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REVIEW ARTICLE

Therapeutic warfarin use and the extrahepatic functions of vitamin K-dependent proteins

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ABSTRACT

The impact of warfarin therapy on the functions of extrahepatic vitamin K-dependent proteins (VKDP) is less clearly understood and less widely recognised in clinical practice than that on the hepatic counterparts (clotting factors II, VII, IX and X). Warfarin inhibits osteocalcin, an abundant extrahepatic VKDP involved in the mineralisation and maturation of bone and thus, primarily by this mechanism, may have an adverse effect on bone health. Whilst some studies do link warfarin use to an increase in osteoporosis and fracture risk others have not. Warfarin also inhibits the extrahepatic VKDP matrix gla protein (MGP) which acts to prevent ectopic calcification of the vasculature. Studies have consistently found a correlation between warfarin use and vascular calcification with inhibition of MGP believed to be the main cause. Inhibition of MGP also appears to explain warfarin's well established teratogenic effect. Further adverse effects may also arise from warfarin's inhibition of other known extrahepatic VKDPs. The available evidence is intriguing, and suggests that the impact of warfarin on the extrahepatic functions of vitamin K-dependent proteins warrants further careful consideration.

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Introduction

Warfarin is a vitamin K antagonist initially licensed in Northern America for use as a rodenticide in 1952 and subsequently, two years later, for use in humans as an anticoagulant [1]. Its use is now indicated to prevent thrombus formation and embolisation in patients with atrial fibrillation and prosthetic heart valve replacement as well as in the treatment and prophylaxis of venous thrombosis and pulmonary embolism [2]. Warfarin is extensively used with at least 1% of the British population estimated to be taking it [1].

Warfarin's therapeutic effect is derived from its inhibition of four hepatically produced vitamin K-dependent proteins (VKDP) that are involved in coagulation. These are clotting factors II, VII, IX and X and as a result of their inhibition the blood's tendency to clot is reduced [3]. Adverse effects, occurring as a result of over anticoagulation, have led to warfarin being consistently ranked amongst the top 10 medications with most adverse drug effects reported for the last two decades [4]. Haemorrhage is relatively common [5] with warfarin dosing a metaphorical two edged sword; too little and the patient is not protected from thrombotic risk, too much and the patient may bleed. However, this is well understood by clinicians with the frequency of adverse events minimised through regular monitoring of the international normalised ratio (INR). Extra hepatically produced VKDPs, involved in a number of different functions, have been more recently discovered [6]. Warfarin also inhibits the post translational modification of these proteins but the potential adverse effects this confers are less clearly understood and less well known to clinicians.

Because of the widespread use of warfarin there is the potential that any adverse effects, caused by inhibition of extra hepatic VKDPs, could pose a heavy burden to patients. As a result it is essential that further effort be made to understand these.

Search procedure

The literature search was completed by inputting the following search terms into pubmed: 'Warfarin, Vitamin K antagonist, Adverse effect, Side effect, Extra hepatic, VKDP, Bone, Vascular Calcification, Matrix gla protein, Osteocalcin, GAS 6, PGRP1, PGRP2, TMG3, TMG4, Periostin, Transthyretin'.

Vitamin K

The hydroquinone form of vitamin K is a co-factor required for the function of the enzyme γ-glutamyl carboxylase [7]. This enzyme converts the amino acid

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glutamate (Glu) to γ-carboxyglutamate (Gla) and in the process vitamin K is converted to vitamin K 2,3-epoxide. Vitamin K is subsequently salvaged by vitamin K 2,3-epoxide reductase (Figure 1), so that it may function again as a co-factor [7].

VKDPs require γ-carboxylation to confer biological activity [6]. Some of these are produced primarily by the liver; factor II, VII, IX and X as well as protein C, S and Z. Others are produced primarily outside of the liver; osteocalcin, matrix gla protein (MGP), growth arrest-specific 6 (GAS6), periostin, transthyretin, proline rich gla proteins (PRGP 1 and 2), gla rich protein (GRP) and transmembrane gla proteins (TMG 3 and 4, also known as PRGP3 and PRGP 4) [6]. It should be noted that whilst VKDPs can be split roughly into hepatic and extra hepatic proteins some hepatic proteins are produced to a lesser extent in other tissues whilst some extra hepatic proteins are produced at low levels in the liver [8].

Vitamin K antagonists

Vitamin K antagonists work by inhibiting a key enzyme that drives the vitamin K cycle; vitamin K epoxide reductase [9]. The action of warfarin causes a decrease in the production of active VKDPs, the extent of which is dependent on the dose of vitamin K antagonist administered and the susceptibility of the patient to the vitamin K antagonist [9]. Warfarin is by far the most common vitamin K antagonist used in medicine [10].

It has been known since the early 1930s that vitamin K had a role in coagulation [11]. However, since the discovery of a Gla sequence in osteocalcin in the mid-1970s [12] and in MGP in 1983 [13], it has also been known that there are a range of extra hepatic VKDPs that, owing to the same dependence on reduced vitamin K to act as a cofactor for enzymatic activation, could also be inhibited by warfarin. Since the main role of all hepatically produced VKDPs is coagulation the most visible side effect of warfarin on this action is haemorrhage [5]. The extra hepatic VKDPs however have a much wider range of function and as a result a much more varied array of adverse effects can occur when inhibited by warfarin. Due to the functions that were established for the first extra hepatic VKDPs to be identified, the adverse effects that have been most widely considered are the effect of warfarin upon bone health, calcification of vessels and development of the foetus [14,15]. Other potential effects warfarin may have, by inhibition of extra hepatic VKDPs, are being investigated and as more of these proteins are discovered the scope for identifying adverse effects increases.

Effect of warfarin on bone health

Bone is comprised of cells; osteoblasts, osteocytes and osteoclasts, within a mineralised organic matrix. The mineral component of bone is primarily made of hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$) whilst the organic component is comprised 90% of type 1 collagen along with smaller matrix proteins and proteoglycans [16]. A number of VKDPs have been identified in the non-collagenous organic bone matrix including osteocalcin [12], MGP [13] and protein S [17]. Osteocalcin is the most prevalent of these VKDPs accounting for 80% of the Gla content of mature bone [18].

Osteocalcin has three Gla residues in the first helical region. These Gla residues bind calcium and give osteocalcin its high affinity for hydroxyapatite resulting in accumulation of osteocalcin in the bone [14]. A C terminus is also present in osteocalcin which faces outwards and is available for interaction with other molecules and cells [19]. The role of osteocalcin in bone metabolism is not completely clear but it is thought to play a role in regulation of bone mineralisation, maturation and remodelling. Studies showing inhibition of hydroxyapatite growth as a result of decreased levels of osteocalcin [20] support its role in mineralisation. Osteocalcin knock-out mice were produced to study osteocalcin's effects further and a small increase in bone growth was seen compared to the wild type mice. Cortical bone width in wild type mice at 6 months was 169μm whilst in knock-out mice width was 257 μm at the same time point [21]. However, in knock-out mice that also underwent ovariectomy, and therefore, had lower oestrogen levels, there was an increased loss of bone density compared to the wild type. Bone marrow cavity expanded by 0.02 mm^2 in wild type mice post ovariectomy whilst cavity expanded by 0.05 mm^2 in knock-out mice [21]. Further, and more advanced, analysis of the bone in osteocalcin knock-out mice revealed there to be a decrease in mineral maturation [22]. These findings show

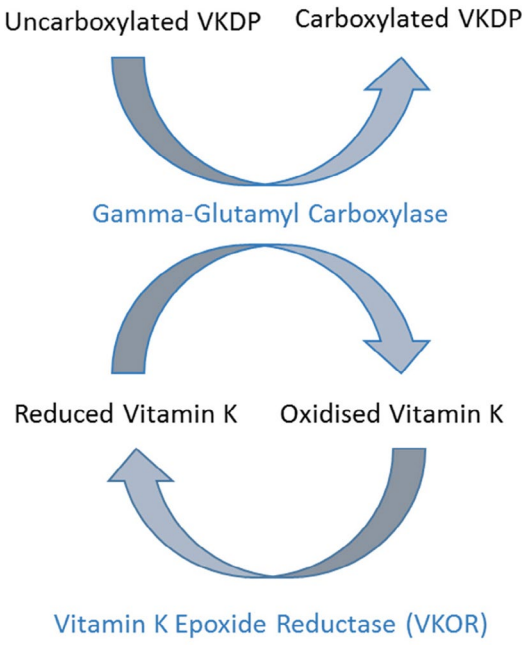

Figure 1. A simplification of the vitamin K cycle to illustrate the role vitamin K plays in the production of active VKDPs. Produced from the literature.

osteocalcin's role in regulation of mineralisation and maturation. There have also been reports that the C terminus of osteocalcin is involved in attracting osteoclast precursors. Studies finding that osteocalcin deficient bone, subcutaneously implanted in rats, results in a lower presence of surrounding osteoclasts than the number surrounding normal osteocalcin replete bone supports this and thus osteocalcin's role in bone remodelling [19]. The body of evidence points to a complex picture of action for osteocalcin with potential modification by oestrogen but, whilst further research is required, it does strongly suggest that osteocalcin is an important factor in bone metabolism. In addition, despite precise understanding of its physiological action, measurement of osteocalcin levels is widely accepted as a marker of bone formation [23].

Whilst osteocalcin is the most abundant of the VKDPs found in bone, reduced vitamin K is also required to produce the other Gla containing bone proteins. These proteins may have different actions culminating in a complex and multi-faceted effect on bone integrity. In addition, vitamin K is believed to positively influence the calcium levels present in the body by decreasing calcium excretion. It is possible this may occur, at least in part, due to vitamin K dependent carboxylation of certain proteins in the kidney [24]. Post-menopausal women given vitamin K supplementation of 1 mg per day for 2 weeks showed reduction in calcium excretion of up to 50% [25]. A subsequent study, looking at healthy young individuals, found a similar reduction in urinary calcium excretion simply with a high regular dietary intake of vitamin K [26]. This is important due to calcium's salience in bone metabolism.

Warfarin inhibits carboxylation of osteocalcin and so, as it lacks Gla residues, it loses its affinity for hydroxyapatite. Treatment of a one-month old rat with warfarin showed fraction of osteocalcin able to bind hydroxyapatite to decrease from 95% to just 10% [27]. As a result osteocalcin does not accumulate in the bone matrix and bone mineralisation is lessened [27]. In addition, warfarin inhibits the carboxylation of other VKDPs in the bone matrix and so, whilst their role is still not entirely understood, will disrupt their normal physiological action on bone metabolism. Moreover, it is possible that warfarin may inhibit vitamin K-induced calcium retention, potentially by inhibition of VKDPs in the kidney. One study looked at rats given an unrestricted diet with vitamin K supplementation that were either treated with warfarin or received no treatment for a period of 2 weeks. Serum levels of calcium were marginally lower (11.1 ± 0.1 mg/dl) in rats treated with warfarin compared to those not treated with warfarin (11.7 ± 0.2 mg/dl) [28] whilst another study found a decrease in calcium levels as INR increased [29]. Therefore, by a combination of these effects, a mechanism for warfarin to negatively impact bone health is apparent.

Many studies have indeed shown adverse effects of warfarin on bone health by a number of outcomes. One study found statistically significant decrease in bone mineral density of greater than 50% in the second metacarpals of patients receiving warfarin, as secondary prophylaxis after stroke, in comparison to controls [30]. A review of nine cross-sectional studies found similar results with adults on warfarin therapy showing loss of bone density in the ultradistal radius (standardised mean difference, warfarin vs. control = −0.39), although it should be noted not in the spine or hip [31]. Warfarin can also be used for thromboprophylaxis in congenital heart disease. Owing to improved survival of this population warfarin use in children, whilst still uncommon, is on the increase [32]. Given childhood is a critical period for the development of peak bone mass and the long-term nature of warfarin use in such patients, any effect on bone density may be particularly pronounced in such patients. A case-control study, in which mean case age was 14.7 years with warfarin therapy initiated on average at 6.8 years, found a statistically significant reduction of 17% in lumbar spine bone density in those taking warfarin [33]. Moreover, there is evidence to suggest warfarin use can increase fracture rate. It was found in women over the age of 35 that the longer they were on warfarin therapy the greater the risk of vertebral and rib fracture. Patients taking warfarin for more than year were found to have 5.3 times the risk of a vertebral fracture and 3.4 times the risk of a rib fracture compared to the age and sex specific population norm [34].

In contrast, other studies have not found a correlation between warfarin therapy and adverse bone health. The largest retrospective study carried out so far investigating the risk of hip fracture in patients on warfarin, using 52701 patients on warfarin therapy and 60383 controls, found no increased risk [35]. In addition, no increased loss of bone mineral density was found when comparing 50 cardiology patients on warfarin to age, sex and race matched controls [36].

Studies into warfarin's effect on bone show that bone mineral density loss, if it occurs, can occur in a number of diverse skeletal sites but may do so with particular preference for areas containing a high amount of trabecular bone, such as the axial skeleton, and in patients that have been on warfarin therapy for a longer period of time [14]. Many of the studies carried out have been criticised for the failure to control other variables, such as vitamin K and calcium intake, and inability to show causality due to the high co morbidity in patients traditionally prescribed warfarin [14]. The evidence suggests bone mineral density loss and increased fracture risk can occur in prolonged warfarin use but further investigation must still be undertaken to clearly establish this.

Effect of warfarin on vascular calcification

Vascular calcification occurs with age. Most people over 60 will have a degree of calcification in their major arteries

[37]. It is now recognised not as a passive degenerative process but as a pathobiological process governed by the mechanical, metabolic, endocrine, morphogenetic and inflammatory signals cells are subject to [38]. It results in increased mortality by facilitating development of aortic stenosis, hypertension, ischaemic heart disease and heart failure among other complications [37].

MGP was first discovered in bone [13] where it was established as an inhibitor of cartilaginous calcification. It requires vitamin K for activation to produce its 5 Gla residues [13]. Physiological calcification is a requirement for the formation of bone. In endochondral ossification, mesenchymal cells differentiate into chondrocytes, a cartilaginous matrix is formed and it subsequently becomes calcified to produce bone whilst in intramembranous ossification there is direct calcification of mesenchymal cells [39]. However, premature completion of ossification, due to advanced calcification, can lead to stunted bone growth and thus inhibitory proteins such as MGP are required. When mice were produced with knock out of the MGP gene they developed short stature due to inappropriate calcification of cartilage, specifically including that of the growth plate [40]. Similarly, in humans Keutel syndrome, first described in 1971 [41], has been determined to result from mutations in the MGP gene leading to absence or non-functional production of the MGP protein [42]. One of the defining features of Keutel syndrome is diffuse calcification of cartilage.

MGP is now known to be produced by a range of other tissues, in addition to bone, including vascular smooth muscle cells (VSMCs) [28] where it also has an important role in preventing calcification. MGP knock-out mice died unexpectedly within 8 weeks due to arterial calcification resulting in blood vessel rupture [40]. When MGP expression was restored in the arteries it rescued MGP knock-out mice from arterial calcification [43]. However, returning hepatic MGP expression and subsequently raising MGP serum levels did not serve to rescue the mice from arterial calcification [43]. This shows that MGP, produced locally in VSMCs, is vital in inhibiting ectopic calcification of the vasculature. It has also been postulated that two other VKDPs; GRP and osteocalcin, may have a smaller, and as of yet less defined, roles in inhibition of vascular calcification [44]. It has been suggested that lower levels of vascular calcification that occur in MGP deficient humans, for example those with Keutel syndrome, compared to MGP knock-out mice may be due to GRP acting as a backup inhibitor of vascular calcification in humans which is not present in mice [44].

Warfarin's inhibition of MGP and, to a lesser extent, GRP and osteocalcin will lead to a loss of these proteins calcification countering effects in blood vessels. As such it is plausible that warfarin may increase calcification of the vasculature. Such effect was seen when rats were dosed with warfarin at a level sufficient to inhibit MGP carboxylation. There was rapid calcification of the aorta and aortic heart valves with the appearance of linear calcified structures in the aortic media after only 2 weeks of treatment [45]. Only as recently as 2004 did a study confirm that vitamin K antagonist use also leads to increased vascular calcification in humans. Aortic valves, removed during valve replacement surgery, from people who had taken vitamin K antagonist pre-operatively and those who hadn't taken vitamin K antagonist pre-operatively were compared. Mean length of treatment in patients taking vitamin K antagonist pre-operatively was 25 months with target INR of 2–3 for all included patients. Calcifications in those taking vitamin K antagonists pre-operatively were up to twice as large as in those who hadn't [46]. Further investigations have had similar findings. Degree of coronary artery calcification was compared, by use of CT scan, between patients with atrial fibrillation taking or not taking warfarin [47]. Mean duration of warfarin treatment was 46 months. Patients taking warfarin had significantly increased amounts of calcium present in the coronary arteries compared to patients not on warfarin therapy. Sixty-two per cent of patients not taking warfarin had no coronary artery calcification whilst only 35% of those taking warfarin had no calcification present. Calcification also increased significantly with duration of warfarin therapy. Mean coronary calcification score in patients taking warfarin for over 60 months was 2.5× that of patients taking warfarin for 6–60 months.

Studies have almost unanimously resulted in findings that support the occurrence of warfarin induced vascular calcification with many studies finding large and significant increases in vascular calcification due to warfarin use. This vascular calcification has been strongly correlated to an increase in morbidity and mortality due to cardiovascular sequelae [37].

Effects of warfarin on the developing foetus

The first case of congenital malformation occurring due to warfarin administration during pregnancy was reported in 1966 [48]. Since then the teratogenic effects of warfarin have been well characterised. Administration of warfarin during the first trimester, a critical period for organogenesis, carries a 20% risk of still birth and a further 20% risk of congenital anomaly [49]. Congenital anomalies often occur together in a syndromic manner; this is termed 'warfarin embryopathy'. Whilst malformations can effect multiple systems the most common and characteristic occur in the skeletal system and include nasal hypoplasia with vertebral and epiphyseal stippling [50]. Warfarin embryopathy is widely recognised and as such use of warfarin is generally contra-indicated during pregnancy, particularly in the first trimester, where use of alternative anticoagulants is recommended. In certain high risk cases, however, such as patients with mechanical heart valves, warfarin use may be unavoidable [51].

Table 1. Possible adverse effects resulting from warfarin's inhibition of specific extrahepatic VKDPs. Table compiled from the literature.

Extra hepatic VKDP	Possible adverse effect due to warfarin inhibition
Osteocalcin	Osteoporosis, Bone fracture, Vascular Calcification
MGP	Vascular Calcification Teratogenicity
GRP	Vascular Calcification
GAS6*	Wide range of potential effects e.g. increased CNS demyelination
Periostin*	Exacerbation of inflammatory disease
Transthyretin*	Multiple pathologies, mainly related to amyloid deposition
PRGPs**	Interference with signalling pathways
TMGs	Currently unknown

*Possible adverse effect predicted from response when VKDP function is interfered with by factor other than warfarin.
**Possible adverse effect predicted from function of VKDP.

There is good evidence to suggest a role for extra-hepatic VKDP inhibition in warfarin embryopathy. Abnormalities within the skeletal system are thought to occur primarily due to warfarin inhibition of MGP [52]. As previously discussed, the lack of MGP's inhibiting role in calcification can lead to ossification and early closure of the growth plates, subsequently stunting growth, as well as causing ectopic calcification giving the classic manifestation of skeletal stippling. A role for osteocalcin in such skeletal malformation has also been suggested [50] although a number of studies have suggested its effect to be minimal in comparison to that of MGP [52].

In contrast to warfarin's effect on bone health and vasculature, teratogenicity represents a case where warfarin's adverse effect via the inhibition of extra hepatic VKDPs is well recognised and has influenced clinical practice.

Other adverse effects

Whilst osteoporosis, vascular calcification and teratogenicity comprise the main stay of recognised extra hepatic VKDP related adverse effects of warfarin this is not to say there aren't others. GAS6 is known to have a wide range of functions in the body including regulation of apoptosis, proliferation, white blood cell migration and platelet aggregation [53]. As a result, inhibition of this protein could lead to a variety of consequences. Indeed, one study found that there was increased oligodendrocyte loss with greater demyelination, following CNS insult, in GAS6 deficient mice compared to controls [54]. Similarly, periostin is known to be involved in inflammatory processes and regulates both angiogenesis and cell movement. It was recently found that significantly greater inflammation and joint destruction occurred due to rheumatoid arthritis in periostin knock-out mice than in wild type mice [55]. It is feasible that warfarin inhibition could produce comparable effects in patients. Transthyretin's function has been established as a carrier protein, responsible for the transport of both thyroid hormones and retinol. Mutation of this protein, leading to loss of function, can cause a wide range of diseases including amyloidotic polyneuropathy, cardiomyopathy and carpal tunnel syndrome [56]. Once again, we can postulate that inhibition of this protein by warfarin may cause similar effects.

PRGP1 and PGRP2 contain proline motifs found in a number of signalling proteins [57] whilst PRGP2 has been found specifically to interact with the oncogene yes-associated protein 1 (YAP1) [58]. As such PRGPs may have a role in signal transduction with any adverse effects due to warfarin use resulting from interference with these pathways. Despite being widely distributed in both adult and foetal tissue the role of the most recently discovered VKDPS; TMG3 and TMG4, is not known and is under investigation [6]. As a result, any number of adverse effects could occur due to warfarin's action on these VKDPs. All of the possible adverse effects discussed are summarised in Table 1.

Overall the physiology of extra hepatic VKDPs is poorly understood. In addition to the possible adverse effects mentioned, further adverse effects may result from inhibition of presently unknown functions carried out by these proteins. Adverse effects may also result from inhibition of extra hepatic VKDPs yet to be identified.

Conclusion

Whilst there is evidence linking warfarin use to impaired bone health, leading to osteoporosis and increased fracture rates, it is as of yet inconclusive and further research must be undertaken to determine the precise relationship between the two. There is however a much stronger evidence base showing that the use of warfarin results in increased vascular calcification. Cardiovascular related morbidity and mortality are well established consequences of this process. Teratogenicity is a widely recognised adverse effect of warfarin and there is good evidence to show this occurs through inhibition of extra hepatic VKDPs. Furthermore, it must be considered that we do not presently understand the full side effect profile of warfarin. Its use may be causing adverse effects that will only be elucidated in the future.

When considering the use of any medication, risk must be weighed against benefit. Warfarin has a history of over 60 years of successful use as a medical anticoagulant. Its benefit has been deemed so significant its use has been continued despite the recognised and serious adverse effect of haemorrhage. Hence, whilst there is evidence to show that warfarin has adverse effects via inhibition of extra hepatic VKDPs, unbeknownst to many clinicians, it is likely to be insufficient to suggest that, in general, warfarin should not be prescribed.

It should however perhaps be considered on a case by case basis, especially with the advent of novel

anticoagulants giving a viable option to anticoagulation in many cases with no impact on VKDPs. In those with known pre-existing osteoporosis or vascular calcification the decision could be taken to use one of these alternative therapies. Alternatively, patients on warfarin, known to have a baseline susceptibility, could be monitored for the development of extra hepatic VKDP related side effects.

Disclosure statement

No potential conflict of interest was reported by the authors.

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