

# Thrombomodulin as a Marker for Bleeding Complications During Warfarin Treatment

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**Background:** The major adverse effect of warfarin treatment is hemorrhage. Several risk factors for bleeding complications are also risk factors for thromboembolic events, making the clinical decision to initiate or withhold anticoagulant treatment difficult. Specific markers that solely identify patients at high risk of bleeding would have great clinical impact. This study aimed to test if thrombomodulin (TM) concentrations were associated with bleeding complications, cardiovascular events, or mortality in long-term anticoagulant-treated patients.

**Methods:** In a longitudinal cohort study we followed up 719 patients receiving warfarin treatment for a mean duration of 4.2 years. All bleeding complications causing hospitalization were registered and classified. Soluble TM antigen (sTM) concentration in plasma was measured with an enzyme-linked immunosorbent assay method.

**Results:** During the follow-up time, 113 clinically relevant bleeding events and 73 major bleeding events occurred. Increased concentration of sTM was associated with both clinically relevant bleeding and major bleeding events after adjustment for age. In the multivariable models, hazard ratios for the highest tertiles compared with the lowest were 2.29 (95% confidence interval, 1.35-3.89) and 2.33 (95% confidence interval, 1.21-4.48), respectively. No association between sTM concentration and nonfatal ischemic cardiovascular events or all-cause mortality was found.

**Conclusions:** Increased levels of sTM are associated with bleeding complications during warfarin treatment but not with cardiovascular events or all-cause mortality. Soluble TM antigen concentration has potential as a new specific marker to identify patients at high risk of bleeding during warfarin treatment.

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TREATMENT WITH VITAMIN K antagonists (VKAs) is widely accepted for primary and secondary prevention of thromboembolic events.<sup>1,2</sup> Warfarin is by far the most common VKA used today. The major adverse effect of warfarin treatment is the increased risk for bleeding complications. In a population in northern Sweden, we have observed a risk for clinically relevant bleeding of 3.9% per treatment year.<sup>3</sup> Attempts to predict the risk of bleeding complications during warfarin treatment have been made, but only a few risk factors have been described. Several risk factors for bleeding have been proposed including age,<sup>3</sup> prior gastrointestinal tract bleeding, and hypertension.<sup>4</sup> However, many of the risk factors for bleeding are also associated with increased risk for thromboembolic events, making the clinical decision to initiate or withhold anticoagulant treatment difficult. Therefore, identifying specific biochemical markers that solely identify patients at high risk of bleeding would have great clinical impact.

Thrombomodulin (TM) is a transmembrane glycoprotein expressed on the endothelial cell surface<sup>5</sup> that plays an important role in coagulation and fibrinolysis. Smaller heterogeneous, soluble TM fragments circulate in plasma of healthy individuals.<sup>6</sup> Studies have demonstrated that the variously sized TM molecular subspecies retain functional activity.<sup>7</sup> Thus, it is reasonable to assume that TM may influence the risk of bleeding and thrombosis. In an earlier prospective study, we have suggested that soluble TM antigen (sTM) concentration could identify patients at high risk of bleeding.<sup>8</sup> In addition, a case-control study of VKA-treated patients found a similar association between sTM and bleeding complications.<sup>9</sup> These studies were small or retrospective in design and should be confirmed in larger trials, which, in addition to bleeding complications during warfarin treatment, also take cardiovascular morbidity and mortality into account.

The primary aim of the present longitudinal cohort study of warfarin-treated patients was to test the hypothesis that increased concentrations of sTM are asso-

ciated with the risk of bleeding complications. The secondary objective was to evaluate the relationship between sTM and risk of nonfatal ischemic cardiovascular events and all-cause mortality.

## METHODS

### PATIENTS

In this longitudinal cohort study of 719 VKA-treated patients, blood samples were obtained at study inclusion, and patients were thereafter observed for bleeding complications and/or thrombotic events. Patients were recruited from the warfarin clinics at Skellefteå County Hospital, Skellefteå, Sweden, and Umeå University Hospital, Umeå, Sweden, in June 1996. There are approximately 80 000 and 120 000 inhabitants in the Skellefteå and the Umeå regions, respectively. In the Skellefteå region, more than 90% of the patients receiving warfarin treatment were monitored at a specialized warfarin treatment clinic. The corresponding number for the Umeå region was approximately 80%. Indications for warfarin treatment were obtained from registries at the clinics.

In total, 1204 patients attended one of the warfarin treatment clinics. Planned treatment duration of more than 3 months was defined as long-term treatment. Consent forms were sent to all 957 patients receiving long-term warfarin treatment, of whom 64 failed to answer and 46 declined to participate, leaving 847 patients eligible for blood sampling. Because of missing blood samples ( $n=102$ ), treatment stoppage ( $n=15$ ), or death ( $n=11$ ), 128 patients were excluded. Blood samples were ultimately obtained from 719 patients, who were thus included in the study (356 from Skellefteå and 363 from the Umeå warfarin treatment clinic). From the Skellefteå warfarin treatment clinic, international normalized ratios (INRs) at blood sampling were obtained from the warfarin registry, and data about diabetes, prior peptic ulcer, prior bleeding peptic ulcer, diabetes, hypertension, and body mass index were available through questionnaires. All patients received warfarin treatment for at least 2 months prior to blood sampling and study inclusion. The study was approved by the Research Ethics Committee of Umeå University.

### BLOOD SAMPLING AND LABORATORY PROCEDURES

Venous blood samples were drawn with a minimum of stasis and collected in siliconized, routine citrated plasma tubes containing 0.13M of sodium citrate. After centrifugation, the plasma samples were frozen and stored at  $-70^{\circ}\text{C}$  until analyzed. Both study cohorts were analyzed at the same time and location. The laboratory staff had no knowledge of event status.

The measurement of sTM concentration in plasma was performed using an in-house enzyme-linked immunosorbent assay method in which the monoclonal antibodies TM43b and TM531 were used.<sup>10</sup> Intra-assay and interassay coefficients of variation were lower than 5% and lower than 6%, respectively. High sensitivity C-reactive protein (hsCRP) levels were determined with an automated hsCRP method (IMMULITE Diagnostic Products Corporation, Los Angeles, California). The interassay coefficient of variation was lower than 6%, and INRs were determined at each hospital laboratory.

### FOLLOW-UP STUDY PROTOCOL

The date of inclusion was set as the date of blood sampling, with the earliest inclusion date being June 1, 1996. All patients were followed up prospectively until death, bleeding, cessation of war-

farin treatment, or until January 1, 2002. In an effort to identify all bleeding events causing hospital admission or death, medical records from the departments of medicine, surgery, otolaryngology, ophthalmology, urology, neurology, gynecology, oncology, neurosurgery, and orthopedic surgery were reviewed from June 1, 1996, to January 1, 2002. One patient moved out of the region during the study period and was followed up to the date of migration. During the study period, 136 patients were withdrawn from warfarin treatment.

All bleeding complications causing admission to hospital or death were recorded and classified by a panel of 3 researchers (M.L., L.J., and J.-H.J.). Major bleeding events were defined according to Schulman et al<sup>11</sup> as fatal bleeding and/or symptomatic bleeding in a critical area or organ and/or bleeding causing a fall in hemoglobin level of 2 g/dL or more (to convert to grams per liter, multiply by 10) or leading to transfusion of 2 or more units of whole blood. Clinically relevant bleeding was defined as major bleeding or an overt bleeding event based on objective investigations that caused admission to hospital or prolonged hospital care but did not meet the criteria for major bleeding. All other bleeding events were classified as minor and were excluded. Myocardial infarction, ischemic stroke, and peripheral arterial emboli were recorded. The cause of death was registered and classified according to the death certificate. In all but 1 case, the cause of death could be classified. Investigators classifying events were blinded to the biochemical results.

### STATISTICAL ANALYSIS

Spearman correlation coefficients were used to evaluate potential relations between variables. Analyses of sTM were performed as a continuous variable. To investigate a nonlinear association between sTM concentration and bleeding events, we also compared low, medium, and high values of sTM by categorizing sTM into tertiles, with the lowest tertile as the reference group. The distribution of sTM and hsCRP values was skewed and transformed using the natural logarithm, with the increments of hazard ratios (HRs) presented for the standard deviations. The assumption of proportional hazard was verified graphically using Kaplan-Meier survival curves. Univariable Cox regression analysis was performed on each of the variables to estimate the HR and 95% confidence interval (CI). Multivariable Cox regression analysis was performed to estimate the effects on different determinants when controlling for other factors. Factors were excluded from the multivariable model if the univariable Cox regression resulted in a clearly nonsignificant HR ( $P > .20$ ). Survival plots with the proportion of patients free of major bleeding events were calculated. In addition, data on hypertension, diabetes, body mass index, previous peptic ulcer, and previous bleeding peptic ulcer were available from the Skellefteå warfarin treatment clinic and were tested for association with bleeding with univariable Cox regression analysis. Direct age-adjustment was performed in 10-year intervals.

$P < .05$  (2 sided) was considered statistically significant. SPSS software version 15.0 (SPSS Inc, Chicago, Illinois) was used for all statistical analyses. Individuals with missing values (30 for sTM level and 2 for hsCRP level) were excluded from the statistical analyses.

## RESULTS

Clinical characteristics at baseline and indications for treatment with warfarin are given in **Table 1**. The mean age was 70 years, and 37% of the subjects were female. The most common indication for warfarin treatment was prosthetic heart valve, followed by atrial fibrillation. The maximum follow-up time was 5.6 years. Mean follow-up time

was 4.2 years, with 3001 treatment years in 719 patients. In total, 113 clinically relevant bleeding events were registered and 73 major bleeding events occurred. Median time from blood sampling to a clinically relevant bleeding event was 758 days (range, 2-2032 days). The principal area of clinically relevant bleeding was the gastrointestinal tract (34%), followed by intracranial (16%) and soft (13%) tissue. Fatal bleeding occurred in 11 patients (0.4% per treatment year). Soluble TM antigen concentrations were categorized into tertiles ( $\leq 5.0$ , ng/mL; 5.1-6.1 ng/mL; and  $> 6.1$  ng/mL). Soluble TM antigen concentration was significantly correlated with hsCRP level ( $r=0.15$ ;  $P<.001$ ) and age ( $r=0.23$ ;  $P<.001$ ). International normalized ratios at the time of sampling were available from the Skellefteå warfarin treatment clinic. Of the patients, 9% had an INR below 2.0, 5% had an INR above

3.5, and 86% had a therapeutic INR. No significant correlation between sTM concentration and INR at the time of sampling was found ( $r=0.05$ ;  $P=.39$ ).

## BLEEDING EVENTS

The annual risk of clinically relevant bleeding was 3.9% and of major bleeding, 2.4%. Clinically relevant bleeding and major bleeding occurred at INRs greater than 3.5 in 24% and 27% of cases, respectively.

The univariable analyses showed age and sTM concentration as continuous variables, and the second and third tertiles of sTM were significantly associated with clinically relevant bleeding and major bleeding. Sex and hsCRP level were not related to the risk of clinically relevant bleeding or major bleeding (**Table 2**). Hypertension, diabetes, body mass index, previous peptic ulcer, and previous bleeding peptic ulcer were not significantly associated with clinically relevant bleeding or major bleeding. International normalized ratio at baseline was not associated with bleeding events ( $P=.35$ ).

The multivariable models showed age and sTM as continuous variables, and the third tertile of sTM concentration was significantly associated with both clinically relevant bleeding and major bleeding. Age-adjusted incidence of clinically relevant bleeding was 2.4 per 100 treatment years in the lowest tertile of sTM concentration, 4.5 in the middle tertile, and 5.6 in the highest tertile. The corresponding incidence of major bleeding per 100 treatment years was 1.5 in the lowest tertile of sTM concentration, 2.7 in the middle tertile, and 3.7 in the highest tertile.

Age-adjusted survival plots illustrating the proportion of patients free of major bleeding in different tertiles of sTM concentration are shown in **Figure 1**. A possible interaction between age and sTM concentration was investigated with clinically relevant bleeding as an outcome. Older patients ( $\geq 70$  years) with high sTM levels (above the median of 5.61 ng/mL) had an HR of 3.68 compared with controls, that is younger patients ( $< 70$  years) with low sTM (below the median). The HR for combined exposure of older age and low sTM was 2.44 com-

**Table 1. Baseline Characteristics of the Study Cohort<sup>a</sup>**

Characteristic	Study Cohort (N=719)
Age at inclusion, mean (SD), y	70 (11)
INR at sampling, %	
<2.0	9
2.0-3.5	86
>3.5	5
Female, No. (%)	268 (37)
Follow-up time, mean (SD), y	4.2 (1.8)
sTM, mean (SD), ng/mL	6.1 (2.8)
hsCRP, mean (SD), mg/L	7.0 (14.9)
Indications for warfarin treatment, No. (%)	
Prosthetic heart valve	248 (35)
Atrial fibrillation	228 (32)
Venous thromboembolism	83 (11)
Ischemic stroke	73 (10)
Peripheral arterial thromboembolism	40 (6)
Miscellaneous	40 (6)
Not defined	7 (1)

Abbreviations: hsCRP, high sensitivity C-reactive protein; INR, international normalized ratio; sTM, soluble thrombomodulin antigen.

SI conversion factor: To convert hsCRP to nanomoles per liter, multiply by 9.524.

<sup>a</sup>Data from the Skellefteå warfarin treatment clinic.

**Table 2. Univariable and Multivariable Cox Regression Analysis Showing the Hazard Ratios for Clinically Relevant and Major Bleeding Events<sup>a</sup>**

Variable	Hazard Ratio (95% Confidence Interval)			
	Clinically Relevant Bleeding Event		Major Bleeding Event	
	Univariable	Multivariable	Univariable	Multivariable
Age per 10 y	1.57 (1.28-1.92)	1.45 (1.19-1.78) <sup>b</sup>	1.50 (1.17-1.93)	1.36 (1.06-1.74) <sup>b</sup>
Female	1.09 (0.75-1.59)	...	1.31 (0.82-2.08)	...
sTM, per 1 SD	1.39 (1.20-1.60)	1.33 (1.14-1.54) <sup>b</sup>	1.44 (1.21-1.71)	1.39 (1.16-1.66) <sup>b</sup>
sTM				
First tertile (n=221)	1 [Reference]	1 [Reference]	1 [Reference]	1 [Reference]
Second tertile (n=241)	2.11 (1.24-3.59)	1.87 (1.09-3.19) <sup>c</sup>	1.99 (1.02-3.88)	1.82 (0.93-3.55) <sup>c</sup>
Third tertile (n=227)	2.74 (1.63-4.61)	2.29 (1.35-3.89) <sup>c</sup>	2.69 (1.41-5.14)	2.33 (1.21-4.48) <sup>c</sup>
hsCRP, per 1 SD	1.00 (0.83-1.20)	...	1.02 (0.81-1.28)	...

Abbreviations: hsCRP, high sensitivity C-reactive protein; sTM, soluble thrombomodulin antigen; ellipses, factor was excluded from the multivariable model because the  $P$  value in the univariable analysis was greater than .20.

<sup>a</sup>Soluble TM antigen and hsCRP were logarithmically transformed and presented per 1-SD increment.

<sup>b</sup>Multivariable model included age and sTM as continuous variable.

<sup>c</sup>Multivariable model included age and tertiles of sTM.

pared with controls. The HR for combined exposure of younger age and high sTM was 1.64 compared with controls. No synergistic effect was observed between age and sTM concentration.

Stratification for sex showed that women with an sTM concentration in the third tertile had an HR of 3.21 (95% CI, 1.34-7.67) for clinically relevant bleeding compared with the first tertile ( $P = .01$ ). Age-adjusted incidence of clinically relevant bleeding per 100 treatment years were 2.0% in the lowest tertile and 7.1% in the highest tertile. In men, the corresponding HR for sTM concentration was 1.85 (95% CI, 0.95-3.61) for clinically relevant bleeding ( $P = .07$ ). Stratification by warfarin treatment clinic showed similar HRs between sTM concentration and clinically relevant bleeding in both populations. After adjustment for age, the HR for the third tertile compared with the first was 2.16 in Skellefteå and 2.47 in Umeå.

In patients with atrial fibrillation ( $n = 228$ ), 29 clinically relevant bleeding events and 15 major bleeding events occurred. Multivariable Cox regression analyses showed a significant association between sTM concentration as a continuous variable and clinically relevant bleeding (HR, 1.70 [95% CI, 1.10-2.62]). For major bleeding, multivariable Cox regression analysis showed a significant association for sTM concentration as a continuous variable (HR, 2.06 [95% CI, 1.15-3.71]).

When excluding patients with bleeding events at an INR greater than 3.5 at the time of bleeding, 88 clinically relevant bleeding events remained. Multivariable Cox regression analysis showed significant associations for sTM concentration as a continuous variable (HR, 1.32 [95% CI, 1.11-1.57]).

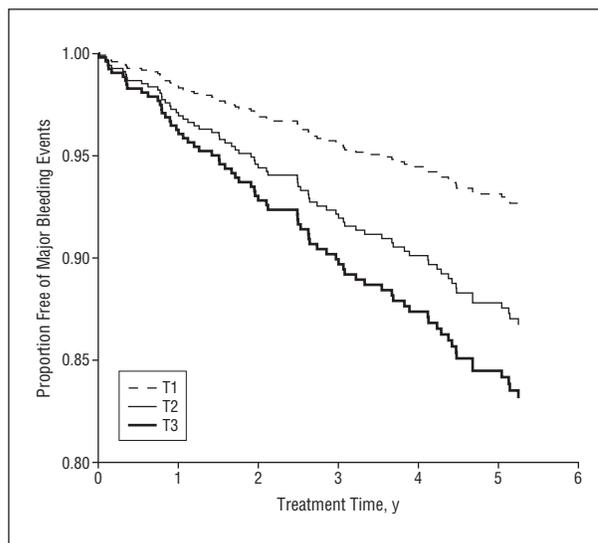
### NONFATAL ISCHEMIC CARDIOVASCULAR EVENTS AND ALL-CAUSE MORTALITY

In total, 161 patients died during warfarin treatment. The most common cause of death was ischemic heart disease followed by stroke. A total of 110 cardiovascular deaths and 75 nonfatal cardiovascular events (myocardial infarction, stroke, and peripheral arterial emboli) occurred.

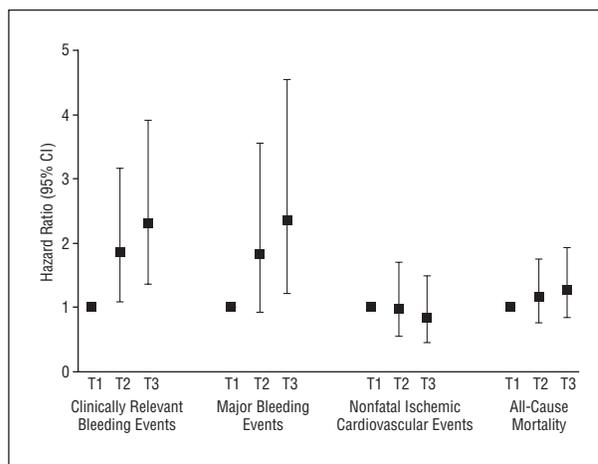
Age was significantly associated with all-cause mortality (HR per 10-year increment, 1.76; 95% CI, 1.47-2.10) and nonfatal ischemic cardiovascular events (HR per 10-year increment, 1.48; 95% CI, 1.16-1.89). After age adjustment, no significant association for sTM concentration and all-cause mortality or nonfatal ischemic cardiovascular events were found. Age-adjusted regression analyses for tertiles of sTM concentration with clinically relevant and major bleeding events, all-cause mortality, and nonfatal cardiovascular events as outcomes are shown in **Figure 2**.

### COMMENT

Thrombomodulin is known to affect both coagulation and fibrinolysis. The anticoagulant function of TM is achieved by its binding to thrombin and by the activation of protein C.<sup>12</sup> Thrombomodulin also activates thrombin-activated fibrinolysis inhibitor (TAFI), thereby affecting fibrinolysis.<sup>13</sup> It is reasonable to assume that TM could



**Figure 1.** Age-adjusted survival plots showing proportions of patients free of major bleeding events for tertiles (T1-T3) of soluble thrombomodulin antigen concentration.



**Figure 2.** Age-adjusted hazard ratios for clinically relevant bleeding events, major bleeding events, nonfatal ischemic cardiovascular events, and all-cause mortality for tertiles (T1-T3) of soluble thrombomodulin antigen concentration.

influence the risk of both bleeding and thrombosis during warfarin treatment.

In this longitudinal cohort study, we found that sTM concentration, expressed either as tertiles or as a continuous variable, had a significant association with both major and clinically relevant bleeding events during warfarin treatment. No relationship between sTM concentration and nonfatal ischemic cardiovascular events or all-cause mortality was found. After 5 years of treatment, 23% of patients in the highest tertile of sTM concentration had experienced a clinically relevant bleeding event compared with 10% in the lowest tertile. Furthermore, we found that women with high levels of sTM showed an even higher risk of clinically relevant bleeding (HR, 3.21). A subgroup analysis of patients with atrial fibrillation as an indication for warfarin treatment showed a significant HR similar to the whole population. To our knowledge, only 2 previous studies of sTM

concentration and risk of bleeding in warfarin-treated patients have been published. The first was a prospective study with 22 bleeding events in which a significant association between sTM concentration and bleeding was found despite the low number of events.<sup>8</sup> A second retrospective study including 110 patients with bleeding events and 220 controls showed significant associations with risk of bleeding (odds ratio, 3.25) in the highest quartile of sTM concentration when compared with the lowest quartile.<sup>9</sup> The present study confirms the previous findings and strengthens the evidence for sTM concentration as a predictor for bleeding during warfarin treatment.

High-sensitivity CRP is an inflammatory marker associated with cardiovascular disease.<sup>14</sup> In the present study, we found no association between bleeding events and hsCRP level, which may indicate that the risk of bleeding during warfarin treatment is not primarily related to an inflammatory process.

Prior studies have yielded conflicting results regarding sTM concentration and the risk of cardiovascular disease. Salomaa et al<sup>15</sup> showed a decreased risk for cardiovascular disease in previously healthy patients with high levels of sTM. However, Thøgersen et al<sup>16</sup> showed that an increase in sTM concentration was associated with first-ever myocardial infarction. A high level of soluble TM activity and sTM has been associated with a personal history of heart disease in patients with type 2 diabetes mellitus.<sup>17</sup> Other studies have shown that sTM concentration can predict cardiovascular events in patients with prior myocardial infarction or peripheral arterial disease.<sup>18,19</sup> An association between sTM concentration and both vascular mortality and total mortality has been suggested.<sup>20,21</sup>

High levels of sTM are also believed to reflect endothelial damage.<sup>22-24</sup> Endothelial cell damage would be expected to increase the risk of ischemic cardiovascular events, as well as bleeding events. In the present study, we could not find any relationship between sTM concentration and nonfatal ischemic cardiovascular events or all-cause mortality.

As demonstrated in previous studies,<sup>25,26</sup> our study showed that age is a predictor of bleeding during warfarin treatment. Age was also found to predict total mortality and nonfatal ischemic cardiovascular events during warfarin treatment. A correlation between sTM concentration and age has previously been shown.<sup>27</sup> Traditional risk factors for bleeding such as hypertension, diabetes, and previous bleeding peptic ulcer<sup>4</sup> were not significantly associated with bleeding events in our study, possibly owing to lack of power.

There are limitations to this study. The blood samples were collected during warfarin treatment; therefore, it is not possible to draw any conclusions regarding the predictive value of sTM measured before the initiation of warfarin therapy. The time dependence of bleeding complications could alter study results. Previous studies have shown that the risk of bleeding might be highest the first month after initiation of treatment.<sup>25</sup> The bleeding frequency in our study may therefore be underestimated. Furthermore, sTM concentration was determined on only 1 occasion, and it is possible that repeated testing could increase the predictive value of sTM. In this study, we showed that sTM levels were associated with bleeding

events in the 2 different clinical settings, a university and a county hospital, with a high proportion (80%-90%) of all warfarin-treated patients in the catchment area. Further studies on other populations are needed to confirm the generalizability of these results

Determining which patients at highest risk of thromboembolic complications should be considered for long-term anticoagulation treatment is a relevant clinical problem. One often weighs the risk of bleeding against the risk of thromboembolic events, especially in patients with atrial fibrillation. Traditional risk factors for hemorrhagic complications such as hypertension and advanced age are also risk factors for thromboembolic events<sup>2</sup> and are, therefore, of limited clinical value when balancing the risk to benefit ratio of warfarin treatment. Soluble TM antigen concentration differs from previously defined risk factors for bleeding in that, while not associated with nonfatal ischemic cardiovascular events or all-cause mortality, it identifies patients at high risk of bleeding complications during warfarin treatment.

In conclusion, sTM concentration in warfarin-treated patients has the potential of being a useful and specific predictor of bleeding complications beyond previously known risk factors. Further studies are needed to increase the clinical usefulness of sTM. Such studies ought to include sampling before and after the initiation of warfarin therapy to investigate the predictive value of sTM concentration in warfarin therapy in a clinical setting. Focus should also be placed on determining clinically relevant cutoff values and investigating if sTM concentration has an even higher predictive value in women.

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**Author Contributions:** Dr Lind had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. *Study concept and design:* Nilsson and Jansson. *Acquisition of data:* Lind, Johansson, Nilsson, Birgander, and Jansson. *Analysis and interpretation of data:* Lind, Boman, Johansson, Nilsson, Öhlin, and Jansson. *Drafting of the manuscript:* Lind and Jansson. *Critical revision of the manuscript for important intellectual content:* Lind, Boman, Johansson, Nilsson, Öhlin, Birgander, and Jansson. *Statistical analysis:* Lind, Johansson, and Jansson. *Obtained funding:* Lind, Johansson, Öhlin, and Jansson. *Administrative, technical, and material support:* Lind, Boman, Johansson, Nilsson, Öhlin, Birgander, and Jansson. *Study supervision:* Boman, Johansson, and Jansson.

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