

Corn oil-induced decrease in arterial thrombosis tendency may be related to altered plasma vitamin K transport

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Abstract In this article we report the effects of low and high fat diets on the arterial thrombosis tendency in rats. The animal system used was the aorta loop model, in which we compared the effect of saturated (hardened coconut oil, HCO) and unsaturated (sunflower seed oil, SSO; corn oil, CO) fatty acids on the arterial thrombosis tendency at high fat intake (50 energy%, 45 energy% of which was either HCO, SSO, or CO). Under these conditions both SSO and CO had a beneficial effect (relative to HCO) on the arterial thrombosis tendency. In a subsequent study we compared these high fat diets with a low fat diet (5 energy%). As compared with the low fat diet, only CO significantly decreased the thrombosis risk. Serum vitamin K and triglycerides had decreased substantially after the CO diet, and to a much lesser extent after the SSO diet. It is concluded that corn oil may have a mildly anticoagulant effect, the potential benefit of which is discussed.—Schurgers, L. J., and C. Vermeer. Corn oil-induced decrease in arterial thrombosis tendency may be related to altered plasma vitamin K transport. *J. Lipid Res.* 2001. 42: 1120–1124.

Supplementary key words PUFA • vessel wall • atherosclerosis • phylloquinone • aorta loop • nutritional oils • fatty acid

It is well known that a diet rich in saturated fatty acids (SAFA) forms a risk factor for atherosclerosis in humans (1, 2), and that their inclusion in the diet increases the arterial thrombosis tendency (ATT) in rats (3). At our institute we have an operational animal model for ATT, known as the aorta loop model (4). In this model, thrombogenic diets such as those rich in hardened coconut oil (HCO) induce an increase of the ATT. Diets rich in monounsaturated fatty acids (MUFA) and PUFA, such as are found in corn oil (CO), sunflower seed oil (SSO), linseed oil, or safflower oil, exhibit this effect to a much lesser extent, but most of these oils will show a beneficial effect only if they are used to replace SAFA in a high fat diet (1). In humans, several studies have shown that fasting factor VIIc, which is regarded as an independent risk factor for cardiovascular disease, is decreased by low fat diets. A similar effect has been reported for diets in which the SAFA composition

was substituted by MUFA or PUFA (5). Exceptions in this respect may be fish oil (rich in n–3 PUFA) and CO (n–6 PUFA). Several studies have shown that both fish oil and CO consumption leads to a reduction of blood cholesterol and triglycerides (6–9). The mechanism underlying such beneficial effect is still a matter of debate (10, 11), but one hypothesis is that the protective effect against cardiovascular disease is due to a decrease in the serum cholesterol and triglyceride levels (12), and to a mild reduction in the circulating vitamin K-dependent blood coagulation factor concentrations (12, 13). The latter phenomenon may be mediated by a lipid-lowering effect that affected the lipoprotein transport and delivery of vitamin K to the liver, thereby reducing the synthesis of active vitamin K-dependent coagulation proteins. The credence of such a hypothesis is reinforced by findings that plasma concentrations of phylloquinone (vitamin K₁), the predominant circulating and dietary form of vitamin K, show a strong positive correlation with plasma triglycerides (14–16), reflecting the fact that triglyceride-rich lipoproteins (TGRLP) are the major transporters of vitamin K₁ in both the postprandial and fasting states (17, 18). This suggests that factors that influence the metabolism of TGRLP may also affect the transport, tissue distribution, and bioavailability of vitamin K (17–19).

To test this hypothesis we have compared whether lipid-lowering diets based on n–6 PUFA may affect the ATT (in rats only) and vitamin K metabolism (rats and humans). CO and SSO were the experimental fats selected for this study because, in contrast to most other plant oils, their vitamin K content is low. Moreover, these oils are commonly used for food preparation in The Netherlands. First, we have compared in experimental animal studies the effects of CO, SSO, and HCO on the ATT, and we

Abbreviations: ATT, arterial thrombosis tendency; CO, corn oil; HCO, hardened coconut oil; OT, obstruction time of aorta loop; SSO, sunflower seed oil.

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have tried to find an explanation for the observed differences from a number of biomarkers in serum.

MATERIALS AND METHODS

Materials

Phylloquinone was purchased from Sigma (St. Louis, MO). HCO and SSO were purchased from Chempri (Raamsdonksveer, The Netherlands). CO was a kind gift from CPC-Bestfoods (Heilbronn, Germany). All other chemicals and reagents were of analytical or HPLC grade. Powdered stock rat food was from Hope Farms (Woerden, The Netherlands), and had been made vitamin K deficient by irradiation (0.9 mrad). Stocks for preparing high fat (25%, w/w) stocks contained 23% cerelose/glucose, whereas the low fat (5% w/w) stocks contained 54% cerelose/glucose. In this way the caloric values for all diets had been made similar. Other dietary components (protein, fiber, and trace elements) were present as described previously (20). Before each study the fats (as indicated) were mixed with the stocks at our experimental animal department. The fatty acid composition of the oils is shown in **Table 1**.

Animals and diets

Male Wistar rats were housed individually in wire-bottom cages with a 12-h light-dark cycle and controlled temperature ($20 \pm 2^\circ\text{C}$) and humidity ($50 \pm 10\%$). The vitamin K-deficient food was supplemented with vitamin K₁ (2 $\mu\text{g/g}$) as an oil solution, whereas the added oils provided less than 5 ng of K₁ per g of food. On the basis of these data the vitamin K intake was estimated to be 39 $\mu\text{g/day}$. Food for the low fat regimen contained 5 energy% of SSO as the only fat compound, food for the high fat regimen contained 5 energy% of SSO plus 45 energy% of either HCO, SSO, or CO (as indicated). To protect the foods from lipid peroxidation, the diets were prepared freshly each week and stored at -20°C under nitrogen until use. During the experiment the animals were allowed to eat ad libitum. No significant differences between the various groups were found with respect to their mean ($\pm\text{SD}$) daily food intake (19 ± 5 g) and weight gain (17 ± 7 g/week). The protocol for this experiment was approved by the Maastricht University Committee for Animal Experiments.

ATT in rats

The ATT was measured using the rat aorta loop model. This model is based on the surgical insertion of a loop-shaped polyethylene cannula in the abdominal aorta of the animals (3). The wound is closed in such a way that the loop protrudes outside the abdomen and remains available for visual inspection. At places where the cannula is in permanent contact with the vessel wall endothelial damage and flow disturbances result in the formation of a thrombus, which is accompanied by a color change of the loop. Eventually, a thrombus will develop in all cases, and the time required for complete obstruction of the loop is called the obstruction time (OT). It has been well documented that in rats the OT is inversely correlated with the ATT, which may depend on either diet or drugs (4, 21). In the experimental setup groups of 24 rats each entered the study at the age of 5 weeks, and received the indicated diets until completion of the experiment. After a feeding period of 8 weeks the loops were implanted, and the OT values were recorded. Citrated blood was collected shortly before implantation of the loops.

Various analytical techniques

Prothrombin concentrations were assessed with the one-stage coagulation assay, using Thromborel S and human clotting

TABLE 1. Fatty acid compositions of the different oils

| | Type of Diet | | | |
|--|--------------|------|------|------|
| | Low Fat | HCO | SSO | CO |
| Caloric intake additional to standard diet (energy%) | 0 | 45 | 45 | 45 |
| SAFA (% of total fat) | 12 | 100 | 12 | 13 |
| MUFA (% of total fat) | 25 | 0 | 25 | 33 |
| PUFA (% of total fat) | 63 | 0 | 63 | 54 |
| Vitamin E (mg/day) | | | | |
| α -Tocopherol | 0.48 | 0 | 4.35 | 1.23 |
| γ -Tocopherol | 0.97 | 0 | 0.45 | 6.31 |
| Vitamin K ($\mu\text{g/day}$) | 0.09 | 0.05 | 0.79 | 0.14 |
| Ubiquinone-9 ($\mu\text{g/day}$) | 0.05 | 0 | 0.42 | 3.72 |

The standard diet (low fat) for rats contained 5 energy% of SSO. In the high fat diets (HCO, SSO, or CO) 45 energy% of either HCO, SSO, or CO was added to the standard diet to a final 50 energy%. HCO, hardened coconut oil; SSO, sunflower seed oil; CO, corn oil; SAFA, saturated fatty acids; MUFA, monounsaturated fatty acids.

prothrombin-deficient plasma from Behringwerke (Marburg, Germany). Plasma vitamin K concentrations were assessed by HPLC, with on-line electrochemical detection of the effluent and fluorescence detection (22, 23). Triglycerides and cholesterol were determined by standard enzymatic techniques (Boehringer Mannheim, Germany) on a Beckman (Fullerton, CA) Synchron CX 7-2 autoanalyzer.

Statistics

Because OT values show a log-normal distribution pattern, logarithmic transformation of these values was performed before the differences between the groups were tested by one-way ANOVA using the Bonferroni post-hoc procedure for multiple group analysis. Mean OT values were calculated from the mean log OT values. Effects on blood coagulation were tested non-parametrically for each group, using the Mann-Whitney U test. All statistics were performed with the statistical program StatView 4.1 (Abacus Concepts, Cary, NC).

RESULTS

ATT in rats at high intake of different oils

The effects of high intake of three different oils were compared in three groups of 24 rats each. The data are shown in **Fig. 1** and demonstrate that at high fat intake both unsaturated oils (SO and CO) induced a significantly decreased arterial thrombosis risk as compared with the saturated oil. Remarkably, there was a significant difference between both unsaturated oils as well, with the lowest arterial thrombosis risk for the CO group.

Effects of high and low intake of dietary oils in rats

In a second, independent study, we compared the effects of high and low fat intake. One group of animals received a low fat diet (5 energy% of SSO), and served as a reference group in this experiment. The other groups received high fat diets as described above. The data of this experiment are summarized in **Table 2**. It was found that the OT values in the HCO group were significantly less than those in the low fat group, indicating an increased ATT due to high intake of saturated oil. No such effect was observed at high intake of SSO,

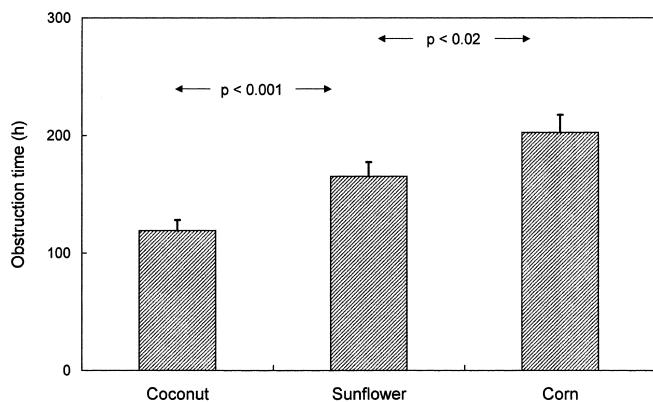


Fig. 1. ATT at high fat intake: comparison of different fat types. The time required for obstruction of the aorta loop (ATT) is inversely correlated with thrombosis tendency, and was expressed in hours \pm SD. Statistical analysis was performed after log transformation of the data (see Materials and Methods).

which resulted in OT values comparable to those of the low fat group. Consistent with the data in Fig. 1, high intake of CO resulted in very long OT values, suggesting that high intake of CO significantly reduced the ATT both as compared with low fat intake and high SSO intake.

In an attempt to explain these data, we have measured a number of variables in the blood taken shortly before the implantation of the loops (Table 2). None of the oils affected the circulating prothrombin concentration. It should be mentioned here that the assays were performed with human reagents, which may have affected assay sensitivity in a negative way. The HCO diet resulted in relatively high circulating levels of triglycerides and vitamin K, whereas in the CO and SSO groups the triglycerides were significantly lower than in the low fat group. This effect was stronger in the CO group than in the SSO group, but whether this difference is sufficient to explain the strongly reduced ATT in the CO group is not clear. Also, circulating vitamin K was reduced during high intake of unsaturated oil, but the effect was statistically significant only for the CO group.

DISCUSSION

This article is based on the initial discovery that in an animal model system the ATT is decreased by replacing

HCO in the diet by either SSO or CO. Whereas these first experiments were performed exclusively in animals on a high fat regimen (50 energy%), later studies demonstrated that the beneficial effect of SSO is lost if the high fat diet is compared with low fat diets (5 energy%). For high intake of CO, however, a substantial and significant protective effect remained even when the diet was compared with a low fat regimen (Table 2). The difference between CO and HCO was comparable to that obtained in rats treated with low doses of vitamin K antagonist (G. Hornstra, unpublished data) or with high doses of aspirin (24). The difference between HCO and both plant oils may be explained by the fact that the former is fully saturated, whereas both CO and SSO contain a large fraction of unsaturated fatty acids. Because the fatty acid composition of CO and SSO is remarkably similar, other explanations must be found for their different effects on the ATT. Substantial differences between CO and SSO were reported with respect to their nonfat fraction. CO, for instance, was reported to contain 10- to 20-fold more ubiquinone and γ -tocopherol than did SSO (6). Because these compounds have a marked structural analogy with vitamin K, our group and others have tested whether either ubiquinone (25) or tocopherol (26) may act as competitive inhibitors for the vitamin K-dependent carboxylase, thus inhibiting the biosynthesis of blood coagulation factors. Although in vitro some inhibition was found, the effect was weak and it is unlikely that it can explain the marked in vivo differences between both oils.

We have tested this hypothesis in rats as well as in a human nutrition experiment. In rats we found no significant effect of any of the oils on the circulating prothrombin concentration. It should be kept in mind, however, that the prothrombin assay is not particularly sensitive in detecting small fluctuations. A direct assay for descarboxyprothrombin would be far more accurate, but this test is not available for rats. As compared with the low fat group, serum vitamin K had remained constant in rats receiving SSO, but had significantly decreased in the CO group. Circulating triglycerides, which play a major role in vitamin K transport, had decreased in both plant oils, but to a much larger extent in the CO than in the SSO group. Hence it is at least feasible that the type of oil affects the bioavailability and tissue distribution of vitamin K.

TABLE 2. Comparison of high and low dietary fat in the rat

| | Low Fat | HCO | SSO | CO |
|--|---------------|----------------|-----------------|-----------------|
| Number of Rats/Dietary Fat (Energy%) | 24/5 | 48/50 | 48/50 | 48/50 |
| Obstruction time (h) | 120 \pm 8 | 91 \pm 11* | 123 \pm 10 | 144 \pm 13* |
| Plasma prothrombin (% of reference) | 96 \pm 7.6 | 99 \pm 9.2 | 102 \pm 9.1 | 101 \pm 5.5 |
| Serum vitamin K ₁ (μ g/l) | 0.8 \pm 0.2 | 1.9 \pm 0.3* | 0.8 \pm 0.27 | 0.7 \pm 0.2* |
| Serum triglycerides (mM) | 1.0 \pm 0.1 | 1.8 \pm 0.1* | 0.9 \pm 0.03* | 0.6 \pm 0.05* |
| K ₁ /triglyceride (μ g/mmol) | 0.8 \pm 0.2 | 1.1 \pm 0.2 | 0.9 \pm 0.2 | 1.1 \pm 0.2 |

Rats were fed various diets for 8 weeks before blood was taken and aorta loops were implanted. Determinations in plasma and serum were performed in duplicate and mean values for each animal were used to calculate the mean of means (\pm SD) given here. The values obtained in the three high fat groups were compared with the low fat group; differences were regarded to be significant at $P < 0.05$ (*).

When trying to find an explanation for the observed effects of CO it is important to realize that after intestinal absorption, vitamin K is transported in the blood via lipoproteins and that it is liberated therefrom during the hydrolysis of the chylomicron triglycerides in a variety of tissues (including the liver) before the chylomicron remnants are cleared by the liver (27). Only a little vitamin K is found in the LDL and HDL fractions (18). Obviously, the postprandial fatty acid content of the chylomicrons will depend on the composition of the diet. Animal and some human studies suggest an increased activity of endothelial lipoprotein lipase toward the hydrolysis of PUFA-rich compared with SAFA-rich chylomicrons (8, 28). In the context of vitamin K and vitamin K-dependent coagulation proteins, a study of rats showed that the PUFA-rich fish oil not only decreased serum cholesterol and triglyceride levels, but also led to decreased levels of the vitamin K-dependent coagulation factors prothrombin and factor VII (12). These authors postulated that this hypocoagulable effect may be mediated by limited delivery of vitamin K to the liver and thereby the synthesis of vitamin K-dependent coagulation proteins. Our data support the view that circulating levels of vitamin K-dependent coagulation proteins are linked to lipid metabolism in normal physiology (29), with the intriguing possibility that part of this interaction with lipids may be mediated via an effect on vitamin K metabolism (12). An explanation for such an effect could be that CO induces a different transport for vitamin K with more extrahepatic vitamin K uptake rather than accumulation in the liver, but this is speculative at this time.

We and others have demonstrated that subclinical vitamin K deficiency is common in the healthy adult population and may be associated with low bone mass (30–32), increased fracture rate in postmenopausal osteoporosis (33), and increased vascular calcification (34). These phenomena may be related to the synthesis of incompletely carboxylated Gla proteins such as osteocalcin in bones (35), and matrix Gla protein (MGP) in the arterial vessel wall (36). Therefore, nutrients capable of shifting the balance between hepatic and extrahepatic vitamin K uptake may have a dual effect. On the one hand, they might induce a mildly anticoagulant effect by decreasing the hepatic vitamin K status, thus lowering the ATT. On the other hand, they might improve vascular MGP carboxylation and thus contribute to the prevention of arterial calcification. ■

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REFERENCES

- Hornstra, G., and A. D. Kester. 1997. Effect of the dietary fat type on arterial thrombosis tendency: systematic studies with a rat model. *Atherosclerosis*. **131**: 25–33.
- Gurr, M. I. 1992. Dietary lipids and coronary heart disease: old evidence, new perspective. *Prog. Lipid Res.* **31**: 195–243.
- Hornstra, G., and A. Vendelmans-Starrenburg. 1973. Induction of experimental arterial occlusive thrombi in rats. *Atherosclerosis*. **17**: 369–382.
- Hornstra, G., and R. N. Lussenburg. 1975. Relationship between the type of dietary fatty acid and arterial thrombosis tendency in rats. *Atherosclerosis*. **22**: 499–516.
- Mennen, L. I., E. G. Schouten, D. E. Grobbee, and C. Kluit. 1996. Coagulation factor VII, dietary fat and blood lipids: a review. *Thromb. Haemost.* **76**: 492–499.
- Kohlmeier, M., W. Riesen, and G. Schlierf. 1988. Metabolic changes in healthy men using fat-modified diets. I. Disposition of serum cholesterol. *Ann. Nutr. Metab.* **32**: 1–9.
- Wardlaw, G. M., and J. T. Snook. 1990. Effect of diets high in butter, corn oil, or high-oleic acid sunflower oil on serum lipids and apolipoproteins in men. *Am. J. Clin. Nutr.* **51**: 815–821.
- Weintraub, M. S., R. Zechner, A. Brown, S. Eisenberg, and J. L. Breslow. 1988. Dietary polyunsaturated fats of the W-6 and W-3 series reduce postprandial lipoprotein levels. Chronic and acute effects of fat saturation on postprandial lipoprotein metabolism. *J. Clin. Invest.* **82**: 1884–1893.
- Dupont, J., P. J. White, M. P. Carpenter, E. J. Schaefer, S. N. Meydani, C. E. Elson, M. Woods, and S. L. Gorbach. 1990. Food uses and health effects of corn oil. *J. Am. Coll. Nutr.* **9**: 438–470.
- Goto, Y., H. Tamachi, and E. H. Moriguchi. 1993. Eicosapentaenoic acid and atherosclerosis. *Prostagl. Leukot. Essent. Fatty Acids*. **48**: 337–342.
- Chin, J. P. F. 1994. Marine oils and cardiovascular reactivity. *Prostaglandins Leukot. Essent. Fatty Acids*. **50**: 211–222.
- Nieuwenhuys, C. M., S. Beguin, R. F. Offermans, J. J. Emeis, G. Hornstra, and J. W. Heemskerk. 1998. Hypocoagulant and lipid-lowering effects of dietary n-3 polyunsaturated fatty acids with unchanged platelet activation in rats. *Arterioscler. Thromb. Vasc. Biol.* **18**: 1480–1489.
- Shahar, E., A. R. Folsom, K. K. Wu, B. H. Dennis, T. Shimakawa, M. G. Conlan, C. E. Davis, and O. D. Williams. 1993. Associations of fish intake and dietary n-3 polyunsaturated fatty acids with a hypocoagulable profile. The Atherosclerosis Risk in Communities (ARIC) Study. *Arterioscler. Thromb.* **13**: 1205–1212.
- Sadowski, J. A., S. J. Hood, G. E. Dallal, and P. J. Garry. 1989. Phylloquinone in plasma from elderly and young adults: factors influencing its concentration. *Am. J. Clin. Nutr.* **50**: 100–108.
- Saupe, J., M. J. Shearer, and M. Kohlmeier. 1993. Phylloquinone transport and its influence on gamma-carboxyglutamate residues of osteocalcin in patients on maintenance hemodialysis. *Am. J. Clin. Nutr.* **58**: 204–208.
- Kohlmeier, M., J. Saupe, H. J. Drossel, and M. J. Shearer. 1995. Variation of phylloquinone (vitamin K₁) concentrations in hemodialysis patients. *Thromb. Haemost.* **74**: 1252–1254.
- Kohlmeier, M., A. Salomon, J. Saupe, and M. J. Shearer. 1996. Transport of vitamin K to bone in humans. *J. Nutr.* **126(Suppl.)**: 1192S–1196S.
- Lamon-Fava, S., J. A. Sadowski, K. W. Davidson, M. E. O'Brien, J. R. McNamara, and E. J. Schaefer. 1998. Plasma lipoproteins as carriers of phylloquinone (vitamin K₁) in humans. *Am. J. Clin. Nutr.* **67**: 1226–1231.
- Shearer, M. J., P. Barkhan, and G. R. Webster. 1970. Absorption and excretion of an oral dose of tritiated vitamin K₁ in man. *Br. J. Haematol.* **18**: 297–308.
- Groenen-van Dooren, M. M. C. L., B. A. M. Soute, K. S-G. Jie, H. H. W. Thijssen, and C. Vermeer. 1993. The relative effects of phylloquinone and menaquinone on the blood coagulation factor synthesis in vitamin K-deficient rats. *Biochem. Pharmacol.* **46**: 433–437.
- Roncaglioni, M. C., G. di Minno, J. Pangrazzi, I. Reyers, L. Mussoni, G. de Gaetano, and M. B. Donati. 1980. Plasmatic and vascular factors of the hemostatic system in rats receiving an estrogen-progestogen combination. *Contraception*. **22**: 249–257.
- Thijssen, H. H. W., and M. J. Drittij-Reijnders. 1993. Vitamin K metabolism and vitamin K₁ status in human liver samples: a search for inter-individual differences in warfarin sensitivity. *Br. J. Haematol.* **84**: 681–685.
- Gijssbers, B. L. M. G., G. K-S. Jie, and C. Vermeer. 1996. Effect of food composition on vitamin K absorption in human volunteers. *Br. J. Nutr.* **76**: 223–229.
- Reyers, I., A. Hennissen, M. B. Donati, G. Hornstra, and G. de

- Gaetano. 1985. Aspirin and the prevention of experimental thrombosis: difficulty in establishing unequivocal effectiveness. *Thromb. Haemost.* **54**: 619–621.
25. Rondan, J. E., B. A. M. Soute, H. H. W. Thijssen, J. Saupe, and C. Vermeer. 1996. Natural prenylquinones inhibit the enzymes of the vitamin K cycle in vitro. *Biochim. Biophys. Acta.* **1298**: 87–94.
26. Uotila, L. 1988. Inhibition of vitamin K dependent carboxylase by vitamin E and its derivatives. In *Current Advances in Vitamin K Research*. J.W. Suttie, editor. Elsevier, New York. 59–64.
27. Havel, R. J., and J. P. Kane. 1995. Introduction: structure and metabolism of plasma lipoproteins. In *The Metabolic and Molecular Bases of Inherited Disease*. C. R. Scriver, A. L. Beaudet, W. S. Sly, and D. Valle, editors. McGraw-Hill, New York. 1841–1851.
28. Williams, C. M. 1997. Postprandial lipid metabolism: effects of dietary fatty acids. *Proc. Nutr. Soc.* **56**: 679–692.
29. Hoffman, C. J., W. E. Lawson, R. H. Miller, and M. B. Hultin. 1994. Correlation of vitamin K-dependent clotting factors with cholesterol and triglycerides in healthy young adults. *Arterioscler. Thromb.* **14**: 1737–1740.
30. Szulc, P., M. Arlot, M-C. Chapuy, F. Duboeuf, P. J. Meunier, and P. D. Delmas. 1994. Serum undercarboxylated osteocalcin correlates with hip bone mineral density in elderly women. *J. Bone Miner. Res.* **9**: 1591–1595.
31. Knapen, M. H. J., A. C. Nieuwenhuijzen Kruseman, R. S. M. E. Wouters, and C. Vermeer. 1998. Correlation of serum osteocalcin with bone mineral density in women during the first 10 years after menopause. *Calcif. Tissue Int.* **63**: 375–379.
32. Väänänen, H. K., H. Luukinen, S-M. Käkönen, K. Pettersson, K. Koski, P. Laippala, T. Lövgren, and S-L. Kivelä. 1999. Strong prediction of fractures among the elderly by the ratio of carboxylated and total serum osteocalcin. *Calcif. Tissue Int.* **64**: S79.
33. Szulc, P., M-C. Chapuy, P. J. Meunier, and P. D. Delmas. 1996. Serum undercarboxylated osteocalcin is a marker of the risk of hip fracture: a three year follow-up study. *Bone.* **18**: 487–488.
34. Jie, K-S. G., M. L. Bots, C. Vermeer, J. C. M. Witteman, and D. E. Grobbee. 1995. Vitamin K intake and osteocalcin levels in women with and without aortic atherosclerosis: a population-based study. *Atherosclerosis.* **116**: 117–123.
35. Cairns, J. R., and P. A. Price. 1994. Direct demonstration that the vitamin K-dependent bone Gla protein is incompletely γ -carboxylated in humans. *J. Bone Miner. Res.* **9**: 1989–1997.
36. Price, P. A., S. A. Faus, and M. K. Williamson. 1998. Warfarin causes rapid calcification of the elastic lamellae in rat arteries and heart valves. *Arterioscler. Thromb. Vasc. Biol.* **18**: 1400–1407.