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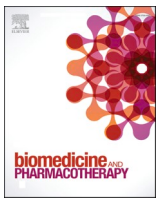

The interplay between tamoxifen and endoxifen plasma concentrations and coagulation parameters in patients with primary breast cancer

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ABSTRACT

Background: Tamoxifen is an effective treatment for primary breast cancer but increases the risk for venous thromboembolism. Tamoxifen decreases anticoagulant proteins, including antithrombin (AT), protein C (PC) and tissue factor (TF) pathway inhibitor, and enhances thrombin generation (TG). However, the relation between plasma concentrations of both tamoxifen and its active metabolite endoxifen and coagulation remains unknown.
Methods: Tamoxifen and endoxifen were measured in 141 patients from the prospective open-label intervention TOTAM-study after 3 months (m) and 6 m of tamoxifen treatment. Levels of AT and PC, the procoagulant TF, and TG parameters were determined at both timepoints if samples were available (n = 53–135 per analysis). Levels of coagulation proteins and TG parameters were correlated and compared between: 1) quartiles of tamoxifen and endoxifen levels, and 2) 3 m and 6 m of treatment.
Results: At 3 m, levels of AT, PC, TF and TG parameters were not associated with tamoxifen nor endoxifen levels. At 6 m, median TF levels were lower in patients in the 3rd (56.6 [33] pg/mL), and 4th (50.1 [19] pg/mL) endoxifen quartiles compared to the 1st (lowest) quartile (76 [69] pg/mL) (P=0.027 and P=0.018, respectively), but no differences in anticoagulant proteins or TG parameters were observed. An increase in circulating TF levels (3 m: 46.0 [15] versus 6 m: 54.4 [39] pg/mL, $P < 0.001$) and TG parameters was observed at the 6 m treatment timepoint, while AT and PC levels remained stable.
Conclusions: Our results indicate that higher tamoxifen and endoxifen levels are not correlated with an increased procoagulant state, suggesting tamoxifen dose escalation does not further promote hypercoagulability.

What is known on this topic?

- Patients who receive tamoxifen have increased risk of venous thromboembolism, which could be mediated by direct effects of tamoxifen and its primary metabolite endoxifen on various coagulation factors.
- Therapeutic drug monitoring (TDM)-directed dose escalation of tamoxifen might be required in case of low endoxifen levels to yield superior treatment efficacy.

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- Higher doses of tamoxifen do not lead to an increase in patient-reported side effects, but effects on coagulation remain unknown.

What does this paper add?

- Higher tamoxifen plasma concentrations do not correlate with an increased procoagulant state, whereas higher endoxifen concentrations correlate with

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lower circulating tissue factor levels, providing a first indication that tamoxifen dose escalation does not further increase VTE risk.
- Over time, tamoxifen may increase tissue factor levels. This requires further study.

1. Introduction

Tamoxifen is indicated for the adjuvant treatment of estrogen-receptor (ER) positive breast cancer, effectively reducing the annual breast cancer death rate with almost one-third. [1] Tamoxifen and its metabolites act as selective ER modulators (SERM) and have antagonistic effects on the ER in breast cancer cells, yielding anti-tumor effects by prevention of estrogen-mediated tumor cell growth. [2] However, tamoxifen can act as an ER agonist in other tissues. [2] These tissue-specific ER agonistic or antagonistic effects of tamoxifen are determined by several factors including tissue-specific expression of the two ER subtypes (ERα and ERβ) and availability of intracellular coactivators and corepressors for ER-dependent target genes. [3].

Tamoxifen treatment is associated with various side effects, of which hot flashes, joint pain, vaginal dryness and insomnia are most commonly reported. [4] These side effects are caused by the ER agonistic or antagonistic effects of tamoxifen and its metabolites in tissues other than breast cancer cells. For example, tamoxifen treatment can stimulate endometrial cell growth by agonistic effects on endometrial tissue, whereas it can cause hot flashes by its antagonistic effects in the central nervous system. An alarming observation is that tamoxifen increases the risk of venous thromboembolism (VTE): tamoxifen-treated patients have a 2–3.5 fold increased risk of developing a VTE compared to breast cancer patients without adjuvant tamoxifen treatment. The reported VTE incidence is 1–3% during tamoxifen therapy and most events occur within the first 2 years of treatment. [5,6] Next to being potentially life-threatening in severe cases, VTE can lead to significant morbidity, a lower quality of life and psychological stress. [7,8] Moreover, anticoagulant therapy for treatment and secondary prevention of VTE can increase the risk of bleeding. Therefore, a better understanding of tamoxifen-associated VTE is essential to optimize patient treatment.

Currently, the mechanisms underlying the prothrombotic properties of tamoxifen treatment remain largely unclear. Some studies have shown that tamoxifen treatment is associated with a reduction in plasma levels of various anticoagulant proteins, including protein C, antithrombin and tissue factor pathway inhibitor (TFPI), and an increase in thrombin generation potential, suggestive of a procoagulant state. [9–11] Although there is currently no direct evidence for a dose-dependent effect of tamoxifen on VTE risk, one study found higher levels of the anticoagulant antithrombin in patients who received low daily tamoxifen doses (1 mg or 5 mg) compared with the standard of 20 mg. [12] While a higher tamoxifen dose is not associated with an increase in patient-reported side effects such as hot flashes and vaginal dryness [13–15], it is essential to determine if higher levels of tamoxifen and its metabolites are linked to an increased procoagulant state, which could possibly further increase VTE risk.

Tamoxifen itself has relatively low affinity for the ER and is converted into 4-hydroxytamoxifen or n-desmethyltamoxifen and subsequently to endoxifen by various hepatic cytochrome P450 (CYP) enzymes, mainly CYP2D6 and CYP3A4. [16] Endoxifen is considered the most important metabolite for treatment efficacy. [17] Endoxifen has a much higher affinity for the ER than n-desmethyltamoxifen [17,18] and a similar affinity as 4-hydroxytamoxifen, but endoxifen plasma concentrations are up to 14-fold higher than the latter. [17,19] Since low endoxifen plasma levels are associated with increased breast cancer recurrence rates [20], an efficacy threshold of minimally 16 nM endoxifen is generally accepted for tamoxifen precision dosing. [21,22] Given that one out of five patients do not reach this threshold on the standard daily dose of 20 mg tamoxifen, therapeutic drug monitoring (TDM) of tamoxifen and endoxifen plasma levels could be useful to select patients who require an increase in tamoxifen dose. [13] Particularly tamoxifen plasma levels often become significantly higher than population average upon tamoxifen dose escalation [14] and both tamoxifen and endoxifen levels have a high interpatient variability regardless of dose. [23] Therefore, it is essential to determine the possible implications of higher concentrations of both tamoxifen and its primary metabolite endoxifen on VTE risk.

Here we investigated whether higher plasma levels of tamoxifen and endoxifen are associated with a procoagulant state of the coagulation system. For this, we assessed if tamoxifen and endoxifen plasma levels correlated with 1) levels of various pro- and anti-coagulant proteins which were previously demonstrated to be affected by tamoxifen [9–11], and 2) thrombin generation parameters in patients undergoing TDM of adjuvant tamoxifen treatment for primary breast cancer. In addition, we investigated the time-dependent effects of tamoxifen on coagulation parameters.

2. Materials and methods

The current study was a secondary analysis from the TOTAM (Therapeutic drug monitoring Of TAMoxifen) study: a prospective intervention study on the feasibility of TDM of tamoxifen coordinated by the Erasmus MC Cancer Institute in Rotterdam, the Netherlands. [13] This study was approved by the local Medical Ethics Committee in January 2018 (MEC 2017–548) and registered in the International Clinical Trial Registry Platform (ICTRP; https://trialsearch.who.int; NL6918). Patients were included in this specific part of the study between November 2020 and November 2021. Informed consent was obtained from all participants.

2.1. Study design

As described in the original study, female patients who used adjuvant tamoxifen 20 mg daily for primary breast cancer were included after 3 months (3 m) of therapy. [13,24,25] Steady-state tamoxifen and endoxifen levels were measured at study inclusion. If endoxifen levels were below the treatment threshold of 16 nM, tamoxifen dose was increased to 30 mg or 40 mg daily. If endoxifen levels were above or equal to 32 nM and patients reported bothersome side effects, tamoxifen dose could be reduced to 10 mg daily. Tamoxifen and endoxifen levels were measured again after 6 months (6 m) of tamoxifen therapy. At both the 3 m and 6 m timepoints, coagulation analyses were performed. Patients who were diagnosed with recurrence of breast cancer or a new primary cancer within 1 year after start of tamoxifen were excluded to eliminate the effect of a (new) active malignancy on coagulation protein measurements. Also, measurements were excluded from analyses if patients were using anticoagulant therapy (direct oral anticoagulants, vitamin K antagonist or low molecular weight heparins) at the time of sampling. VTE events within 1 year of tamoxifen therapy initiation were identified by manual chart review of the electronic medical record and all VTE events were diagnosed using radiologic imaging.

2.2. Pharmacokinetic analysis

Tamoxifen and endoxifen trough (C_{min}) plasma concentrations were measured in blood samples after 3 m and 6 m of tamoxifen therapy, using a validated ultra-performance liquid chromatography with a tandem mass spectrometry method (UP-LCMS/MS). [26].

2.3. Coagulation analyses

In all available blood samples, protein C, antithrombin, tissue factor and thrombin generation parameters were determined after 3 m and 6 m of tamoxifen therapy. For protein C, antithrombin and thrombin

generation analyses blood was collected in citrate tubes, while for tissue factor determination blood was sampled in lithium heparin tubes. Plasma levels of protein C and antithrombin were determined using a chromogenic assay (respectively Berichrom® Protein C and INNOVANCE® Antithrombin) on a Sysmex CS5100 (Siemens Healthineers). Circulating tissue factor was assessed using an enzyme-linked immunosorbent assay (ELISA) (Human Coagulation Factor III/Tissue factor Quantikine ELISA; R&D systems). Thrombin generation was adapted from protocols using low plasma volumes as previously described. [27,28] Thrombin generation curves were obtained from reactions of patient plasma supplemented with either PPPlow reagent (Stago) containing tissue factor and phospholipids (i.e. with exogenous tissue factor) or with phospholipids only (phospholipid-TGT, Rossix; final concentration 4 μM; i.e. without exogenous tissue factor). Thrombin formation was initiated by the addition of substrate buffer (FluCa, Stago). The final reaction volume was 60 μL, of which 40 μL was plasma. Thrombin formation was determined every 15 s for 90–120 min and corrected for the calibrator using Thrombinoscope software. The thrombin generation parameters determined were: endogenous thrombin potential (ETP or area under the curve), thrombin peak, lag time, time to peak, and velocity index. [29] The ETP represents the total amount of thrombin generated over time; the thrombin peak is the maximum concentration of thrombin generated; the lag time is defined as the time between the addition of the trigger until the initiation of thrombin generation; the time to peak is the time required to reach the peak of thrombin generation, and the velocity index is a composite index defined as [peak height / (time to peak – lag time)].

2.4. Statistical analysis

Normal distribution of the data was assessed using the Shapiro Wilk test. Patients were stratified to quartiles (Q1-Q4) based on their tamoxifen and endoxifen plasma levels at the 3 m and 6 m timepoints separately. Subsequently, levels of coagulation proteins and thrombin generation were compared between quartiles, with the lowest quartile (Q1) serving as the reference group, using ANOVA with Dunnett's test or Kruskall-Wallis with a Bonferroni correction approach (p-value times 3, i.e. the number of comparisons) to reduce the risk of type-1 error associated with multiple comparisons. Correlations between coagulation proteins and absolute tamoxifen and endoxifen concentrations were determined using Spearman's rank correlation. To assess the time-effect of tamoxifen treatment, levels of coagulation proteins and thrombin generation were compared between 3 m and 6 m with the paired samples t-test or Wilcoxon signed rank test. Also, coagulation parameters were compared between patients who received chemotherapy and patient who did not receive chemotherapy with unpaired samples t-test or Mann-Whitney U test. If data was missing for specific measurements patients were excluded from these analyses. Data were analysed using SPSS Statistics (IBM version 28.0.1.0) and P values < 0.05 were considered statistically significant.

3. Results

From the total cohort of 144 patients, three patients were excluded because of the development of a second malignancy (n = 2) or diagnosis of metastatic breast cancer (n = 1) within one year after initiation of tamoxifen treatment. In total, 141 patients were eligible for this study. Patient characteristics are summarized in Table 1. Median age was 58 [IQR 49–67] and most patients had stage 1 or 2 disease with the no special type as the most common subtype (78%). The majority of patients had received both breast conserving surgery and radiotherapy prior to the start of tamoxifen treatment (60%), almost half received (neo)adjuvant chemotherapy (45%) and approximately 10% of patients received adjuvant anti-HER2 therapy.

Table 1
Baseline characteristics of the study participants.

Baseline characteristics (n = 141)		Median [IQR] or n (%)
Age		58 [49–67]
BMI		26.4 [23.7–30.2]
Tumor stage		
	T1	70 (49.6)
	T2	57 (40.4)
	T3	12 (8.5)
	T4	2 (1.4)
Nodal stage		
	N0	80 (56.7)
	N1	45 (31.9)
	N2	13 (9.2)
	N3	3 (2.1)
Tumor pathology		
	NST	110 (78.0)
	Lobular	25 (17.7)
	Other	6 (4.3)
Histological grade (BR)		
	I	14 (9.9)
	II	101 (71.6)
	III	26 (18.4)
Local treatment		
	BCS only	2 (1.4)
	BCS + RTx	85 (60.3)
	Mastectomy only	28 (19.9)
	Mastectomy + RTx	26 (18.4)
(Neo)adjuvant chemotherapy		
	Yes	63 (44.7)
	No	78 (55.3)
(Neo)adjuvant anti-HER2 therapy		
	Yes	13 (9.2)
	No	128 (90.8)
Smoking status		
	Current smoker	13 (9.2)
	Former smoker	46 (32.6)
	Never smoker	79 (56.0)
	Unknown	3 (2.1)
History of VTE		3 (2.1)

Age and BMI were determined at the time of first blood sampling (after 3 months of tamoxifen therapy). Abbreviations: BMI: body mass index, BCS: breast conserving surgery, IQR: interquartile range, NST: no special type, RTx: radiotherapy, VTE: venous thromboembolism

3.1. VTE occurrence

VTE occurred in 7 (5.0%) of the included patients within one year after start of tamoxifen treatment. These VTE consisted of: deep venous thrombosis (n = 3), superficial thromboflebitis (n = 3) and pulmonary embolism (n = 1). The characteristics of these VTE events are specified in Supplementary Table **A**. All three patients with a medical history of VTE in our cohort experienced a VTE event again. None of the patients used anticoagulation during tamoxifen therapy given that these previous events of VTE had occurred more than 5 years prior to initiation of tamoxifen treatment.

3.2. Correlation between tamoxifen or endoxifen levels and coagulation parameters

Coagulation parameters were available of 53–135 patients, depending on the specific parameter assessed and duration of tamoxifen treatment. The levels of coagulation parameters and tamoxifen and endoxifen plasma levels for the total study population can be found in Supplementary table **B**. The plasma tamoxifen levels ranged from 91 to 962 nM and correlated weakly with protein C at 3 m of treatment ($r = 0.180$, $P = 0.039$, Fig. 1**A**), but not at 6 m of therapy ($r = 0.090$, $P = 0.364$, Fig. 1**B**). When stratifying to tamoxifen plasma levels, no significant difference was observed when comparing protein C levels in the higher quartiles with those of the lowest quartile of patients at 3 m or 6

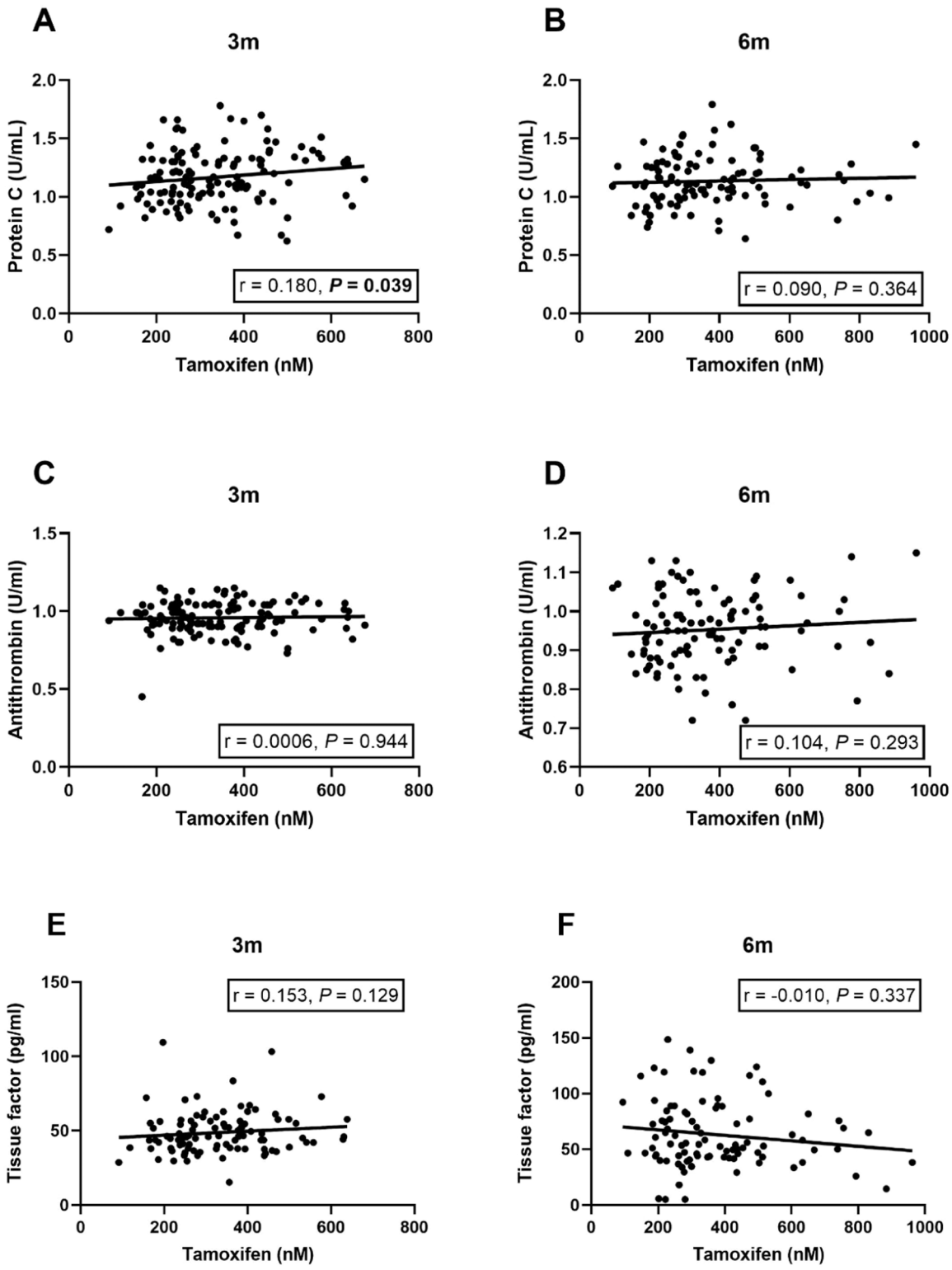

Fig. 1. Correlation of tamoxifen plasma levels after 3 months and 6 months of tamoxifen treatment with **A+B,** protein C (n = 133 and n = 104, respectively), **C+D** antithrombin (n = 135 and n = 104, respectively), and **E + F** tissue factor (n = 100 and n = 95, respectively).

m of treatment (Table 2). No correlation was observed between the tamoxifen plasma levels and those of antithrombin or tissue factor (Fig. 1**C-F**), and no significant difference was observed for the latter when comparing these based on quartiles of tamoxifen plasma levels (Table 2). In addition, tamoxifen levels did not correlate with parameters of thrombin generation triggered with either exogenously added or endogenously present tissue factor (Table 3). Overall, these data indicate that higher tamoxifen levels are weakly associated with higher levels of the anticoagulant protein C after 3 m of treatment, while no correlation indicative of a procoagulant state was observed.

The plasma endoxifen levels at 3 months ranged from 4 to 70 nM. For both 3 m and 6 m of tamoxifen therapy, no correlation was observed between plasma endoxifen levels and protein C or antithrombin levels (Fig. 2**A-D**), neither when comparing these factors based on quartiles of

Table 2
Levels of coagulation factors by quartiles of tamoxifen plasma levels.

3 months of tamoxifen				
	Q1	**Q2**	**Q3**	**Q4**
Tamoxifen (nM)	**91–246**	**246–325**	**325–432**	**432–676**
	n = 34	**n = 35**	**n = 33**	**n = 33**
Protein C (U/mL)	1.122 (0.21)	1.169 (0.21)	1.153 (0.25)	1.233 (0.25)
	n = 34	n = 34	n = 33	n = 32
Antithrombin (U/mL)	0.955 (0.12)	0.950 (0.08)	0.965 (0.11)	0.963 (0.09)
	n = 34	n = 35	n = 33	n = 33
Tissue factor (pg/mL)	44.1 [14]	46.0 [21]	50.1 [13]	44.6 [20]
	n = 26	n = 25	n = 29	n = 20
6 months of tamoxifen				
	Q1	**Q2**	**Q3**	**Q4**
Tamoxifen (nM)	**94–238**	**238–331**	**331–469**	**469–962**
	n = 26	**n = 27**	**n = 27**	**n = 26**
Protein C (U/mL)	1.067 (0.19)	1.162 (0.20)	1.177 (0.24)	1.125 (0.20)
	n = 26	n = 27	n = 26	n = 25
Antithrombin (U/mL)	0.947 (0.09)	0.953 (0.10)	0.938 (0.08)	0.974 (0.11)
	n = 26	n = 27	n = 26	n = 25
Tissue factor (pg/mL)	66.6 [45]	53.5 [40]	52.0 [45]	55.6 [40]
	n = 24	n = 25	n = 24	n = 22

Data are displayed as mean (SD) or median [IQR]. Data were missing for some participants in some subgroups. All comparisons were non-significant.

Table 3
Thrombin generation parameters by quartiles of tamoxifen plasma levels.

3 months of tamoxifen				
	Q1	**Q2**	**Q3**	**Q4**
Tamoxifen (nM)	**91–246**	**246–325**	**325–432**	**432–676**
	n = 34	**n = 35**	**n = 33**	**n = 33**
With exogenous tissue factor				
ETP (nM*min)	2238 (1070)	2139 (357)	2178 (388)	2040 (279)
Thrombin peak (nM)	236 (59)	267 (60)	260 (59)	238 (51)
Lag time (min)	7.2 (1.6)	6.6 (1.3)	7.1 (1.6)	7.3 (1.4)
Time to peak (min)	11.6 [3]	10.5 [2]	11.5 [3]	11.8 [3]
Velocity index (nM/min)	53.0 (19)	66.5 (23)	61.5 (21)	59.5 (21)
Without exogenous tissue factor				
ETP (nM*min)	1083 (563)	1170 (361)	1260 (343)	1118 (435)
Thrombin peak (nM)	41.4 (39)	67.5 (36)	73.7 (43)	58.2 (35)
Lag time (min)	37.2 [63]	37.4 [23]	35.0 [39]	36.8 [48]
Time to peak (min)	58.5 [50]	44.0 [21]	43.1 [29]	44.9 [29]
Velocity index (nM/min)	4.0 [9]	9.8 [10]	9.9 [18]	11.3 [15]
6 months of tamoxifen				
	Q1	**Q2**	**Q3**	**Q4**
Tamoxifen (nM)	**94–238**	**238–331**	**331–469**	**469–962**
	n = 26	**n = 27**	**n = 27**	**n = 26**
With exogenous tissue factor				
ETP (nM*min)	2160 (450)	2128 (379)	2118 (504)	2028 (312)
Thrombin peak (nM)	248 (67)	257 (66)	253 (78)	248 (58)
Lag time (min)	6.1 [2]	6.0 [2]	6.8 [5]	6.1 [5]
Time to peak (min)	10.5 [2]	10.5 [2]	11.0 [3]	10.0 [2]
Velocity index (nM/min)	56 (20)	60 (23)	59 (26)	59 (22)
Without exogenous tissue factor				
ETP (nM*min)	1301 (442)	945 (467)	1433 (391)	1095 (550)
Thrombin peak (nM)	69 [69]	68 [62]	79 [110]	68 [127]
Lag time (min)	32.0 (13)	31.6 (13)	26.0 (14)	31.3 (19)
Time to peak (min)	43 [18]	39 [11]	33 [17]	40 [23]
Velocity index (nM/min)	12.8 [13]	10.4 [15]	15.1 [28]	11.7 [39]

Data are displayed as mean (SD) or median [IQR]. Data were missing for some participants in some subgroups. All comparisons were non-significant. Abbreviations: ETP: endogenous thrombin potential.

endoxifen plasma levels (Table 4). In contrast, endoxifen levels correlated negatively with tissue factor at 6 m of treatment ($r = -0.290$, $P = 0.004$, Fig. 2**F**). When stratified to quartiles of endoxifen levels, patients with higher endoxifen concentrations had lower tissue factor levels at 6 m of therapy (Q3: 56.6 [33] pg/mL and Q4: 50.1 [19] pg/mL versus Q1: 75.6 [69] pg/mL, adjusted P values of 0.027 and 0.018, respectively) (Table 4). No correlation with tissue factor levels was observed at 3 m of tamoxifen treatment. Thrombin generation parameters did not correlate with endoxifen levels at any timepoint (Table 5). These data indicate that higher endoxifen levels are associated with lower circulating levels of the procoagulant tissue factor, which is not associated with a procoagulant state.

3.3. Time-dependent effect of tamoxifen treatment on coagulation

The time-dependent effect of tamoxifen treatment on coagulation was determined in patients who remained on 20 mg tamoxifen daily (n = 80) during the study period (Table 6). Compared to the 3 m timepoint, median tissue factor levels were significantly higher after 6 m of therapy (46.0 versus 54.4 pg/mL, respectively, $P < 0.001$). In line with this, thrombin generation initiated by endogenous tissue factor was enhanced at 6 m relative to 3 m of therapy, reflected by a significant increase in thrombin peak and velocity index, and shortened lag time and time to peak. Parameters of thrombin generation triggered by exogenous tissue factor and levels of protein C and antithrombin were similar between 3 m and 6 m of therapy. This significant increase in circulating tissue factor levels after 6 m of treatment was also observed in patients who received a tamoxifen dose increase to 30 or 40 mg daily (Supplementary Table **C**) as well as in patients in whom the tamoxifen dose was decreased to 10 mg daily (Supplementary Table **D**). This coincided with a significant increase in thrombin peak in endogenously triggered thrombin generation for patients who switched to 10 mg tamoxifen (Supplementary Table **D**). The levels of protein C and antithrombin remained similar in both patient groups.

3.4. Coagulation parameters in chemotherapy-treated patients versus patients who did not receive chemotherapy

All coagulation parameters were analysed in the group of patients that received (neo-)adjuvant chemotherapy for their breast cancer and in patients who did not receive chemotherapy separately (Supplementary table **E + F**). Patients who received chemotherapy did not demonstrate an increase in procoagulant parameters or decrease in anticoagulant parameters, except for a slightly shorter lag time after 6 m of treatment only.

4. Discussion

This study is the first to assess if the procoagulant effects of tamoxifen are associated with plasma levels of tamoxifen and its primary active metabolite endoxifen in a representative cohort of patients with primary breast cancer receiving adjuvant tamoxifen. By measurement of both various coagulation proteins previously shown to be affected by tamoxifen [9,10] and thrombin generation parameters, we demonstrate that higher plasma levels of tamoxifen and endoxifen are not associated with higher procoagulant or lower anticoagulant parameters. These findings provide a first indication that higher tamoxifen or endoxifen levels do not have an additional procoagulant effect and therefore might not lead to an further increased risk of tamoxifen-related VTE.

Levels of antithrombin and protein C were previously demonstrated to decrease during tamoxifen therapy, but these studies did not measure tamoxifen and endoxifen plasma levels. [9,10] Our study shows that endoxifen levels do not correlate with these anticoagulant factors. Protein C correlated positively with tamoxifen levels after 3 months of

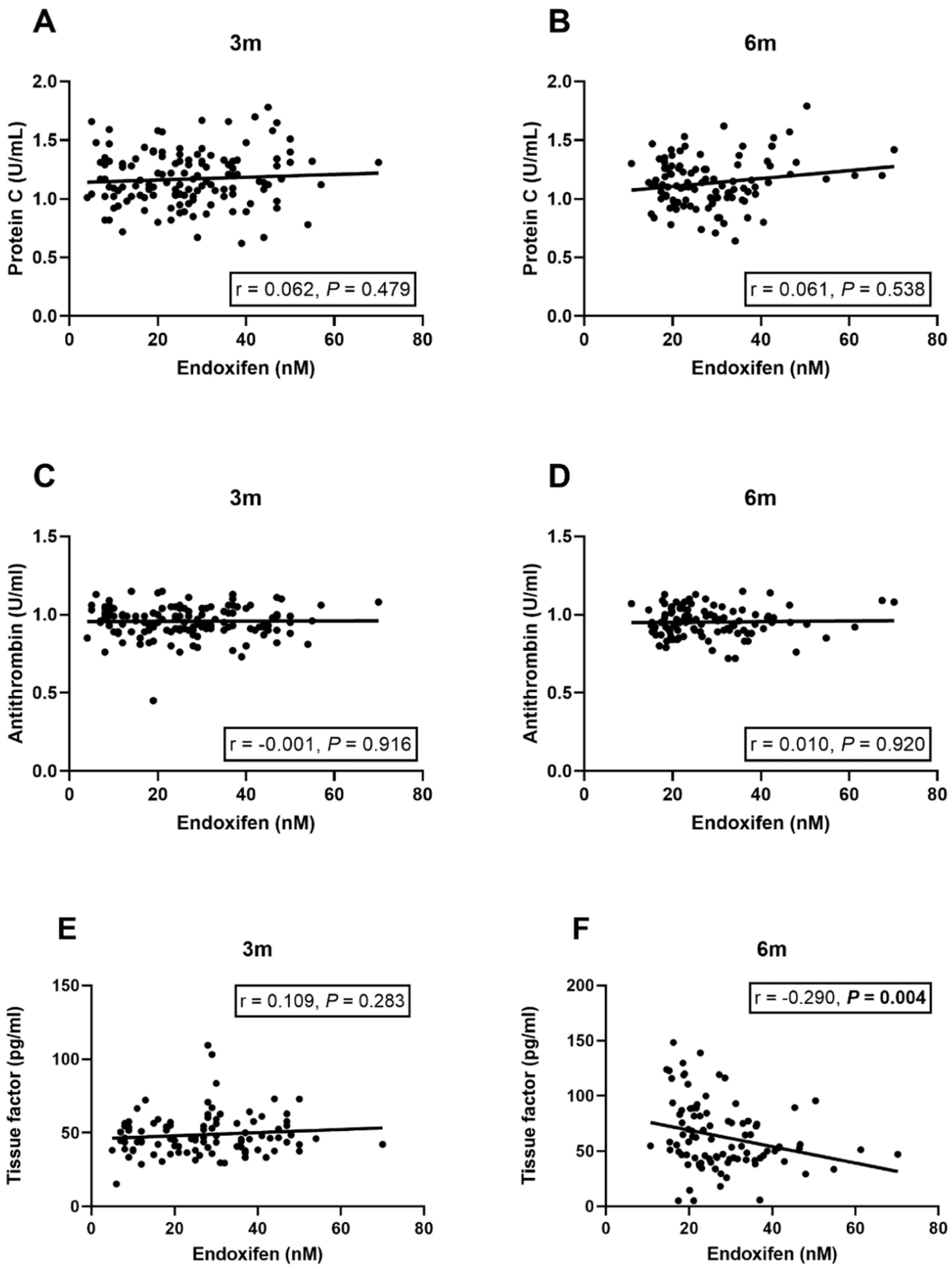

Fig. 2. Correlation of endoxifen plasma levels after 3 m and 6 m of tamoxifen treatment with **A+B,** protein C (n = 133 and n = 104, respectively), **C+D** antithrombin (n = 135 and n = 104, respectively), and **E + F** tissue factor (n = 100 and n = 95, respectively).

therapy. Despite this weak correlation, this observation points to a possible anticoagulant effect that did not persist at 6 months of treatment. Normal levels of protein C range from approximately 0.70 to 1.40 IU/mL and levels approximately below are associated with a significant increase in VTE risk. [30] Most protein C levels observed in our cohort fall within this normal range and are therefore not considered specifically low. [31] Although antithrombin levels in our cohort were slightly lower than earlier described in healthy controls [median 1.04 IU/mL], most still fell within the normal range of 0.80–1.20 IU/mL and were above the lower limit earlier associated with an increased VTE risk. [30,32] In addition, antithrombin did not have any association with higher concentrations of tamoxifen nor endoxifen.

Table 4
Levels of coagulation factors by quartiles of endoxifen.

3 months of tamoxifen				
	Q1	**Q2**	**Q3**	**Q4**
Endoxifen (nM)	**4–17**	**17–26**	**26–37**	**37–70**
	n = 34	**n = 35**	**n = 33**	**n = 33**
Protein C (U/mL)	1.155	1.162	1.155	1.202
	(0.22)	(0.21)	(0.23)	(0.28)
	n = 34	n = 34	n = 32	n = 33
Antithrombin (U/mL)	0.967	0.944	0.959	0.960
	(0.09)	(0.12)	(0.08)	(0.11)
	n = 34	n = 35	n = 33	n = 33
Tissue factor (pg/mL)	44.7 [16]	43.0 [10]	52.2 [19]	46.5 [19]
	n = 27	n = 20	n = 27	n = 26
6 months of tamoxifen				
	Q1	**Q2**	**Q3**	**Q4**
Endoxifen (nM)	**11–20**	**20–25**	**25–34**	**34–70**
	n = 26	**n = 27**	**n = 27**	**n = 26**
Protein C (U/mL)	1.144	1.137	1.032	1.230
	(0.18)	(0.17)	(0.21)	(0.23)
	n = 25	n = 27	n = 27	n = 25
Antithrombin (U/mL)	0.941	0.979	0.935	0.955
	(0.09)	(0.08)	(0.09)	(0.10)
	n = 25	n = 27	n = 27	n = 25
Tissue factor (pg/mL)	75.6 [69]	65.7 [46]	**56.6 [33]** *	**50.1 [19]** *
	n = 23	n = 26	n = 25	n = 21

Data are displayed as mean (SD) or median [IQR]. Data were missing for some participants in some subgroups. *P-value < 0.05 versus Q1, all other comparisons were non-significant. Abbreviations: ETP: endogenous thrombin potential

Table 5
Thrombin generation parameters by quartiles of endoxifen.

3 months of tamoxifen				
	Q1	**Q2**	**Q3**	**Q4**
Endoxifen (nM)	**4–17**	**17–26**	**26–37**	**37–70**
	n = 34	**n = 35**	**n = 33**	**n = 33**
With exogenous tissue factor				
ETP (nM*min)	2133 (369)	2292 (1177)	2057 (282)	2206 (442)
Thrombin peak (nM)	261 (59)	245 (65)	244 (51)	255 (65)
Lag time (min)	7.0 (1.5)	7.3 (1.8)	6.8 (1.4)	7.0 (1.2)
Time to peak (min)	10.9 [2.0]	11.6 [3.7]	10.9 [2.3]	12.1 [2.4]
Velocity index (U/mL)	66 (25)	56 (21)	56 (18)	59 (20)
Without exogenous tissue factor				
ETP (nM*min)	1304 (339)	1351 (431)	977 (399)	1122 (387)
Thrombin peak (nM)	75 (45)	77 (47)	48 (32)	48 (26)
Lag time (min)	31 [44]	34 [21]	37 [36]	50 [28]
Time to peak (min)	45 [21]	43 [22]	48 [32]	62 [22]
Velocity index (U/mL)	11.3 [17]	14.5 [13]	5.5 [5.8]	6.3 [8.2]
6 months of tamoxifen				
	Q1	**Q2**	**Q3**	**Q4**
Endoxifen level (nM)	**11–20**	**20–25**	**25–34**	**34–70**
	n = 26	**n = 27**	**n = 27**	**n = 26**
With exogenous tissue factor				
ETP (nM*min)	2156 (463)	1975 (396)	2109 (325)	2271 (436)
Thrombin peak (nM)	270 (84)	235 (65)	236 (49)	271 (52)
Lag time (min)	6.0 [6]	6.1 [3]	6.1 [2]	7.0 [6]
Time to peak (min)	9.9 [1]	10.9 [3]	10.9 [2]	10.8 [2]
Velocity index	67 (30)	54 (20)	50 (16)	62 (15)
Without exogenous tissue factor				
ETP (nM*min)	1252 (534)	996 (383)	1083 (478)	1463 (499)
Thrombin peak (nM)	87 [146]	51 [24]	79 [68]	80 [147]
Lag time (min)	26.8 (18)	34.9 (14)	32.3 (8.5)	26.7 (14)
Time to peak (min)	36 [21]	41 [10]	36 [17]	33 [24]
Velocity index (U/mL)	16.3 [42]	9.0 [7]	15.1 [15]	15.1 [34]

Data are displayed as mean (SD) or median [IQR]. Data were missing for some participants in some subgroups. All comparisons were non-significant. Abbreviations: ETP: endogenous thrombin potential.

To the best of our knowledge, the procoagulant protein tissue factor has not been directly measured in the context of tamoxifen therapy before. It has been shown that levels of the anticoagulant protein TFPI decrease during treatment with tamoxifen. [11] Given that this factor inhibits the activity of the tissue factor FVIIa complex in a FXa-dependent manner, this tamoxifen-induced TFPI decrease potentially leads to a hypercoagulable state. [33] Here we found that the endoxifen levels are negatively, albeit modestly, correlated with circulating tissue factor levels after 6 months of tamoxifen treatment. Thus, if tissue factor has any correlation with plasma levels during tamoxifen therapy at all, this is most likely in the direction of anti-coagulation. Importantly, the effect of tamoxifen and endoxifen levels on TFPI has not been studied yet, and the eventual net outcome on tissue factor / TFPI signalling remains therefore currently unclear.

Table 6
Time-dependent effect of tamoxifen treatment on coagulation parameters in patients who remained on 20 mg tamoxifen daily during the study.

		3 months	6 months
Protein C (U/mL)	n = 66	1.19 (0.25)	1.16 (0.22)
Antithrombin (U/mL)	n = 67	0.95 (0.08)	0.94 (0.09)
Tissue factor (pg/mL)	n = 55	46.0 [15.4]	**54.4 [38.8]** ***
Thrombin generation parameters			
With exogenous tissue factor			
ETP (nM*min)	n = 40	2080 (369)	2170 (387)
Thrombin peak (nM)	n = 40	245 (58)	256 (65)
Lag time (min)	n = 40	6.9 (1.4)	9.0 (7.9)
Time to peak (min)	n = 40	11.3 [2.8]	10.5 [2.0]
Velocity index (nM/min)	n = 40	56.3 (21)	59.12 (19)
Without exogenous tissue factor			
ETP (nM*min)	n = 29	1137 (414)	1276 (463)
Thrombin peak (nM)	n = 30	59.5 (35)	**97.0 (66)** ***
Lag time (min)	n = 30	38.7 [20]	**29.7 [19]** ***
Time to peak (min)	n = 30	44.0 [21]	**38.8 [18]** **
Velocity index (nM/min)	n = 30	9.10 [9.4]	**12.9 [23]** **

Data are displayed as mean (SD) or median [IQR]. Data were missing for some participants in some subgroups. Abbreviations: ETP: endogenous thrombin potential. P value indicates results of paired t-test or Wilcoxon signed rank test. **P value < 0.01, *** P value < 0.001, all other comparisons were non-significant.

To gain a better understanding of the possible effect of tamoxifen and endoxifen levels on a procoagulant state during tamoxifen, we performed thrombin generation assays which provide a more comprehensive evaluation of coagulation relative to the prothrombin time (PT) and activated partial thromboplastin time (APTT) clotting assays. [29] Given that the parameters of thrombin generation were similar between all patients stratified for tamoxifen and endoxifen plasma concentrations, this further indicates that an increase in plasma levels of tamoxifen and endoxifen does not coincide with a procoagulant potential. Interestingly, we found increased thrombin generation after 6 months compared to 3 months of treatment, independent from any tamoxifen dose adjustments. This was only observed in the condition without exogenous addition of tissue factor suggesting that this enhanced thrombin generation is tissue factor-mediated. Indeed, tissue factor increased after 6 months compared to 3 months of treatment. Although our observed number of VTE events was small, the majority of patients (4 out of 7 patients) experienced an event between approximately 3 and 6 months after start of tamoxifen therapy. This could indicate that patients, within the first year of tamoxifen treatment, experience the highest thrombotic risk during this time period. Of note, all patients with a previous history of VTE developed a VTE again during tamoxifen therapy. Although the small number of events has to be taken into account, this observation might suggest that patients with a history of VTE have the highest prothrombotic risk during tamoxifen treatment. Further studies on the effects of tamoxifen on tissue factor and tissue factor signalling at different timepoints would be interesting to gain a better insight in the general prothrombotic effects of tamoxifen and to study if tamoxifen increases VTE risk in a time-dependent manner. Moreover, in patients with a previous history of VTE, the possible extra risk of developing a VTE during tamoxifen therapy requires further study and warrants extra caution in clinical practice.

In patients with breast cancer who receive adjuvant tamoxifen, there

are other factors that can determine prothrombotic risk. For example, chemotherapy, radiotherapy and surgery are all independent risk factors for VTE. [34–36] Since most patients had completed their chemotherapy and radiotherapy treatments before the start of tamoxifen, the influence of these treatments on VTE risk was probably minimal and even further diminished over time. Although these other treatments might directly affect various coagulation factors including a possible increase in the procoagulant tissue factor as well, we found an increase rather than a decrease in tissue factor levels over time (i,e, longer after completion of the other treatments). This makes it more likely that tamoxifen is directly responsible for the observed increase in tissue factor in this study. Also, no consistent trend towards an increase in procoagulant or a decrease in anticoagulant factors was observed in patients who received chemotherapy compared to patients who did not receive chemotherapy. Lastly, although the presence of (recurrent) cancer is an independent risk factor for VTE [37], the recurrence rate for ER-positive breast cancer is generally low, especially in the first year. [1] Also, patients who developed clinical breast cancer recurrence ($n = 1$) or a new primary cancer ($n = 2$) within one year after start of tamoxifen therapy were excluded from our analyses. Therefore, it is very unlikely that any of the included patients had (recurrent) cancer at time of measurements and status of cancer did thus probably not influence the observed time-dependent effect of tamoxifen treatment on tissue factor and thrombin generation levels.

Given that treatment with aromatase inhibitors, another adjuvant endocrine treatment for ER-positive breast cancer, does not predispose to VTE [38], we hypothesized that the prothrombotic effects of tamoxifen are predominately mediated via the ER, rather than estrogenic effects specifically. Endoxifen and 4-hydroxytamoxifen are the metabolites with the highest affinity for the ER. [17] However, tamoxifen and endoxifen reach up to respectively 14- and 40-fold higher plasma concentrations than 4-hydroxytamoxifen. [10,18] Although n-desmethyltamoxifen reaches slightly higher plasma concentrations than tamoxifen, its affinity for the ER is 100 times lower and this metabolite is therefore considered to be of minor importance. [18] Hence, the inclusion of both tamoxifen and its primary metabolite endoxifen levels was a strength of the current study. Although more research is needed to definitely rule out a role for the other metabolites in the increased VTE risk, tamoxifen has linear pharmacokinetics, indicating that higher levels of tamoxifen and endoxifen were most likely paralleled by higher levels of 4-hydroxytamoxifen and n-desmethyltamoxifen as well. Unfortunately, our study was underpowered to investigate if higher tamoxifen or endoxifen levels predispose to VTE since the number of events was limited. However, our observations provide a first indication that levels of tamoxifen and endoxifen are not associated with increased VTE risk. Also, tamoxifen and endoxifen levels from patients who experienced a VTE did not differ substantially from the median tamoxifen and endoxifen levels in our total study population. In fact, in three out of five patients tamoxifen and endoxifen levels before the occurrence of the VTE belonged to the lowest quartiles. A previous phase I study in which endoxifen was administered as a drug itself (rather than tamoxifen) found that only one out of the 38 included patients with metastatic breast cancer developed a VTE (2.6%). [39] This low VTE incidence despite endoxifen plasma levels 10–100 times higher (360–5200 nM) than in our current study [39] and the fact that included patients had metastatic breast cancer, which is an independent VTE risk factor [40], further suggest that higher endoxifen levels do not predispose to higher VTE risk.

The current study has some limitations. First, samples before start of tamoxifen therapy were not available. Therefore, the direct relationship between the included coagulation proteins and tamoxifen treatment could not be validated. However, we focused on the relationship between tamoxifen and endoxifen concentrations and coagulation system activation. In addition, we performed intra-patient comparisons. Therefore, baseline samples were not essential to answer our primary research questions. Secondly, although we carefully selected the measured coagulation parameters based on previous studies and additionally performed thrombin generation assays, a selection of surrogate markers for a procoagulant state was used. In future studies, the direct correlation between tamoxifen and endoxifen plasma levels and VTE should be investigated. Thirdly, samples were missing for some measurements which could limit statistical power. Fourthly, no definite conclusions can be drawn for substantially higher or lower tamoxifen levels than observed in our study. As the standard dose of 20 mg daily is most frequently prescribed and is thus representative for the current clinical practice, the number of patients using higher or lower tamoxifen doses was limited here. Therefore, more research in patients using non-standard tamoxifen doses is required.

In conclusion, our study indicates that higher tamoxifen and endoxifen levels are not correlated with an increased procoagulant state. Although adequate monitoring of VTE remains important, this provides a first indication that a TDM-directed tamoxifen dose escalation does not additionally increase VTE risk.

Author contributions

SMB, DCHvD, SLWK, MJHAK, MHAB, RHJM and HHV conceived the study design and idea. SMB, RP and RHJM included patients. RFPvdA, KLC, RB, HHV and MHAB acquired data. SMB, DvD, EOdH and MHAB performed the statistical analyses. SMB, DCHvD, MJHAK, AJ, SLWK, JV, AHJD, HHV, MHAB and RHJM mainly interpreted the results. SMB and DCHvD drafted the manuscript. All authors revised the manuscript, approved the final version and agreed with its submission.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data will be made available on request.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the

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