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Monitoring of vitamin K-dependent proteins in perioperative and critical care patients

Sofia Dahlberg



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DOCTORAL DISSERTATION

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To be defended on Friday, the 11th of June, 2021, at 9 a.m.

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Title: Monitoring of vitamin K-dependent proteins in perioperative and critical care patients		
<p>Background: In addition to its indisputable role in coagulation, vitamin K has several other effects mediated by extrahepatic vitamin K-dependent proteins (VKDPs), such as Matrix Gla protein (MGP), osteocalcin (OC) and growth arrest-specific gene 6 protein (Gas6). Research has mainly focused on the long-term effects of the suboptimal function of VKDPs, but accumulating evidence suggests that they may also be involved in acute conditions such as post-myocardial infarction remodelling and the development of heart failure, sepsis and pneumonia-induced pulmonary fibrosis. However, well-designed studies are scarce.</p> <p>Aims: The aim of this dissertation is to help clarify how VKDPs are affected by major surgery and severe illness (Studies I–III) and whether vitamin K supplementation could modify their natural course (Studies IV and V).</p> <p>Methods: Studies I and II prospectively investigated changes in routine coagulation assays (prothrombin time [PT] and activated partial thromboplastin time [APTT]) and the activity of VKDPs prothrombin (protein induced by vitamin K absence/antagonist-II [PIVKA-II], Study I), desphospho-uncarboxylated MGP (dp-ucMGP, Study II) and uncarboxylated OC (ucOC, Study II) in the perioperative period. Study III prospectively analysed changes in PIVKA-II, PT-INR and intensive care unit (ICU) scoring system sequential organ failure assessment (SOFA) score at admission and every third day during the first week in the ICU. Study IV was a retrospective registry study that investigated the effect of intravenous vitamin K on PT-INR in critically ill patients with a prolonged PT-INR. Patients were propensity score matched to controls. Study V was a prospective analysis of how intravenous vitamin K affects both routine and advanced coagulation assays as well as the activity of VKDPs (PIVKA-II and dp-ucMGP).</p> <p>Results: Studies I and II indicate that VKDP activity is low in many patients preoperatively and further decreases in response to surgery, despite mostly normal coagulation assays. These changes were particularly pronounced in patients with high cardiovascular risk. In Study III an elevated PIVKA-II was demonstrated at admission to the ICU, which further increased at every time point. This change was accompanied by a slightly abnormal but stable PT-INR and improving SOFA score. Patients who had suffered myocardial infarctions were especially prone to low VKDP protein activity during the first week in the ICU. In Study IV, PT-INR decreased in both groups. The decrease in PT-INR was more pronounced in patients who received vitamin K. In Study V, intravenous vitamin K reduced but did not normalise PT-INR, Quick PT, dp-ucMGP and PIVKA-II. Also, increased activity of FII, VII, IX and X as well as increased thrombin generation were demonstrated. A sensitivity analysis indicated that this was a true vitamin K effect regardless of whether the patient's condition had improved or deteriorated as measured by the SOFA score.</p> <p>Conclusions: The suboptimal activity of VKDPs is common in critically ill patients and in the perioperative period. The clinical significance of this is not well established, but several potential involvements in disease pathways have been suggested. Intravenous vitamin K had a small but significant effect on routine coagulation assays. This was accompanied by the increased activity of coagulation factors and increased thrombin generation. It was also shown that it is possible to affect the activity of VKDPs with a single dose of vitamin K. Whether there are any true beneficial effects exerted by VKDPs in critically ill patients should be investigated in future studies.</p>		
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Monitoring of vitamin K-dependent proteins in perioperative and critical care patients

Sofia Dahlberg



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Populärvetenskaplig sammanfattning

K-vitamin är ett livsviktigt näringsämne som upptäcktes för drygt 100 år sedan av den danska biokemisten Henrik Dam. Hans forskningsstudier påvisade att djur som fick en fettsnål diet utvecklade allvarliga inre blödningar. Han döpte sin upptäckt till K-vitamin där K:et står för Koagulation, det vill säga blodets förmåga att levra sig. Normal koagulation är en förutsättning för att den blödning som uppstår vid till exempel en sårskada ska upphöra. På 1960-talet kunde en forskningsgrupp i Malmö under ledning av Johan Stenflo påvisa mekanismen för hur K-vitamin aktiverade ett leverproducerat koagulationsprotein - koagulationsfaktor (F)II (protrombin). En så kallad karboxylgrupp bands in till proteinet via specifika enzymer i den så kallade K-vitamincykeln. Protrombin ändrade därigenom sin tredimensionella struktur och kunde binda in kalcium – först då kunde proteinet fungera. Samma mekanism gällde för alla de andra K vitaminberoende koagulationsfaktorerna (FVII, IX, X, protein C och S).

Under senare år har ytterligare ett flertal K-vitaminberoende proteiner upptäckts som produceras utanför levern. De har andra effekter än koagulation, men är också beroende av karboxylering för sin funktion. En del av dessa tros vara involverade i hjärt-kärlsjukdom, benskörhet, neurodegeneration, blodförgiftning och cancer. Gemensamt för samtliga K-vitaminberoende protein är att deras funktion minskar om tillgången på K-vitamin är låg. Man kan uppskatta deras aktivitet med hjälp av speciella blodprover som mäter karboxyleringsgraden. Kroppens förråd av K-vitamin är förhållandevis litet och minskar snabbt om intaget av K-vitamin är lågt, trots att K-vitamincykeln återanvänder K-vitamin. Genom stora epidemiologiska studier har man sett att långvarig K-vitaminbrist är kopplat till ökad förekomst av kärlförkalkning och hjärtsjukdom, samt att ett lågt K-vitaminintag är kopplat till ökad risk för cancer. Det finns teorier om att K-vitaminberoende proteiner även är involverade i akuta sjukdomsprocesser, såsom blodförgiftning, hjärtinfarkt och försämrad lungfunktion efter lunginflammation. Det finns dock väldigt få studier kring hur aktiviteten av K-vitaminberoende protein påverkas på kort sikt under förhållanden av stor fysisk påfrestning såsom vid större kirurgiska ingrepp samt hos svårt sjuka patienter som vårdas på intensivvårdsavdelningar.

Den här avhandlingen utgörs av fem delarbeten och syftar till att öka kunskapen kring hur aktiviteten av olika typer av K-vitaminberoende protein påverkas hos svårt sjuka patienter, samt om tillförsel av K-vitamin har någon mätbar effekt.

I delarbete I och II mättes aktiviteten av tre olika K-vitaminberoende protein före och efter stora kirurgiska ingrepp på hjärnan, bukorganen och skelettet. Studierna omfattade 35 respektive 40 patienter. I tillägg analyserades rutinkoagulationsprover. I båda studierna visade resultaten att karboxyleringsdefekten av både leverproducerat (protrombin (metod: PIVKA-II)) och icke leverproducerat (Matrix Gla protein (metod: dp-ucMGP)) K-vitaminberoende protein är ökad redan före ingreppet och ökar ytterligare efter operationen. I studie II påvisades en trend mot att ökningen av dp-ucMGP var särskilt uttalad hos patienter med känd hjärt-kärlsjukdom. I delarbete III mättes PIVKA-II vid inläggning på intensivvårdsavdelning och därefter var tredje dag under första veckan. Utöver detta mättes rutinkoagulationsprover och en väletablerad skattningsskala användes för att bedöma om patienterna befann sig i förbättringsfas eller inte. 95 patienter inkluderades. Resultaten visade att karboxyleringen hos det K-vitaminberoende proteinet var lågt redan vid inläggning och sjönk stadigt under första veckan på intensivvårdsavdelning. Rutinkoagulationsproverna låg stabila och patienterna var generellt i förbättringsfas, trots detta ökade PIVKA-II. Försämringen var mest uttalad hos patienter som genomgått akut hjärtinfarkt.

I avhandlingens två sista delarbeten analyserades effekten av K-vitamintillförsel. Studie IV var en retrospektiv analys där effekten av K-vitamin studeras hos patienter med ett lätt förlängt koagulationsprov (PK-värde). PK-värdet är ett rutinkoagulationsprov som stiger vid ökad blödningsbenägenhet. En kontrollgrupp som inte fått K-vitamin inkluderades för jämförelse. Patienterna i de olika grupperna matchades för att säkerställa att det inte var för stora skillnader mellan grupperna. 129 patienter inkluderades både i K-vitamingruppen och i kontrollgruppen. Resultaten visade att tillförsel av K-vitamin gav snabbare korrigering av PK-värdet jämfört med kontrollgruppen, dock var skillnaden i absoluta tal förhållandevis liten. I studie V analyserades effekten av K-vitamintillförsel prospektivt på ett flertal koagulationsprover samt PIVKA-II och dp-ucMGP. Patienter med lätt förlängda PK-värden inkluderades, liksom i studie IV. Blodprover togs före och 24 timmar efter K-vitaminet gavs. 52 patienter inkluderades. K-vitamin gav en mätbar positiv effekt på majoriteten av koagulationsproverna samt minskade dp-ucMGP, dock ej till normala nivåer.

Sammanfattningsvis indikerar avhandlingens resultat att aktiviteten av olika K-vitaminberoende protein är låg hos svårt sjuka patienter, och att utan intervention försämras den ytterligare postoperativt och under intensivvård. Att tillföra K-vitamin tycks ha positiva effekter både på koagulation och på aktiviteten av det K-vitaminberoende proteinet MGP. Den kliniska betydelsen av att korrigera K-vitaminberoende proteiners aktivitet hos svårt sjuka patienter med normala eller lätt förlängda rutinkoagulationsprover är oklar. Det finns dock ett flertal studier och teorier om positiva effekter men detta behöver klarläggas ytterligare i större, väldesignade kliniska studier.

Abstract

Background: Vitamin K is essential for maintaining normal haemostasis as it is needed for the activation of factors II, VII, IX and X as well as proteins C and S. In addition to its indisputable role in coagulation, vitamin K has been implicated in cardiovascular disease, sepsis, cancer and neurodegenerative and pulmonary diseases. These effects are thought to be mediated through extrahepatic vitamin K-dependent proteins (VKDPs) such as Matrix Gla protein (MGP), osteocalcin (OC) and growth arrest-specific gene 6 protein (Gas6).

Research has mainly focused on the long-term effects of the suboptimal function of VKDPs, but accumulating evidence suggests that they may also be involved in acute conditions such as post-myocardial infarction remodelling and development of heart failure, sepsis and pneumonia-induced pulmonary fibrosis. However, well-designed studies are scarce, and little is known about how the activity of VKDPs changes during critical illness and in the perioperative period.

Aims: The aim of this dissertation is to help clarify how VKDP activity and routine coagulation assays are affected by major surgery and severe illness (Studies I–III) and whether vitamin K supplementation could modify their natural course (Studies IV and V).

Methods: Studies I and II prospectively investigated changes in routine coagulation assays (prothrombin time [PT-INR] and activated partial thromboplastin time [APTT]) and the activity of VKDPs prothrombin (protein induced by vitamin K absence/antagonist-II [PIVKA-II], Study I), desphospho-uncarboxylated MGP (dp-ucMGP, Study II) and uncarboxylated OC (ucOC, Study II) in the perioperative period. Study III prospectively analysed changes in PIVKA-II, PT-INR and the intensive care unit (ICU) scoring system sequential organ failure assessment (SOFA) score at admission and every third day during the first week in the ICU. Study IV was a retrospective registry study that investigated the effect of intravenous vitamin K on PT-INR in critically ill patients with a prolonged PT-INR. Patients were propensity score matched to controls. Study V was a prospective analysis of how intravenous vitamin K affects both routine and advanced coagulation assays as well as the activity of VKDPs (PIVKA-II and dp-ucMGP).

Results: Studies I and II indicate that VKDP activity is low in many patients preoperatively and further decreases in response to surgery despite mostly normal coagulation assays. These changes were particularly pronounced in patients with

high cardiovascular risk. In Study III, an elevated PIVKA-II was demonstrated at admission to the ICU, which further increased at every time point. This change was accompanied by a slightly abnormal but stable PT-INR and improving SOFA score. Patients who had suffered myocardial infarctions were especially prone to low VKDP protein activity during the first week in the ICU. In Study IV, PT-INR decreased in both groups. The decrease in PT-INR was more pronounced in patients who received vitamin K. In Study V, intravenous vitamin K reduced but did not normalise PT-INR, Quick PT, dp-ucMGP and PIVKA-II. Also, the increased activity of FII, VII, IX and X as well as increased thrombin generation were demonstrated. A sensitivity analysis indicated that this was a true vitamin K effect regardless of whether the patient's condition had improved or deteriorated as measured by the SOFA score.

Conclusions: The suboptimal activity of VKDPs is common in critically ill patients and in the perioperative period. The clinical significance of this is not well established, but several potential involvements in disease pathways have been suggested. It is possible that vitamin K supplementation would be beneficial, especially in at-risk populations. Intravenous vitamin K had a small but significant effect on routine coagulation assays. This was accompanied by an increased activity of coagulation factors and increased thrombin generation. It was also shown that it is possible to affect the activity of VKDPs with a single dose of vitamin K. Whether there are any true beneficial effects exerted by VKDPs in critically ill patients should be investigated in future studies.

List of publications

- I. **Dahlberg S**, Nilsson CU, Kander T, Schött U. Detection of subclinical vitamin K deficiency in neurosurgery with PIVKA-II. *Scand J Clin Lab Invest.* 2017;77:267-74.
- II. **Dahlberg S**, Ede J, Schurgers L, Vermeer C, Kander T, Klarin B, Schött U. Desphospho-Uncarboxylated Matrix-Gla protein is increased postoperatively in cardiovascular risk patients. *Nutrients.* 2018;5:10.
- III. **Dahlberg S**, Schurgers L, Schott U, Kander T. Vitamin K deficiency in critically ill patients: A prospective observational study. *J Crit Care.* 2019;49:105-9.
- IV. **Dahlberg S**, Schött U, Kander T. The effect of vitamin K on prothrombin time in critically ill patients: An observational registry study. *J Intensive Care.* 2021;9:11.
- V. **Dahlberg S**, Schött U, Ängeby Eriksson E, Tahirsylaj Y, Schurgers L, Kander T. The effect of intravenously given vitamin K to critically ill patients with spontaneously prolonged prothrombin time: A prospective observational study. Submitted to *Journal of Intensive Care.*

Abbreviations

APTT	Activated partial thromboplastin time
BMI	Body mass index
CRP	C-reactive protein
DIC	Disseminated intravascular coagulation
dp-ucMGP	desphospho-uncarboxylated Matrix Gla protein
EMR	Expected mortality rate
EPT	Endstage prothrombin time
F	Factor
Gas6	Growth arrest-specific gene 6 protein
GGCX	Gamma-glutamyl carboxylase
GRP	Gla rich protein
Hb	Haemoglobin
HDL	High density lipoproteins
HES	Hydroxyethyl starch
INR	International normalised ratio
IQR	Interquartile range
KH2	Vitamin K hydroquinone
KO	Vitamin K 2,3-epoxide
LDL	Low density lipoproteins
LVAD	Left ventricular assist device
MK	Menaquinone
NOAC	Novel oral anticoagulants
OC	Osteocalcin
PIVKA-II	Protein induced by vitamin K absence/antagonist-II
PT	Prothrombin time
ROTEM	Rotational thromboelastometry
SAPS	Simplified acute physiology score
SOFA	Sequential organ failure assessment
TAM	Tyro3, AXL, MerTK
TF	Tissue factor
TGA	Thrombin generation assay
TRL	Triglyceride-rich lipoproteins
WISN	Warfarin-induced skin necrosis
VKA	Vitamin K antagonist
VKDP	Vitamin K-dependent protein
VKOR	Vitamin K epoxide reductase
VKR	Vitamin K reductase

Introduction

History

The history of vitamin K and its medical application dates back to the early 1900s and is the result of collective efforts from biochemists, veterinarians and animals. The fat-soluble micronutrient vitamin K was identified in the 1930s by Danish biochemist Henrik Dam, who discovered that chickens who were fed a low-fat diet developed bleeding complications. Initially, the symptoms were believed to derive from vitamin C deficiency, but after unsuccessful attempts to treat the animals with high doses of vitamin C and other experimental diets, Dam concluded that there was an unknown compound separate from the other fat-soluble nutrients A, D and E. He named his discovery vitamin K, where the K stands for koagulation (coagulation with German and Scandinavian spelling) to emphasise its importance for haemostasis. Less than a year later, the biochemical structure of vitamin K was demonstrated by American researcher Edward Doisy. For this, Dam and Doisy shared the Nobel prize in 1943 [1]. Even though the precise mechanism of action remained unknown, vitamin K was soon introduced in medical practice to treat bleeding conditions and prevent haemorrhagic disease in new-borns [2].

Concomitantly, farmers were facing a new malady called sweet clover disease, in which large numbers of cattle developed spontaneous and fatal bleeding events. Sweet clover disease was first described in the 1920s by Doctor Schofield, a veterinary pathologist who linked the condition to spoiled sweet clover hay and also demonstrated that withdrawal of the feed and transfusions from healthy animals resulted in a regression of symptoms. Later, it was discovered that the animals had a severe prothrombin deficiency and an elevated prothrombin time (PT) through a test developed by Quick et al. in 1935. Further research by biochemist Karl Paul Link revealed that the spoiling process resulted in the oxidation of the natural coumarins to form the haemorrhagic agent, dicoumarol, and that supplementation with vitamin K reversed the bleeding complications of sweet clover disease. This subsequently led to the development of warfarin, named after the Wisconsin Alumni Research Foundation, with the suffix -arin representing its association with coumarin [3]. Vitamin K antagonists (VKAs) were incorporated into medical practice to treat acute myocardial infarction; for instance, President Eisenhower was treated with warfarin following a heart attack in the 1950s.

Decades later, additional insights were provided by Swedish professor Johan Stenflo et al., who demonstrated that prothrombin Glu residues were modified by vitamin K [4]. The precise mechanism of action was later elucidated when the vitamin K-dependent γ -carboxylation of Glu residues was demonstrated by the same research group. The carboxylation of Glu to Gla induced a conformational change that allowed the protein to bind to calcium ions and thus become biologically active [5].

Different forms of vitamin K

There are two naturally occurring forms of vitamin K, vitamin K₁ (phylloquinone) and vitamin K₂ (menaquinone). In addition, the synthetic forms vitamins K₃–K₅ exist.

Vitamin K₁ is produced by photosynthetic plants that utilise it as an electron carrier. In contrast, vitamin K₂ is synthesised by anaerobic bacteria and occurs in meat products and fermented foods such as cheese, curd and the Japanese specialty natto [6]. Vitamin K₃ is cheap to produce and commonly used in animal feed. It is toxic in high doses due to the generation of reactive oxygen species [7], something that has been utilised in cancer therapy [8] but may also cause severe haemolytic anaemia in infants [9]. It is, however, still used in parts of the world to prevent haemorrhagic disease in neonates [10]. Vitamins K₄ and K₅ have gained some research interest due to presumed fungistatic [11] and anticarcinogenic effects [12–14]. Vitamins K₃–K₅ will not be considered further in the scope of this dissertation.

All subspecies share a polar naphthoquinone ring structure, to which a hydrophobic side chain of varying length and saturation is attached. Vitamin K₂ is further divided into menaquinone-n (MK-n), where n corresponds to the side chains' number of prenyl units with repeating unsaturated bonds [15]. The forms of vitamin K differ in synthesis, metabolism and tissue distribution.

The molecular structure of different vitamin K species is shown in Figure 1.

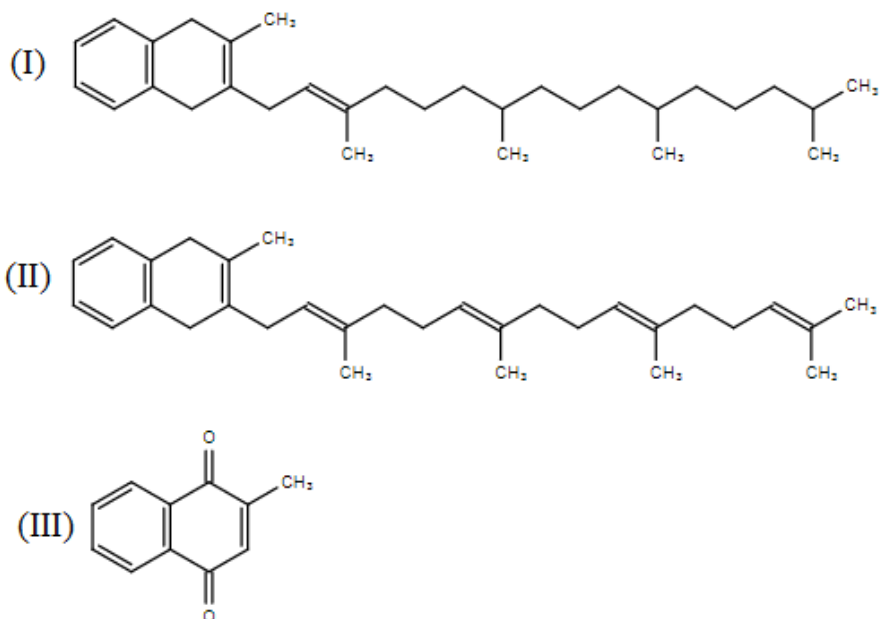


Figure 1. Vitamin K species

(I) Vitamin K₁, (II) vitamin K₂, (III) vitamin K₃

In general, tissue stores of vitamin K are low. Vitamin K₁ is preferentially utilised by the liver, whereas vitamin K₂ is the dominating form in extrahepatic tissues [6]. Vitamins K₁ and K₃ can be converted to vitamin K₂ (MK-4) *in vivo*, which had already been demonstrated by the late 1950s when increased levels of vitamin K₂ were found in several organs in animals that were fed vitamin K₃ or vitamin K₁ [16].

Sources of vitamin K and recommended daily intake

Dietary sources

Leafy vegetables constitute the main source of vitamin K₁, with reported concentrations ranging from 400–700 µg vitamin K₁ per 100 grams (g). The darker green the leaves, the higher the vitamin K₁ content [17]. Another source is vegetable-based oils, which provide concentrations in the range 50–200 µg vitamin K₁ per 100 g.

Vitamin K₂ is found in meat products and fermented foods. However, a significant variability in the vitamin K₂ content between the different products has been demonstrated. This could be due to the fact that bacterial strains differ in their capacity for vitamin K₂ synthesis. One example of this is the Japanese soy dish natto,

which contains very high levels of vitamin K₂ due to *Bacillus subtilis*, which also produces vitamin K₂ in the intestines for days after ingestion. This effect was obliterated by boiling the natto, presumably since this eliminated the bacillus species [18].

Vitamin K production by intestinal flora

The human intestinal bacterial flora contain species that are capable of vitamin K₂ synthesis. Because of this, the intestines were initially believed to be an important source of vitamin K. This view was reinforced when studies reported hypoprothrombinaemia secondary to antibiotic treatment [19]. However, subsequent research concluded that the intestinal contribution to the vitamin K status was much smaller than initially thought, since most vitamin K-producing bacteria reside in the colon and the absorption happens mainly in the small intestines [20].

Recommended daily intake

American guidelines recommend a daily intake of vitamin K ranging from 2 µg/day in infants to 75 µg/day in teenagers, with no gender difference. For adults, the recommendation is a daily intake of 120 µg/day (males) and 90 µg/day (females) [21]. The current recommendations for vitamin K intake are based on what is needed to maintain normal coagulation and may not be sufficient to fully carboxylate the extrahepatic Gla proteins. The recommendations are also not adjusted for potential differences in age and ethnicity [22].

Synthetic vitamin K concentrates

In addition to naturally occurring vitamin K, there are also synthetic forms that are used in medical practice and as supplements. Vitamin K₁ is commonly administered as Konakion, which may be given orally, intramuscularly or intravenously. However, intramuscular administration is discouraged due to unpredictable absorption [23]. Studies have shown that the intravenous route of administration is considerably faster in reversing VKAs [24]. Oral administration should be avoided in patients with cholestatic disease as the absorption is significantly decreased [25]. Vitamin K₂ (MK-4 and MK-7) exist as capsules, and there are, to our knowledge, currently no intravenous formulas.

The vitamin K cycle

To facilitate protein gamma-carboxylation, vitamin K undergoes a series of enzymatic reactions, referred to as the vitamin K cycle (Figure 2).

Initially, vitamin K is reduced by vitamin K reductase (VKR), resulting in vitamin K hydroquinone (KH₂). KH₂ is the substrate used by gamma-glutamyl carboxylase (GGCX) in the step where glutamic acid residues are carboxylated to form gamma-carboxy glutamic acid in Gla proteins, which is the active form. This reaction is dependent on the presence of oxygen and carbon dioxide. Concomitantly, through oxidation, KH₂ is transformed to vitamin K 2,3-epoxide (KO). The cycle is completed through the action of the enzyme vitamin K epoxide reductase, which regenerates vitamin K from KO. VKOR is the target of VKAs, such as warfarin [26].

GGCX recognises the Gla proteins through the so-called propeptide [27]. Even though propeptides share comparatively similar structure, their affinity for GGCX varies significantly [28].

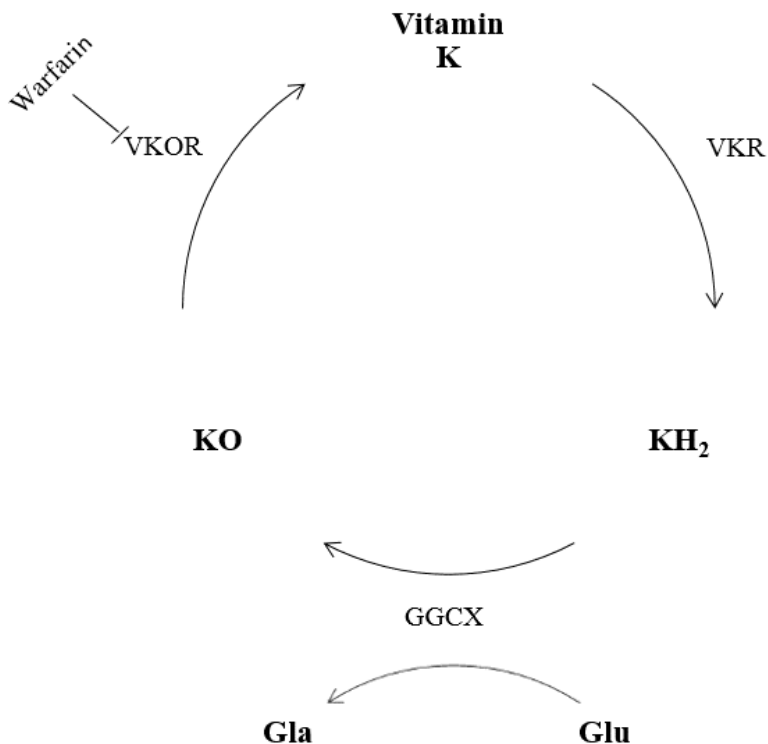


Figure 2. The vitamin K cycle

VKR: vitamin K reductase; KH₂: vitamin K hydroquinone; GGCX: gamma-glutamyl carboxylase; KO: vitamin K epoxide; VKOR: vitamin K epoxide reductase

Intestinal absorption and transport of vitamin K

The intestinal absorption of vitamin K require pancreatic enzymes and gall salts to form micelles that are available for enterocyte uptake.

Vitamin K is integrated into chylomicrons following intestinal absorption, and subsequently enters the circulation. The chylomicrons are broken down into chylomicron remnants by lipoprotein lipases that reside in the endothelium. Smaller amounts of vitamin K are transported using low density lipoproteins (LDL) and high density lipoproteins (HDL).

The plasma vitamin K₁ concentration have been studied using deuterium-labelled greens. The results showed that the concentration reaches its highest point after approximately 6 to 9 hours, and then falls down to baseline levels within 24 hours [29]. Vitamin K₂ species vary significantly in terms of bioavailability and lipoprotein transport. For instance, MK-4 is transported by triglyceride-rich lipoproteins (TRLs), LDLs and HDLs and is cleared from the circulation within a few hours. MK-9 in the other hand remained in the circulation for up to two days and predominantly found in TRLs and LDLs [30]. MK-7 has shown the longest half-life up to 96 hours, which is believed to contribute to its higher bioavailability for VKDPs [31].

Vitamin K-dependent proteins (VKDPs)

Coagulation factors

Vitamin K is essential for the coagulation cascade as it activates both pro- and anticoagulant proteins, namely factors (F) II, VII, IX and X as well as proteins C, S and Z. Their individual functions are discussed in coming sections. The coagulation proteins display significant variation in half-life, see Table 1. This is believed to be a contributing factor to the rare complication of warfarin-induced skin necrosis (WISN), which develops in roughly 1:10000 of warfarin-treated patients. In WISN, patients exhibit a paradoxical prothrombotic state in response to warfarin, which might be explained by the fact that protein C levels drop more rapidly compared to the other vitamin K-dependent coagulation proteins [32].

TABLE 1. Coagulation factors' half-life in plasma

Coagulation factor number	Coagulation factor name	Half-life (hours)
I	Fibrinogen	70–120
II	Prothrombin	70
III	Tissue factor	
IV	Calcium	
V	Pro-accelerlin	20
VI	Unassigned	
VII	Pro-converlin	3–6
VIII	Anti-haemophilic factor A	10
IX	Anti-haemophilic factor B or Christmas factor	25
X	Stuart-Prower factor	40
XI	Plasma thromboplastin antecedent	80
XII	Hageman factor	
XIII	Fibrin-stabilising factor	120–200
Protein C	Protein C	10
Protein S	Protein S	40

Matrix Gla protein (MGP)

MGP was isolated from bovine bone matrix in the 1980s, and MGP expression has since been demonstrated in several organs, such as the kidney, lung, brain, cartilage, blood vessels and heart. Substantial evidence suggests that MGP functions as a calcification inhibitor, but its exact mechanism has yet to be discovered. MGP knock-out in mice resulted in massive arterial calcification and premature death [33], a process that appeared to be regulated locally as it was not affected by systemic MGP secretion. In addition, MGP has been shown to interact with vitronectin and bone morphogenic protein 2 [34], which are present in arteries and atherosclerotic lesions. Increased concentrations of inactive, uncarboxylated MGP have been demonstrated in both medial and intimal calcification.

In humans, mutations in the MGP gene may result in the rare condition Keutel syndrome, which is associated with bone and cartilage abnormalities, pulmonary hypertension and hearing loss [35]. Vascular abnormalities do not seem to develop as early compared to mice, perhaps due to the upregulation of other calcification inhibitors, but a post-mortem examination of a 38-year old patient with Keutel syndrome revealed widespread vascular abnormalities [36].

Like other VKDPs, MGP is dependent on gamma carboxylation to become biologically active. In addition, MGP undergoes phosphorylation, which is believed to affect its secretion from the extracellular matrix. Hence, MGP may be found in different states of carboxylation and/or phosphorylation.

Osteocalcin (OC)

OC was identified in the 1970s and was the first extrahepatic Gla protein to be discovered. It is synthesised mainly by osteoblasts and is involved in bone mineralisation. Research has mainly focused on its potential involvement in the development of osteoporosis. Pre-clinical data also suggest that OC plays a role in cardiovascular disease, but high-quality data are lacking, and a causal relationship has not been established [37].

Growth arrest-specific gene 6 protein (Gas6)

Gas6 shares a structural similarity with protein S, and they both have the capacity to bind to Tyro3, Axl and MerTK (TAM) receptors [38]. In turn, TAM receptors activate several downstream signalling pathways involved in inflammation and cell proliferation.

Gla rich protein (GRP)

As implied by its name, GRP is the most densely populated with respect to Gla residues among the VKDPs. Similar to MGP, GRP is believed to be involved in vascular calcification. Studies have shown calcification inhibitory properties that are gamma carboxylation-dependent. To date, there are only a few studies that have investigated the role of GRP, but low GRP has been suggested as an early marker for vascular calcification in patients with chronic kidney disease [39].

Markers of vitamin K status

Vitamin K status may be estimated in several ways, and the lack of a gold standard measure is a complicating factor [40].

Perhaps the most intuitive approach would be to measure circulating vitamin K levels in plasma. Methods such as high performance liquid chromatography and mass spectrometry are technically challenging and require separate analyses for the various vitamin K subspecies. The samples should be analysed fasting and preferably adjusted for triglycerides. Also, there are no established reference intervals, which further complicates interpretation.

Vitamin K status may also be estimated by analysing the fraction of circulating uncarboxylated VKDPs. High levels of inactive, uncarboxylated proteins could be interpreted as a functional vitamin K deficiency. There are several commercially available assays.

Proteins induced by vitamin K absence for factor II (PIVKA-II) is a measure of uncarboxylated prothrombin (FII) and is believed to reflect hepatic vitamin K status. Increased levels of PIVKA-II have been demonstrated both in patients on VKA treatment [41] and in response to the dietary depletion of vitamin K [42]. PIVKA-II has been suggested to be a more sensitive marker for increased bleeding diathesis compared to standard coagulation assays, which require a substantial decrease in clotting factor levels before prolongation.

It is also possible to measure the circulating levels of different MGP species, in particular the uncarboxylated unphosphorylated variant (dp-ucMGP), which is biologically inactive. Accumulating evidence suggests that dp-ucMGP functions as a marker of vascular vitamin K status, and more recently, studies have shown elevated dp-ucMGP levels in pulmonary disease, such as emphysema and COVID-19. Increased dp-ucMGP levels have been demonstrated in patients with normal PIVKA-II levels, which might suggest that the body prioritises the carboxylation of coagulation factors to sustain normal haemostasis. It could also be a result of low vitamin K₂ intake, which is preferentially utilised by the extrahepatic VKDPs.

Implication of vitamin K's involvement in disease

Vitamin K-related research has traditionally focused on its involvement in coagulation homeostasis. However, the research focus has shifted in recent decades due to the discovery of extrahepatic VKDPs as well as accumulating results suggesting that vitamin K in itself may have beneficial health effects that extend beyond supporting coagulation. It should, however, be noted that high-quality data are scarce and that most arguments are on a theoretical or preclinical basis and need to be verified in large-scale studies. It has also not been established whether there is a causality between altered levels of circulating VKDPs and disease or if abnormal levels of VKDPs are rather a consequence. Nevertheless, below is a brief review of the pathophysiological processes in which vitamin K may be involved.

Cardiovascular disease

Cardiovascular disease is a leading cause of death globally and has a significant impact both on the individual and societal levels [43]. In the early 2000s, a study which indicated that treatment with VKAs may induce cardiovascular calcification in humans was published [44], something that had been demonstrated in animals a few years earlier [45]. Since then, several studies that support a plausible link between VKAs and cardiovascular disease have been published [46].

The pathophysiological explanation for vitamin K's involvement in cardiovascular disease is mainly attributed to MGP activity. Circulating levels of dp-ucMGP have

been associated with increased mortality and the incidence of cardiovascular events in uremic patients [47, 48]. In larger population studies, dp-ucMGP has been associated with vascular stiffness [49] and cardiovascular mortality [50].

In addition, it is possible that vitamin K in itself exerts positive cardiovascular effects. In a small randomised study in which healthy subjects were given vitamin K₂ or placebo for 8 weeks, a 12% increase in cardiac output during exercise was seen in the supplemented group [51]. One of the theoretical explanations for this is vitamin K₂'s ability to function as an electron carrier and support the mitochondrial production of adenosine triphosphate, which in turn could increase contractability in cardiac myocytes. Whether these results may be extrapolated to critically ill patients remain unclear, and there are, to our knowledge, currently no studies on vitamin K₂ supplementation in critically ill patients.

Since cardiovascular disease typically is slow-progressive, this research might not seem overly relevant for the ICU population, whose typical length of stay ranges from days to weeks. However, there are pre-clinical studies that have demonstrated an increased gene expression of MGP 24 hours after myocardial infarction which peaks after 4 weeks, perhaps indicating that MGP plays a role in post-infarction myocardial remodelling [52].

Research interest in this area sparked further when animal studies showed that the cardiovascular abnormalities were partly reversible through vitamin K supplementation [53]. Substantial research effort has since been put into investigating whether this can be extrapolated to humans. So far, results have been inconclusive. Some studies suggest a beneficial effect on vascular [54, 55] and valvular [56] calcification, whereas others do not [57, 58]. Several systematic reviews on the matter have been published [59-61], and most of them conclude that it is premature to say whether vitamin K might prevent or reduce vascular calcification. Larger, well-designed studies are needed, and the supplementation efforts should likely be directed towards at-risk populations, such as uremic and diabetic patients. Currently, several larger randomised controlled trials are in progress [62].

Sepsis

Sepsis is caused by a dysregulated host response to an infection and is a condition associated with high mortality [63]. Sepsis is a common reason for ICU admission as well as a common complication of prolonged ICU care. Gas6 is involved in inflammatory signalling, and pre-clinical studies have shown potential therapeutic roles for Gas6 in sepsis-induced organ damage to the kidneys [64] and lungs [65].

Thus far, human studies are scarce, but increased circulating levels of Gas6 have been demonstrated in critically ill patients [66]. Furthermore, the levels of Gas6 within 24 hours of ICU admission have been shown to outperform both CRP and

procalcitonin as predictors of mortality [67]. Gas6 also has the added benefit of remaining steadily increased over a longer period compared to pro-inflammatory cytokines, where the increase is transient, complicating the interpretation. In a post-mortem examination, Gas6 levels could not be used to distinguish sepsis-related deaths from other causes, such as trauma or massive pulmonary embolism [68], suggesting that Gas6 may not be a specific marker for sepsis but rather represents high inflammatory burden. To date, there are, to the best of our knowledge, no studies on whether vitamin K supplementation affects Gas6 levels.

Pulmonary disease

Recent studies have identified pulmonary disease as another potential disease pathway in which MGP might be involved. In a study on COVID-19 patients, pronounced elevations of dp-ucMGP, which correlated to a poorer prognosis, were demonstrated [69]. Researchers have hypothesised that macrophage activation and subsequent changes in calcium content in elastic fibres result in the upregulation of MGP synthesis and therefore increased vitamin K demand. Pulmonary damage and the development of fibrosis may occur after long-standing mechanical ventilation and acute respiratory distress syndrome, two conditions that ICU patients are at risk for.

Osteoporosis

The discovery of the bone derived VKDP OC in combination with data from observational studies linking an increased fracture rate and lower bone mineral density to higher levels of circulating inactive, uncarboxylated OC have motivated several clinical trials. Carboxylated OC is believed to affect the interaction between osteoblasts and osteoclasts through its high affinity for calcium and hydroxyapatite. Furthermore, vitamin K has been suggested to promote bone health via interaction with several different receptors [70], such as nuclear factor Kappa B and receptor activator of nuclear factor Kappa B ligand. Even though some studies suggest protective effects of vitamin K supplementation in certain patient categories [71], there is insufficient evidence to draw any definitive conclusions [72].

Cancer

Similar to cardiovascular disease, cancer is one of the leading causes of death worldwide [73]. In large population-based studies, dietary vitamin K intake has been associated with decreased cancer risk [74, 75]. However, in these studies, a high vitamin K intake was also associated with determinants of high socio-economic status, and no causal relationship has been established. Nevertheless, vitamin K-mediated anti-tumour effects against some cancer cell lines have been demonstrated [76] using several distinct pathways reviewed elsewhere [77]. As with the other

diseases previously discussed in this chapter, the effects are mediated through both vitamin K species and VKDPs.

Neurodegenerative disease

In the 1980s, researchers proposed a possible link between vitamin K and cognition after demonstrating impaired psychomotor function in warfarin-treated rats [78]. Succeeding studies have suggested that both Gas6 and protein S have neuroprotective effects [79] and that vitamin K might induce sphingolipid synthesis [80, 81]. Impaired sphingolipid metabolism has been observed in neurodegenerative maladies such as Alzheimer's disease [82]. Vitamin K concentration has been positively associated with cognitive status in patients aged above 75 years [83], and when comparing geriatric patients on VKA treatment with age-matched controls, a more pronounced executive dysfunction at baseline and a more rapid decline over 24 months were observed [84].

Normal haemostasis

Haemostasis is a tightly regulated process that is balanced by pro- and anticoagulant factors to achieve homeostasis.

The initial step in the coagulation cascade is the so-called primary haemostasis, in which platelets adhere to the subendothelial collagen in the injured vascular wall. The platelet-collagen interaction is mediated either by directly binding the glycoprotein (GP) Ia/IIa receptor or indirectly via von Willebrand factor, which crosslinks platelets and collagen through the GPIb (non-activated platelets) or GPIIb/IIIa (activated platelets) receptor [85]. The activated platelets change shape and aggregate to form a platelet plug that is stabilised by fibrinogen. The platelets also release granular contents from α granules and dense bodies that recruit more platelets, support platelet activation and promote the formation of a stable thrombus. Finally, the platelets express receptors that allow the accumulation of tissue factor (TF) on their surface, which initiates and reinforces thrombin generation, referred to as the thrombin burst.

The next part of haemostasis is the plasma coagulation, in which a series of coagulation factor interactions result in the generation of thrombin. This is mainly initiated by the exposure of subendothelial TF, which forms a complex with circulating FVII, which in turn activates FIX and, in the presence of FVIII, FX. FX forms a complex with FV (the prothrombinase complex), which converts prothrombin to thrombin. Initially, only small amounts of thrombin are generated, but through self-enhancing feedback loops, increasing amounts of thrombin are

generated. Finally, fibrinogen is cleaved to monomeric fibrin, which is crosslinked by FXIIIa to form a stable clot.

To counteract the procoagulant system and avoid excessive thrombosis, haemostasis is negatively regulated by fibrinolysis and anticoagulant factors. The most important negative regulator of haemostasis is antithrombin, which has the ability to inactivate thrombin as well as FXa, FIXa, FXIa and FXIIa. Furthermore, VKDPs C and S are negative regulators that inactivate FVa and FVIIIa [86]. Fibrinolysis is a self-regulated process in which plasminogen (PLG) is converted to plasmin by tissue PLG activator (t-PA) or urokinase. In turn, plasmin dissolves fibrin into soluble degradation products [87].

Coagulopathy in perioperative and critically ill patients

Coagulation abnormalities occur frequently in conjunction with critical illness or major surgery and are independently associated with increased mortality [88, 89]. The underlying cause is multi-factorial and may derive from blood loss, haemodilution and/or a dysregulated immune response. Unfavourable conditions such as hypothermia, hypocalcaemia and acidosis also contribute to coagulation abnormalities in critically ill patients.

Sepsis is commonly associated with haemostatic changes that result from an increased consumption of coagulation factors combined with an immoderate activation of the coagulation cascade [90]. The interaction between the immune system and the coagulation is complex, but a core part is increased TF expression with the subsequent downstream activation of coagulation proteins [91, 92]. In addition, there are bacteria- and cytokine-mediated pathways that enhance fibrinolysis and inhibit the activity of proteins C and S [93, 94].

Perioperative coagulation abnormalities could result from the combined effects of intraoperative bleeding and fluid administration. This results in a dilutional coagulopathy with decreased levels of fibrinogen and coagulation factors [95]. Low fibrinogen has been suggested to be a key component in acquired coagulopathy following cardiac surgery, trauma and post-partum bleeding [96].

Critically ill and surgical patients are at high risk for developing coagulopathies from these mechanisms. A previous study has shown low levels of the vitamin K-dependent FII, FVII, FIX and FX in patients admitted to the ICU which were temporarily corrected by the administration of prothrombin complex concentrate [97]. However, after 24 hours, the levels had decreased back to baseline. Another study on patients undergoing major abdominal surgery showed that prolonged preoperative fasting resulted in decreased preoperative levels of vitamin K-dependent clotting factors compared to controls. One week after surgery, the levels

of vitamin K-dependent clotting factors, mainly FVII and FX, were still lower compared to controls, accompanied by an increased PIVKA-II [98].

Little is known about the vitamin K status in critically ill patients, and generally accepted guidelines are missing. In a review by Hunt et al. published in the *New England Journal of Medicine*, the authors recommended a weekly dose of 10 mg but also reflected on the lack of knowledge in this area [88]. Previous studies on healthy subjects have shown that vitamin K stores are rapidly depleted and that vitamin K status might drastically alter over a few days with low intake [42]. Critically ill patients are at risk of vitamin K deficiency for several reasons, such as low intake while ill or in the time leading up to falling ill, decreased bowel absorption due to paralytic ileus or bowel oedema and increased demand due to excessive coagulation activation.

Aims

The motivation behind the research that forms this thesis was to increase the knowledge about how VKDP activity is affected by critical illness, major surgery and supplementation with vitamin K. The specific aims are listed below.

Study I

To prospectively investigate PIVKA-II changes in elective neurosurgery.

Study II

To prospectively investigate changes in dp-ucMGP and ucOC after major surgery.

Study III

To investigate how PIVKA-II develops over the first week of intensive care.

Study IV

To retrospectively analyse the extent to which 10 mg of intravenous vitamin K affects PT-INR in critically ill patients.

Study V

To prospectively investigate the extent to which 10 mg of intravenous vitamin K affects routine and advanced coagulation assays as well as PIVKA-II and dp-ucMGP in critically ill patients.

Material and methods

Laboratory analyses

Prothrombin time (PT)

PT may be analysed using the international normalised ratio (INR), referred to as Owren PT-INR and commonly used in Sweden, or the internationally used Quick PT method. PT-INR is calibrated using reference plasma samples from Equalis (Uppsala, Sweden).

The Owren PT-INR is sensitive to changes in F FII, FVII and FX. The sample is diluted using citrated bovine plasma, after which a reagent is added and the time it takes to form a clot is measured (seconds). The result is given as a ratio of the patient's clotting time relative to the clotting time of a healthy reference population. The reference interval was 0.9–1.2, with a coefficient of variation (CV) < 4%.

The Quick PT is a similar method, with the exception that it is also sensitive to changes in fibrinogen and FV. The result is expressed in seconds. The reference interval was 10–13s, with a CV < 4%.

Activated partial thromboplastin time (APTT)

APTT is sensitive to changes in FII, FV, FVIII–FXII and fibrinogen. APTT was measured at the central accredited laboratory at the hospital using a reagent that activates the intrinsic pathway and phospholipids. The results were expressed in seconds, and the reference interval was 26–33s, with a CV < 4%.

Fibrinogen

Fibrinogen was measured using a photometric assay (Multifibren U, Siemens, AG, Gerlangen, Germany). Excess thrombin (50 U/mL) was added to plasma samples, and clotting time was registered using an automated coagulometer (Symex CA 7000, Siemens AG, Gerlangen, Germany). The results were compared to clotting times with known fibrinogen concentrations. The reference interval was 2–4 g/L, CV was < 5%.

Coagulation factor activities

Coagulation factor activity was determined with the Thromborel S reagent, and dilutions were made in factor-deficient plasma on the BCS-XP coagulation analyser (Siemens Healthcare) at our accredited Department of Laboratory Medicine. The results were given as kIE/L, and the reference intervals were FII 0.70-1.50 kIE/L, FVII 0.40-1.60 kIE/L, FIX 0.80-1.50 kIE/L, FX 0.70-1.54 kIE/L protein C 0.70-1.30 kIE/L and protein S 0.65-1.40 kIE/L. All had CVs < 8%.

Thrombin generation assay

TGA was analysed using Ceveron alpha®, an automatic system which measures the formation of thrombin over time. Coagulation was initiated by TF. Two reagents were used: TGA RB, with a low concentration of phospholipids and TF, and TGA RC, with a high concentration of TF. The reference intervals with the TGA RC reagent were area under curve (AUC) 1195–2568 nM × min and for TGA RB AUC 1538–2652 nM × min.

ROTEM

ROTEM (EXTEM assay) (Pentapharm GmbH, Munich, Germany) was used to measure clot formation and elasticity. The analyses were performed in line with the manufacturer's instructions. Reference values: for clotting time (CT) 38–79 s, for clot formation time (CFT) 34–159 s, for α -angle 63–83°, for maximal clot firmness (MCF) 50–77 mm and for maximum lysis (ML) 0–18 %. All samples were analysed in duplicate, and mean values were calculated.

Matrix Gla protein

The levels of circulating MGP species in plasma were determined using commercially available assays. The carboxylated fraction (dp-cMGP) was analysed using a monoclonal antibody directed towards the carboxylated MGP sequence 35–53. The normal reference was 1763 ± 478 pmol/L, with a CV of 9.4%.

To analyse the uncarboxylated fraction, a two-step method was used. In brief, samples were exposed to magnetic particles coated with monoclonal antibodies against dp-MGP and uc-MGP. Secondly, trigger reagents were added, resulting in light emission that was directly proportional to the level of dp-ucMGP in the sample. The range of the assay was between 200 and 12,000 pmol/L and was linear up to 11,651 pmol/L. The upper reference value was 300 pmol/L, with a CV of 8.2%.

Osteocalcin

The different OC species were analysed using the enzyme immunoassays directed towards uncarboxylated OC (ucOC) and carboxylated OC (cOC). Both were obtained from Takara Shuzo Co. Ltd., Shiga, Japan, and analyses were carried out according to the manufacturer's protocol. The intra- and inter-assay variation coefficients for ucOC were 5.2% and 8.5%, respectively; those for cOC were 6.9% and 9.4%, respectively.

The normal reference range for ucOC has been defined as 2–4 ng/mL and for cOC as 5–9 ng/mL in healthy subjects older than 40 years.

PIVKA-II

In Studies I and III, PIVKA-II analyses were performed using a commercially available kit (Stago, Asnieres-sur-Seine, France). The method used an enzyme-linked immunosorbent assay (ELISA) technique, which detects monoclonal antibodies towards hypocarboxylated prothrombin. The upper reference was set by the manufacturer to $> 2 \mu\text{g/L}$, with a CV of $< 4\%$.

In Study V, another ELISA-based method using a different antibody was used. Results were given as arbitrary units per litre (AU/L). Using electrophoretic techniques, 1 AU is equivalent to 1 mg of purified uncarboxylated FII. The detection limit and normal upper reference was 0.20 AU/ml.

Study design

Study I

Study I was a prospective observational study that included patients undergoing elective neurosurgery. The study was approved by the Lund Regional Ethical Review Board (Protocol DNR 2012/43) and was conducted at the University Hospital in Lund, Sweden. The inclusion period was January to June 2012.

The study included patients above 18 years of age undergoing elective craniotomy and brain tumour resection. All patients gave informed written consent to participate. Blood samples were drawn before surgery, immediately after surgery and in the morning of the first postoperative day. On all occasions, PT-INR, Quick PT, FII activity and APTT were analysed. Exclusion criteria were known coagulation disorders, treatment with anticoagulants within 5 days of surgery, renal failure or abnormal preoperative routine coagulation tests. Most patients had glucocorticoid treatment prior to surgery. All patients received preoperative prophylactic antibiotic rifampicin.

Study II

Study II was a prospective observational study that included patients undergoing elective or emergency orthopaedic, urological, or abdominal surgery. The study participants gave their informed and written consent to participate, and the study was approved by the Regional Ethical Board, Lund, Sweden (DNR 2013/374). No power analysis was performed.

Blood samples were collected the day before surgery and in the morning of the fourth postoperative day. At both time points, dp-ucMGP, dp-cMGP, ucOC and cOC were analysed.

Patients with hepatocellular carcinoma, known liver cirrhosis, bleeding disorders, elevated plasma bilirubin or plasma liver transaminases, or treatment with either strong platelet inhibitors or warfarin with a PT-INR of more than 1.2 were excluded from participation.

Study III

The study was approved by the Regional Ethical Review Board in Lund (DNR 2014/916 and 2010/482). Adults who were admitted to the general ICU or cared for in the postoperative unit were eligible for inclusion. Exclusion criteria were pre-existing bleeding disorders and known hepatocellular carcinoma.

The inclusion period was July to December 2011. Blood samples were taken during office hours. PIVKA-II, PT-INR and the sequential organ failure assessment (SOFA) score were analysed near admission and every third day until three samples were obtained or until the patient was discharged or deceased.

Patients were treated according to the standard of care at the ICU. The nutritional regime was set to 15–25 kcal/kg/day, preferentially using the enteral route of administration. Patients were grouped by reason for admission to enable subgroup analysis. Warfarin-treated individuals were analysed separately as VKAs affect both routine coagulation assays and the circulating levels of PIVKA-II [99]. The remaining subgroups were cardiac arrest, trauma and sepsis, respiratory insufficiency, surgical, postoperative and other. The ‘other’ subgroup consisted of patients with conditions that were not frequent enough to form a subgroup ($n < 5$ patients). The difference between the surgical and postoperative subgroups were their postoperative level of care. Patients in the surgical subgroup remained in the ICU > 24 hours after surgery, whereas postoperative patients were discharged ≤ 24 hours after surgery.

The expected mortality rate (EMR) calibrated for Swedish ICUs was computed using the simplified acute physiology score (SAPS3) with the formula $EMR = \exp(-32,06302 + \ln(SAPS\ 3\ score + 10,34,171) * 7,199,704) / (1 + \exp(-32,06302 + \ln(SAPS\ 3\ score + 10,34,171) * 7,199,704))$ [100].

Study IV

Study IV was a single-centre retrospective registry study. It was approved by the Regional Ethical Review Board in Lund, Sweden (registration numbers 2014/916 and 2018/866) and was performed in conformity with the Declaration of Helsinki. The study was conducted at a nine-bed general ICU at Lund University Hospital. The inclusion period was September 2013 to May 2019. The manuscript was written in accordance with the STROBE guidelines [101].

PT was analysed using the Owren PT-INR. Patients admitted to the ICU with a PT-INR in the range of 1.3–1.9 at any time during their stay were identified. Patients who were given intravenous vitamin K₁ (Konakion Novum®, Cheplapharm Arzneimittel GmbH Ziegelhof 24, Greifswald, Germany) and who had had a PT-INR analysed less than 12 hours before vitamin K administration (pre-treatment value) and 12–36 hours following vitamin K administration (post-treatment value) were eligible. Exclusion criteria were any plasma or platelet transfusions or a > 1 unit erythrocyte concentrate transfusion between PT-INR samplings, vitamin K administration within 72 hours prior to the pre-treatment sample and known liver cirrhosis [102]. Vitamin K was given upon the decision of the treating physician as no local guidelines for vitamin K administration were available.

From the registry, patients with PT-INR in the same range who were not given vitamin K were included in the control group. These were defined as patients with prolonged PT-INR at their initial sampling (pre-treatment) at any time during the length of stay, with a follow-up sample within 12–36 hours (post-treatment). The groups are referred to as the vitamin K (VK) group and the control group, respectively.

A schematic flow chart of the study design is shown in Figure 4.

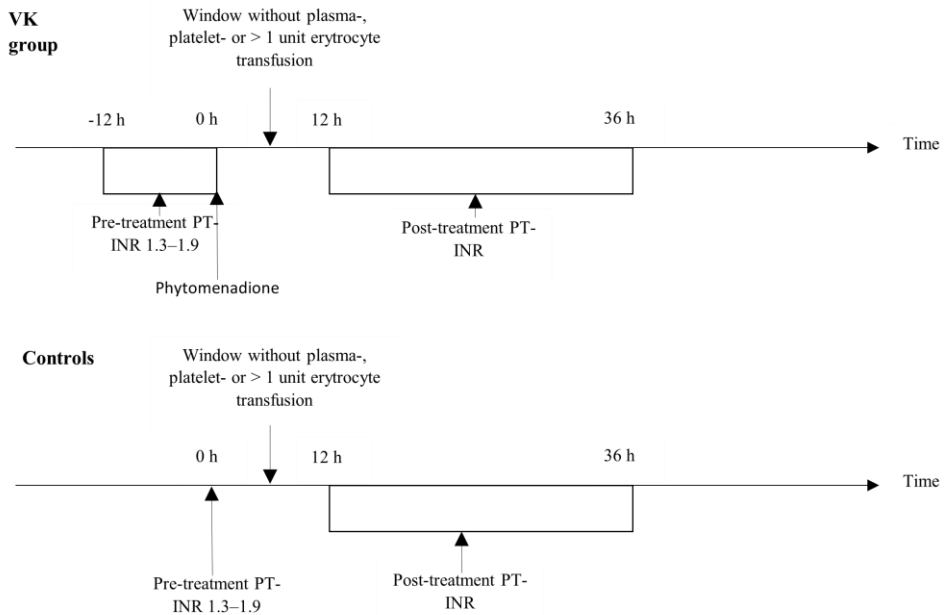


Figure 4. Study IV design

Study V

Study V was a prospective observational study performed at Lund University Hospital, Sweden. It was approved by the Lund Regional Ethical Review Board (DNR 2018/1010) and registered at ClinicalTrials.gov Identifier: NTC3782025. Written and informed consent was obtained from all study participants.

Non-bleeding adult patients admitted to the ICU or the postoperative care unit after all-day surgery with a PT-INR of more than 1.2 who were ordered 10 mg intravenous vitamin K₁ (Konaktion Novum[®], Cheplapharm Arzneimittel GmbH, Greifswald, Germany) were included. The exclusion criteria were absence of consent, hepatocellular carcinoma, liver resection within 6 months, treatment with VKAs or novel oral anticoagulants (NOAC), pre-existing coagulation disorders and administration of vitamin K less than 36 hours before inclusion. Patients were included in two separate periods – February to April 2019 and September 2019 to March 2020. All included patients were treated according to the standard of care at the ICU and the postoperative care unit.

Ethical considerations

All studies were conducted in accordance with the principles of the Declaration of Helsinki and approved by the Regional Ethical Review Board.

Written and informed consent was obtained from all participants either prior to the sampling or, in cases where this was not possible, afterwards at the earliest moment when the patient was competent to make decisions.

Statistics

Study I

The data were analysed using GraphPad Prism 7 (GraphPad Software, La Jolla, CA, USA).

Since normal distribution was not assumed, non-parametric statistical tests were used. All distributions were summarised using the median with 25th and 75th percentiles. For repeated measures, the Wilcoxon signed-rank test for paired samples was used. After Bonferroni correction a p value < 0.0125 ($0.05/4$) was considered statistically significant. Correlation tests were performed using the Spearman rank correlation method.

Study II

The statistical calculations were performed using GraphPad Prism (GraphPad Software, La Jolla, CA, US) for Windows, version 7. Variables were non-parametric, and the Wilcoxon signed-rank test was used for comparisons between day 1 and day 5. Correlations were analysed using Spearman's correlation test.

The significance level was set to $p < 0.05$.

Study III

Data were analysed using IBM SPSS for Windows, version 24.0 (IBM Corp., Armonk, NY, USA). Non-parametric methods were used since variables did not assume a Gaussian distribution. Change over time was analysed using the Wilcoxon signed-rank test. Correlations were analysed using Spearman's rank correlation.

The statistical significance level was set to $p < 0.05$.

Study IV

Data were analysed using IBM SPSS for Windows, version 26.0 (IBM Corp., Armonk, NY, USA) and R, version 3.5.0 (Auckland, New Zealand).

The repeated measures were analysed using the Wilcoxon signed-rank test. Nominal data (gender, reasons for admission, comorbidities, mortality) were analysed using the chi-squared test. Continuous variables are presented as a median (interquartile range [IQR]). Categorical variables are presented as numbers (percentages). Delta values were defined as the difference between post- and pre-treatment PT-INR and were compared using the Mann–Whitney U test.

To increase inter-group comparability, propensity score matching was used. The matching variables were age, gender, admission SAPS 3 EMR and pre-treatment PT-INR. The propensity score was generated through logistic regression and a 1:1 nearest neighbour algorithm. A maximum allowable difference between two participants (calliper) was defined to ensure a good match. If no match was found, the case was excluded.

A p-value of < 0.05 was considered statistically significant.

Study V

Analyses were processed using IBM SPSS for Windows, version 27.0 (IBM Corp., Armonk, NY, USA). Non-parametric statistical methods were used as Gaussian distribution was not assumed.

Sample size calculation was performed using G*Power version 3.1 (Heinrich Heine Universität, Düsseldorf, Germany) for the Wilcoxon signed-rank test. It was based on a pilot study from our department in which critically ill patients with prolonged PT-INR and no overt bleeding were given intravenous vitamin K. The observed effect size was -0.48, which combined with a two-tailed alpha value of 0.05 and 90% power rendered a sample size of 50 patients.

Continuous variables were presented using median (IQR), and numbers are given with (%). The differences between the samples before and after vitamin K were calculated using the two-tailed Wilcoxon signed-rank test, and the comparison of changes in the sensitivity analyses were performed using the Mann–Whitney U test.

The significance level was set to $p < 0.05$.

Results

Study I

The study included 35 patients. The sample was predominantly female (21 female, 14 male), with a mean age of 57 years (SD 13). Patient characteristics are presented in Table I.1.

Preoperatively, the median PIVKA-II plasma level was 1.97 $\mu\text{g/L}$. Seventeen patients had PIVKA-II levels above the normal reference of 2 $\mu\text{g/L}$ before going into surgery. Most of them still had a pathologically elevated PIVKA-II at day 1 postoperatively, with a further increase in 11 of the patients.

For the entire cohort, PIVKA-II was unchanged at the end of surgery but had increased when comparing preoperative levels with the morning after surgery ($p < 0.01$, Figure I.1).

In contrast, factor II was decreased at the end of surgery but had returned to preoperative levels at day 1 postoperatively.

PT-INR was higher immediately after surgery compared to preoperatively. At day 1 after surgery, PT-INR had decreased but was still higher than it was before surgery ($p < 0.001$, Figure I.2).

EPT had decreased both at the end of surgery and at day 1 after surgery compared with preoperative values.

The correlation analysis did not reveal any correlations between PIVKA-II and PT-INR or EPT (correlation coefficients 0.39 and 0.24, respectively). A positive correlation was demonstrated between PIVKA-II and FII activity (correlation coefficient of 0.47, $p < 0.01$) and between PIVKA-II and BMI (correlation coefficient of 0.62, $p < 0.001$).

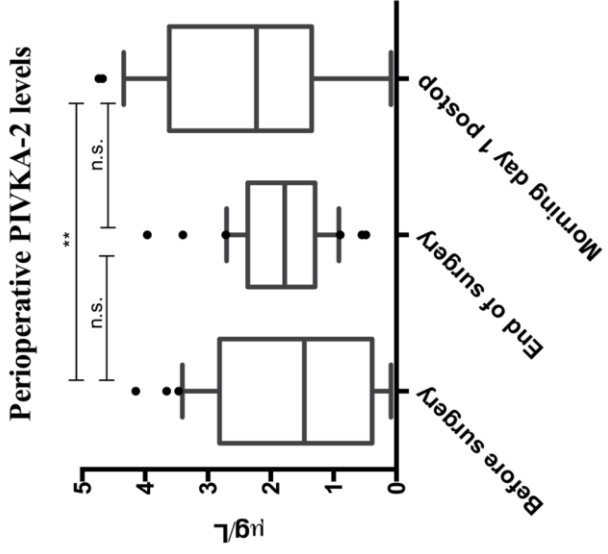
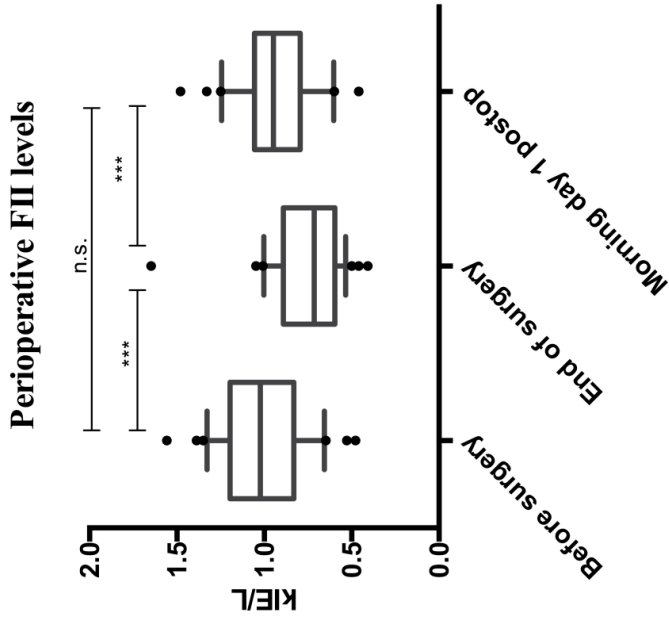


Figure 1.1
Perioperative changes in FII and PIVKA-II

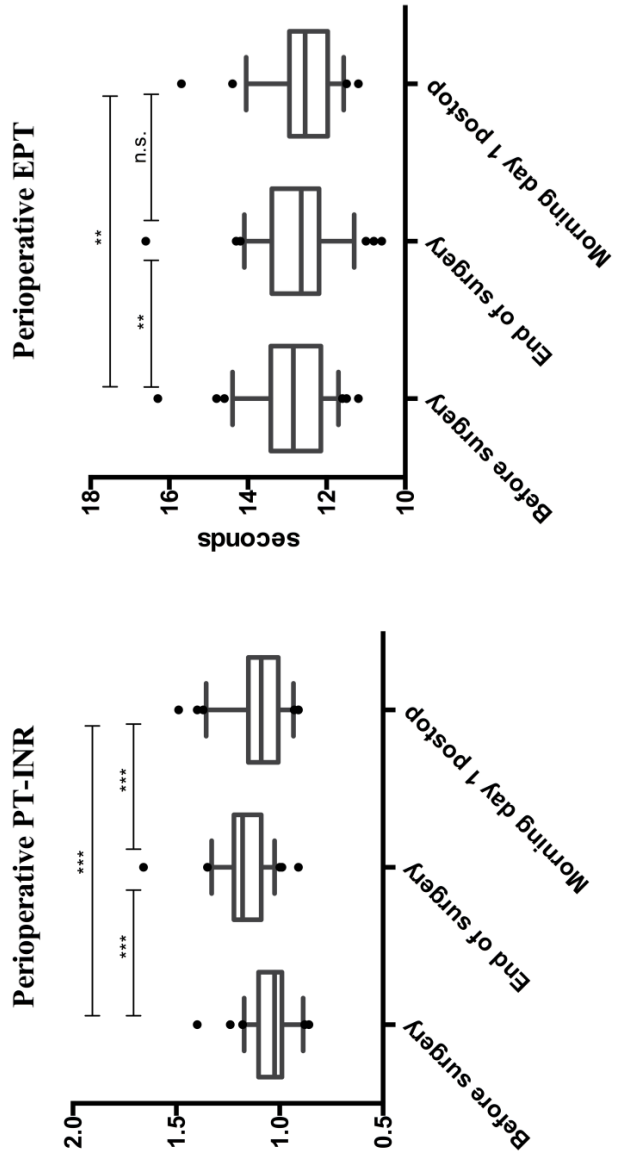


Figure 1.2
Perioperative changes in PT-INR and Quick Endstage prothrombin time (EPT)

Study II

Forty patients were included in the study. The patients underwent elective or emergency gastrointestinal, urological or orthopaedic surgery. The sample consisted of 12 females and 28 males, with a median age of 71 (range 32–88) years.

Patients were assigned to two groups based on the anatomical location of their surgical condition. Patients with gastrointestinal and urological conditions were in one group (A, $n = 23$), whereas patients that underwent orthopaedic procedures formed the second group (O, $n = 17$). Thirty-nine of these patients have previously been described [103].

For the entire cohort, the levels of dp-ucMGP in plasma were increased when comparing baseline with day 5 ($p < 0.0001$, Figure II.1). The same was true when looking at the abdominal and orthopaedic subgroups ($p = 0.008$ and $p = 0.0007$ respectively, Figure II.2). The majority of patients (74%) had an elevated dp-ucMGP compared to the reference range at day 1 (median 887 pmol/L range 210–2027 pmol/L). At day 5, dp-ucMGP had further increased, and only four patients (10%) had non-pathological dp-ucMGP levels (median 1186 pmol/L, range 301–2233 pmol/L). Patients with pre-existing cardiovascular comorbidities ($n = 10$) demonstrated a trend towards a more pronounced increase in dp-ucMGP both at baseline (median 1377 pmol/L, range 587–2008 pmol/L) and at day 5 (Median 1433 pmol/L, range 300–1848 pmol/L). However, it did not reach statistical significance. Cardiovascular comorbidities included previous coronary artery bypass surgery, previous myocardial infarction, known aortic stenosis, stable angina, chronic atrial fibrillation, or pre-existing heart failure. In contrast to the dynamics in dp-ucMGP, dp-cMGP remained unchanged when comparing day 1 and day 5. Roughly half of the patients (46%) had a dp-cMGP above the reference range (> 2241 pmol/L) at day 1.

At baseline, 12 patients equally distributed among the groups had an elevated ucOC. Only three patients had an elevated cOC at baseline. The ratio of ucOC to cOC was elevated in 26% at baseline, with no change over time for the entire cohort or for any of the subgroups. The plasma levels of ucOC decreased postoperatively for the entire cohort ($p = 0.017$, Figure II.3). When analysing subgroups, no change was observed in either ucOC or cOC in the abdominal group. In the orthopaedic group both ucOC and cOC had decreased when comparing baseline with day 5. cOC had decreased when comparing baseline with day 5 for the entire cohort. The same trend was observed in orthopaedic patients, whereas the abdominal group had unchanged cOC levels at day 5.

The correlation analyses did not reveal any correlations between any of the MGP or OC species. At baseline, dp-ucMGP showed no correlation with PIVKA-II; however, at day 5, dp-ucMGP showed a positive correlation with PIVKA-II (correlation coefficient 0.44; $p = 0.005$). No correlations between OC species and PIVKA-II were demonstrated at any time point.

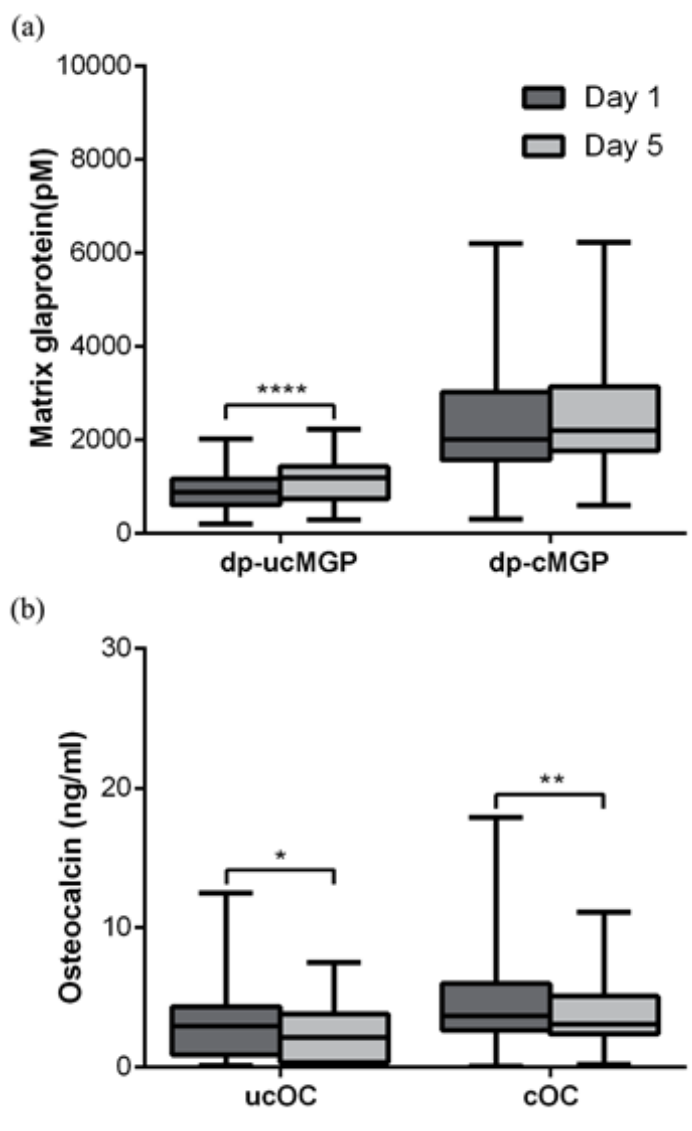


Figure II.1 Perioperative changes in Matrix Gla protein and osteocalcin for the entire cohort. pM pmol/L.

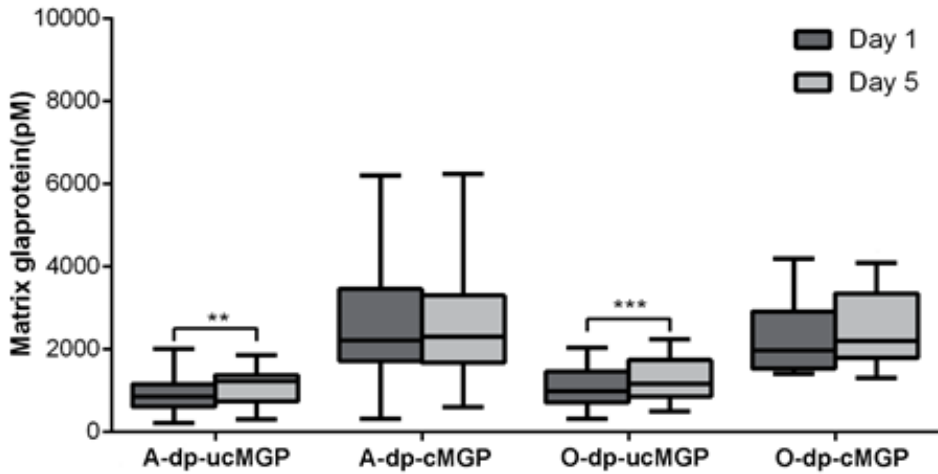


Figure II.2
Perioperative changes in Matrix Gla protein for the abdominal (A) and orthopaedic (O) subgroups. pM pmol/L

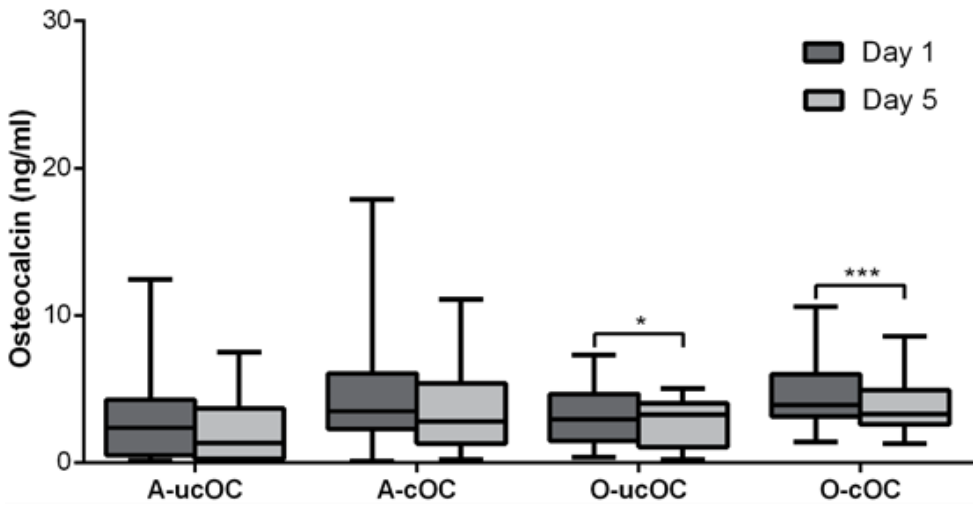


Figure II.3
Perioperative changes in osteocalcin for the abdominal (A) and orthopaedic (O) subgroups

Study III

The study population consisted of 95 patients. The sample was predominantly male (60 males, 35 females) and had a median age of 66 years (range 18–92). Patients were initially considered as one cohort and then divided into subgroups depending on the diagnosis leading up to ICU admission. The subgroups were cardiac arrest (n = 10), trauma (n = 9), sepsis (n = 19), respiratory insufficiency (n = 13), surgical (n = 10), postoperative (n = 23) and other (n = 7) as well as warfarin-treated patients (n = 4). The ‘other’ subgroup included patients with kidney failure, liver bleeding and circulatory insufficiency.

At baseline, the median PIVKA-II in plasma was 4.97 µg/L for patients not on VKA treatment and 73.4 µg/L for the warfarin-treated patients.

At baseline, the median EMR was 29.9%, the median PT-INR was 1.3 and the median SOFA score was 7. SOFA score and EMR were not analysed in postoperative patients. The median length of stay in the ICU was four days. The individual subgroups’ baseline values are shown in Table III.2.

Fifty-two patients had two or more consecutive PIVKA-II values and were hence available for repeated-samples analysis. PIVKA-II had increased when comparing baseline with the second sampling ($p = 0.047$, Figure III.1). Median PIVKA-II at second sampling was 7.88 µg/L for the patients not on VKA treatment and 38.3 µg/L for warfarin-treated patients. When looking at the subgroups, the cardiac arrest patients demonstrated the largest increase in PIVKA-II, with a median value at the second sampling of 21.40 µg/L (median delta value 18.27 µg/L). In decreasing order, the respiratory insufficiency subgroup had a PIVKA-II median value of 9.34 µg/L (median delta value 2.07 µg/L), trauma had a value of 7.09 µg/L (median delta value 1.45 µg/L), sepsis 5.26 µg/L (median delta value 1.93 µg/L) and surgical 4.41 µg/L (median delta value 0.94 µg/L). In two groups, PIVKA-II had decreased at second sampling – the warfarin-treated patients, who had a median value of 38.3 µg/L at second sampling (median delta value –1.6 µg/L), and the other subgroup, who had a median value of 7.01 µg/L (median delta value 2.24 µg/L).

TABLE III.2 Subgroup baseline characteristics

Group	Baseline characteristics	
Cardiac arrest	n	10
	Gender	2 female / 8 male
	Age	68.5 years (29-80)
	PIVKA-II	4.09 µg/L (0.49-8.22)
	PT-INR	1.3 (1.1-2.4)
	SOFA score	8.5 (5-12)
	EMR	41.4% (4.6-60.8)
Trauma	n	9
	Gender	4 female / 5 male
	Age	32 years (18-92)
	PIVKA-II	4.35 µg/L (0.003-54.2)
	PT-INR	1.2 (1.1-1.7)
	SOFA score	5 (2-9)
	EMR	5.2% (0.4-48.8)
Sepsis	n	19
	Gender	10 female / 9 male
	Age	64 years (20-86)
	PIVKA-II	4.33 µg/L (0.37-59.6)
	PT-INR	1.3 (1-1.8)
	SOFA score	10 (0-16)
	EMR	40.3% (1.8-85.8)
Respiratory insufficiency	n	13
	Gender	4 female / 9 male
	Age	68 years (50-80)
	PIVKA-II	4.97 µg/L (2.09-17.5)
	PT-INR	1.2 (1.1-1.4)
	SOFA score	5 (2-9)
	EMR	12.2% (1.4-62.7)
Surgical	n	10
	Gender	3 female / 7 male
	Age	64 years (18-81)
	PIVKA-II	5.37 µg/L (0.8-62)
	PT-INR	1.3 (1.1-1.5)
	SOFA score	7 (3-10)
	EMR	34% (11.1-62.7)
Postoperative	n	23
	Gender	9 female / 14 male
	Age	69 years (21-84)
	PIVKA-II	5.18 µg/L (0.94-184.4)
	PT-INR	1.2 (0.9-1.6)
	SOFA score	N/A
	EMR	N/A
Other	n	7
	Gender	3 female / 4 male
	Age	63 years (22-72)
	PIVKA-II	12.8 µg/L (2.11-18.2)
	PT-INR	1.2 (1-1.4)
	SOFA score	7 (4-14)
	EMR	40.3% (1.6-69.5)
Warfarin-treated	n	4
	Gender	4 male
	Age	70 years (63-76)
	PIVKA-II	73.4 µg/L (13.3-240.2)
	PT-INR	1.25 (1.1-1.5)
	SOFA score	6 (5-7)
	EMR	11.1% (5.2-11.1)

At day 6, 25 patients were available for analysis. PIVKA-II continued to increase when comparing the third sampling both with baseline and the second sampling ($p = 0.011$ and $p = 0.025$, respectively). In cardiac arrest patients, who demonstrated the most drastic increase in PIVKA-II, only one patient was available for the third sampling. In this patient, PIVKA-II levels continued to increase from $0.49 \mu\text{g/L}$ at the first sampling to $50.4 \mu\text{g/L}$ at the second sampling to $166.1 \mu\text{g/L}$ at the third sampling. This patient eventually passed away after 13 days in the ICU.

Thirty-five patients had two or more consecutive SOFA scores. SOFA score had decreased when comparing baseline with the second sampling ($p = 0.014$, Figure III.2), indicating improvement of patient condition. No change in SOFA score was observed between the second and third sampling.

No significant change in PT-INR was observed when comparing the baseline with the second sampling ($n = 31$). No bleeding events were recorded.

Thirteen patients passed away in the ICU, six from the cardiac arrest subgroup, one from the respiratory insufficiency subgroup and six from the sepsis group. All patients, except one, had a baseline PIVKA-II above the reference range, with a median value of $4.34 \mu\text{g/L}$, which further increased in six out of the eight patients available for the second sampling. The median PIVKA-II at the second sampling had increased to $15.8 \mu\text{g/L}$. The median PT-INR at baseline was 1.1, which remained unaffected. The median SOFA score was 9 and had increased in 50% of patients at the second sampling.

No correlations between PIVKA-II and PT-INR or between PIVKA-II and mortality or SOFA score were demonstrated.

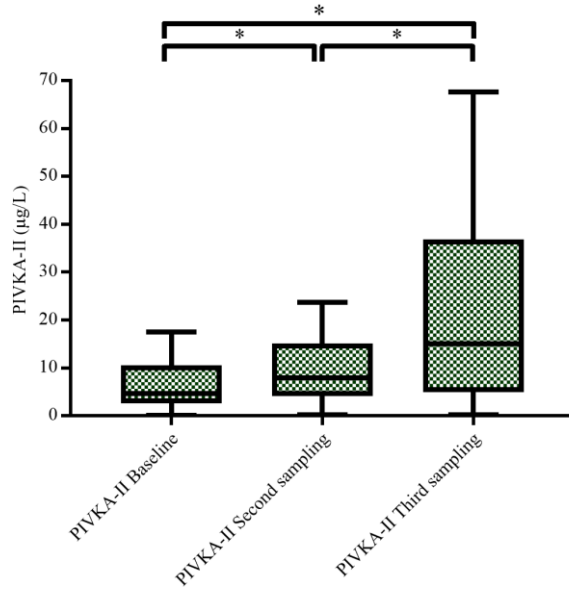


Figure III.1. Boxplot of changes in the PIVKA-II values over time
 n = 52 at baseline, n = 52 at second sampling, n = 25 at third sampling. Patients treated with vitamin K antagonists excluded. Outliers were removed from the picture but were included in the calculations. *p < .05.

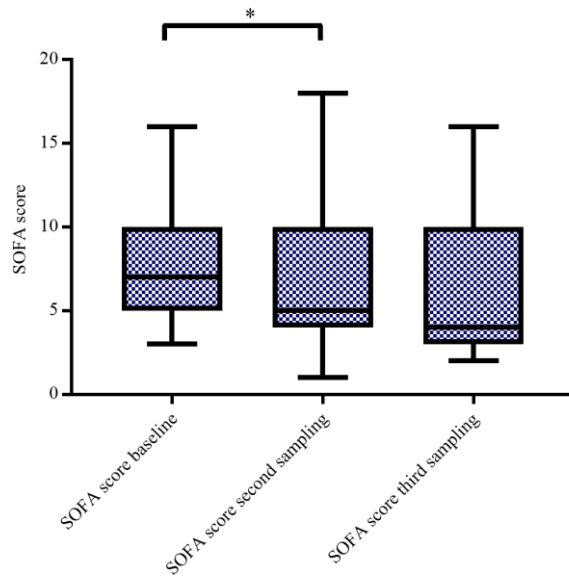


Figure III.2. Boxplot changes in the SOFA score over time
 n = 35 at baseline, n = 35 at second sampling, n = 17 at third sampling. Outliers were removed from the picture but were included in the calculations. *p < .05.

Study IV

The study population was extracted from a database comprising 4,541 records. The enrolment of patients is shown in Figure IV.1. In total, 621 patients with a PT-INR in the range of 1.3–1.9 who had received intravenous vitamin K were identified. After applying exclusion criteria, 134 patients remained and were included in the vitamin K (VK) group. The most common vitamin K dose was 10 mg (128/134, 96%), with a range of 5–20 mg. The control group was extracted from the same database. A total of 1,640 admissions with a PT-INR in the range 1.3–1.9 were identified, and 615 patients remained after applying exclusion criteria.

The propensity score-match rendered two equally sized groups comprising 129 patients each. Matching reduced the standardised difference to less than 10% for all variables, indicating a good match. Post matching, two variables differed between the groups. The VK group had a higher prevalence of trauma as the reason for admission (12% vs. 3%, $p = 0.009$). In contrast, controls were more likely to have had a cardiovascular reason for admission (40% vs. 21%, $p = 0.001$).

Details on the baseline characteristics, propensity scores and standardised differences are shown in Table IV.1.

When comparing delta values, the median decrease of PT-INR was greater in the VK group -0.10 (-0.30 to -0.10) compared to the control group -0.10 (-0.20 to 0.10) ($p = 0.01$).

Before vitamin K administration, PT-INR was 1.5 (1.4–1.5) in the VK group and 1.5 (1.4–1.6) in the control group (N.S.). At 12–36 hours after the administration of vitamin K, the median PT-INR had decreased to 1.4 (1.3–1.4) ($p < 0.001$) in the VK group and to 1.4 (1.3–1.6) in the control group ($p = 0.004$) (Figure IV.2 and Table IV.2).

No difference in mortality between the VK group and the controls was observed at 30 ($n = 37$ vs. $n = 35$, $p = 0.78$), 90 ($n = 51$ vs. $n = 50$, $p = 0.90$) and 180 days ($n = 63$ vs. $n = 58$, $p = 0.53$), respectively.

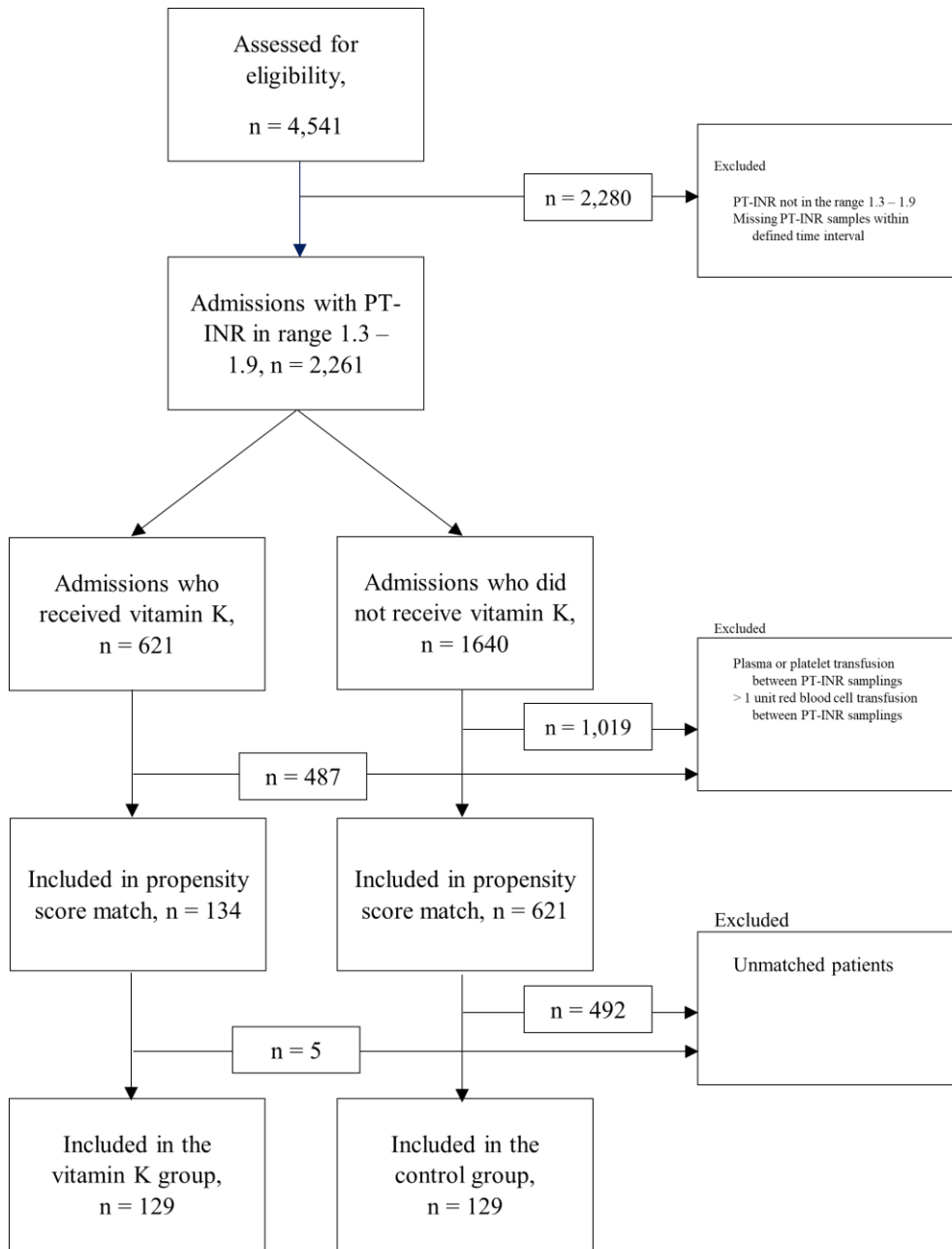


Figure IV.1. Consort diagram

PT-INR = Prothrombin time, international normalised ratio

TABLE IV.1 Baseline characteristics and propensity score matching

	Unmatched groups				Matched groups			
	Controls n = 615	VK group n = 134	SD	p- value	Controls n = 129	VK group n = 129	SD	p- value
Variables included in matching								
SAPS 3 EMR	24 (10-45)	26 (14-45)	0.033	0.38	28 (12-47)	26 (14-42)	-0.088	0.64
Age (years)	67 (54-75)	68 (55-75)	-0.004	0.99	68 (54-75)	67 (55-74)	-0.041	0.62
Pre-treatment PT-INR	1.3 (1.3- 1.4)	1.5 (1.4- 1.6)	0.78	<0.001	1.5 (1.4- 1.6)	1.5 (1.4- 1.6)	0.005	0.96
Sex (female)	204 (33)	43 (30)	-0.023	0.81	43 (33)	42 (33)	-0.017	0.90
Baseline variables not included in matching								
Reasons for admission ⁱ , n (%)								
Severe sepsis or septic shock ⁱⁱ	159 (26)	53 (40)	NA	0.001	53 (41)	52 (40)	NA	0.90
Trauma	61 (10)	16 (12)	NA	0.49	4 (3)	15 (12)	NA	0.009
CNS	254 (41)	33 (25)	NA	<0.001	32 (25)	31 (24)	NA	0.89
Haematologic	41 (7)	9 (7)	NA	0.99	8 (6)	8 (6)	NA	1.0
Gastric	103 (17)	38 (28)	NA	0.002	31 (24)	38 (29)	NA	0.33
Metabolic	100 (16)	17 (13)	NA	0.30	23 (18)	17 (13)	NA	0.30
Respiratory	301 (49)	72 (54)	NA	0.32	69 (53)	69 (53)	NA	1.0
Cardiovascular	226 (37)	28 (21)	NA	<0.001	51 (40)	27 (21)	NA	0.001
Hepatic	30 (5)	9 (7)	NA	0.39	7 (5)	9 (7)	NA	0.61
Renal	130 (21)	27 (20)	NA	0.79	38 (29)	27 (21)	NA	0.12
Other	50 (8)	15 (11)	NA	0.26	12 (9)	14 (11)	NA	0.68
Comorbidities, n (%) ⁱⁱⁱ								
Cancer ^{iv}	85 (14)	26 (19)	NA	0.099	15 (12)	23 (18)	NA	0.15
Congestive heart failure ^v	30 (5)	9 (7)	NA	0.39	10 (8)	8 (6)	NA	0.63
Haematological malignancy ^{vi}	22 (4)	6 (4)	NA	0.62	10 (8)	6 (5)	NA	0.31
Immuno- suppressants ^{vii}	35 (6)	6 (4)	NA	0.58	8 (6)	6 (5)	NA	0.59
Multiple comorbidities	13 (2)	3 (2)	NA	0.93	2 (2)	3 (2)	NA	0.65

ⁱ Some patients had multiple reasons for admission.ⁱⁱ Sepsis 2 definition.ⁱⁱⁱ Some patients had multiple comorbidities.^{iv} Cancer disseminated beyond regional lymph nodes.^v New York Heart Association stadium IV.^{vi} Diagnosis of lymphoma, leukaemia or myeloma.^{vii} Systemic steroid treatment, chemotherapy or external radiotherapy within 6 months.

TABLE IV.2 Laboratory analyses before and after vitamin K administration

Laboratory analyses	VK1 Group pre-treatment n = 129	VK Group post-treatment n = 129	p-value VK Group	Controls pre-treatment n = 129	Controls post-treatment n = 129	p-value controls
PT-INR2	1.50 (1.40–1.60)	1.40 (1.30–1.50)	< 0.001	1.5 (1.4–1.6)	1.4 (1.3–1.6)	0.004
APTT3 (s)	35 (30–42)	34 (30–42)	0.96	36 (31–41)	34 (30–41)	0.41
Platelets (x109/L)	158 (121–224)	150 (103–221)	0.008	167 (104–250)	153 (99–209)	< 0.001
Haemoglobin (g/L)	102 (93–113)	102 (94–112)	0.19	108 (95–119)	103 (94–116)	0.002
Fibrinogen (g/L)	4 (2.9–4.6)	4.6 (3.3–5.9)	< 0.001	3.3 (2.3–4.9)	3.8 (2.9–5.1)	< 0.001
CRP4 (mg/L)	146 (62–245)	181 (106–278)	< 0.001	89 (35–183)	132 (61–232)	< 0.001
Leukocytes (x109/L)	13 (9–18)	13 (9–18)	0.91	12 (8–17)	11 (9–16)	0.24

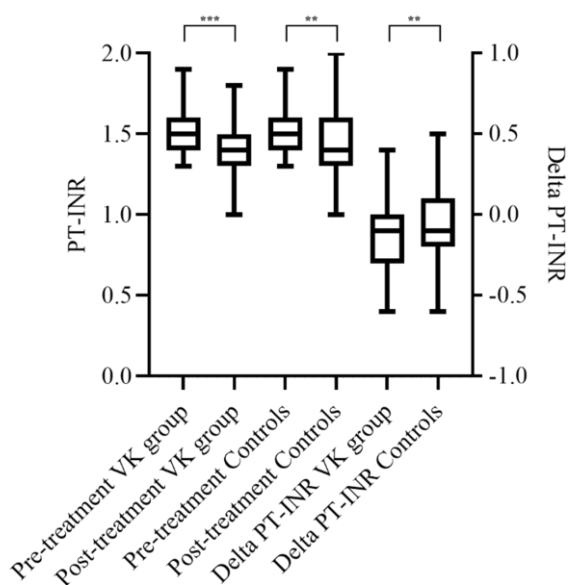


Figure IV.2. Boxplot of changes in PT-INR and delta PT-INR before and after vitamin K administration
 Whiskers represent maximum and minimum values up to 1.5*IQR. Outliers were defined as > 1.5*IQR and are removed from the picture but were included in the calculations. VK = vitamin K, PT-INR = prothrombin time, international normalised ratio. ** p ≤ 0.01, *** p < 0.001

Study V

Ninety-five patients were eligible for inclusion. After applying the exclusion criteria, 52 patients were included in the study (Figure V.1).

The sample consisted mostly of males (69%). The median age was 68 years (55–74). Sepsis was the most frequent reason for ICU admission, followed by postoperative care and cardiovascular disease. The median SOFA score at inclusion was 7 (5–11), and the median SAPS 3 at admission was 65 (52–74). No adverse reactions to vitamin K were reported. A post-hoc power analysis gave an observed power of 91%.

The values of all included assays before and after vitamin K administration are shown in Table V.1.

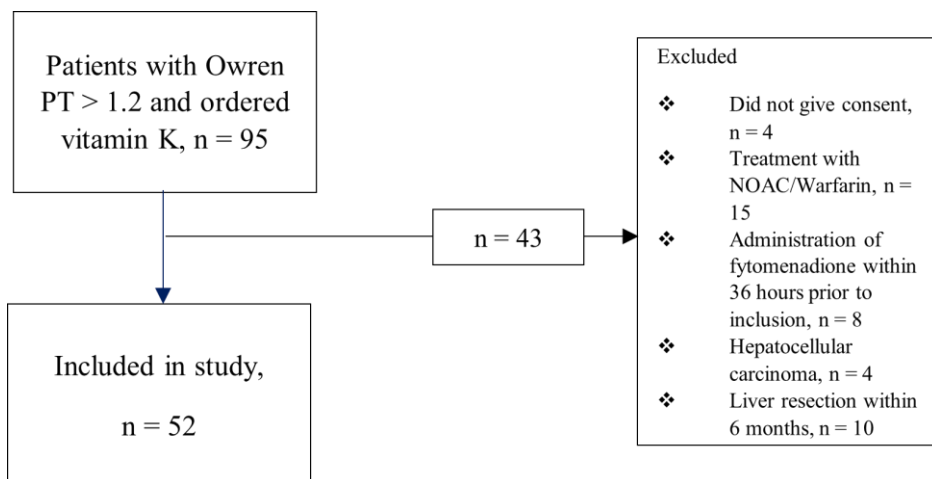


Figure V.1. Consort diagram
PT prothrombin time, NOAC novel oral anticoagulants

TABLE V.1 Laboratory analyses and SOFA before and after vitamin K administration

	Before	After	Reference interval	Trend	p-value
Standard coagulation assays					
APTT ⁱ (s)	31.5 (27–39.5)	31.5 (28.3–39)	26–33	–	0.81
Qwren PT ⁱⁱ (INR ⁱⁱⁱ)	1.4 (1.3–1.6)	1.3 (1.2–1.4)	0.9–1.2	↓	< 0.001
Quick PT (s)	13.7 (12.5–14.4)	12.1 (11.4–13.1)	10–13	↓	< 0.001
Fibrinogen (g/L)	4.2 (3.2–4.7)	5 (4.3–5.9)	2 - 4	↑	< 0.001
Vitamin K-dependent proteins					
dp-ucMGP ^{iv} (pmol/L)	840 (600–1300)	580 (450– 660)	< 300	↓	< 0.001
PIVKA-II ^v (AU/L)	Below detection limit	Below detection limit	< 0.15	NA	NA
Coagulation factor activity					
F ^{vii} (kIE/L)	0.61 (0.47–0.76)	0.70 (0.53–0.86)	0.7–1.5	↑	< 0.001
FVII (kIE/L)	0.48 (0.34–0.57)	0.61 (0.51–0.78)	0.4–1.6	↑	< 0.001
FIX (kIE/L)	0.94 (0.77–1.25)	1.14 (0.89–1.38)	0.8–1.5	↑	< 0.001
FX (kIE/L)	0.62 (0.47–0.79)	0.72 (0.59–0.93)	0.7–1.54	↑	< 0.001
Protein C (kIE/L)	0.65 (0.56–0.80)	0.68 (0.54–0.86)	0.7–1.3	–	0.11
Protein S (kIE/L)	0.56 (0.46–0.78)	0.62 (0.50–0.83)	0.65–1.4	↑	0.024
Thrombin generation assays					
TGA ^{vii} RB ^{viii} , AUC (nM)	2400 (2200–2900)	2600 (2300–3000)	1500 - 2700	↑	0.006
TGA RC ^{ix} , AUC (nM)	2200 (2000–2600)	2400 (2200–2800)	1200–2600	↑	0.005
ROTEM*					
CT ^{xi} (s)	82 (76–97)	86 (75–93)	38–79	–	0.29
CFT ^{xii} (s)	78 (59–99)	76 (64–94)	34–159	–	0.30
α angle (°)	74 (70–78)	75 (71–78)	63–83	–	0.19
MCF ^{xiii} (mm)	67 (60–72)	67 (62–74)	50–77	↑	0.008
ML ^{xiv} (%)	7 (3–10)	5 (2–10)	0 - 18	–	0.39
Other analyses and SOFA^{xv}					
CRP ^{xvi} (mg/L)	99 (64–235)	167 (109–239)	< 5	↑	0.01
Haemoglobin (g/L)	106 (98–113)	98 (93–103)	117–170	↓	0.002
Leukocytes (x10 ⁹ /L)	11 (8.0–13)	10.5 (9.0–14)	3.5–8.8	–	0.73
Platelets (x10 ⁹ /L)	173 (125–229)	164 (116–235)	145–387	–	0.23
SOFA	6 (4–9)	4.5 (3–7)	0	↓	0.005

ⁱ Activated partial thromboplastin timeⁱⁱ Prothrombin timeⁱⁱⁱ International normalised ratio^{iv} Desphospho-uncarboxylated matrix Gla protein. Values are presented with 2 significant figures.^v Protein induced by vitamin K absence/antagonist-II^{vi} Factor^{vii} Thrombin generation assay. Values are presented with 2 significant figures.^{viii} Reagent B^{ix} Reagent C^x Rotational thromboelastometry^{xi} Clotting time^{xii} Clot formation time^{xiii} Maximal clot firmness^{xiv} Maximal lysis^{xv} Sequential organ failure assessment^{xvi} C-reactive protein

Before vitamin K administration, Owren PT-INR was 1.4 (1.3–1.6) and decreased to 1.3 (1.2–1.4), ($p < 0.001$) in response to vitamin K. A similar decrease was observed in Quick PT, while APTT was unchanged (Figure V.2). Fibrinogen concentration increased ($p < 0.001$).

The activity of FII, FVII, FIX and FX was all increased ($p < 0.001$). Only FII and FX were below the normal reference range before vitamin K administration. All coagulation factors were within their normal ranges after vitamin K administration (Figure V.3).

The activity of proteins C and S were both slightly decreased before vitamin K administration. Protein C activity was unchanged ($p = 0.11$), whereas protein S increased ($p = 0.024$) but was still below its normal reference. Thrombin generation assays (TGA RB and TGA RC) increased between samplings ($p = 0.006$ and $p = 0.005$, respectively). The median values were within normal reference ranges at all times.

Except for CT, which was slightly prolonged before vitamin K administration, all ROTEM parameters always had a median value within the normal reference ranges. MCF increased after vitamin K administration ($p = 0.016$), while the remaining ROTEM parameters were unchanged.

PIVKA-II was undetectable in most patients before vitamin K administration (44/50, 88%). In the patients with measurable PIVKA-II levels ($n = 6$), levels decreased after vitamin K administration but not to normal levels ($p = 0.031$). The median dp-ucMGP levels decreased in response to vitamin K administration ($p < 0.001$). dp-ucMGP was elevated compared to the normal reference interval both before and after vitamin K administration.

The changes in the PIVKA-II and dp-ucMGP are shown in Figure V.4.

For the additional laboratory analyses and SOFA scores in response to vitamin K administration, haemoglobin and SOFA score decreased ($p = 0.002$ and $p = 0.005$, respectively), whereas CRP increased ($p = 0.01$). Leukocytes and platelets were unchanged ($p = 0.73$ and $p = 0.23$, respectively); see Table V.1.

To better clarify whether the observed changes between sample occasions may be explained by a general improvement over time, patients were divided in two groups based on whether the SOFA score had decreased ($n = 27$) or increased (or remained unchanged) ($n = 25$) between the two time points (Table V.2).

In brief, patients with a decreasing SOFA score, indicating clinical improvement, did not demonstrate a response compared to the group with increased or unchanged SOFA scores. By contrast, the delta changes indicated a stronger return towards normal Owren PT in the group with increased or unchanged SOFA scores.

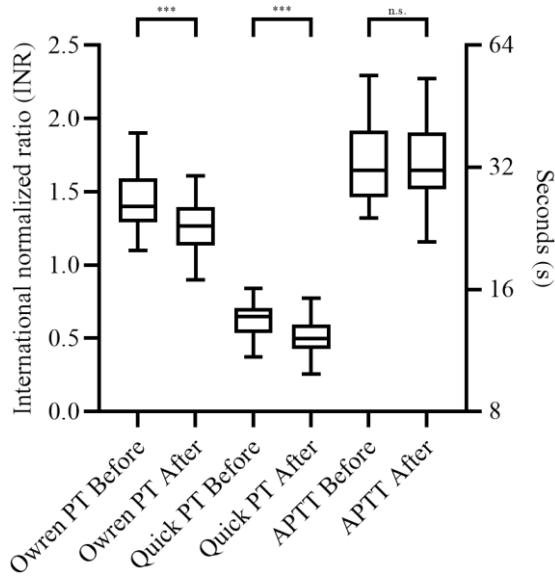


Figure V.2. Owren prothrom time (INR) and Quick prothrombin time (seconds) and Activated partial thromboplastin time (seconds) before and after vitamin K administration

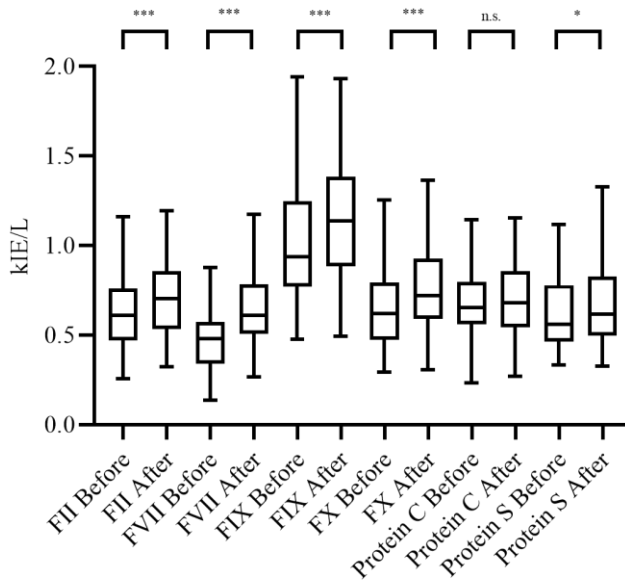


Figure V.3. Changes in coagulation factor activity before and after vitamin K administration. F = Factor.

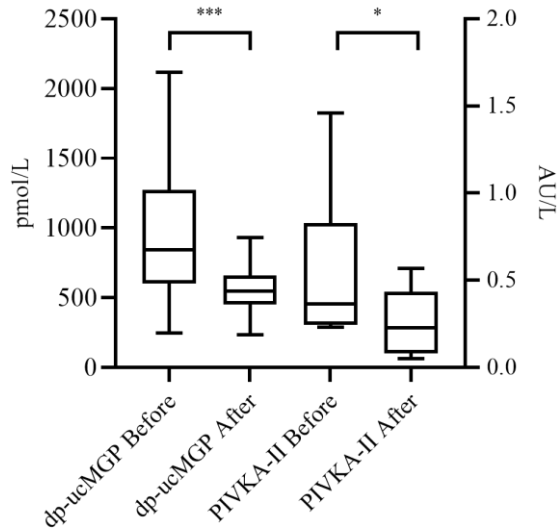


Figure V.4. Changes in vitamin K-dependent proteins before and after vitamin K administration
 For Dp-ucMGP n = 52 and for PIVKA-II n = 6. dp-ucMGP desphospho-uncarboxylated Matrix Gla protein (pmol/L), PIVKA-II proteins induced by vitamin K absence for factor II (AU/L).

TABLE V.2 Sensitivity analysis

	Decreased SOFA ⁱ score, n = 27			Increased or stagnant SOFA score, n = 25			
	Before	After	p-value	Before	After	p-value	p-value delta changes
Standard coagulation assays							
APTT ⁱⁱ (s)	35 (29–40)	32 (30–42)	0.081	30 (28–40)	32 (28–41)	0.588	0.883
Qwren PT ⁱⁱⁱ (INR ^{iv})	1.4 (1.3–1.6)	1.4 (1.2–1.5)	0.046	1.4 (1.4–1.6)	1.3 (1.2–1.4)	< 0.001	0.027 ^v
Quick PT (s)	14 (12–15)	12 (12–13)	< 0.001	14 (12–14)	12(11–13)	< 0.001	0.963
Fibrinogen (g/L)	4.2 (3.2–4.5)	5.0 (4.1–5.5)	0.001	4.2 (3.3–4.9)	5.0 (4.4–6.4)	0.005	0.647
Vitamin K-dependent proteins							
dp-ucMGP ^{vi} (pmol/L)	800 (600–1400)	560 (450–680)	0.001	970 (610–1270)	520 (410–660)	<0.001	0.589
PIVKA-II ^{vii} (AU/L)	NA	NA	NA	NA	NA	NA	NA
Coagulation factor activity							
F ^{viii} III (kIE/L)	0.61 (0.48–0.76)	0.68 (0.50–0.84)	0.011	0.62 (0.47–0.75)	0.73 (0.57–0.87)	0.002	0.203
FVII (kIE/L)	0.48 (0.39–0.56)	0.60 (0.50–0.79)	< 0.001	0.48 (0.31–0.68)	0.62 (0.51–0.81)	0.001	0.920
FIX (kIE/L)	0.89 (0.79–1.09)	1.01 (0.88–1.23)	0.001	1.06 (0.71–1.41)	1.35 (1.00–1.47)	0.001	0.436
FX (kIE/L)	0.53 (0.44–0.70)	0.66 (0.54–0.83)	0.001	0.66 (0.51–0.88)	0.88 (0.63–1.10)	< 0.001	0.047 ⁹
Protein C (kIE/L)	0.60 (0.54–0.75)	0.64 (0.53–0.76)	0.775	0.70 (0.59–0.89)	0.79 (0.61–0.98)	0.013	0.041 ⁹
Protein S (kIE/L)	0.51 (0.44–0.59)	0.53 (0.47–0.63)	0.093	0.68 (0.56–0.85)	0.65 (0.56–1.00)	0.122	0.521
Thrombin generation assays							
TGA RB ^x , AUC (nM)	2500 (2200–3000)	2600 (2500–3000)	0.121	2400 (2100–2900)	2600 (2300–3000)	0.020	0.405
TGA RC ^x , AUC (nM)	2200 (2000–2500)	2400 (2300–2700)	0.052	2200 (2000–2600)	2300 (2100–2800)	0.049	0.706
ROTEM^{xi}							
CT ^{xii} (s)	82 (75–100)	87 (74–102)	0.801	82 (78–96)	84 (75–89)	0.045	0.181
CFT ^{xiii} (s)	79 (64–99)	76 (66–97)	0.461	76 (57–104)	71 (59–92)	0.510	0.769
α angle (°)	74 (70–77)	74 (70–77)	0.282	74 (69–79)	75 (72–78)	0.531	0.854
MCF ^{xiv} (mm)	65 (60–71)	67 (62–73)	0.010	67 (57–74)	68 (62–74)	0.229	0.373
ML ^{xv} (%)	7 (3–11)	6 (3–13)	0.220	5 (2–9)	5 (2–9)	0.760	0.373
Other analyses and SOFA							
CRP ^{xvi} (mg/L)	94 (64–234)	161 (119–218)	0.012	145 (56–275)	183 (102–262)	0.426	0.240
Haemoglobin (g/L)	108 (100–113)	96 (93–102)	0.001	105 (93–114)	101 (93–112)	0.162	0.047 ^{xvii}
Leukocytes (x10 ⁹ /L)	9.8 (8.5–12)	9.8 (8.5–12)	0.684	11 (8.6–15)	12 (9.6–14)	0.795	0.984
Platelets (x10 ⁹ /L)	166 (122–231)	152 (120–289)	0.289	190 (125–228)	177 (115–222)	0.455	0.984
SOFA	6 (4–10)	3 (2–5)	< 0.001	4 (3–8)	6 (3–11)	0.005	< 0.001

i. Sequential organ failure assessment

ii. Activated partial thromboplastin time

iii. Prothrombin time

iv. International normalised ratio

v. Greater decrease in the group with increased SOFA score

vi. Desphospho-uncarboxylated matrix Gla protein

vii. Protein induced by vitamin K absence/antagonist-II

viii. Coagulation factor

ix. Thrombin generation assay, Reagent B

x. Thrombin generation assay, Reagent C

xi. Rotational thromboelastometry

xii. Clotting time

xiii. Clot formation time

xiv. Maximal clot firmness

xv. Maximal lysis

xvi. C-reactive protein

xvii. Greater decrease in the group with decreased SOFA score

Discussion

Perioperative changes in vitamin K-dependent proteins

Studies I and II investigated perioperative carboxylation changes in both hepatic and extrahepatic VKDPs. This is a previously unexplored field of research.

In Study I, changes in PIVKA-II, FII activity and PT were measured before and after surgery as well as on the first postoperative day. The results showed an increase in PIVKA-II and PT-INR on the morning after surgery, accompanied by unchanged FII activity and a decrease in Quick PT.

A positive correlation between preoperative PIVKA-II levels and body mass index was also demonstrated. This is in line with results from studies on bariatric surgery, where vitamin K insufficiency was present in 40% of patients [104].

In Study II, perioperative changes in the extrahepatic Gla proteins MGP and OC were studied. Results showed increased preoperative levels of dp-ucMGP compared to the reference range for healthy individuals, which increased a further 4 days following major orthopaedic, urological or gastrointestinal surgery. The increase in dp-ucMGP was more pronounced in the limited number of patients with pre-existing cardiovascular disease. In contrast, both active (cOC) and inactive (ucOC) decreased in response to surgery.

The preoperative increase in dp-ucMGP may be related to cardiovascular comorbidities. Previous studies have shown increased levels of dp-ucMGP in atherosclerotic patients. dp-ucMGP has also been associated with left ventricle dysfunction and N-terminal prohormone of brain natriuretic peptide, suggesting a potential involvement in the development of heart failure and myocardial remodelling [105, 106].

As previously mentioned, body stores of vitamin K are low. Previous studies have induced vitamin K deficiency in healthy subjects, with a subsequent drop in plasma levels by half after only a few days accompanied by a rising PIVKA-II [42]. Similarly, in surgical patients, vitamin K levels have been shown to drop by two-thirds over three days [107]. These results indicate that a patient's vitamin K status may drastically alter over a short period of time with low intake, such as during perioperative fasting or severe illness. Many patients in Studies I and II were of older age and/or had an underlying malignancy, both of which are known risk

factors for malnutrition [108, 109] and may have contributed to the lowered activity of VKDPs observed preoperatively.

The significance of measuring the activity (carboxylation) of VKDPs in the perioperative setting may not be immediately obvious. The results of Studies I and II indicate that the levels of PIVKA-II and dp-ucMGP are commonly elevated before surgery and even more pronounced in the postoperative period. Another recent study with a similar study design replicated these findings [110]. That certain patient categories, such as those undergoing bariatric surgery, are at risk for developing a postoperative vitamin K deficiency was concluded by a systematic review that also reflected on the general lack of knowledge in this area and need for further studies to investigate whether vitamin K supplementation has any clinical benefit [111].

Another patient category previously studied comprises those with advanced heart failure requiring the implantation of a left ventricular assist device (LVAD). These patients may have impaired coagulation due to long-standing backward failure and subsequent congestion of the liver. Perioperative supplementation with vitamin K has shown reduced frequency of reoperation due to bleeding [112] and reduced need for blood product transfusion [113], without increasing the risk of stroke or pump thrombosis. In addition to the immediate benefits of reduced bleeding, there are additional gains with less blood product transfusions, such as lower risk for transfusion-related complications [114] and potentially reduced risk for allosensitisation in patients supported by LVAD while awaiting heart transplant [115].

Furthermore, a perioperative and ICU setting in which vitamin K supplementation might be beneficial is during prophylactic or therapeutic use of certain antibiotics that as a side effect might induce vitamin K deficiency. This complication may be fatal [116] and is perhaps avoidable with vitamin K administration [117]. Certain antibiotics contain a side chain that inhibits the effect of GGCX or VKOR, thus halting the vitamin K cycle and inducing vitamin K deficiency [118]. Previous studies have shown that patients on total parenteral nutrition have lower vitamin K₁ levels and are more prone to develop cephalosporin-induced hypoprothrombinaemia, increased PIVKA-II and decreased protein C [119]. The coagulopathy was reversible with vitamin K administration but recurred within days in some patients. The optimal supplementation dose and interval is not known. In a study of critically ill children on prolonged antibiotic therapy, a single prophylactic dose of up to 10 mg vitamin K at admission was not effective in preventing hypoprothrombinaemia. In most of the children, the coagulopathy developed more than 10 days into treatment and more frequently in those with severe malnutrition. When it was diagnosed, it was successfully reversed using the same dose of vitamin K as given as prophylaxis. These results reinforce that vitamin K status rapidly deteriorates when intake is low, such as during critical illness, and that efforts should be directed towards developing supplementation guidelines.

Finally, there are some reports that suggest vitamin K might promote wound healing, which is essential after surgery. In pre-clinical studies, vitamin K-supplemented animals exhibited less weight loss, bowel oedema and abscess formation as well as increased collagen formation compared to non-supplemented animals following abdominal surgery [120]. Topical vitamin K has been shown to reduce healing time in both pre-clinical [121] and randomised human studies [122]. However, the number of studies available on this matter is very limited.

In summary, the results of Studies I and II indicate that VKDP activity decreases in the perioperative period. It is possible that vitamin K supplementation would be beneficial in certain patient populations, which should be investigated in future studies.

Vitamin K-dependent protein activity during critical illness

In Study III, PIVKA-II, Owren PT-INR and SOFA score were measured at ICU admission and every third day during the first week of intensive care. The study results demonstrated elevated levels of PIVKA-II near admission, which further increased during the ICU stay, while Owren PT-INR remained unaffected, and SOFA score indicated overall patient improvement.

The dynamics in PIVKA-II may represent several things and are addressed in two parts. Firstly, there was an elevated level of PIVKA-II at baseline compared with a healthy population. The majority of the study participants had conditions that did not develop instantaneously but rather worsened over the course of a few days. It is plausible that the patients' vitamin K intake during this time was suboptimal, and as previously described, that time frame is sufficient to significantly alter vitamin K levels. The pathological PIVKA-II at baseline could also reflect pre-existing conditions as the study population was elderly. This would be consistent with previous studies in which low activity of VKDPs has been observed in common comorbidities, such as hypertension [123], chronic kidney disease [124], type 2 diabetes [125] and ischemic heart disease [125]. Unfortunately, no data on patient comorbidities other than the SAPS3 were analysed.

Secondly, PIVKA-II consistently increased at each time point despite normal PT-INR and the improvement of patient condition measured by SOFA score. This might be explained by several things. PIVKA-II is considered a marker of hepatic vitamin K status, and the increase might reflect a depletion of hepatic stores, namely failure to meet an increased demand during critical illness. It is also possible that during severe illness the liver prioritises sustaining other functions over carboxylation, with increasing PIVKA-II despite normal vitamin K levels. To the best of our knowledge, there are no studies that have measured circulating vitamin K levels in critically ill

patients, perhaps due to the technical challenges previously discussed. Finally, PIVKA-II might simply be a marker for an inflammatory response as FII has previously been described as an acute phase reactant [126]. However, previous studies have failed to demonstrate any correlation between PIVKA-II and the inflammatory marker C-reactive protein (CRP) [127] and in Study I the increase in PIVKA-II was not accompanied by an increase in FII activity.

An interesting finding from Study III is the tangible increase in PIVKA-II observed in cardiac arrest patients, who demonstrated the most pronounced increase between the first and second time points. The reason why this subset of critically ill patients seems to be more at risk for decreased VKDP activity is unclear. It could be reflective of some pathophysiological process associated with cardiac arrest, such as transient systemic ischemia, which could potentially decrease the oxygen-dependent recycling of vitamin K [128]. In previous studies, the hypoxia-induced production of PIVKA-II has been demonstrated in cancer cell lines [129], and hypoxia in infants has been shown to reduce vitamin K-dependent clotting factor activity [130, 131]. A similar trend was also observed for some of the patients with sepsis, which is associated both with coagulation abnormalities [132] and hypoperfusion [133], but it was not confirmed on a group level. The present study did not include any measures of perfusion such as lactate, which otherwise would have been interesting.

Similar to Studies I and II, the clinical relevance of measuring VKDP activity in this setting may be questionable. None of the patients developed any bleeding complications, which would be the most imminent complication of vitamin K deficiency. Instead, these were patients with stable and only slightly abnormal coagulation, who showed an overall tendency toward improvement. However, the results of Study III show that, despite these favourable conditions, the VKDP activity steadily decreased during the first week of intensive care. It is possible that this puts the patient at risk for developing future bleeding complications. Furthermore, the decreased activity of prothrombin observed in Study III might be accompanied by decreased activity in extrahepatic VKDPs as a positive correlation between these has been demonstrated [134]. As previously mentioned, studies on vitamin K levels in critically ill patients are scarce. In severely burned paediatric patients, circulating levels of vitamin K were found to be below normal in 90% of cases [135]. Coagulopathies occur frequently after thermal injury and have been reported to develop within 24 hours and be associated with increased transfusion requirement and prolonged mechanical ventilation [136]. In critically ill adults, markedly deranged levels of VKDPs C and S have been observed after trauma or sepsis [137].

There are no generally accepted guidelines for vitamin K supplementation in critically ill patients. The results of Study III and others [138] support the need for further investigation aimed at development of substitution regimes, especially for at-risk patients.

Effect of vitamin K supplementation on coagulation assays and vitamin K-dependent protein activity in critically ill patients

Studies IV and V analysed the effect of vitamin K supplementation on both coagulation assays and VKDP activity.

Study IV retrospectively investigated whether vitamin K supplementation was beneficial in critically ill patients with a spontaneously prolonged Owren PT-INR (range 1.3–1.9) and showed that supplemented patients demonstrated a larger decrease in PT compared to controls.

It should, however, be mentioned that the difference in absolute numbers was relatively small when comparing supplemented patients and controls, which is why the clinical relevance of the results may be questioned. One could argue that due to the insidious nature of critical illness, even small improvements should be encouraged. Also, it is possible that the trend observed in Study III, where VKDP activity steadily decreased, could have been avoided with vitamin K supplementation. The scarce amount of research in this area is also reflected in that no optimal dose or time interval for vitamin K administration is known. Previous research has, however, shown that two consecutive doses of 10 mg vitamin K were more effective in reversing PT-INR compared to a single dose [139].

Whether an increased Owren PT-INR in a non-bleeding patient carries any clinical significance is unclear [140]. A PT-INR below 1.8 does not generally increase the risk for clinically significant bleeding events as it corresponds to coagulation factor levels above 30% [141]. However, in certain situations, such as before epidural or spinal anaesthesia, a PT-INR below 1.5 is usually desired [142]. In patients with cerebral contusions, an increased Owren PT-INR is a risk factor for haemorrhagic progression [143], and in patients with traumatic brain injuries, recent Nordic guidelines recommend an Owren PT-INR below 1.3 [144]. Patients with trauma-induced coagulopathy have an increased mortality when Owren PT-INR is greater than 1.5 [145]. Furthermore, many patients with an increased Owren PT-INR are treated with prophylactic plasma transfusions before invasive procedures, but the evidence is low [146, 147]. It is possible that vitamin K might be an option before non-urgent surgery and invasive procedures.

Study V replicated the findings of Study IV with respect to changes in Owren PT-INR, with a concomitant increase in coagulation factor activity. Simultaneously, dp-ucMGP and PIVKA-II decreased in response to vitamin K however not to normal levels, which may be interpreted as an improved but not restored vitamin K status. Anticoagulative proteins C and S were not increased as much, suggesting that vitamin K triggered a mainly pro-coagulative response. A post-hoc sensitivity analysis showed that the observed effects were not stronger in patients with clinical

improvement between the sampling occasions, indicating that the results were not confounded by a less pronounced coagulopathy over time.

To our knowledge, this is the first study to investigate changes in dp-ucMGP in response to vitamin K supplementation in critically ill patients. As previously mentioned, research has been focused on its potential involvement in cardiovascular disease and in uremic vascular calcification [148, 149]. dp-ucMGP levels have been positively associated with increased mortality in diabetic patients with chronic kidney disease [47]. The relevance of measuring dp-ucMGP in an ICU population is less clear. As previously mentioned, upregulated MGP gene expression has been observed 24 hours after myocardial infarction [52], and a potential involvement in myocardial remodelling and the development of heart failure has been postulated. This is in line with the results from Studies II and III, where the most pronounced deviations in VKDP activity were observed in patients with cardiovascular disease.

More recently, studies have suggested the potential involvement of MGP in pulmonary disease [150-152], with pronounced elevations of dp-ucMGP in patients with Coronavirus 2019 (COVID-19) infection [69]. The theoretical explanation for this phenomenon is the pneumonia-induced activation of inflammatory cytokines, with the subsequent degradation and calcification of elastic fibres. To counteract this, MGP synthesis is increased, which in turn results in an increased vitamin K demand. When this demand is not met, dp-ucMGP increases. As vitamin K deficiency first affects the extrahepatic proteins, it is possible that the levels of protein S, which is partly synthesised by endothelial cells, would decline, which might contribute to the thrombosis complications associated with COVID-19 [153]. There are studies suggesting that vitamin K deficiency is common in COVID-19 patients and might be considered a modifiable risk factor [154]. It is possible that these results could be partly extrapolated to pulmonary damage and the development of fibrosis that may occur in critically ill patients following long-standing mechanical ventilation or acute respiratory distress syndrome [155].

The prolonged PT-INR at baseline in Study V may be partly explained by vitamin K deficiency. The pathophysiology behind vitamin K deficiency in hospitalised patients is multifactorial and may derive from inadequate supply (malnutrition, prolonged intravenous nutrition), malabsorption (bowel inflammation, gastric retention or ischemic intestinal wall damage) and drugs [103]. Most likely, the main culprits are insufficient supply and malabsorption in critically ill patients.

Unexpectedly, the PIVKA-II assay was below the detection limit for most patients. This contrasts with Studies I, II and III as well as with other studies where elevated PIVKA-II levels have been demonstrated in ICU [138] and perioperative patients [103]. It should be noted that a different method was used to analyse PIVKA-II in Study V compared to Studies I, II and III, but the extent to which it might have affected the results is unclear. It is also possible that the choice of vitamin K species is of relevance. In Studies IV and V, vitamin K₁ was used, which is preferentially

utilised by hepatic VKDPs [156], whereas vitamin K₂ has a higher bioavailability for extrahepatic proteins due to its different distribution in the body. This could explain why dp-ucMGP levels were only partially reduced by vitamin K and that protein S levels were not as affected compared to the exclusively liver-derived coagulation factors.

In summary, the results of Studies IV and V suggest that vitamin K administration to critically ill patients has positive effects both in terms of improved coagulation assays and VKDP activity. This might have beneficial effects, as discussed above, although the procoagulative response is a concern for an increased risk for thromboembolic complications that needs to be addressed in future studies. This is, to our knowledge, the first study to show that it is possible to alter VKDP activity with a single dose of vitamin K. Naturally, a more extensive study protocol would be needed to evaluate whether vitamin K supplementation could affect conditions such as myocardial remodelling, pulmonary disease and sepsis.

Limitations

There are several limitations to the studies included in this dissertation that warrant mentioning. Some general limitations that apply to all studies are relatively small sample sizes, heterogeneous study populations and short follow-up times. Furthermore, all studies are single-centre, which might limit the generalisability of the results.

Studies I and II investigated perioperative changes in VKDPs and found high levels of inactive VKDPs preoperatively, which further increased after surgery. It would have been interesting to include measures from matched controls to better elucidate the extent to which the surgical trauma affected the VKDPs. The diagnosis and surgical indication were also very different in the study populations, and both elective and emergent patients were included. Also, information about intraoperative blood product transfusions or the administration of drugs that affect coagulation were not analysed in either Study I or Study II.

Study III included patients admitted to the ICU both due to single- or multiple-organ failure but also postoperative patients who were coming out of general anaesthesia. The postoperative patients were never meant to stay in the ICU for longer than a few hours and should probably in retrospect have been omitted. In Study III, patients were lost to follow-up at each time point due to discharge or death, introducing a possible selection bias. There was also an issue with missing data with respect to Owren PT-INR and SOFA score.

Study IV was a retrospective analysis, and thus it is affected by the inherent flaws of this design. It would naturally have been better to do a randomised study, but, as

discussed in the ethics section, this is challenging in ICU patients due to the current interpretation of the law in Sweden. Furthermore, the study design does not detect whether transfusions had been given in close proximity to the pre-treatment blood draw other than the predefined transfusion free period of 24 h. There was also no record of whether pro-coagulant drugs such as factor concentrate was administered.

Study V would have been improved if a control group had been included as this would have allowed us to better distinguish what was a true vitamin K effect. It should also be mentioned that even though positive effects on coagulation were observed, they were relatively small, and the clinical significance might be debatable. The study population was heterogeneous with respect to diagnosis, which in turn means that the coagulation abnormalities may derive from different mechanisms (such as consumption coagulopathy, dilution or malnutrition/malabsorption). As the subgroups were not large enough to allow for any meaningful statistical analysis, this was not investigated.

Conclusions

The main conclusions to be drawn from the study findings are as follows.

VKDP activity, measured by the carboxylation degree of prothrombin and MGP, is commonly decreased preoperatively and further decreases in response to different types of surgery, even though standard coagulation assays fail to detect it. Patients with high cardiovascular burden seem to be more at risk. (Papers I and II)

Similarly, VKDP activity measured by PIVKA-II is decreased at ICU admission and further deteriorates in the first week of intensive care regardless of stable coagulation assays and overall improvement in patient condition. This trend was especially pronounced in patients admitted following cardiac arrest. (Paper III)

The administration of 10 mg vitamin K₁ reinforces the spontaneous normalisation of a prolonged Owren PT-INR in critically ill patients (Papers IV and V).

In addition to positive effects on several coagulation assays, it is also possible to affect the activity of the extrahepatic VKDP MGP with a single dose of vitamin K₁. (Paper V)

Future perspectives

Over the past decades, thanks to the discovery of extrahepatic VKDPs and accumulating evidence supporting their involvement in several disease pathways, the research interest in VKDP activity has increased. Even though efforts are mainly directed towards studying the long-term effects of suboptimal protein activity, the findings from the studies presented in this thesis and several others indicate that there might be short-term benefits as well. However, this is a poorly researched area, and much remains to be clarified. Examples of areas where future research is needed are as follows:

Development of perioperative supplementation guidelines – for instance, in patients with poor nutritional status, malabsorption, those undergoing bariatric surgery or patients with increased risk for coagulopathies, such as those with severe right-sided heart failure. It is possible that certain patient populations would benefit from vitamin K supplementation both before and after surgery.

Development of supplementation guidelines for patients treated at the ICU – especially for those receiving parenteral nutrition or long-standing antibiotic therapy.

Vitamin K to reverse prolonged PT due to dilutional or consumptive coagulopathies. This approach has not been studied in disseminated intravascular or trauma-induced coagulation defects.

Vitamin K to reverse slightly prolonged PT to reduce unnecessary use of plasma transfusions or procoagulative factor concentrates before invasive procedures in non-emergent situations.

Randomised controlled trials to establish whether there is any merit to the theories on the potential involvement of VKDPs in conditions such as myocardial remodelling, sepsis and pulmonary damage following infection or mechanical ventilation. This could help to clarify whether VKDPs are involved in disease progression or if they might function as markers for disease.

Since several studies suggest that *vitamin K₂* has a better bioavailability for extrahepatic VKDPs, it would be of interest to *investigate its effect on VKDP activity in perioperative and critically ill patients.* However, there are currently no intravenous formulas available.

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