

Oral vitamin K2 (menaquinone 7; MK-7) supplementation and effect on vitamin K status of breastfed term infants in early infancy; a four-cohort prospective, part-randomised, part-blinded study.

The OK Daily Study



Protocol Version 1.1

24th April 2026

FULL TITLE OF THE STUDY

Oral vitamin K2 (menaquinone 7; MK-7) supplementation and effect on vitamin K status of breastfed term infants in early infancy; a four-cohort prospective, part-randomised, part-blinded study.

SHORT STUDY TITLE / ACRONYM

'OK Daily Study' – Oral Vitamin K Daily Study

PROTOCOL VERSION NUMBER AND DATE

Version 1.1, 24th April 2026

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SIGNATURE PAGE

The undersigned confirm that the following protocol has been agreed and accepted and that the Chief Investigators agree to conduct the study in compliance with the approved protocol and will adhere to the principles outlined in the Declaration of Helsinki, the Sponsor's SOPs, and other regulatory requirement.

We agree to ensure that the confidential information contained in this document will not be used for any other purpose other than the evaluation or conduct of the investigation without the prior written consent of the Sponsor

We also confirm that we will make the findings of the study publicly available through publication or other dissemination tools without any unnecessary delay and that an honest accurate and transparent account of the study will be given; and that any discrepancies from the study as planned in this protocol will be explained.

For and on behalf of the Study Sponsor:

Signature:

Date:

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Name (please print):

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Position:

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Chief Investigator:

Signature:

Date:

...../...../.....

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Name: (please print):

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Joint Chief Investigator:

Signature:

Date:

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Name: (please print):

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Funder(s)	PharmaLinea Ltd. (commercial partner) Norfolk and Norwich University Hospital NICU Research Fund Norfolk and Norwich University Hospital Charitable Fund
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STUDY SUMMARY

Study Title	Oral vitamin K2 (menaquinone 7; MK-7) supplementation and effect on vitamin K status of breastfed term infants in early infancy; a four-cohort prospective, part-randomised, part-blinded study
Short Title	The OK Daily Study
Study Design	Four cohort, prospective, part-randomised, part-blinded study
Study Participants	Babies born ≥ 37 weeks gestational age and their mothers.
Number of Subjects	~134 infant and mother dyads would be recruited to three groups (four cohorts) to follow up a minimum of 77 mother/infant dyads. This will achieve 80% power for the primary outcome (biochemical deficiency defined as a PIVKA-II >0.05 AU/mL) compared between supplemented and unsupplemented infants (group 1).
Hypothesis	Daily oral vitamin K2 in the form of menaquinone-7 (MK-7) supplementation of infants or their mothers will significantly reduce biochemical vitamin K deficiency in breastfed infants at 2-3 months postnatal age.

Aims, Outcomes and Outcome Measures:

	Aim	Outcome Measure(s)
Primary	To examine the efficacy of daily oral MK-7 dietary supplementation for the reduction of sub-clinical vitamin K deficiency in early infancy.	Proportion of infants who have a PIVKA-II concentration of >0.05 AU/mL at ~ 2 months postnatal age in the supplemented versus placebo cohorts in group 1.
Secondary	I To compare the relative efficacy of maternal oral MK-7 dietary supplementation versus infant daily oral MK-7 dietary supplementation for the reduction of sub-clinical vitamin K deficiency in infants in early infancy	i) Proportion of infants with a PIVKA-II concentration of >0.05 AU/mL at age ~ 2 months in the MK-7 maternal supplemented group versus infant MK-7 supplemented cohort. ii) Percentage undercarboxylated osteocalcin as a proportion of total osteocalcin (%GluOC) in infants in the MK-7 maternal supplemented group versus infant MK-7 supplemented cohort at age ~ 2 months. iii) Comparative serum vitamin K concentrations of infants at ~ 2 months postnatal in the maternal MK-7 supplemented infant group versus infant MK-7 supplemented cohort at age ~ 2 months. iv) Comparative desphospho-uncarboxylated matrix Gla-protein (dp-ucMGP) concentrations of infants at ~ 2 months in the maternal MK-7 supplemented infant group versus the infant MK-7 supplemented cohort at age ~ 2 months.
	II To examine the utility of maternal oral supplementation with MK-7 for the reduction of subclinical vitamin K deficiency in breastfed infants.	i) Proportion of infants in the maternal MK-7 supplemented group with a PIVKA-II concentration of >0.05 AU/mL at age ~ 2 months. ii) Comparison of proportions of infants with a PIVKA-II concentration of >0.05 AU/mL at age ~ 2 months in the maternal MK-7 supplemented group versus infants in the placebo cohort. iii) Comparison of %GluOC in the maternal MK-7 supplemented group versus infants in the placebo cohort at age ~ 2 months. iv) Comparative dp-ucMGP concentrations of infants in the maternal MK-7 supplemented infant group versus the infant placebo cohort at age ~ 2 months.
	III To compare daily MK-7 dietary supplementation of breastfed infants with that of vitamin K1 supplementation via formula milk fed infants for the reduction of sub-	i) Comparison of proportions of infants with a PIVKA-II concentration of >0.05 AU/mL at age ~ 2 months in the infant MK-7 supplemented group versus formula fed infant group.

		clinical vitamin K deficiency in early infancy.	ii) Comparison of %GluOC in infants in the infant MK-7 supplemented group versus formula fed infant group at age ~2 months. iii) Comparative dp-ucMGP concentrations of infants in the infant supplemented group versus the formula fed infant group at age ~2 months.
	IV	To assess the feasibility, acceptance and compliance by the mothers of providing a daily oral MK-7 supplement to breastfed babies or themselves.	i) Assessment of mothers' opinions on acceptability of maternal daily MK-7 supplementation at follow up at ~2 months postpartum. ii) Assessment of parents' opinions on maternal versus infant vitamin supplementation follow up at ~2 months postpartum. iii) Assessment of compliance with maternal and infant supplementation by reviewing the amount of supplement/placebo remaining at the time of follow up and/or review of completed administration log at ~2 months postpartum.
	V	To assess the effect of daily maternal MK-7 supplementation on the maternal vitamin K status of breastfeeding mothers.	i) Proportion of MK-7 supplemented breastfeeding mothers with an elevated PIVKA-II level (>0.05 AU/mL) at ~2 months postpartum. ii) Proportions of mothers with an elevated PIVKA-II level (>0.05 AU/mL) at ~2 months postpartum in MK-7 supplemented group versus unsupplemented group. iii) %GluOC in MK-7 supplemented breastfeeding mothers at age ~2 months postpartum. iv) dp-ucMGP in MK-7 supplemented breastfeeding mothers at age ~2 months postpartum. v) Comparison of %GluOC in MK-7 supplemented breastfeeding mothers versus unsupplemented breastfeeding mothers at age ~2 months postpartum. vi) dp-ucMGP concentrations in MK-7 supplemented breastfeeding mothers versus unsupplemented breastfeeding mothers at postnatal age ~2 months.
	VI	To assess the effect of maternal daily MK-7 supplementation on vitamin K secretory content of human breastmilk	i) Concentrations of K vitamers in the breastmilk of supplemented breastfeeding mothers at ~2 months postpartum. ii) Comparison of K vitamers concentrations in the breast milk of lactating mothers at ~2 months postpartum in the maternal MK-7 supplementation group versus unsupplemented lactating mothers.

Rationale	The most common risk factor associated with late vitamin K deficiency bleeding is breastfeeding, which is primarily due to the significantly lower vitamin K levels (vitamin K1 concentration: 0.1-0.3 µg/100 mL) in breastmilk when compared to the adequately fortified formula milks (vitamin K1 concentration: 3.6-8 µg/100 mL). Late vitamin K deficiency bleeding (VKDB), although rare, most frequently presents with intracranial haemorrhage, which is often fatal and is due to a lack of vitamin K impairing coagulation. Most breastfed infants (term and preterm) will become biochemically vitamin K deficient by 2-3 months postnatal age, despite being given the nationally recommended dose of vitamin K1 at birth. This state inevitably renders them more vulnerable to late VKDB and its associated sequelae. Supplementation with 50 µg/day of phyloquinone (vitamin K1) has been shown to reduce the prevalence of biochemical deficiency from 70% to 20% in early infancy. There has been much recent interest in the menaquinones (forms of vitamin K2), particularly menaquinone-7 (MK-7). MK-7 has a longer half-life (3 days) as compared with phyloquinone (1-2 hours) and therefore it has been hypothesised that use of MK-7 instead of vitamin K1 would lead to more stable serum stores of vitamin K. There has so far been no research looking at menaquinone-7 supplementation in the infant population. The present study will compare both neonatal (45 µg/day) and maternal (2 mg/day) supplementation of MK-7 versus a placebo (infant supplementation containing no vitamin K but providing the recommended 10 µg vitamin D3/day). A cohort of formula fed infants receiving the recommended daily vitamin K1 supplementation provided by their infant formulas will act as an adequately supplemented comparator group. We hypothesise that MK-7 supplementation will significantly reduce the expected biochemical vitamin K deficiency in early infancy that otherwise develops in the majority of unsupplemented, predominantly breastfed babies.
Methodology	Participants will be recruited antenatally and postnatally from the participating institutions and will initially be assigned to study arms based on the parents' chosen method of infant feeding. Breastfed infant and mother dyads will then be randomised 2:1, in a part-blinded fashion, into one of two groups. The breastfeeding groups are as follows: - Group 1: Infant group. Infants in this group will receive either the active vitamin K2 supplement (45 µg/day MK-7 and 10 µg/day vitamin D3; cohort 1) or a 'placebo' supplement (10 µg/day of vitamin D3 and no vitamin K; cohort 2). This allocation of Group 1 infants will be randomised and double blinded. - Group 2: Maternal supplementation. Mothers in this group will receive 2 mg/day MK-7 supplementation. Infants will receive the recommended 10 µg/day of vitamin D3.

	A further sub-group of $n \geq 19$ breastfeeding, unsupplemented mothers will be followed up from group 1 to act as control for comparison with the MK-7 supplemented mothers. Infants who are formula fed will be adequately supplemented comparator infants, as they will receive the currently recommended amount of vitamin K1 and vitamin D in their formula milk. The intended formula feeding population will therefore be allocated to Group 3 without randomisation. This group will not receive, or require, any additional supplementation as part of the study. Supplementation will begin within 7 days of birth and will be given with a fat containing meal. Supplementation will continue until follow up at 2 months (range 2-3 months). A total of 3 months' worth of supplements will be provided to each participant. Based on UK average rate of breastfeeding at 6-8 weeks, we could expect up to a 50% attrition rate. For this study, predominantly breastfed is defined a priori as an infant receiving more than ~two thirds ($\geq 67\%$) of their total daily milk feeds comprised of breastmilk. A telephone call will occur at ~1 month of age which will assess breastfeeding amounts, compliance with study supplementation and confirmation of willingness to continue to participate in the study. A single return-to-hospital follow up visit will occur at 2 months postnatal age (range 2-3 months). Blood samples will be taken from all infants. Breastmilk and blood samples will be collected from the supplemented mothers and the maternal unsupplemented comparators. Mothers will also be asked their views on acceptability of daily oral supplements for infants, and their perceived preference for infant versus maternal daily supplementation. Once the follow up visit has been completed, the study will end for an individual participant. At the end of the study overall, once analysis has been completed, consented participants will be contacted with individual results, along with the overall findings from the study should they wish to receive them. Their General Practitioners will also be informed of participation and copied into any results.
Inclusion Criteria	• Infants, and mothers of infants, born ≥ 37 weeks gestational age • Standard dose of intramuscular (IM) vitamin K prophylaxis given to the neonate following birth • For breastfeeding mothers: the mother intends to exclusively/predominantly breastfeed their baby for at least 2 months
Exclusion Criteria	• Inability or refusal to provide informed consent • Inability to adhere to or comply with study procedures • Babies who meet the inclusion gestation but would otherwise, as part of routine neonatal care, be provided with daily vitamin K drops (i.e. NeoKay drops) at discharge • IM vitamin K prophylaxis not received at birth (parents declined vitamin K prophylaxis or parents chose for neonate to receive an oral course of vitamin K prophylaxis instead) • Evidence of conjugated hyperbilirubinaemia (these babies are at higher risk of VKDB)

	Mothers, and babies born to mothers, who are taking any medication that could affect or antagonise vitamin K metabolism (i.e. warfarin, the anti-epileptic drugs phenytoin or carbamazepine, and cephalosporin, rifampicin and isoniazid antimicrobials)
Analysis	<u>Primary Outcome Measure: serum PIVKA-II concentration</u> The primary analysis will be based on the per-protocol population. Individuals who are no longer predominantly breast feed will be excluded. The primary outcome will be rates of biochemical vitamin K deficiency at the 2 month follow up visit, indicated by a raised serum PIVKA-II concentration >0.05 arbitrary units per mL (AU/mL) between the placebo and infant MK-7 supplementation cohorts. Proportions with a raised PIVKA-II will be compared via two-group comparisons using the χ^2 and Fisher exact tests as appropriate. The estimated effect will be the difference in proportions (and the number needed to treat) and the odds ratio; if baseline differences exist then a sensitivity analysis will be performed using logistic regression. <u>Secondary Outcome Measures</u> Secondary outcome measures, including GlaOC:GluOC ratio, dp-ucMGP, serum K vitamers and breastmilk vitamin K analysis, will also be analysed for all infants followed up and mothers in the two breastfeeding cohorts. All data collected will be reported with a median and interquartile range. Concentrations of osteocalcin will be reported in ng/mL. Serum K vitamers will be reported in units of $\mu\text{g/L}$. Presuming all concentrations will be non-normally distributed (as per Clarke et al., 2022), concentrations will be log-transformed towards normality then compared using the two-sample t test. The effect size will be the ratio of the geometric means. Levels of K vitamers in the breastmilk will be reported units of ng/mL. dp-ucMGP will be reported in units of pmol/L. Should these data be normally distributed, then an independent t test will be used for analysis. Should the data be non-normally distributed, a Mann-Whitney test will be used. A two tailed P value of <0.05 will be considered statistically significant
Study End	The study end shall be defined for individual participants as the completion of the 2-month postnatal follow up visit. The overall study end shall be defined as the date of the final database lock, including results of analysis of all biological samples taken as part of the study.

FUNDING AND SUPPORT IN KIND

FUNDERS	FINANCIAL AND NON-FINANCIAL SUPPORT GIVEN
PharmaLinea Ltd.	Provision of all supplements used in this study. Funding provided for laboratory analysis in Professor Schurgers' laboratory at University of Maastricht, The Netherlands. Funding support for a research nurse.

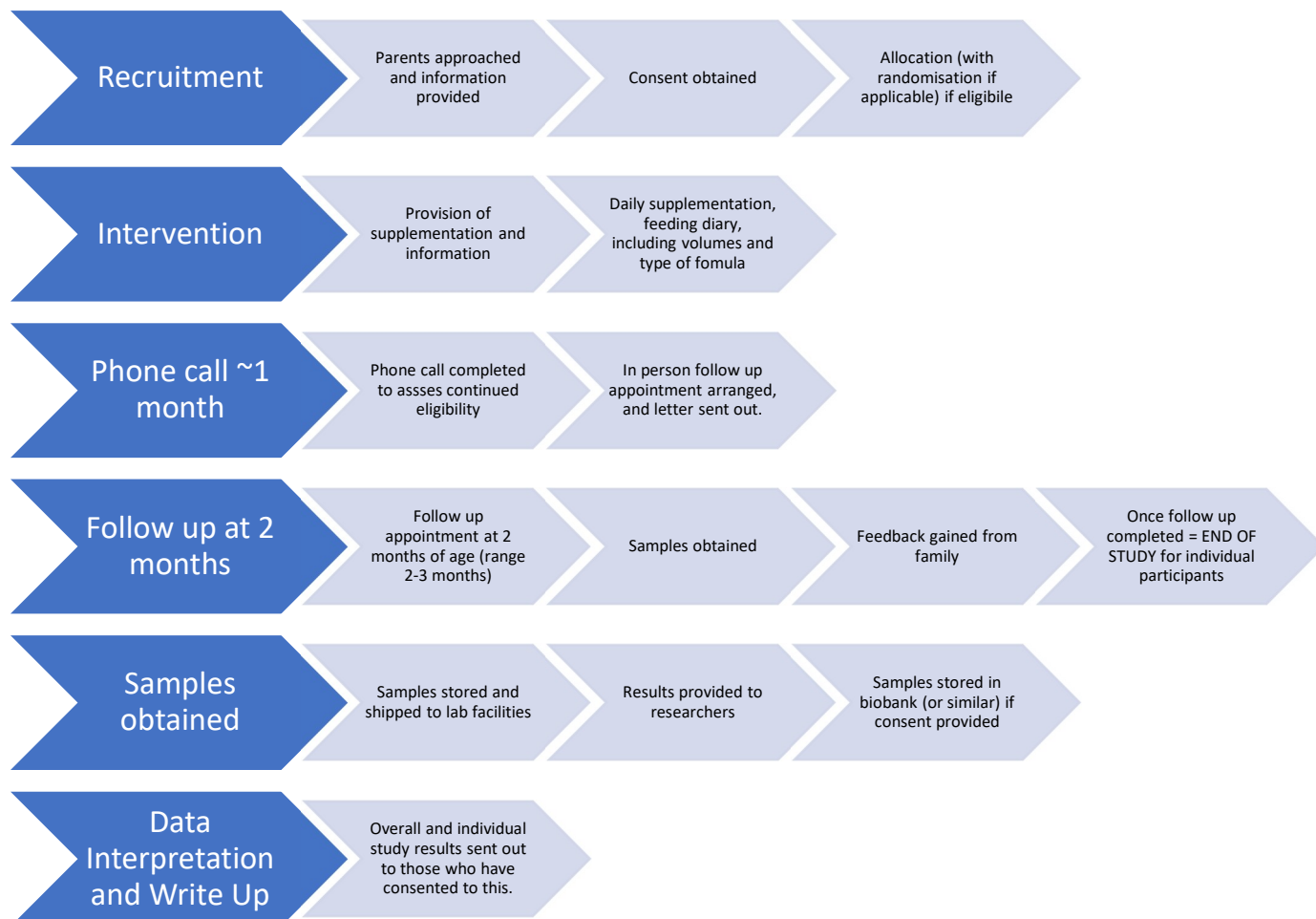
Norfolk and Norwich NICU Research Fund	Funding for to support a research nurse Funding for consumables, pharmacy input, sample storage, sample transport to laboratories and funding for the reasonable travel costs for parents
NNUH Charitable Fund	Funding support for CI time for Prof. Paul Clarke

PROTOCOL CONTRIBUTORS

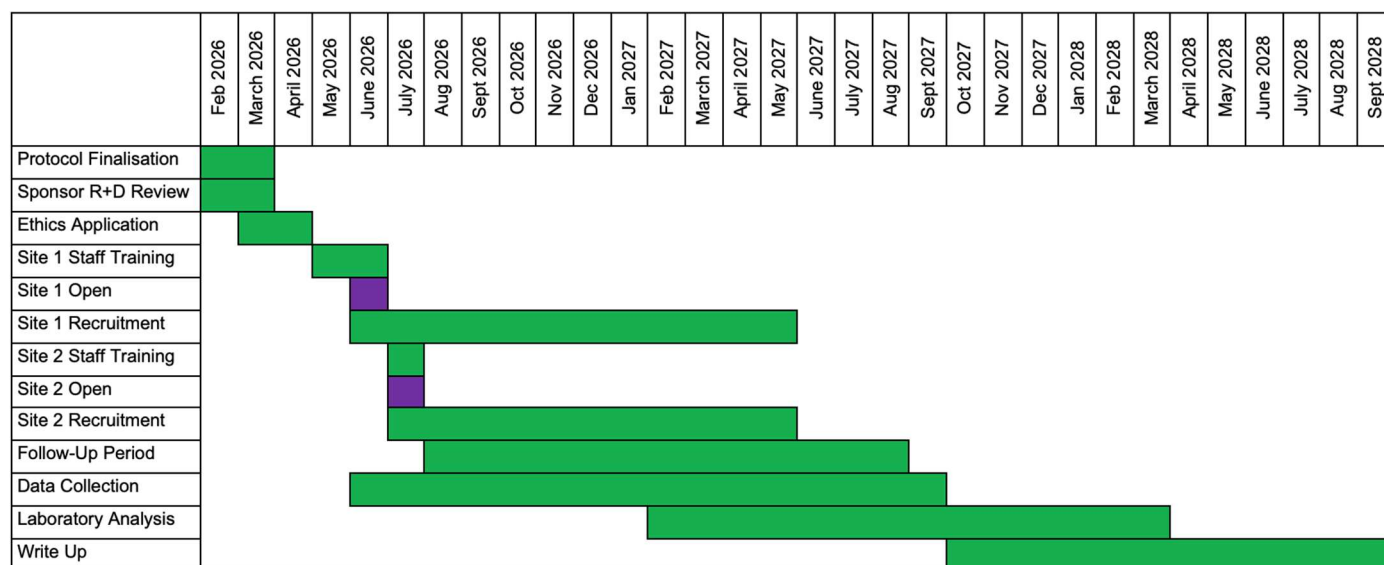
Contributor	Institution(s)	Protocol Contribution
Professor Paul Clarke, Consultant Neonatologist	Norwich Medical School, University of East Anglia; Neonatal Intensive Care Unit, Norfolk and Norwich University Hospitals NHS Foundation Trust	Chief Investigator, intellectual input, study protocol design.
Dr Carianne Lewis, NICU Clinical Research Fellow	Neonatal Intensive Care Unit, Norfolk and Norwich University Hospitals NHS Foundation Trust	Co-Chief Investigator, intellectual input, study protocol design, PPIE and creation of parent facing materials
Professor Leon Schurgers, Chair and Professor of Biochemistry	Department of Biochemistry, CARIM, University of Maastricht, The Netherlands	Intellectual input, study protocol design, laboratory analysis
Dr Martin Shearer, Retired Consultant Scientist	Centre for Haemostasis and Thrombosis, Guy's and St Thomas's Hospitals NHS Foundation Trust	Intellectual input, study protocol design.
Professor Dominic Harrington, Consultant Clinical Scientist	Synnovis and The Human Nutristasis Unit	Intellectual input, laboratory measurement of PIVKA-II
Dr Allan Clark, Statistician	Norwich Clinical Trials Unit, University of East Anglia, Norwich	Support with statistical methods and analysis
Dr Isabel Iglesias-Platas, Senior NICU Clinical Research Fellow	Neonatal Intensive Care Unit, Norfolk and Norwich University Hospitals NHS Foundation Trust	Intellectual input
Dr Vennila Ponnusamy, Consultant Neonatologist	Neonatal Intensive Care Unit, Ashford and St. Peter's Hospitals NHS Foundation Trust, Chertsey	Intellectual input, local Principal Investigator for Ashford and St. Peters NICU.
PharmaLinea, Nutraceutical Company	Cesta v Mestni log 88a, 1000 Ljubljana, SI – European Union, info@pharmalineia.com	Provision of study supplements and funding support. Had an opportunity to comment on the study protocol.

KEY WORDS: Oral vitamin K, vitamin K1 vs K2, menaquinone-7, MK-7, vitamin K status, PIVKA-II, undercarboxylated/carboxylated osteocalcin ratio, neonate, infant, breastfeeding

STUDY FLOW CHART



Gantt Chart of Predicted Study Timeline



STUDY PROTOCOL

Oral Vitamin K2 (menaquinone-7; MK-7) supplementation and effect on vitamin K status of breastfed infants in early infancy; a four-cohort prospective, part-randomised, part-blinded study.

1 BACKGROUND AND RATIONALE

At birth, neonates have borderline vitamin K status. They are at increased risk of developing vitamin K deficiency, and subsequently vitamin K deficiency bleeding (VKDB), if they are not supplemented with vitamin K (1). VKDB is categorised as “early” (within 24 hours of birth), “classical” (within 1 week of birth), and “late” (from the second week up to 6 months of age) (2). Late VKDB is rare with an estimated incidence of 0.67 in 100,000 in the UK (3). Up to 60% of infants with late VKDB present with intracranial haemorrhage which can cause brain damage or be fatal (1,4). Adequate vitamin K prophylaxis at birth, and ongoing dietary vitamin K intake, are necessary to maintain vitamin K stores of babies, and therefore reduce the risk of this essentially preventable condition (1,5–8).

The most common risk factor associated with VKDB is exclusive breastfeeding. Infant formula milks are adequately fortified with micronutrients to the currently recommended levels, with vitamin K content in the range 3.6-8.0 µg/100 mL (7,9–13). In comparison, breastmilk contains only 0.1-0.3 µg/100 mL (6,7), some 12-80 times less, strongly implying that extra supplementation is routinely required for exclusively breastfed infants in early infancy. A recent study by Perrone et al. (14) compared various phyloquinone (vitamin K1) supplementation regimens in exclusively breastfed term neonates. Even after the recommended prophylactic 1 mg phyloquinone injection at birth, approximately 70% of included neonates developed sub-clinical vitamin K deficiency by age 3 months postnatal (14). Providing 50 µg phyloquinone supplementation daily decreased the prevalence of subclinical deficiency in the included babies to around 20% by 3 months post-natal (14). These findings were mirrored in an observational study in preterm neonates (8). A similar proportion of exclusively breastfed babies who did not receive ongoing dietary vitamin K supplementation post discharge developed subclinical vitamin K deficiency by 2-3 months postnatal (8). Virtually no preterm infants fed with sub-clinical vitamin K-supplemented infant formulas developed subclinical deficiency (8).

Vitamin K is found in nature in two forms, phyloquinone (vitamin K1) and menaquinone (vitamin K2). The latter is further subdivided into menaquinone analogues, namely menaquinone-4 through to menaquinone-13 (MK-4 to MK-13) (15). Current infant vitamin K prophylaxis and supplementation in the UK is universally with phyloquinone (16,17). There is interest in using MK-7 as an oral vitamin K supplement (18–22). Although both vitamin K1 and K2 are absorbed in the intestine by the same mechanism (15) MK-7 has a longer half-life: around 3 days when compared to 1-2 hours for phyloquinone. This may imply that vitamin K2 should lead to more stable serum stores (23).

Adult studies looking at dietary MK-7 supplementation have shown that it may be effective at improving cardiovascular and bone health (18,24). MK-7 also improves overall vitamin K status, demonstrated using the sensitive biochemical markers of vitamin K status protein induced by vitamin K absence/antagonism clotting factor II (PIVKA-II), desphospho-uncarboxylated matrix Gla-protein and undercarboxylated: carboxylated osteocalcin ratio (%GluOC) (20,23). However, these were studies done in adults with specific clinical demographics or conditions.

To date, there are no studies exploring MK-7 supplementation in infants or neonates. In the paediatric population, a trial looking at MK-7 in pre-pubertal children found MK-7 to be safe and improve osteocalcin

measurements (19). There have been maternal supplementation trials with menaquinones, using MK-4, which have assessed infant outcomes with varied results (25,26). The primary trials exploring maternal supplementation have used phyloquinone. Greer et al. (1997) looked at giving doses of 2 mg/day and 5 mg/day to mothers to see the effect of this on both the breastmilk vitamin K concentration and the infants' vitamin K status (27). Infants of mothers who were supplemented with daily vitamin K1 were found to have significantly improved PIVKA-II levels when compared to infants of unsupplemented mothers (27). Bolisetty et al. (1998) also examined breastmilk excretion of vitamin K in preterm infants, finding the vitamin K excretion plateaued on day 6 of daily vitamin K1 supplementation (28). No studies to date have examined the effect of maternal MK-7 supplementation on infant outcomes.

There is also a significant lack of comparator studies comparing phyloquinone versus MK-7. Schurgers et al. (23) compared the two forms in an adult study and demonstrated that MK-7 had a much longer half-life than vitamin K1, more stable serum stores and improved carboxylated osteocalcin levels (23).

The present study proposes to compare both infant and maternal oral supplementation of MK-7 versus a 'placebo' and phyloquinone to reduce the expected high prevalence of sub-clinical vitamin K deficiency that would otherwise develop in early infancy in most non-supplemented exclusively breastfed babies.

2 HYPOTHESIS AND AIMS

2.1 Hypothesis

Daily oral menaquinone-7 (MK-7) supplementation of infants or their mothers will abolish the development of biochemical vitamin K deficiency in breastfed infants by 2-3 months postnatal age.

2.2 Aim, Outcomes and Outcome Measures

	Aim	Outcome Measure(s)
Primary	To examine the efficacy of daily oral MK-7 dietary supplementation for the reduction of sub-clinical vitamin K deficiency in early infancy.	Proportion of infants who have a PIVKA-II concentration of >0.05 AU/mL at ~2 months postnatal age in the supplemented versus placebo cohorts (<i>Group 1</i>).
Secondary	I To compare the relative efficacy of maternal oral MK-7 dietary supplementation versus infant daily oral MK-7 dietary supplementation for the reduction of sub-clinical vitamin K deficiency in infants in early infancy	i) Proportion of infants with a PIVKA-II concentration of >0.05 AU/mL at age ~2 months in the MK-7 maternal supplemented group (<i>Group 2 infants</i>) versus infant MK-7 supplemented cohort (<i>Group 1 supplemented infant cohort</i>). ii) Percentage undercarboxylated osteocalcin as a proportion of total osteocalcin (%GluOC) in infants in the MK-7 maternal supplemented group (<i>Group 2 infants</i>) versus infant MK-7 supplemented cohort (<i>Group 1 supplemented infant cohort</i>) at age ~2 months. iii) Comparative serum vitamin K concentrations of infants at ~2 months postnatal in the maternal MK-7

		supplemented infant group (<i>Group 2 infants</i>) versus infant MK-7 supplemented cohort (<i>Group 1 supplemented infant cohort</i>) at age ~2 months. iv) Comparative dephosphorylated undercarboxylated matrix Gla Protein (dp-ucMGP) concentrations of infants at ~2 months in the maternal MK-7 supplemented infant group (<i>Group 2 infants</i>) versus the infant MK-7 supplemented cohort (<i>Group 1 supplemented infant cohort</i>) at age ~2 months.
II	To examine the utility of maternal oral supplementation with MK-7 for the reduction of subclinical vitamin K deficiency in breastfed infants.	i) Proportion of infants in the maternal MK-7 supplemented group (<i>Group 2 infants</i>) with a PIVKA-II concentration of >0.05 AU/mL at age ~2 months. ii) Comparison of proportions of infants with a PIVKA-II concentration of >0.05 AU/mL at age ~2 months in the maternal MK-7 supplemented group (<i>Group 2 infants</i>) versus infants in the placebo cohort (<i>Group 1 placebo cohort</i>). iii) Comparison of %GluOC in infants in the maternal MK-7 supplemented group (<i>Group 2 infants</i>) versus infants in the placebo cohort (<i>Group 1 placebo cohort</i>) at age ~2 months. iv) Comparative dp-ucMGP concentrations of infants in the maternal MK-7 supplemented infant group (<i>Group 2 infants</i>) versus the infant placebo cohort (<i>Group 1 placebo cohort</i>) at age ~2 months.
III	To compare daily MK-7 dietary supplementation of breastfed infants with that of vitamin K1 supplementation via formula milk fed infants for the reduction of sub-clinical vitamin K deficiency in early infancy.	i) Comparison of proportions of infants with a PIVKA-II concentration of >0.05 AU/mL at age ~2 months in the infant MK-7 supplemented group (<i>Group 1 supplemented infant cohort</i>) versus formula fed infant group (<i>Group 3 infants</i>). ii) Comparison of %GluOC in the infant MK-7 supplemented group (<i>Group 1 supplemented infant cohort</i>) versus formula fed infant group (<i>Group 3 infants</i>) at age ~2 months.

			iii) Comparative dp-ucMGP concentrations of infants in the infant supplemented group (<i>Group 1 supplemented infants</i>) versus the formula fed infant group (<i>Group 3 infants</i>) at age ~2 months.
	IV	To assess the feasibility, acceptance and compliance by the mothers of providing a daily oral MK-7 supplement to breastfed babies or themselves.	i) Assessment of mothers' opinions on acceptability of maternal daily MK-7 supplementation at follow up at ~2 months postpartum. (<i>Group 2 mothers</i>) ii) Assessment of parents' opinions on maternal versus infant vitamin supplementation at follow up at ~2 months postpartum. (<i>Mothers in Group 2 and mothers of Group 1 infants</i>) iii) Assessment of compliance with maternal and infant supplementation by reviewing the amount of supplement/placebo remaining at the time of follow up and/or review of completed administration log at ~2 months postpartum (<i>Mothers of Group 1 infants and mothers in Group 2</i>)
	V	To assess the effect of daily maternal MK-7 supplementation on the maternal vitamin K status of breastfeeding mothers.	i) Proportion of MK-7 supplemented breastfeeding mothers (<i>Group 2 mothers</i>) with an elevated PIVKA-II level (>0.05 AU/mL) at ~2 months postpartum. ii) Proportions of mothers with an elevated PIVKA-II level (>0.05 AU/mL) at ~2 months postpartum in the MK-7 supplemented group (<i>Group 2 mothers</i>) versus unsupplemented group (<i>Group 1 mothers</i>) iii) %GluOC in MK-7 supplemented breastfeeding mothers (<i>Group 2 mothers</i>) at age ~2 months postpartum. iv) dp-ucMGP in MK-7 supplemented breastfeeding mothers (<i>Group 2 mothers</i>) at age ~2 months postpartum. v) %GluOC in MK-7 supplemented breastfeeding mothers (<i>Group 2 mothers</i>) versus unsupplemented breastfeeding mothers (<i>Group 1 mothers</i>) at age ~2 months postpartum. vi) dp-ucMGP concentrations in MK-7 supplemented breastfeeding mothers (<i>Group 2 mothers</i>) versus unsupplemented

			breastfeeding mothers (<i>Group 1 mothers</i>) at postnatal age ~2 months.
	VI	To assess the effect of maternal daily MK-7 supplementation on vitamin K secretory content of human breastmilk	i) Concentrations of K vitamers in the breastmilk of supplemented breastfeeding mothers (<i>Group 2 mothers</i>) at ~2 months postpartum. ii) Comparison of K vitamers concentrations in the breast milk of lactating mothers at ~2 months postpartum in the maternal MK-7 supplementation group (<i>Group 2 mothers</i>) versus unsupplemented lactating mothers (<i>Group 1 mothers</i>).

NB: Serum and breastmilk K vitamers will include, but not be limited to, vitamin K1 and menaquinone-7 concentration.

Compliance with the study will be monitored by estimating the remaining volume of drops left (measured with a syringe) and the number of capsules left at follow up. This will be compared to the expected volume/number of capsules remaining based on the date the supplementation was started and the date of the follow up appointment.

3 STUDY DESIGN

3.1 Overall Study Design

This will be a prospective, part-randomised, part-blinded, two-centre study comprising four cohorts and enrolling up to 134 infant and mother dyads. This is with the aim to follow up a minimum of 77 infants, up to 19 supplemented mothers (range ~10-20) and up to 19 unsupplemented control mothers (range ~10-20).

Participants will be split into three groups (four cohorts):

Groups 1 and 2: Exclusively breastfeeding infants. These will be randomised 2:1 to one of two groups:

- Group 1: Infant Group – infants either receive MK-7 supplementation or a placebo in a double-blind fashion (recruit ≥ 76 mother and infant dyads, to follow up the minimum of $n=38$ (minimum of $n=19$ to each cohort) required to achieve 80% power for the primary outcome measure)
- Group 2: Maternal Group – mothers receive MK-7 supplementation (recruit $n \geq 38$ to follow up $n \geq 10-20$ dyads)

Group 3: Formula-feeding infants. Infants do not receive, or require, additional supplementation as part of this study (Recruit $n \geq 20$ to follow up $n \geq 10-20$ infants)

For the breastfeeding group we will aim to selectively enrol mothers who intend to exclusively or predominantly breastfeed their baby for at least 2-3 months Recruitment of mothers who have already successfully breastfed another child is preferred, to improve follow up rate.

Breastfed infants will be randomised 2:1 (group one: group two). For group 2 recruitment, an initial target of $n \leq 19$ will be set. However, the number of dyads followed up in group 2 will depend on how recruitment is proceeding, with an aim to follow up $n \leq 10-20$ dyads. All breastfed infants will be supplemented with 10 µg/day of vitamin D3, meeting the current UK national guidelines (29).

Infants in Group 1 will be randomly allocated to receive either the active supplement, 45 µg/day of vitamin K2 and 10 µg of vitamin D3 via a K2D3 product (6 drops), or the placebo supplement (with respect to vitamin K), 10 µg vitamin D3 only (6 drops). Allocation of infant supplements in group 1 will be double-blinded.

Maternal supplementation in group 2 will be provided open label. Mothers will receive 2 mg per day oral MK-7 (via two 1 mg capsules of MK-7 per day). Infants in group 2 will also receive 10 µg/day of vitamin D3 (6 drops), meeting the current UK national guidelines (29).

Formula fed infants will represent a group of infants adequately supplemented with ~4-8 µg/100 mL vitamin K1, meeting current recommended daily vitamin K intakes (9–13). Infant formulas also contain additional vitamin D, at around ~1.5 µg/100 mL meeting the currently recommended daily vitamin D requirement (9–13,29). Infants in Group 3 therefore do not require, or receive, any additional vitamin K or vitamin D supplementation as part of this study.

4 ELIGIBILITY CRITERIA

4.1 Inclusion criteria

- Infants, and mothers of infants, born ≥ 37 weeks gestational age
- Standard dose of intramuscular (IM) vitamin K prophylaxis given to the neonate following birth
- For breastfeeding mothers: the mother intends to exclusively/predominantly breastfeed their baby for at least 2 months

4.2 Exclusion criteria

- Inability or refusal to provide informed consent
- Inability to adhere to or comply with study procedures
- Babies who meet the inclusion gestation but would otherwise, as part of routine neonatal care, be provided with daily vitamin K drops (i.e. NeoKay drops) at discharge
- IM vitamin K prophylaxis **not** received at birth (parents declined vitamin K prophylaxis or parents chose for neonate to receive an oral course of vitamin K prophylaxis instead)
- Evidence of conjugated hyperbilirubinaemia (these babies are at higher risk of VKDB)
- Mothers, and babies born to mothers, who are taking any medication that could affect or antagonise vitamin K metabolism (i.e. warfarin, the anti-epileptic drugs phenytoin or carbamazepine, and cephalosporin, rifampicin and isoniazid antimicrobials)

5 SAMPLE SIZE, RECRUITMENT AND RANDOMISATION

5.1 Sample Size

The primary outcome for this study is the comparison of infant PIVKA-II levels at 2 months' postnatal age (range 2-3 months) between infants who have been supplemented with vitamin K2 (in the form MK-7) vs infant placebo (group 1). Based on a 70% versus 20% rate of sub-clinical vitamin K deficiency (defined as a PIVKA-II > 0.05 AU/L) seen in a recent previous study (14), the present study would need $n = 19$ individual

infants per cohort in group 1 to have 80% power to detect a difference between the two cohorts at the 5% level of significance using a Chi-squared test with continuity correction.

Groups 2 and 3 (maternal supplementation and infant formula groups respectively) are designed to examine experimental concepts, therefore power calculations are not possible.

For group 2, an initial aim will be set to follow up $n \approx 19$ dyads to allow 2:1 randomisation (group one: group two). However, depending on recruitment, this may be reduced, with an aim to finally follow up $n \approx 10-20$. A substantial amendment may be submitted to this effect.

For group 3, up to $n \approx 20$ will be recruited, to follow up $n \approx 10-20$.

Nationally, in 2023-2024, 52.7% of infants were fully or partially breastfeeding at 6-8 weeks postnatal age (30). We would therefore assume ~50% of randomised breastfeeding dyads will drop-out or be excluded from the final analysis due to no longer predominantly breast feeding (defined as feeding less than two thirds or <67% of the total daily milk feed volume comprised by breast milk). Based on a previous study (8), an expected 20% of formula fed infants will not return for follow up.

Therefore, allowing for these anticipated losses to follow up we aim to enrol up to 134 mother and infant dyads.

Numbers to be enrolled in each group:

Group 1 – infant group (infant MK-7 supplementation vs placebo) – each cohort (supplementation and placebo) needs a minimum of $n=19$ to achieve 80% power. Therefore, $n \approx 76$ dyads will be recruited, to account for an expected 50% loss to follow up.

From this group, $n \approx 38$ mothers will also be recruited to provide blood and a breastmilk sample as a control.

Group 2 – maternal group (maternal MK-7 supplementation) – this is exploratory, so an initial aim of $n \approx 19$ will be followed up. Again, allowing for an up to 50% loss to follow up due to anticipated breastfeeding rates, we will aim to recruit $n \approx 38$ to this group. However, depending on how recruitment is proceeding, the number of participants successfully followed up may be reduced to $n \approx 10-20$.

Group 3 – formula group (infants fed with formula) – this is again exploratory, so a minimum of $n \approx 10$ dyads will be followed up. Based on a previous study (8), we expect may ~20% loss to follow up. Therefore, a minimum of $n \approx 10-20$ dyads will be enrolled.

5.2 Screening for eligibility

Identification of potential participants will be completed by the clinical and midwifery team or members of the Neonatal Intensive Care Unit (NICU) clinical research team (nurses or doctors). This may involve use of clinical systems such as the Newborn Infant Physical Examination system and BadgerNet electronic record. Mothers of infants who both fit the inclusion criteria and who have none of the exclusion criteria will be invited to consider the study and would need to provide written informed consent for inclusion.

Only basic information of those who have been approached will be stored to indicate pre-screening numbers. This will be stored securely, within each trust's information governance guidelines, and will not be included in the study data. This information will only be accessible to members of the research team who have been granted permission by the line manager and are delegated to the study.

5.3 Recruitment and Randomisation

Recruitment will be completed before discharge from the hospital. This will include provision of information about the study and obtaining informed consent.

As this is not a clinical trial of an investigational medicinal product (CTIMP), consent can be gained by any healthcare professional who is trained, familiar and confident with the information contained within the protocol, who holds an up to date Good Medical Practice certification and who have been appropriately delegated by a Chief Investigator (CI) or local Principal Investigator (PI).

Once informed written maternal consent has been obtained, participants will be provided with a unique study identifier and baseline information will be collected about the pregnancy and maternal medical history, primarily the family's choice to feed their infant.

Mother and infant pairs who are exclusively/predominantly breastfeeding will be randomised 2:1 to either Group 1 or 2 (Figure 1). Infants who are formula fed will be allocated to Group 3 without randomisation (Figure 1).

Randomisation will be completed using a web-based randomisation service, with the neonatal allocation to active MK-7 or placebo (Group 1) being blinded to the study team and the family. Once randomisation has been completed, sufficient supplement will be provided to cover up to 3 months (minimum target supplementation period: 2 months), and information about the study, and administration of the supplement, will be given prior to hospital discharge.

Follow up will be completed at 2.0 months (range: 2-3 months). Where possible, the follow up visit will be incorporated to coincide with any other routine planned follow-up return visit to the hospital.

Parents will be asked to record feed quantities. Estimations of both phyloquinone amount from infant formula and percentage of breastfeeding to formula feeding for combination-fed babies will be made according to the baby's weight and parent-reported milk intake volumes.

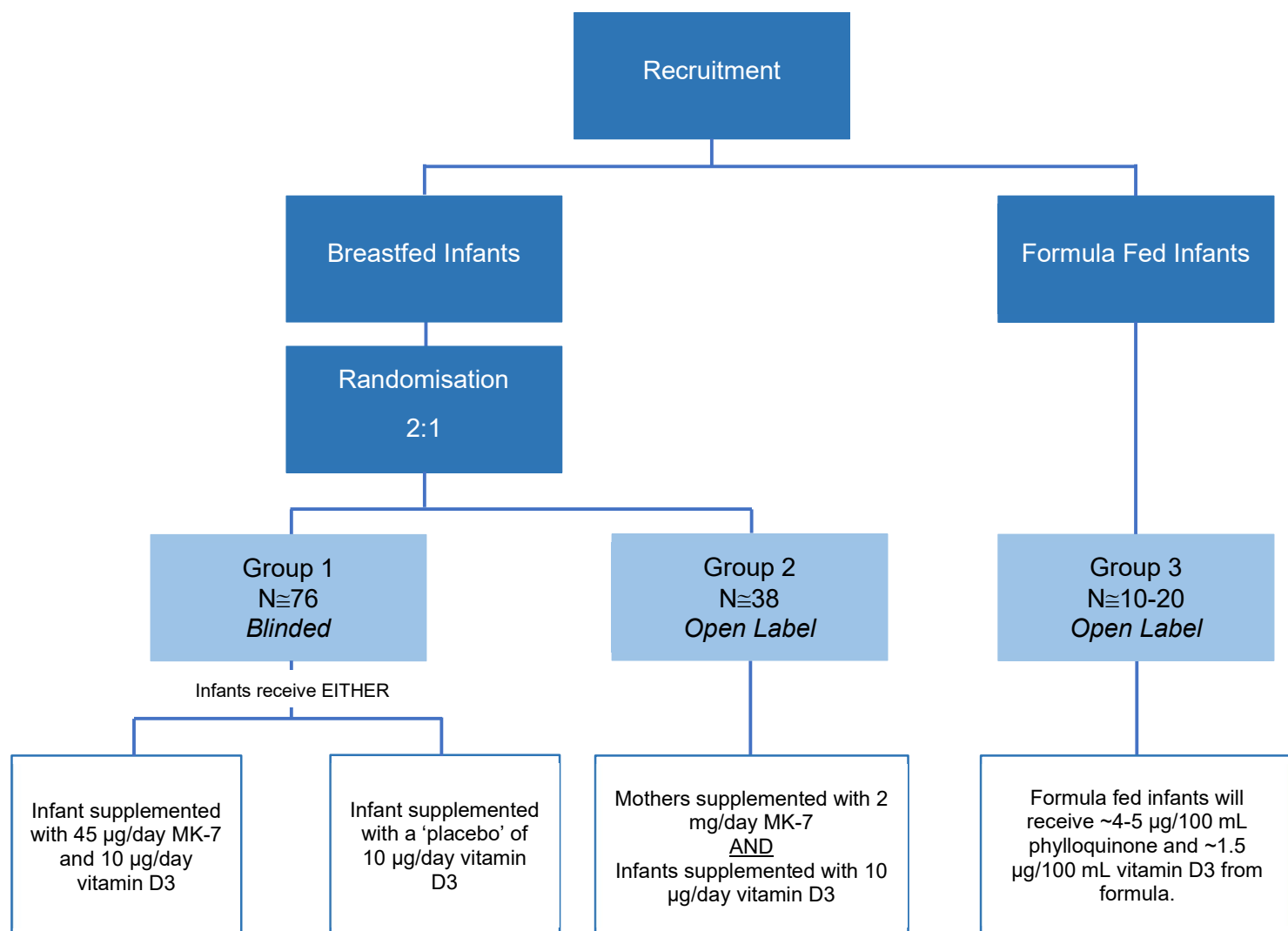


Figure 1: Recruitment and Randomisation: Numbers representative of mother and infant dyads being recruited to meet follow up requirements

5.3.1 Rationale for Menaquinone-7 Dosing

All vitamin K supplementation will be required to be given with a fat containing meal to ensure optimal vitamin K absorption. This will be specified to the parents upon recruitment and will also be advised in the study materials. Reminders about daily dosing and taking the supplementation with the feed will be completed at the phone call to organise follow up at around 1 month of age.

Neonatal Dosing

The rationale for neonatal vitamin K dosing is based on the average intake from infant formula, along with a comparator amount for vitamin K1 supplementation. In a recent Italian study, Perrone et al. (2024) used a

dose of 50 µg/day of vitamin K1 in one of their infant trial groups, demonstrating a significant reduction in the number of participants who had biochemical vitamin K deficiency as shown by a raised PIVKA-II (14).

Maternal Dosing

Previous studies of maternal supplementation with vitamin K1 have shown a variety of doses have been used to increase vitamin K1 levels in the maternal milk. A dose range of 2-5 mg/day of vitamin K1 has been shown to increase concentrations in breast milk to comparable concentrations as found in formula fed infants without causing side effects to the mother (27,28). A dose of 2 mg/day of MK-7 was chosen with an aim to increase breastmilk vitamin K levels similarly, in a safe manner. Neither vitamin K1 nor K2 have any known toxic effects in humans, including when given in very large pharmacological doses.

5.3.2 Rationale for Placebo Group (group 1: control cohort)

The current standard care for breastfed term babies in the UK is provision of a single large dose of vitamin K soon after birth (0.5-1 mg given IM or an equivalent oral vitamin K course) (17). Further supplementation with extra dietary vitamin K is not yet recommended or routinely given in most centres. National guidance is that breastfed babies take 10 µg/day of vitamin D. The 'placebo' control cohort of group 1 infants will therefore reflect standard current UK practice with respect to breastfeeding babies. This group will receive (blinded) the recommended 10 µg/day vitamin D via the 'placebo' supplement, while still forming a control cohort of babies who are unsupplemented with vitamin K for comparison. Vitamin D3 will also be provided (unblinded) daily to the infants in group 2 (maternal supplementation group) to align with national guidance.

5.3.3 Rationale for Control 'Formula fed' Group (group 3)

Group 3, a group of neonates who will remain formula fed, will act as an expectedly vitamin K-replete comparator cohort. Formula milks in the UK are adequately supplemented with vitamin K1 to meet recommended daily intakes. Therefore, this group of formula fed infants will provide a useful comparison between currently recommended daily vitamin K1 intake (provided via infant formula) and a comparable MK-7 intake. PharmaLinea Ltd., who provide MK-7 preparations, do not manufacture a vitamin K1 preparation, therefore blinding would become difficult if using a different additional manufacturer.

5.3.4 Loss to Follow up and Withdrawals from Follow up

At ~ 1 month of age, all participating families will be contacted to assess compliance with ongoing supplementation (Groups 1 and 2), willingness to continue participation and confirmation of intention to return for the follow up study visit at age 2-3 months (all Groups).

Mother and infant dyads will be excluded from the 2 month follow up visit if:

- The study supplement, either infant or maternal, has been ceased prematurely by the family (non-compliance)
- For breastfeeding infants: the infant is taking less than two thirds ($\leq 67\%$) of the total daily milk feeds as breastmilk. As previously mentioned, infant formula contains vitamin K1 and the addition of infant formula would likely skew the results of the study.
- Infants who have been found to have conjugated hyperbilirubinaemia on routine midwifery follow up. Conjugated hyperbilirubinaemia is an indicator of liver pathology and puts them at much higher risk of VKDB. It is routine that these infants are provided with supraphysiological doses of vitamin K as part of their care, which would, therefore, affect the study outcomes.
- The family wish to no longer be part of the study.

- If the infant has recently had any clinical blood tests completed, then invitation to the 2 month follow up visit will be at the investigators' discretion. No research blood tests will be taken if the infant's haemoglobin is known to be less than 100 g/L.
- There are any other reasons found by the investigator that would make the mother and infant dyad unsuitable for follow up.

Reasons for all losses to follow up will be recorded.

5.3.5 Supplement Product Information

All vitamin supplements used in this study will be provided by PharmLinea Ltd. (Ljubljana, Slovenia; info@pharmalineia.com). PharmaLinea will supply:

- Vitamin D3 drops: 10 µg/day vitamin D3 in 6 drops
- K2D3 infant drops: 45 µg/day menaquinone-7 + 10 µg/day vitamin D3 in 6 drops
- Maternal capsules: 1 mg menaquinone-7 per capsule – dose 2 mg/day, therefore two capsules to be taken daily

All supplements will be manufactured to FSSC 22000, and therefore Good Manufacturing Practice (GMP) food production standards, and will be provided for the study gratis. Standard manufacturer quality control testing to confirm the quantities of the active compound(s) along with microbial testing will be performed on each batch of supplement. Further enhanced testing, including but not limited to heavy metal, pesticide and aflatoxin testing for each lot, can also be completed according to regulatory requirements.

Similar K2D3 drops manufactured by PharmaLinea as will be used in this study are currently marketed in Vietnam, United Arab Emirates and Saudi Arabia. K2D3 products marketed to adults and children are sold in Vietnam, Korea, Kazakhstan, Moldova, Romania, Ukraine and Germany.

5.4 Consent

Informed consent will be obtained by a trained, delegated member of staff. This will be completed after the parents have received information, had time to consider the information and had all their questions answered to their satisfaction.

6 METHODS OF DATA COLLECTION AND DATA ANALYSIS

6.1 Information Governance

All participants in the study will be given a unique study number once informed written consent has been obtained. This study number will be used to pseudonymise study samples and data where required.

Personal identifying information will only be accessible to the hospital research team who has been appropriately delegated to the study after full, informed consent has been received. Anyone outside of the delegated research team, i.e. statisticians or laboratory analysts, will have access to the pseudonymised data using the unique study number only, and will not have any access to personal identifying information.

All personal identifying information will be stored securely within NHS computer systems, with research data accessible only by the relevant research study personnel. Paper copies of any study data will be stored in locked offices accessible to the research team.

6.2 Study Setting

Mother/infant dyads can be approached ante- or post-natally, but participants will be enrolled before discharge from the hospital. Initial recruitment will include information giving, gaining informed consent, an initial questionnaire about maternal health and pregnancy, randomisation, and provision of supplementation supplies and study information, and will be completed by delegated study staff.

The follow-up visit will be completed in an outpatient facility that has the correct equipment in place and will allow samples to be taken, processed, stored, and shipped in an accurate and efficient manner. During the clinic return visit appointment acceptability and dietary history questions will be asked, along with the blood and/or breastmilk samples being obtained. Breastmilk samples may be collected at this appointment but may also be collected by the mother at home within 24 hours of the follow up appointment and provided to the study team at this visit.

6.3 Follow-up

An initial phone call (and/or email contact where preferred) will take place at ~1 months of age (range 1-2 months), to check that the participant is still eligible for follow up. Eligibility will be assessed by confirming:

- The participants remain happy to participate
- The participants are compliant with the study supplements
- For the breastfeeding cohorts, the infant is still predominantly breastfed, defined as taking at least than two thirds ($\leq 67\%$) of their feeds in a 24-hour period as breastmilk
- There is no evidence of significant conjugated hyperbilirubinaemia (usually identified by the community midwifery team)
- Whether the infant has had any recent blood tests; if recent blood tests have been performed, then research bloods will be taken at the investigators' discretion. If any blood results are available, the infant haemoglobin must be more than 100 g/L before taking bloods for research.
- There are no other reasons why follow up should not be completed.

Follow up appointments will subsequently be arranged and may include an invitation letter. For mothers providing breastmilk samples, sample collection containers will also be provided. Should the parents not be able to be contacted by phone or email, then a letter with a follow up appointment will still be sent and eligibility will be checked at the follow up appointment.

The follow up appointment will be held at the recruiting hospital or clinical research facility.

6.4 Data Collection

Data Collection Forms

Data collection will be completed by the delegated research staff. Collection will be completed on paper forms, and/or via a secure electronic database. Data collection forms will be labelled with the unique study number. Should this be completed on a computer-based system, then hard copies of the data collected will be downloaded and archived at the end of the study.

Any paper copies will be kept in folders in locked research offices. Only the delegated research team will be able to access these. Should an online platform be used, then hard copies of the data retained will be stored in a secure area. Retention of research materials will be for a minimum of 5 years, as per the Sponsor's standard operating procedure for research relating to women and their babies.

Samples

Samples will be labelled with the unique study number, along with the date and time the sample was taken. No personal identification information will be placed on the samples collected, keeping them blinded to the laboratory staff.

Results will be provided to the CI once available, which will only be identified by the unique study number. As these are pseudonymised, the data can also be provided for statistical analysis while maintaining confidentiality.

6.5 Laboratory Samples

Blood Samples

Because vitamin K is photosensitive, blood samples will be protected from light at all stages of processing and analysis. This will be most easily executed by immediately wrapping the blood bottles in silver foil upon obtainment.

Phlebotomy will be completed by those trained and competent in phlebotomy. All efforts will be made to make the procedure as painless as possible. For infants, an oral sucrose preparation or a topical local anaesthetic will be offered.

Infant Blood Samples

A 3-4 mL (minimum volume = 3 mL) blood sample ('just over half a teaspoon') will be taken from all infants. After centrifugation and red cell separation, this will provide an approximate total volume of 1.5 - 2 mL of plasma/serum to allow measurements of PIVKA-II (0.5 mL serum, primary outcome measure), GluOC and GlaOC (0.3 mL serum), dp-ucMGP (0.25 mL plasma) and serum vitamin K1 and vitamin K2 analysis (including, but not limited to menaquinone-7 serum concentrations) (0.5 mL serum). Infant blood samples will be taken at 2 months postnatal age (range 2-3 months).

Maternal Blood Samples

A minimum 6 mL blood sample will be taken from mothers. Once again, after centrifugation and red cell separation, this will allow ~3 mL of plasma/serum to be available for testing (8). Maternal samples will be taken from the supplemented mothers in group 2, along with a control group of unsupplemented mothers from group 1. Serum samples will be analysed to measure maternal PIVKA-II, GluOC and GlaOC, dp-ucMGP and serum vitamin K1 and vitamin K2 concentrations (including but not limited to menaquinone-7 serum concentrations).

Breastmilk Samples

Breastmilk samples will be requested from the mothers who are taking the MK-7 supplement (Group 2), as well as from a comparator group of unsupplemented breastfeeding mothers for control (Group 1). All mothers will be provided with instructions on how to collect and store breast milk samples at home. The breastmilk sample will also be protected from light upon collection (by covering the sample container with silver foil).

Breastmilk samples will be analysed for total vitamin K levels, vitamin K1 levels, total vitamin K2 levels, menaquinone-4 levels and menaquinone-7 levels.

Laboratory Analysis of Samples

All samples will be analysed by specialist laboratories: in The Nutristasis Unit of Professor Harrington, Synnovis, Guy's and St Thomas' Hospital, London, and in Professor Schurgers' laboratory in the

Department of Biochemistry at the University of Maastricht. Both laboratories have expertise in vitamin K analysis. The Nutristasis Unit is a member of The United Kingdom National External Quality Assessment Service for vitamin K (KEQAS), an accredited international scheme set up to help improve the harmonisation and accuracy of vitamin K analysis.

PIVKA-II

PIVKA-II concentrations will be assayed in the Synnovis laboratory using the Abbott Alinity chemiluminescence immunoassay. For this study, PIVKA-II concentrations >0.05 AU/mL, which is the upper limit of the adult range, will be classed as indicating biochemical vitamin K deficiency.

Serum K Vitamers

Serum vitamin K concentrations (including, but not limited to, vitamin K1 and menaquinone-7 serum concentrations) will be analysed using liquid chromatography-tandem mass spectrometry (LC-MS/MS) at the University of Maastricht. Serum MK-7 analysis will also be done at the University of Maastricht and will be completed using LC with post-column fluorometric detection of MK-7 hydroquinone.

Osteocalcin

GluOC and GlaOC will be measured in Prof. Schurgers' Maastricht laboratory using separate enzyme-linked immunosorbent assays (8). The sum of GluOC and GlaOC will be used to determine total OC. The vitamin K status of bone will be assessed by calculating at the percentage of GluOC compared to the total (8).

Desphospho-uncarboxylated Matrix Gla-Protein

dp-ucMGP will also be completed in Prof. Schurgers' Maastricht Laboratory. dp-ucMGP will be measured with enzyme-linked immune sorbent assay (ELISA) testing (18).

Breastmilk Vitamin K1 and MK-7

Breastmilk analysis for MK-7 and vitamin K1 concentrations in breast milk will be undertaken in the Department of Biochemistry, University of Maastricht, The Netherlands. A modification of the process used by von Kries et al. (1987) (31) will be used. Other K vitamers in the breastmilk, including menaquinone-4 concentration, may be analysed depending on availability.

Other follow-up

Questions about supplement acceptability will also be asked of parents at the time of the follow up visit (range 2-3 months postnatal age). This is to gain understanding about the acceptability of providing and administering vitamin K supplementation to their infants, including, where appropriate, whether maternal or infant supplementation might be preferred. A brief dietary history will also be taken at the follow up visit.

Once mother and infant dyads have completed the 2 months follow up visit, no further follow up will be required.

6.6 Sample Management

Laboratory samples will be stored securely and confidentially, with the only identifier being the participants' unique study number and the date/time the sample had been taken. Only delegated researchers who are involved in the study and those who have been delegated to sample management will have access to the specimens. Each laboratory's named researcher will have ultimate responsibility for temporary local storage of the samples.

Blood samples will be taken at 2-3 months postnatal age. Blood will be centrifuged locally, separated, and serum/plasma divided into labelled aliquots will be stored deep frozen at -70°C or lower. Batches of samples will be sent on dry ice by an approved courier service to the respective analysis laboratories.

Breastmilk samples will be collected into the provided specimen collection containers in the mother's homes or at the study follow up visit. 2.5-5mL hindmilk will need to be collected for analysis a minimum of 12 hours after the mother has taken vitamin K supplementation and within 24 hours of the follow up appointment. Information about collecting samples will be provided. Breastmilk will be stored in a fridge in the patients' home for <24 hours and then frozen at -70°C or lower.

After all analysis has been completed for the study, and specific written consent provided, any surplus samples will be retained in long term storage at an approved facility for use in future, ethically approved nutrition studies. Where written consent was not explicitly provided for residual sample use in this way, any surplus samples will be destroyed as per local laboratory standard operating procedures.

6.7 Recording Safety Data

It is not expected that the study supplements will cause safety issues. Recording of adverse events will take place throughout the study. Serious adverse events (SAEs) will be monitored via maternal and infant medical records (hospital and primary care) during follow-up and until the final follow up visit.

Parents will be encouraged to report any perceived side effects that they or the infant has. This should include gastrointestinal side effects, such as colic, alongside any other concerns that the parents may have. Participants will be provided with contact information for the local study team, should they have any questions or concerns.

Safety data will be collected during a telephone call with the family at around 1 months of age, when confirmation of continued eligibility and willingness for ongoing participation will be made. Final collection of relevant safety data will occur at the follow up visit.

6.8 Statistical Analysis

PIVKA-II: Primary Outcome Measure

The primary analysis will be based on the per-protocol population. This will exclude infants who are no longer predominantly breast feeding (defined as having ~two thirds ($\geq 67\%$) of the overall daily milk feed by volume comprised by breast milk).

The primary outcome of proportions of infants with a raised PIVKA-II at the 2-month follow up visit (primary outcome measure indicating biochemical vitamin K deficiency) will be defined as the proportion of infants in each group/subgroup with a PIVKA-II concentration >0.05 AU/mL. Proportions with a raised PIVKA-II will be compared between each two-group comparison using the χ^2 and Fisher exact tests as appropriate. The estimated effect will be the difference in proportions (and the number needed to treat) and the odds ratio; if baseline differences exist then a sensitivity analysis will use logistic regression.

Secondary Outcome Measures

Concentrations of carboxylated osteocalcin (GlaOC), undercarboxylated osteocalcin (GluOC), and total osteocalcin will also be reported (median and interquartile range) for infants in each of the four groups and mothers in the two maternal groups in units of ng/mL. Total osteocalcin is comprised by the sum of GluOC and GlaOC and measures biochemical vitamin K status of bone (8). It will be assessed for each group by the percentage that undercarboxylated osteocalcin forms out of the total osteocalcin (%GluOC), which is

the most widely accepted indicator of bone vitamin K status (8). Higher percentage values for this measure represents relative deficiency of bone vitamin K status compared to lower %GluOC values (8).

Serum levels of various K vitamers will be reported (median and range) for each of the four infant groups and the two maternal groups in units of $\mu\text{g/L}$

Presuming all concentrations will be non-normally distributed (8), concentrations will be log-transformed towards normality then compared using the two-sample t test. Data will be presented as geometric means (95% confidence intervals) for (log-transformed) skewed distributions, or n (%). The effect size will be the ratio of the geometric means. A two-tailed p value <0.05 will be considered statistically significant.

The levels of K vitamers in the breastmilk will be reported (median and interquartile range) for both the supplemented and unsupplemented mothers in units of ng/mL . The levels of desphospho-uncarboxylated matrix Gla-protein will be reported (median and interquartile range) in pmol/L . Should these data be normally distributed, then an independent t test will be used for analysis, and the mean and standard deviation will be reported. Should the data be non-normally distributed, a Mann-Whitney test will be used. A two-tailed P value of <0.05 will be considered statistically significant.

6.9 END OF THE STUDY

The end of the study will be defined as follows.

Individual Participants

The end of the study for each individual mother and infant dyad will be defined as the moment that the 2 month follow up visit has been completed.

Overall Study

The end of the study overall will be defined as the date of the final database lock. This database will include the results of all biological samples (blood and breastmilk) taken as part of the study. The research ethics committee will be informed of the end of the study within 90 days of the end of the study.

7 ETHICAL AND REGULATORY CONSIDERATIONS

7.1 Assessment and management of risk

Risks for this study are anticipated as minimal. Literature demonstrates the safety and tolerability of MK-7 supplementation at various doses (23). PharmaLinea Ltd. have brought a similar MK-7 product to market on a private label in the United Arab Emirates, Vietnam and Saudi Arabia.

As this study will be conducted in healthy infants, completing phlebotomy for research purposes is additional to routine care and an important ethical consideration. The only way to investigate the hypothesis in question is to assess blood biomarkers of vitamin K status in the infants. PIVKA-II, dp-ucMGP and GluOC:GlaOC ratio are tests of functional activity of vitamin K in the liver, vessels and bone respectively and are excellent biomarkers of vitamin K status (15).

Coagulation status will not be measured in this study. Coagulation screens lack sensitivity and specificity as a marker of vitamin K status (15). Including these tests would increase the volume of blood required along with significantly adding to study costs without adding much scientific value. Similarly baseline samples pre-discharge are not included. All eligible infants will have received a large IM dose of vitamin K at birth as per

standard recommendations, therefore, have satisfactory vitamin K status making baseline testing redundant and unnecessary.

The lowest necessary volume of blood required will be taken from the neonate – an estimated 3-4 mL of whole blood. This will be taken by a healthcare professional who is trained in infant phlebotomy, to enable correct samples to be obtained in the least stressful way. Consideration will be taken to pain relief options for infants, including timing feeding with phlebotomy, oral sucrose preparations and topical local anaesthetic cream use.

7.2 Assessment of benefits

A potentially improved infant vitamin K status would be one potential benefit of this study (for infants allocated to receive active MK-7 in group 1; infants of MK-7 supplemented mothers, group 2), however this would not be the case for the placebo supplemented neonates in group 1.

All babies in the breastfeeding group will be provided with vitamin D3, at the UK nationally recommended dose (10 µg/day) (29). This will be provided as part of the study and would otherwise be something that parents would have to purchase over the counter at their own expense.

A further benefit is the knowledge that the families are helping with research that may improve treatment and outcomes for future babies.

Parents will be reimbursed for all their reasonable costs of travel to return for the single follow up visit.

7.2 Ethics

7.2.1 Declaration of Helsinki

The investigators will ensure that this study is conducted in accordance with the principles of the Declaration of Helsinki.

7.2.2 ICH Guidelines for Good Clinical Practice

The investigators will ensure that this study is conducted in accordance with relevant regulations and with Good Clinical Practice.

7.2.3 Approvals

The study will only start after gaining the requisite approvals from the NHS Trust Research and Development Offices and a UK national REC. Discussions will also be completed with the MHRA, and approvals gained where necessary.

Applications will be completed through the Integrated Research Application System (IRAS), submitting a copy of the protocol, parent information literature and informed consent forms and consenting materials for approvals.

Substantial amendments are defined as any amendments that affect:

- The safety or physical or mental wellbeing of the participants
- The scientific value of the study
- The conduct or management of the study
- The quality or safety of any products used in study

Any substantial amendments will be submitted to the REC and/or MHRA (if applicable) for approval.

All correspondence with the REC will be retained in the Study Master File/Investigator Site File.

Responsibility for the maintenance of the Study Master File has been delegated by the Sponsor to the CIs.

An annual progress report will be submitted, where applicable, to the REC within 30 days of the anniversary date on which the favourable opinion was given, and annually until the study is declared ended. The CIs will be responsible for submitting progress reports. The CI will also notify the REC, and any other approving body, of the end of the study. If the study is terminated early, then the REC will also be notified, along with the reasons behind this.

Within 1 year of the end of study, defined as the time of final database lock, the CI will submit a final Clinical Trial Report with the results of the study, including details of any presentations or publications arising, to the REC and any other approving body.

7.3 Peer review

This study protocol has been developed and internally reviewed within the research team.

7.4 Clinical Trials Authorisation

Discussion within the research team as to whether this study categorised as a Clinical Trial of Investigational Medicinal Product (CTIMP), and therefore required a Clinical Trials Authorisation, was conducted.

The research team had recourse to the MHRA guidelines and '*Is it a Clinical Trial of a Medicinal Product?*' document (Appendix 1), (32) and sought advice from the MHRA clinical trials advice helpline (clinicaltrialhelpline@mhra.gov.uk) along with the Combined Ways of Working advice team (cwow@hra.gov.uk) and the Clinical Trials Helpline for Manufacturing (ctdhelpline@mhra.gov.uk). A response was received that we should proceed as a non-CTIMP, as per the MHRA algorithm (Appendix 1) (32).

Similar vitamin supplement products are already available on the UK market, particularly a K2D3 product manufactured by Sunday Natural Products GmbH (Vegan Vitamin D3 + K2 MK-7 200 IU + 15 µg Baby and Child: <https://www.sunday-natural.co.uk/vitamin-d3-k2-mk7-baby-child-200iu-15mcg-vegan.html>). There are also already maternal menaquinone-7 products available as part of antenatal vitamin supplementation, albeit at a much lower dose than we are studying (<https://theovaco.com/products/ova-her-fertility-pregnancy-support>)

Although this study is not classed as a CTIMP, the supplement will be prescribed and/or dispensed as per each Trusts' local guidance and standard operating procedure, to allow governance and accountability processes to be followed.

7.5 Patient & Public Involvement

Discussion with parents and the public has been completed to ascertain their opinions about the study during protocol development, the likelihood that they would have taken part and to review of the patient facing materials. Feedback and suggestions have been incorporated into the protocol design and parent facing materials.

7.6 Protocol compliance

The Sponsor will monitor progress of the study and adherence to the protocol.

The study will also be submitted to a relevant clinical trial register, such as ClinicalTrials.gov or the European Clinical Trials Register.

7.7 Data protection and patient confidentiality

Consent for participation in the study will include consent for use of personal data by the study team. It will also include consent for the research team outside the direct clinical team to have limited access to personal identifying data i.e. for data input or regulatory compliance purposes.

Members of the research team outside the direct clinical team will all hold up to date Good Clinical Practice (GCP) and information governance (IG) training as per the Sponsor's requirements. This consent applies to both maternal and infant health data, and without this consent the participant cannot be enrolled.

Access to personal identifying data will be limited to the delegated research team. Any data provided to external collaborators (i.e. laboratory analysts or statisticians) for analysis will be pseudonymised by using the unique study identifier.

Personal data will be stored on secure databases, which might include NHS computer shared drives or secure electronic databases. Any paper copies of personal identifying data will be kept in locked research offices. The study will also meet with General Data Protection Regulations and the Data Protection Act 2018.

The data custodians for this study will be the Chief Investigators.

7.8 Indemnity

Indemnity will be via the Trust NHS indemnity scheme.

7.9 Access to the final study dataset

The final data set with linked personal identifying information will only be available to the delegated research team. Pseudonymised data sets (with the unique study number used) will be available to those involved in analysis and write up and following final publication of the research. Fully anonymised versions of study data may be deposited in a suitable data repository for sharing with other researchers upon reasonable request to the Chief Investigators.

8 DISSEMINATION POLICY

8.1 Dissemination policy

The results of this study will be presented at one or more scientific meetings, and the results will be published in a peer-reviewed journal. No participant identifying information will be used in any presentations or publications. A summary of the study results will also be entered into the relevant clinical trials registry.

Consenting parents will be provided with a summary of the findings at the end of the study.

Results of the study may also be published in a lay summary form to parent groups and on NHS hospital social media. Assistance with the dissemination of lay summaries will be with the assistance of PPIE groups.

8.2 Authorship policy

All collaborators who have made significant contributions to the protocol or study conduct will be included in authorship, providing they fulfil criteria for authorship according to International Committee of Medical Journal Editors (ICMJE) guidelines. It is expected that all named in this Protocol as Key Protocol Contributors will merit co-authorship. Others who have assisted the study but do not fulfil all criteria for co-authorship will be acknowledged in a final publication(s).

The results of this study and a version of the manuscript for publication will be shared with the study's commercial partner, PharmaLinea, prior to submission.

All data analysis, interpretation of the data and final decision to publish will lie with the investigators alone and will be independent of PharmaLinea.

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10 APPENDIX 1 – Medicines and Healthcare Products Regulatory Agency Is it a Clinical Trial of a Medicinal Product

IS IT A CLINICAL TRIAL OF A MEDICINAL PRODUCT?

This algorithm and its endnotes will help you answer that question. Please start in column A and follow the instructions. Additional information is provided in the notes at the end of the table. If you have doubts about the answer to any of the questions, contact the MHRA clinical trials unit.

A	B	C	D	E
A CLINICAL TRIAL OF A MEDICINAL PRODUCT?			A NON-INTERVENTIONAL CLINICAL TRIAL?	
Is it a medicinal product (IMP)? ⁱ	Is it not a medicinal product?	What effects of the medicine are you looking for?	Why are you looking for those effects?	How are you looking for those effects?
If you answer no to all the questions in column A, the activity is not a clinical trial on a MP.	If you answer yes to the question below in column B the activity is not a clinical trial on a MP.	If you answer no to all the questions in column C the activity is not a clinical trial under the scope of SI 1031.	If you answer no to all the questions in column D the activity is not a clinical trial under the scope of SI 1031.	If you answer yes to all these questions the activity is a non-interventional trial which is outside the scope of SI 1031. If your answers in columns A,B,C & D brought you to column E and you answer no to any of these questions the activity is a clinical trial within the scope of the Directive.
If you answer yes to any of the questions below go to column B.	If you answer no to this question below go to column C.	If you answer yes to any of the questions below go to column D.	If you answer yes to any of the questions below go to column E.	
A.1 Is it a substance ⁱⁱ or combination of substances presented as having properties for treating or preventing disease in human beings?	B.1 Are you only administering any of the following substances? • Human whole blood ⁱⁱⁱ ; • Human blood cells; • Human plasma; • Tissues except a somatic cell therapy medicinal product ^{iv} ; • A food product (including dietary supplements) not presented as a medicine; • A cosmetic product ^v ; • A medical device	C.1 To discover or verify/compare its clinical effects? C.2 To discover or verify/compare its pharmacological effects, e.g. pharmacodynamics? C.3 To identify or verify/compare its adverse reactions? C.4 To study or verify/compare its absorption, distribution, metabolism or excretion? This is only relevant to measuring vitamin K2 excretion in breastmilk. No other measures will be taken.	D.1 To ascertain or verify/compare the efficacy ^{vi} of the medicine? D.2 To ascertain or verify/compare the safety of the medicine?	E.1 Is this a study of one or more medicinal products, which have a marketing authorisation in the UK? E.2 Are the products prescribed in the usual manner in accordance with the terms of that authorisation? E.3 Does the assignment of any patient involved in the study to a particular therapeutic strategy fall within current practice and is not decided in advance by a clinical trial protocol ^{viii} ? E.4 Is the decision to prescribe a particular medicinal product clearly separated from the decision to include the patient in the study? E.5 Will no diagnostic or monitoring procedures be applied to the patients included in the study, other than those which are applied in the course of current practice? E.6 Will epidemiological methods be used for the analysis of the data arising from the study?
A.2 Does the substance function as a medicine? i.e. can it be administered to human beings either with a view to restoring, correcting or modifying physiological functions by exerting a pharmacological, immunological or metabolic action or to making a medical diagnosis or is otherwise administered for a medicinal purpose?				
A.3 Is it an active substance in a pharmaceutical form?				