5126.
37 Shea MK, Booth SL. Concepts and Controversies in Evaluating Vitamin K Status in Population-Based Studies. *Nutrients*. 2016; **8**. doi:10.3390/nu8010008.
38 van Summeren M, Braam L, Noirt F, Kuis W, Vermeer C. Pronounced elevation of undercarboxylated osteocalcin in healthy children. *Pediatr Res*. 2007; **61**: 366-70. doi:10.1203/pdr.0b013e318030d0b1.
39 Greer FR, Marshall S, Cherry J, Suttie JW. Vitamin K status of lactating mothers, human milk, and breast-feeding infants. *Pediatrics*. 1991; **88**: 751-6.
40 Tsang RC. *Nutrition of the Preterm Infant: Scientific Basis and Practical Guidelines.* Ohio, USA: Digital Educational Publishing, 2005.
41 Bronsky J, Campoy C, Braegger C, ESPGHAN/ESPEN/ESPR/CSPEN working group on pediatric parenteral nutrition. ESPGHAN/ESPEN/ESPR/CSPEN guidelines on pediatric parenteral nutrition: Vitamins. *Clin Nutr*. 2018; **37**: 2366-78. doi:10.1016/j.clnu.2018.06.951.
42 Suttie JW. Vitamin K and human nutrition. *J Am Diet Assoc*. 1992; **92**: 585-90.
43 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. doi:10.1097/00005176-199911000-00023.
44 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. doi:10.1007/s00431-007-0443-x.
45 McCarthy P, Shearer MJ, Gau G, Crampton OE, Barkhan P. Vitamin K content of human liver at different ages. [Abstract]. *Haemostasis*. 1986; **16**: 84-5. doi:10.1159/000317803.
46 Kayata S, Kindberg C, Greer FR, Suttie JW. Vitamin K1 and K2 in infant human liver. *J Pediatr Gastroenterol Nutr*. 1989; **8**: 304-7. doi:10.1097/00005176-198904000-00007.
47 Suttie JW. The importance of menaquinones in human nutrition. *Annu Rev Nutr*. 1995; **15**: 399-417. doi:10.1146/annurev.nu.15.070195.002151.
48 Usui Y, Tanimura H, Nishimura N, Kobayashi N, Okanoue T, Ozawa K. Vitamin K concentrations in the plasma and liver of surgical patients. *Am J Clin Nutr*. 1990; **51**: 846-52. doi:10.1093/ajcn/51.5.846.
49 Binkley NC, Krueger DC, Engelke JA, Foley AL, Suttie JW. Vitamin K supplementation reduces serum concentrations of under-gamma-carboxylated osteocalcin in

healthy young and elderly adults. *Am J Clin Nutr*. 2000; **72**: 1523-8. doi:10.1093/ajcn/72.6.1523.

50 Binkley NC, Krueger DC, Kawahara TN, Engelke JA, Chappell RJ, Suttie JW. A high phylloquinone intake is required to achieve maximal osteocalcin gamma-carboxylation. *Am J Clin Nutr*. 2002; **76**: 1055-60. doi:10.1093/ajcn/76.5.1055.

51 Sokoll LJ, Booth SL, O'Brien ME, Davidson KW, Tsaioun KI, Sadowski JA. Changes in serum osteocalcin, plasma phylloquinone, and urinary gamma-carboxyglutamic acid in response to altered intakes of dietary phylloquinone in human subjects. *Am J Clin Nutr*. 1997; **65**: 779-84. doi:10.1093/ajcn/65.3.779.

52 Bolton-Smith C, McMurdo ME, Paterson CR, Mole PA, Harvey JM, Fenton ST, Prynne CJ, Mishra GD, Shearer MJ. Two-year randomized controlled trial of vitamin K1 (phylloquinone) and vitamin D3 plus calcium on the bone health of older women. *J Bone Miner Res*. 2007; **22**: 509-19. doi:10.1359/jbmr.070116.

53 van Summeren MJ, Braam LA, Lilien MR, Schurgers LJ, Kuis W, Vermeer C. The effect of menaquinone-7 (vitamin K2) supplementation on osteocalcin carboxylation in healthy prepubertal children. *Br J Nutr*. 2009; **102**: 1171-8. doi:10.1017/S0007114509382100.

54 van Summeren MJ, van Coeverden SC, Schurgers LJ, Braam LA, Noirt F, Uiterwaal CS, Kuis W, Vermeer C. Vitamin K status is associated with childhood bone mineral content. *Br J Nutr*. 2008; **100**: 852-8. doi:10.1017/S0007114508921760.

55 Briana DD, Gourgiotis D, Georgiadis A, Boutsikou M, Baka S, Marmarinos A, Hassiakos D, Malamitsi-Puchner A. Intrauterine growth restriction may not suppress bone formation at term, as indicated by circulating concentrations of undercarboxylated osteocalcin and Dickkopf-1. *Metabolism*. 2012; **61**: 335-40. doi:10.1016/j.metabol.2011.07.008.

56 Shimizu N, Shima M, Hirai H, Nakajima S, Nishimura K, Yamaoka K, Okada S. Shift of serum osteocalcin components between cord blood and blood at day 5 of life. *Pediatr Res*. 2002; **52**: 656-9. doi:10.1203/00006450-200211000-00009.

57 Fewtrell M. Early nutritional predictors of long-term bone health in preterm infants. *Curr Opin Clin Nutr Metab Care*. 2011; **14**: 297-301. doi:10.1097/MCO.0b013e328345361b.

58 Fewtrell MS. Does early nutrition program later bone health in preterm infants? *Am J Clin Nutr*. 2011; **94**: 1870S-3S. doi:10.3945/ajcn.110.000844.

59 Szulc P, Chapuy MC, Meunier PJ, Delmas PD. Serum undercarboxylated osteocalcin is a marker of the risk of hip fracture: a three year follow-up study. *Bone*. 1996; **18**: 487-8. doi:10.1016/8756-3282(96)00037-3.

60 Vergnaud P, Garnero P, Meunier PJ, Breart G, Kamihagi K, Delmas PD. Undercarboxylated osteocalcin measured with a specific immunoassay predicts hip fracture in elderly women: the EPIDOS Study. *J Clin Endocrinol Metab*. 1997; **82**: 719-24. doi:10.1210/jcem.82.3.3805.

61 Luukinen H, Kakonen SM, Pettersson K, Koski K, Laippala P, Lovgren T, Kivela SL, Vaananen HK. Strong prediction of fractures among older adults by the ratio of carboxylated to total serum osteocalcin. *J Bone Miner Res*. 2000; **15**: 2473-8. doi:10.1359/jbmr.2000.15.12.2473.

62 Szulc P, Arlot M, Chapuy MC, Duboeuf F, Meunier PJ, Delmas PD. Serum undercarboxylated osteocalcin correlates with hip bone mineral density in elderly women. *J Bone Miner Res*. 1994; **9**: 1591-5. doi:10.1002/jbmr.5650091012.

63 Knapen MH, Nieuwenhuijzen Kruseman AC, Wouters RS, Vermeer C. Correlation of serum osteocalcin fractions with bone mineral density in women during the first 10 years after menopause. *Calcif Tissue Int*. 1998; **63**: 375-9. doi:10.1007/s002239900543.

64 Mott A, Bradley T, Wright K, Cockayne ES, Shearer MJ, Adamson J, Lanham-New SA, Torgerson DJ. Effect of vitamin K on bone mineral density and fractures in adults: an updated systematic review and meta-analysis of randomised controlled trials. *Osteoporos Int*. 2019; **30**: 1543-59. doi:10.1007/s00198-019-04949-0.
65 Diegel CR, Hann S, Ayturk UM, Hu JCW, Lim KE, Droscha CJ, Madaj ZB, Foxa GE, Izaguirre I, Transgenics Core VVA, Paracha N, Pidhaynyy B, Dowd TL, Robling AG, Warman ML, Williams BO. An osteocalcin-deficient mouse strain without endocrine abnormalities. *PLoS Genet*. 2020; **16**: e1008361. doi:10.1371/journal.pgen.1008361.
66 Manolagas SC. Osteocalcin promotes bone mineralization but is not a hormone. *PLoS Genet*. 2020; **16**: e1008714. doi:10.1371/journal.pgen.1008714.
67 Knapen MH, Schurgers LJ, Vermeer C. Vitamin K2 supplementation improves hip bone geometry and bone strength indices in postmenopausal women. *Osteoporos Int*. 2007; **18**: 963-72. doi:10.1007/s00198-007-0337-9.
68 Tanaka N, Arima K, Nishimura T, Tomita Y, Mizukami S, Okabe T, Abe Y, Kawashiri SY, Uchiyama M, Honda Y, Tsujimoto R, Kanagae M, Osaki M, Aoyagi K. Vitamin K deficiency, evaluated with higher serum ucOC, was correlated with poor bone status in women. *J Physiol Anthropol*. 2020; **39**: 9. doi:10.1186/s40101-020-00221-1.
69 Suzuki Y, Maruyama-Nagao A, Sakuraba K, Kawai S. Level of serum undercarboxylated osteocalcin correlates with bone quality assessed by calcaneal quantitative ultrasound sonometry in young Japanese females. *Exp Ther Med*. 2017; **13**: 1937-43. doi:10.3892/etm.2017.4206.
70 Ferron M, Hinoi E, Karsenty G, Ducy P. Osteocalcin differentially regulates beta cell and adipocyte gene expression and affects the development of metabolic diseases in wild-type mice. *Proc Natl Acad Sci U S A*. 2008; **105**: 5266-70. doi:10.1073/pnas.0711119105.
71 Lee NK, Sowa H, Hinoi E, Ferron M, Ahn JD, Confavreux C, Dacquin R, Mee PJ, McKee MD, Jung DY, Zhang Z, Kim JK, Mauvais-Jarvis F, Ducy P, Karsenty G. Endocrine regulation of energy metabolism by the skeleton. *Cell*. 2007; **130**: 456-69. doi:10.1016/j.cell.2007.05.047.
72 Babey ME, Ewing SK, Strotmeyer ES, Napoli N, Schafer AL, Vittinghoff E, Gundberg CM, Schwartz AV. No Evidence of Association Between Undercarboxylated Osteocalcin and Incident Type 2 Diabetes. *J Bone Miner Res*. 2022. doi:10.1002/jbmr.4519.
73 Moriishi T, Komori T. Lack of reproducibility in osteocalcin-deficient mice. *PLoS Genet*. 2020; **16**: e1008939. doi:10.1371/journal.pgen.1008939.
74 Loughnan PM, McDougall PN. Epidemiology of late onset haemorrhagic disease: a pooled data analysis. *J Paediatr Child Health*. 1993; **29**: 177-81. doi:10.1111/j.1440-1754.1993.tb00480.x.
75 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**: 1822-4. doi:10.1111/j.1651-2227.2005.tb01859.x.

Table 1 Baseline characteristics of the study group, n=45

Birth gestational age, weeks	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 VK_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*, n	10 (5-31)
Postnatal age when fully enterally fed, days	10 (2-35)

Data are median (range) unless indicated
*reported for the 32 infants who received any parenteral nutrition

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]

Baseline data are median (range) [interquartile range] or n (%); concentrations are geometric means (95% CI) for (log-transformed) skewed distributions.
PIVKA-II, undercarboxylated factor II; OC, osteocalcin; GluOC, undercarboxylated osteocalcin; GlaOC, carboxylated osteocalcin.
[a] reported for days prior to visit 1 only as no baby received post-discharge human milk fortifier
[b] Vitamin K_1 <0.15 µg/L based on K_1 reference range in healthy non fasting 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
*P-value = 0.003; **P-value <0.001

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)	<0.001
Sub-normal VK_1 concentration[a], n	6 (50%)	0 (0%)	<0.001
PIVKA-II, AU/mL	0.10 (0.05, 0.19)	0.02 (0.02, 0.03)	<0.001
Raised PIVKA-II[b], n	8 (67%)	1 (4%)	0.001
GluOC, ng/mL	118.5 (83.6, 168.0)[c]	13.4 (7.4, 24.1)[d]	<0.001
GlaOC, ng/mL	63.7 (57.7, 70.3)[c]	142.0 (129.6, 155.5)[d]	<0.001
Total OC[e], ng/mL	186.3 (155.5, 223.3)[c]	164.7 (149.5, 181.4)[d]	0.15
%GluOC of total OC	63.6 (53.1, 76.1)[c]	8.1 (4.8, 13.7)[d]	<0.001

Data are geometric means (95% CI) for (log-transformed) skewed distributions, or n (%)
PIVKA-II, undercarboxylated factor II; OC, osteocalcin; GluOC, undercarboxylated osteocalcin; GlaOC, carboxylated osteocalcin.

[a] Defined as VK_1 <0.15 µg/L based on VK_1 reference range in healthy non-fasting 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

Figure 1 Legend: Study flow diagram

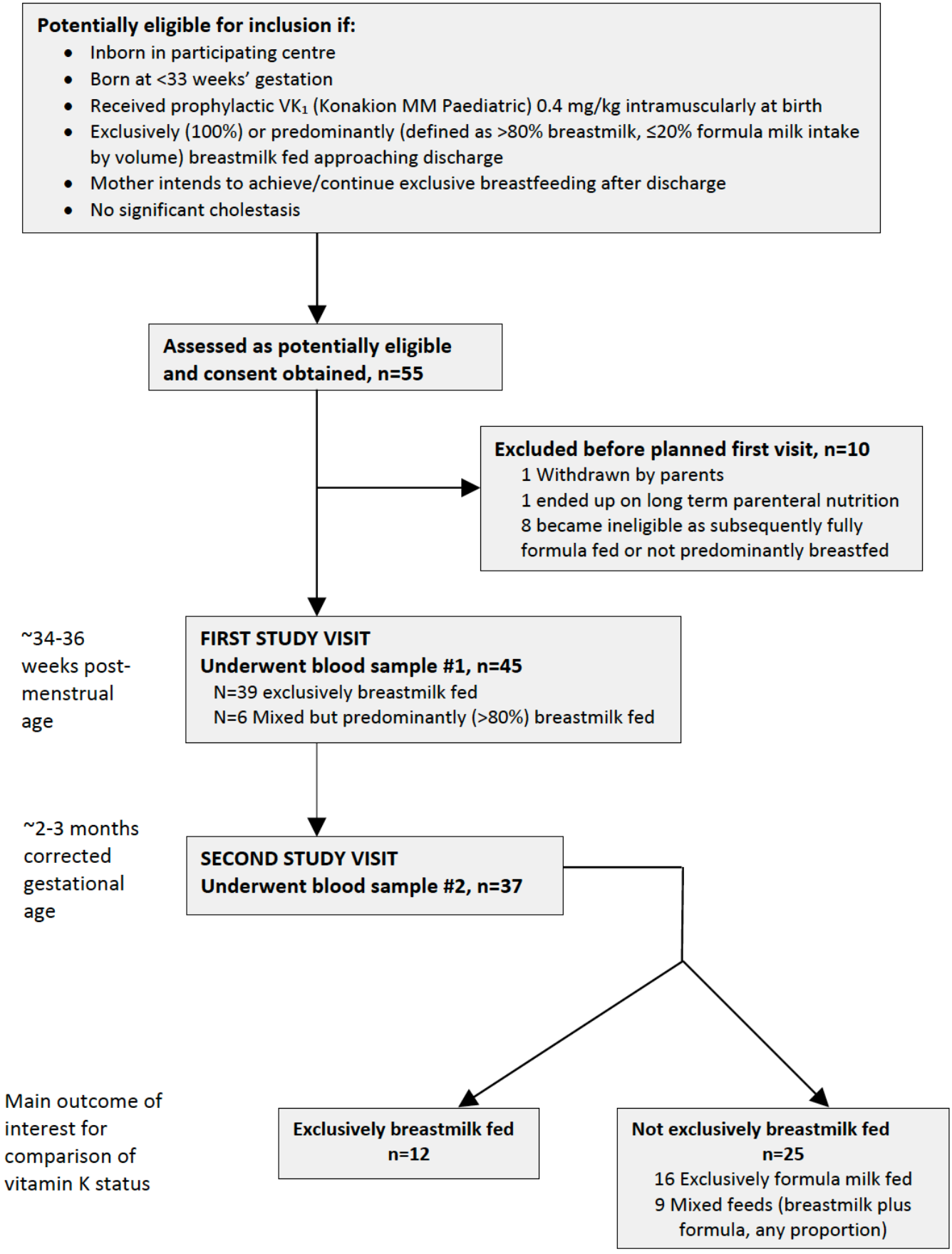

Figure 1: Study flow diagram