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# Vitamin K Supplementation for Prevention of Teratogenic Neurological Calcification in Fetuses with Congenital Zika Syndrome: A Research Protocol

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**Abstract**

**Introduction:** Congenital Zika Syndrome (CZS) manifests in infants exposed to the Zika virus (ZIKV) *in utero*. Recent studies have documented the correlation between bone morphogenetic protein (BMP) and fetal brain calcification in these infants; however, potential treatment avenues remain unaccomplished. Unfortunately, the increase in ZIKV cases has engendered fetal neurodevelopmental defects including brain calcification which permanently impairs neurological function. The dominant pathogenic explanation results from osteogenic factor upregulation. Previous research has identified this underlying factor, highlighting the mechanism to combat the neurodegenerative impacts of ZIKV. Since the correlation between ZIKV and BMP is novel, studies addressing the mitigation of infant brain calcification are limited. Data from established ZIKV research was used to determine the potential utilization of matrix Gla protein (MGP) to inhibit the BMP pathway which calcifies fetal neural tissue. This rationale is founded on evidence showing (a) the efficacy of MGP in combating BMP-dependent calcification, and (b) the activation of MGP with Vitamin $K_2$ (VK2). This study aims to establish a protocol for supplementing pregnant Zika patients with the VK2 derivative menaquinone-4 (MK-4), with the goal of preventing CZS-associated neurological calcification.

**Methods:** A literature search was performed to evaluate the feasibility of VK2 injections for the prevention of neural calcification in CZS patients. The preventative potential of VK2 against CZS-related calcification will be tested using *in vivo* pregnant mouse (*Mus musculus)* models. Treatment groups will receive MK-4 administered with propylene glycol, while the control group will receive a placebo. Neurological calcification of fetuses and neonates will be monitored using pelvic ultrasound and micro-CT. Plasma and cerebral MK-4 content will be quantified using liquid chromatography-tandem mass spectrometry.

**Results:** Anticipated results should demonstrate reduced subcortical calcification in the MK-4-treated murine cohort.

**Discussion:** Protocol implementation precipitates the development of preventative CZS treatments. These findings indicate that low-dose maternal VK2 supplementation could provide a potential avenue for prevention of brain calcification *in utero* after vertical transmission of ZIKV.

**Conclusion:** The proposed treatment would be the first of its kind, providing affected populations with a low-cost intervention for neurological damage caused by CZS, decreasing the burden of disease as ZIKV prevalence grows.

**Keywords:** congenital Zika syndrome; neural calcification; Zika virus; vitamin $K_2$

## Introduction

Zika Virus & Congenital Zika Syndrome

Zika virus (ZIKV) is a single-stranded RNA virus from the *Flaviviridae* family transmitted primarily through *Aedes aegypti* mosquitoes, and secondarily through sexual contact [1]. ZIKV has caused epidemics in Africa, the Pacific, and South America, with Brazil declaring it a national health emergency following a large increase in case-associated deaths in 2015 [1]. As ZIKV continues spreading upward, Asia, North Africa, and North America now face the threat of an imminent epidemic, though there is minimal research funding and limited understanding of disease pathology [1]. Furthermore, there exists no vaccine or antiviral treatments for the infection [1].

ZIKV infection in adults can cause several adverse symptoms including exanthema, arthralgia, non-purulent conjunctivitis, and headache; however, a major concern associated with the ZIKV virion is vertical transmission from mother to fetus [1,2]. Upon maternal infection, the ZIKV traverses the placental barrier and subsequently the fetal blood-brain barrier (BBB), infecting the fetus [3]. The teratogenic contagion, particularly when acquired in the first trimester, can confer neonates with Congenital Zika Syndrome (CZS) [4]. CZS is associated with phenotypic

microcephaly and long-term symptoms including epilepsy, eye defects, and functional impairments, with a highly elevated 36-month post-gestational mortality rate compared to those without the condition [4,5]. Such CZS-associated neurodevelopmental issues are the result of the fetal intracranial calcification produced by ZIKV infection [2].

In the ZIKV virion, seven non-structural proteins are produced, including the essential non-structural protein 3 (NS3). NS3 is a protease that impairs host interferon signalling pathways and apoptotic cell death to allow for replication of viral proteins and evasion of the host immune response [2]. In the fetal brain, NS3 triggers upregulation of bone morphogenetic protein (BMP), a perivascular osteogenic factor [2]. Immature pro-BMP2 is cleaved by NS3 protease to form mature BMP2 [2], which then binds to bone morphogenetic protein receptor (BMPR), to trigger the osteogenic signalling pathway and cause calcification [2]. In patients with CZS, neurological calcifications are present in 88-100% of cases, often occurring at the cortical-subcortical junction, basal ganglia, and periventricular regions [5]. Calcification subsides with age; however, resultant damage is permanent with no established treatment or cure [5]. This article proposes maternal vitamin K supplementation as a novel route to prevent ZIKV-associated neurological calcification present in CZS (see Figure 1).

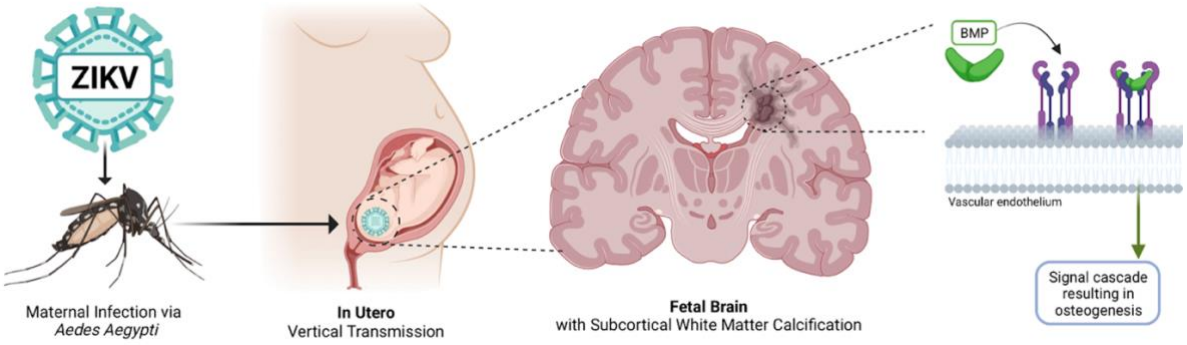


**Figure 1.** Pathophysiology of neurological calcification in CZS (created on BioRender).

Vitamin $K_2$ (VK2), also known as menaquinone, includes a family of fat-soluble nutrients acquired from animal-based dietary sources and bacterial synthesis in the intestinal tract [6]. Variants within the VK2 class include menaquinone-4 (MK-4) which is also known as menatetrenone, and menaquinone-7 (MK-7) [6]. Both variants act as a co-factor of matrix Gla protein (MGP), a protein involved in the inhibition of calcium deposition: VK2-dependent activation of MGP facilitates calcium transport and prevents calcium accumulation in the lining of blood vessel walls [7]. Normally, MGP is activated by post-translational carboxylation of its glutamic acid residues via the gamma-glutamyl carboxylase enzyme (GGCX) in a VK2-dependent reaction [8]. Active MGP sequesters BMP ligands to decrease BMP signalling, and binds to calcium ions to inhibit soft tissue calcification [9]. Through this mechanism, MGP exerts anti-calcification action by inhibiting BMP signal transduction [9], the pathway upregulated by ZIKV to produce the neural calcifications associated with CZS. While this pathway is activated by both variants, MK-4 is preferentially utilized over other VK2 variants for the fetus and is the only variant capable of crossing the placental barrier [10,11]. Since treatment *in utero* is the target of VK2 supplementation for CZS, MK-4 is better suited for supplementation. As such, we propose low-dose maternal MK-4 supplementation could increase MGP activation in the fetal brain, reducing neurological damage caused by CZS-associated calcification (see Figure 2) [9].

The Role of MK-4

MK-4 cofactors activate vitamin K-dependent proteins (VKDPs) through post-translational gamma carboxylation of glutamic acid residues [8,12]. MGP is a VKDP that has proven to be one of the most powerful endogenous inhibitors of calcification [12]. Injections of MK-4 allow for the activation of MGP and subsequent inhibition of BMP, hypothetically preventing brain calcifications for fetuses *in utero*, as demonstrated in Figure 2 [12].

Deficiency of MK-4 has been linked to downregulation of MGP activity, which can lead to the development of vascular and soft tissue calcification. In decreased concentrations of MK-4, the normal carboxylation mechanism is inhibited, preventing MGP activation. This results in increased concentrations of inactive dephosphorylated, uncarboxylated MGP (dp-ucMGP) which is associated with neuropathy [8]. Though novel, MK-4 supplementation is emerging as a vascular mineralization inhibitor in other diseases [8,13]. Research has demonstrated anti-calcification effects of MK-4 supplementation in both arterial calcifications and renal calculi [8,11]. Furthermore, recent studies show an association between dp-ucMGP levels and neuropathy in adult diabetic patients, suggesting MK-4 levels could be pertinent in preventing neurological damage observed in CZS [8].

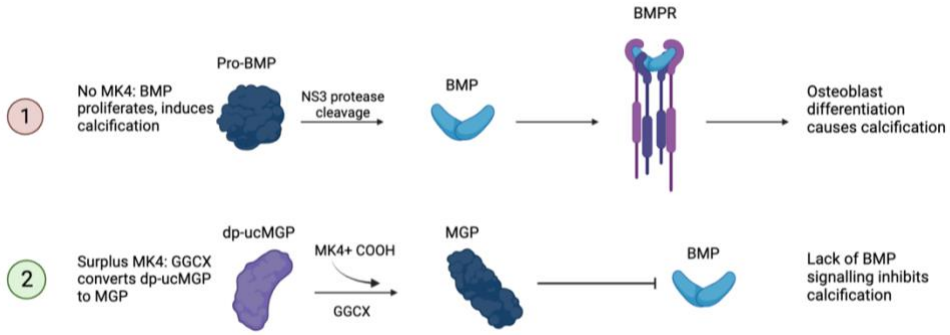


**Figure 2.** i) CZS brain calcification pathway without VK2 and active MGP. (ii) Mechanism of VK2-activation of MGP, inhibiting calcification (created on BioRender).

Crucially, MK-4 can cross placental and brain barriers with potentially sufficient bioavailability to prevent CZS neural calcification. First, maternal intravenous administration increases MK-4 concentration in placental tissue via a carrier protein transport system in the brush border of the placental membrane [10,14]. Subsequently, the placenta serves as a reservoir before release into the umbilical cord blood [11]. ZIKV non-structural protein 1 increases the permeability of vascular endothelial cells in the umbilical cord and brain, causing vascular leakage [15,16]. Additionally, ZIKV disrupts the tight junctions between the syncytiotrophoblast layer of the placental barrier, increasing paracellular permeability by decreasing the number of tight junction proteins ZO-1, occludin, and claudin-4 [2,16,17]. Since syncytiotrophoblasts are implicated in MK-4 transplacental transport and MK-4 enters fetal circulation via the vascular endothelium, the subsequent leakage has potential to accelerate MK-4 transport into the fetal circulation [14]. Second, MK-4 can cross the BBB due to its lipophilic nature and small molecular weight [18]. In contrast, ZIKV crosses the BBB by utilizing exosome-mediated dissemination, inducing an inflammatory response that alters vascular cadherin [19,20]. This increases endothelial permeability, which may accelerate MK-4 transport across the BBB [19,20]. These mechanisms demonstrate the ability of MK-4 in crossing the placental and blood-brain barriers, providing a rationale for its potential effectiveness as a preventative intervention for CZS-associated neurological calcification.

The relevant pathway by which MK-4 could combat CZS combined with the simplicity of its composition makes this intervention an attractive candidate for averting ZIKV teratogenesis in areas of high spread. MK-4 is an easily accessible, low-cost intervention [21], which is beneficial for widespread usage, particularly in developing countries, where ZIKV transmission is most prominent [1]. Furthermore, storage and transport are easily facilitated with this vitamin: MK-4 stored in amber containers can be kept at room temperature (20-25ºC) for approximately 100 days [22,23]. As ZIKV transmission is most prominent in southern, tropical climates [1], this temperature stability gives MK-4 a significant advantage over other interventions requiring cold storage, and its long-lasting shelf life allows for extensive intervention time with less immediacy.

The proposed ability of MK-4 in mitigating CZS development accompanied by the logistical support of its implementation demonstrates the potential utility of MK-4 as a preventative treatment for CZS-related neurological calcifications. Identifying the ability of MK-4 to prevent neural calcification in murine models provides a rationale for its use as a preventative treatment in human CZS.

## Methods

*In vivo* female mouse models *(Mus musculus)* [24], will test the efficacy of MK-4 injections in preventing neural calcifications in CZS as seen in Figure 3. Murine models will be used due to their prior success in modelling ZIKV infection and subsequent neural calcification [2,25]. To produce an immunocompetent mouse model mimicking the human immune response to ZIKV, human signal transducer and activator of transcription 2 (STAT2) knock-in mice will be used. Murine STAT2-dependent interferon (IFN) signalling is not antagonized by ZIKV in wild-type mice, consequently preventing the replication of ZIKV in murine peripheral organs [25]. In contrast, ZIKV NS5 degrades human STAT2 to antagonize human STAT2-dependent IFN signalling, facilitating ZIKV replication. Human STAT2 knock-in mice also do not exhibit high mortality and premature embryonic death associated with the previous IFN-1 knock-out mouse model [2]. Accordingly, pregnant human STAT2 knock-in mice exhibit ZIKV spread in the placenta and fetal brain that models their human counterparts without mortality that would impair specimen analysis [25]. Pregnant dams will be divided via randomized allocation into four equal groups—three treatment groups and one control group. This protocol follows the updated ARRIVE 2.0 guidelines to ensure ethical, reproducible science and transparent reporting in animal research [26]. Criteria for the ARRIVE Essential 10 have been met, and the ARRIVE Recommended Set has been satisfied (excluding 14-16 due to the nature of this protocol).

Pregnancy will be confirmed via pelvic ultrasound in all dams prior to infection. All dams will be inoculated with

ZIKV via subcutaneous (SQ) injection of the active virus on day 1 of gestation [27]. Murine viral spread will be validated through immediate blood sample analysis using available real-time quantitative polymerase chain reaction (qRT-PCR) with appropriately designed ZIKV primers [28]. Following 24 hours of viremia and systemic spread, the three treatment cohorts will be administered the MK-4 intervention at varying concentrations to form a dose-response. Treatment mice will be administered subcutaneous injections of 10, 20, and 40 mg/kg menatetrenone (injectable MK-4) suspended in proportioned propylene glycol vehicles of 30, 60, and 120 µL, administered once daily with breakfast from the date of infection. As 10 mg/kg of menatetrenone will be suspended in 100mL of propylene glycol—mirroring the current composition of preservative-free human newborn Vitamin K1 shots—control mice will receive a weight-adjusted subcutaneous 300 microlitre propylene glycol vehicle injection as a placebo once daily with breakfast from the date of infection [29,30]. Thus, the control group will receive as much propylene glycol as Treatment Group 1. The schedule for infection and treatment of all groups is shown in Table 1. A visual summary of the described methodology is shown in Figure 3.

**Table 1.** Treatment dosages to be administered to groups

| **Group Number** | Treatment 1 | Treatment 2 | Treatment 3 | Control |
|---|---|---|---|---|
| **Treatment Received** | 10 mg/kg SQ inj menatetrenone in 30 µL propylene glycol | 20 mg/kg SQ inj menatetrenone in 60 µL propylene glycol | 40 mg/kg SQ inj menatetrenone in 120 µL propylene glycol | 30 µL SQ inj propylene glycol |

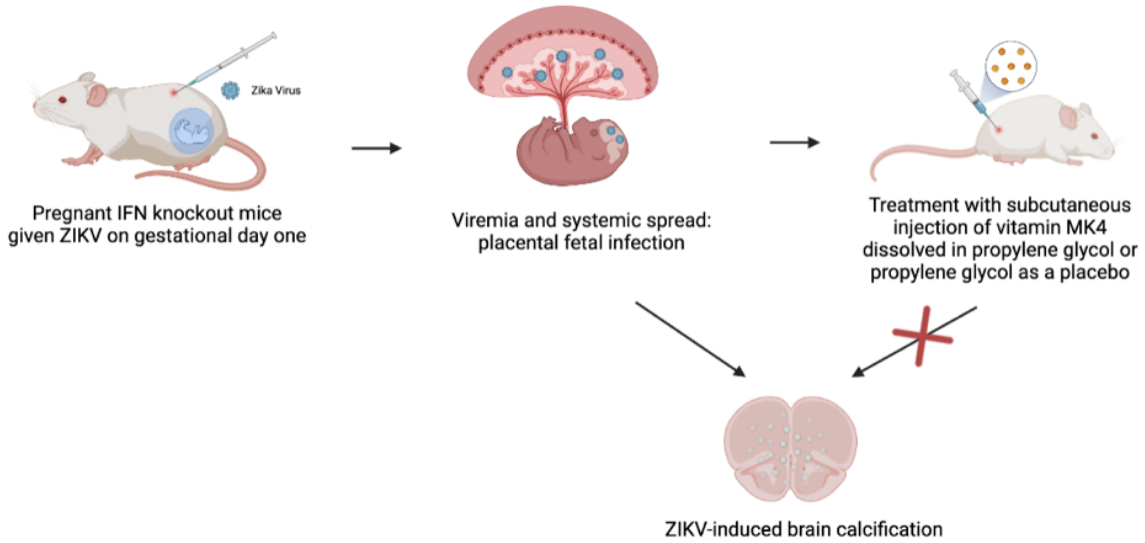


**Figure 3.** Graphic summary of methodology (created on BioRender) [20].

Pre- and Post-Delivery Imaging

Full-term delivery will be induced on day 21 of gestation. Pre-delivery analysis includes pelvic ultrasonography concluding each infected week to track calcification progression [27]. Ultrasonography and Doppler successfully identify early-stage parenchymal and vascular calcification and secondary brain abnormalities, such as enlarged ventricles and cerebral atrophy [31]. Late-term ultrasonography provides the highest degree of sensitivity in the detection of fetal brain calcification [32]. Ultrasound results will be used to track the degree of calcification in fetuses before birth to account for natural variation of CZS pathology as calcification occurs in 88-100% of affected neonates [5,33]. Ultrasound can be used for early identification of potential outliers in cases where calcification may not occur in the fetus.

Pups will be euthanized using a two-step anesthesia and cervical dislocation procedure as per the Canadian Council on Animal Care guidelines before *ex vivo* brain examination [34]. The sample will then be formalin-fixed and immobilized in paraffin wax to facilitate *ex* vivo phenotyping via micron-scale computerized tomography (micro-CT) [35-37]. CT is known as the gold standard method for visualizing calcification of the neural parenchyma and vasculature [35]. In addition to possessing a higher signal-to-noise ratio and spatial resolution than

other imaging technologies such as magnetic resonance imagining or positron emission tomography, micro-CT facilitates three-dimensional visualization of calcifications to definitively ascertain the scope. From the resultant modelling, calcification volume in $mm^3$ will be calculated to yield a measure of calcification load for subsequent statistical analysis [33,36,38]. Positive scans will identify punctate calcification within the cortex, subcortical white matter, and grey-white matter junctions, as well as near the ventricles, where they exist [32,36]. Brain tissue will be preserved for further analysis at 2-8°C [38].

Liquid Chromatography for Plasma and Cerebral MK-4

Post-delivery, pups and dams will be analyzed for MK-4 in plasma and cerebral tissue to confirm vitamin uptake into the placental compartment and fetal brains. Plasma MK-4 can be obtained by anesthetized heart puncture and centrifugation, and prepared using protein precipitation with ethanol, followed by solid phase extraction [39,40]. High-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) using a phenyl-hexyl column and gradient elution allows for isolation and quantification of plasma MK-4 within 14 minutes [40]. After extraction from paraffin wax, cerebral samples will be pulverized and prepared using liquid-liquid extraction in a hexane solvent, followed again by solid-phase extraction [39,41]. For quantification of neural MK-4 a similar HPLC-MS/MS method can be employed but using a reverse-phase C18 column and a mobile phase of methanol/acetic acid (A) and ethanol (B) in lieu of the former method [41]. For both plasma and cerebral samples, MK-4 fraction can be determined via multiple reaction monitoring (MRM) chromatography, an MS/MS scan mode which allows for selection of the predefined and calibrated MK-4 mass/charge ratios for quantification [41].

Qualitative & Quantitative Analysis

Treatment and control group metrics will be compared to determine the efficacy of the MK-4 injections. A one-way between-subjects analysis of variance (ANOVA) followed by Tukey's honestly significant difference (HSD) post hoc tests will be used to compare plasma MK-4 concentrations, cerebral tissue MK-4 concentrations, and volumetric data of neural calcification from micro-CT between each of the four groups. MK-4 values and volumetric data of neural calcification will be expressed as mean ± SD, with significance assessed relative to an alpha value of .05.

**Anticipated Results**

Reduced calcification in treatment pup brains following a full-term pregnancy would support the hypothesis of MK-4 as an effective intervention against neural damage caused by CZS. Based on the literature, the administered MK-4 will perfuse through the placental and blood-brain barriers of treatment group mice, and trigger the carboxylation of dp-ucMGP, activating MGP. If this reaction did occur, post-gestational micro-CT should reveal a greater quantity of vascular and parenchymal subcortical punctate calcifications in control groups compared to treatment groups. Gestational pelvic ultrasound should likewise reveal greater progression of calcification *in utero* compared to treatment groups. Rate and quantity of calcification should follow a dose-response, with the greatest reduction seen in the 40 mg/kg menatetrenone group.

Statistical analysis should reveal greater concentrations of MK-4 in plasma and cerebral tissue for treatment groups compared to control groups. According to the literature, lipophilic menatetrenone should perfuse through the placenta into the fetal compartment, then through the fetal blood-brain barrier to the cerebral tissue [18-20]. Contingent on placental perfusion, treatment pups from the 40 mg/kg group will have the highest plasma MK-4 concentrations. Thus, treatment pups should display a dose-dependent cerebral MK-4 concentration, with the 40 mg/kg group having the highest concentrations due to a higher plasma MK-4 resulting in greater delivery of MK-4 to the neural tissue. Greater plasma and cerebral MK-4 in treatment groups compared to controls would confirm its assumed pharmacokinetic pathway through the dam's circulation to the fetal brain.

**Discussion**

Current literature supports these anticipated results, indicating that maternal supplementation of MK-4 will result in diminished calcifications in the brains of fetuses with CZS. During the gestational period, the carboxylation of dp-ucMGP will activate MGP, thus decreasing calcification. This research supports existing theories surrounding calcification prevention, and the success of these predicted results indicates the practical addition of MK-4 administration into routine CZS therapy. It is integral that these findings are interpreted as a method of diminishing the deleterious effects of CZS, as preventing vertical transmission of the virus is impractical. If successful, this data could be utilized in conjunction with epidemiological findings to alleviate the global burden of CZS-related calcification, improving the quality of life among the affected population. To date, there are no specific alternative treatments for primary brain calcification, aside from general symptom management [42]. Furthermore, no treatments currently exist to prevent calcification onset *in utero*. As ZIKV continues to spread, future implications of this intervention could improve healthcare accessibility—especially in tropical and subtropical regions of the Global South—by providing a low-cost, effective treatment option for neural calcification.

Future directions of investigation include extensions of the therapeutic effect. Extrapolating from recent findings, MK-4 administration was found to be neuroprotective by reducing neurotoxicity, neuroinflammation, and neuronal

necrosis [43]. Accordingly, MK-4 may alleviate ZIKV-induced neuronal inflammation and apoptosis which are contributing factors to microcephaly, meningoencephalitis, and myelitis [44]. Furthermore, continual MK-4 administration may alleviate vitamin K deficiency often present in newborns. At birth, the gut bacteria responsible for menaquinone production are not yet functional [45]. Since MK-4 passes from mother to infant via breastfeeding, the neuroprotective effects of MK-4 against ZIKV may continue after birth.

Literature reviews show that vitamin K administration is relatively safe and well-tolerated, though gaps still exist regarding the ideal form of administration and bioavailability following cross-placental MK-4 transportation. Given that a dose-response relationship exists for MK-4 administration, and biological membrane transportation is successful, the human dosage will be experimentally determined and could vary on a patient-to-patient basis. Additionally, the formulation of this proposed injection may pose a risk for participants due to the adverse effects reported with propylene glycol administration. Though propylene glycol was approved by the Food and Drug Administration in 1982 as an organic solvent for intravenous injection [46], adverse events such as central nervous system toxicity, hyperosmolarity, cardiac arrhythmia, and lactic acidosis have been reported after use. These factors should be monitored accordingly throughout the study progression to determine the safety of the intervention. These elements serve as limitations to this protocol but can be ameliorated with further investigation.

## Conclusions

Menatetrenone shows promise as a preventative treatment for the life-threatening calcification that occurs in congenital Zika syndrome. This research protocol demonstrates the feasibility and clinical implications of a murine model of MK-4-mediated prevention of CZS. Proving the efficacy of menatetrenone in preventing CZS-associated neural calcification provides a rationale for progressing this intervention into clinical trials. Repetition of the protocol in human models would be needed, likely including variable injection solvents and dosages before the ideal formulation is determined. Relatedly, a greater understanding on the long-term effects of menatetrenone supplementation on both mothers and neonates is imperative to understand the safety of this intervention. Following the realization of this protocol, future research should focus on monitoring long-term tolerance of MK-4 and any adverse effects, such as propylene glycol-associated hyperosmolarity and acidosis [47]. MK-4 treatment could ameliorate quality of life in infants affected by CZS by preventing or mitigating detrimental neurological symptoms. Specifically in tropical and subtropical nations prone to ZIKV spread, this feasible and accessible intervention could decrease the burden of this life-threatening disease.

## List of Abbreviations Used

ZIKV: Zika virus
CZS: congenital Zika syndrome
NS3: non-structural protein 3
BBB: blood brain barrier
BMP: bone morphogenetic protein
BMPR: bone morphogenetic protein receptor
VK2: vitamin $K_2$
MK-4: menaquinone-4/menatetrenone
MK-7: menaquinone-7
MGP: matrix Gla protein
GGCX: gamma-glutamyl carboxylase enzyme
VKDP: vitamin K dependent protein
Dp-ucMGP: dephosphorylated, uncarboxylated matrix Gla protein
STAT2: signal transducer and activator of transcription 2
IFN-1: type I interferon
SQ: subcutaneous
Micro-CT: micron-scale computerized tomography
MRI: magnetic resonance imaging
HPLC: high-performance liquid chromatography
MS/MS: tandem mass spectrometry
MRM: multiple reaction monitoring
ANOVA: analysis of variance
HSD: honestly significant difference

## Conflicts of Interest

The authors declare that they have no conflicts of interest.

## Ethics Approval and/or Participant Consent

This study did not require ethical approval or participant consent to propose a study design for a novel treatment of Congenital Zika Syndrome.

## Authors' Contributions

EAF: developed the methods, results, and conclusion, and contributed to the production of figures.
ECG: developed the abstract and discussion and contributed to the introduction and production of figures.
VJHS: developed the introduction and contributed to the discussion, document formatting and production of figures.
SJZB: contributed to the introduction, methods, and discussion.

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