Chapter 7 - Ectopic factor VII expression is an indicator of poor survival in young women with breast cancer

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Abstract

Blood coagulation and cancer progression are engaged in a bidirectional relationship that manifests itself in malignancy-related thrombosis and coagulation factor-dependent tumor progression. Full-length tissue factor (flTF):factor(F)VIIa signaling through Protease Activated Receptor (PAR) 2 determines outcome in murine breast cancer. Whether only canonical liver-derived FVII or also tumor-expressed FVII is involved in cancer progression is unknown. The aim of the present study was to examine the association between tumor-expressed FVII and clinical outcome in breast cancer. Breast cancer specimens collected during primary surgery - at which time radiotherapy or chemotherapy had not been started - were assembled in a tissue micro array (TMA) and immuno-stained for flTF and FVII. Data on flTF expression was available from 507 patients and data on both flTF and FVII expression from 302 patients. The TMA's were independently scored by two observers, and associations with clinical and pathological parameters and survival were analyzed. flTF was abundantly expressed in cancer specimens when compared to normal healthy tissue, while FVII was detected in only 39% of the tumors. Whereas flTF expression was associated with higher tumor grade ($p<0.001$), FVII was also was associated with T status that reflects tumor size and invasiveness ($p=0.009$), and with loss of estrogen ($p=0.011$) and progesterone ($p<0.001$) receptors. Patients with FVII+ tumo(u)rs had a shortened relapse-free survival ($p=0.02$), and when analyzed for double expression of flTF and FVII, we found a similar effect ($p=0.006$). After 10 years, relapse-free rates were 58% for women younger than 55 years with flTF:FVII positive tumors compared to 82% for women > 55 years. These results confirm that flTF expression is a general phenomenon in breast cancer. This report also shows that FVII expression in breast cancer is associated with poor prognosis in young women. Ectopically produced FVII therefore may offer new perspectives in cancer staging or therapeutic options.

Introduction

For more than one century, activation of coagulation is recognized to be tightly interwoven with cancer progression[1]. Patients suffering from various types of cancer may develop venous thrombosis, a condition referred to as Trousseau's syndrome. Tumoral overexpression of full-length tissue factor (flTF), a 47 kDa transmembrane protein, may cause this thrombotic tendency[2]. Under physiological circumstances, flTF expression is limited to the surface of extravascular cells. Upon damage of the endothelium, flTF becomes exposed to the bloodstream and binds the blood-borne zymogen FVII. The flTF:FVIIa complex induces the subsequent activation of factor Xa and thrombin, thus resulting in a fibrin clot[3].

Activation of coagulation also influences various stages of cancerous disease. The flTF:FVIIa complex and thrombin have been implicated in tumor growth and metastasis[4]. flTF expression in human colon cancer is associated with an increased risk of hepatic metastasis[5], and in murine models, flTF expression in colon cancer as well as in breast cancer promotes tumor growth through modulation of angiogenesis[6;7]. Extensive research has uncovered that coagulation proteases activate protease-activated receptors (PARs). The flTF:FVIIa complex specifically activates PAR2 resulting in altered cell behavior such as proliferation, gene expression and migration, eventually leading to enhanced tumor growth[7]. In a spontaneous murine breast cancer model, absence of PAR2 delays the angiogenic switch and as a consequence, tumor growth is delayed[8].

FVII is normally synthesized in the liver and released into the bloodstream. When vessel damage occurs, the flTF:FVII complex initiates coagulation in order to stem bleeding. One report claims that FVII is bound to flTF on fibroblasts even before vascular damage occurs[9]. Whether this FVII came from the circulation or was locally produced is unknown. Furthermore, FVII can be detected in lung macrophages under inflammatory conditions[10-12] and in atherosclerotic plaques[13-15], again suggesting a possible role for ectopic FVII production in pathophysiology.

FVII mRNA has been detected in ovarian, breast, brain, prostate, thyroid, lung and gastric cancer cell lines up to levels comparable with those found in non-malignant hepatoma cell lines, which indicates that FVII may be produced in (patho)physiologically relevant amounts by cancer cells. In ovarian and breast cancer cell lines, ectopically produced FVII is bound to flTF, and FVII levels were sufficient to trigger coagulation and subsequent factor Xa (FXa)-dependent PAR1 activation. Consequently, FVII production in ovarian cancer cells leads to enhanced cancer cell motility and invasiveness[16]. In glioma cell lines, EGFRvIII-driven simultaneous expression of flTF, FVII, PAR1 and PAR2 precedes the production of angiogenic proteins like VEGF and IL-8[17]. This may indicate that ectopic FVII expression facilitates the angiogenic switch in poorly vascularized tumors thus circumventing the requirement for blood-borne FVII.

Hypoxia is an important upstream event that triggers ectopic FVII in tumors. Oxygen depletion leads to the activation of a hypoxia-inducible factor 2α (HIF2)α-dependent pathway in ovarium cancer cells that eventually induces FVII expression[16;18]. A recent study in ovarian cancer cell lines described a non-canonical interaction between hypoxia-driven and epigenetic pathways leading to ectopic FVII production[19]. HIF2α and Sp1 form a complex and bind to a known Sp1-binding site in the FVII promoter region and subsequently recruit histone deacetylase 4 (HDAC4), which induces FVII expression. A different epigenetic mechanism was identified in breast cancer cell lines in which

curcumin-sensitive histone acetyltransferases (HAT) p300 and cyclic AMP-responsive element binding protein (CBP) were exclusively recruited to the promoter region of the FVII gene[20]. Although differences may exist between tumor cells from different tissues, hypoxia alters the acetylation status of the FVII promoter region and thereby induces FVII expression.

The relation between FVII expression in human tumor specimens and patient outcome has not been investigated. In the present study, we examined a large number of human breast cancer samples and matched normal tissue from patients with a long follow-up in order to investigate how tumoral FVII and flTF expression relate to clinical outcome in human breast cancer.

Methods

Reagents- Normal goat serum and EnVision™ anti-rabbit HRP and anti-mouse HRP conjugates were purchased from DAKO (Glostrup, Denmark). FVII polyclonal antibody GTX101238 was purchased from Genetex (Irvine, CA). flTF monoclonal antibody #4509 was purchased from American Diagnostica Stamford, CT).

Study cohort- The patient population comprised patients with non-metastasized breast cancer treated with surgery in the Leiden University Medical Center between 1985 and 1994 of whom tumor material is available (N = 574) as described earlier[21]. Patients with bilateral tumors or a prior history of cancer - other than basal cell carcinoma or cervical carcinoma in situ - were excluded. The following data were recorded: age, tumor grade, histological type, TNM (TNM classification of malignant tumors, Union for International Cancer Control), stage, local and systemic therapy, locoregional or distant tumor recurrence, survival, and expression of estrogen receptor (ER), progesterone receptor (PgR) or human epidermal growth factor receptor 2 (HER2). All tumors were graded according to the Nottingham modification of the Scarff-Bloom-Richardson grading system. Of 266 patients (46%), also normal mammary tissue was available for analysis. Median follow-up was 17.9 years (range: 0.01 to 23.5). Approval was obtained from the Leiden University Medical Center Medical Ethics Committee. All samples were handled in a coded fashion, according to National ethical guidelines (“Code for Proper Secondary Use of Human Tissue”, Dutch Federation of Medical Scientific Societies).

Assessment of expression of FVII and flTF- Tissue sections of 4 µm were cut from a previously constructed tissue microarray of formalin-fixed paraffin-embedded tumors of 574 patients and a tissue microarray of formalin-fixed paraffin-embedded corresponding normal mammary tissue of 266 patients. Immunohistochemical staining was performed

according to standard procedures. Briefly, sections were deparaffinized, rehydrated and endogenous peroxidase activity was blocked with 0.3% H2O2 in MetOH. All sections were subjected to antigen retrieval in sodium citrate buffer for 10 minutes at 100 ˚C. Sections were blocked for 1 hour with 10% normal goat serum in PBS/BSA 1% at room temperature. Sections were then incubated with 1 µg/ml antibody overnight at 4 ˚C (both anti FVII and anti flTF). Sections were washed in PBS, incubated for 30 minutes with Envision, washed and visualized in DAB solution. After counterstaining with hematoxylin, sections were dehydrated and covered. Liver and placenta sections served as a positive control for respectively FVII and flTF. The TMA's contained tumor samples from each patient in triplicate, and were randomly arranged on the array. During the immunohistochemical procedures a random loss of tissue punches occurred, finally resulting in data on flTF expression in 507 patients, on FVII expression in 331 patients and on both flTF and FVII in 302 patients.

FVII was considered to be expressed when one or more tumor cells stained positive. flTF staining was defined by percentage of positive staining tumor cells per microscopic field. Two independent observers performed all scorings in a blinded fashion. flTF staining percentages were arranged in increasing order and on basis of this, the cohort was divided into quartiles. In subsequent analyses, the fist quartile was considered "negative".

Statistical analysis- Statistical analyses were performed using the statistical packages SPSS (version 16.0 for Windows, Spps Inc, Chicago, IL, USA) and Stata (version 10.0 for Windows, StataCorp, College Station, TX, USA). Cohen's kappa coefficient was used to assess the inter-observer agreement in quantification of protein expression. The χ^2 test was used to evaluate associations between protein expression and various clinicopathological parameters.

Relapse-free surival was defined as the time from date of surgery until an event (locoregional recurrence and/or a distance recurrence, whichever came first). Relapse-free survival is reported as cumulative incidence function, after accounting for death as competing risk[22]. The Kaplan–Meier method was used for survival plotting and log-rank test for comparison of curves. Analyses were performed for all patients and stratified for age. Relative survival was calculated by the Hakulinen method as the ratio of the survival observed among the cancer patients and the survival that would have been expected based on the corresponding (age, sex, and year) general population. National life tables were used to estimate expected survival. Relative excess risks of death were estimated using a multivariable generalized linear model with a Poisson distribution, based on collapsed relative survival data, using exact survival times. The Kaplan–Meier method was used for survival plotting and log-rank test for comparison of curves.

Results

Patterns of FVII and flTF expression in human breast cancer- A granular, cytoplasmic staining pattern for human FVII was found in 130 out of 331 patients (39.3%), whereas non-cancerous epithelium showed no FVII staining (figure 1). Inter-observer agreement was high (κ>0.8). Tables 1 and 2 list the associations in the cohort between flTF and FVII expression and several clinical and pathological characteristics. In accordance with previous reports (reviewed in reference 1), we found that expression of flTF in breast cancer is detected in >90% of the cases. We scored the percentage of flTF positive cells, arranged the scored percentages in increasing order, and divided this range into quartiles. The 2nd to the 4th quartile comprised tumor specimens in which all cells per microscopic field were positive for flTF.

Higher histological grades were associated with flTF expression. FVII-positive tumors display a higher histological grade, advanced T-status and loss of estrogen and progesterone receptors, which are unfavorable tumor characteristics[23]. Similar associations, but also a loss of Her2 receptor (p=0.05), was found when both flTF and FVII were positive, thus suggesting that expression of flTF and FVII coincides with loss of the estrogen, progesterone and Her2 receptor. The latter is clinically relevant as the so-called triple negative tumors are considered to have the most unfavorable phenotype in breast cancer patients.

FVII positivity is associated with impaired relapse-free surival and relative survival- The percentage of flTF expressing tumor cells was not associated with the length of the relapse-free period. Analysis of the relapse-free period in patients with FVII positive versus FVII negative tumors revealed that the length of the relapse-free surivial was reduced in patients with FVII positive tumors. After 10 years of follow-up, 72% of the patients with FVII negative tumors were relapse-free compared to 60% of patients with FVII positive tumors (absolute difference 12%, log rank P = .02, Figure 2).

Since flTF serves as the receptor for FVII, we next determined survival in patients with flTF/FVII double positive tumors, compared to single positive or negative tumors. Different lengths of the relapse-free period were observed for these categories (either or both negative 72% relapse-free, versus 58% relapse-free when both positive, P=.006). The overall survival was similar to the relapse-free surival (Figure 2) as well as the differences in survival after 10 years.

Combined flTF and FVII expression is an independent risk factor for decreased RFP and relative survival- In order to investigate whether combined flTF and FVII expression is an

independent risk determinant of relapse-free survival and relative survival, we performed a Cox univariate and multivariate analysis (Table 3). As combined flTF and FVII expression were associated with grade, T-status, presence of lymph node metastasis and histological subtype, we included these determinants in our analysis. Combined flTF and FVII expression indeed independently predicted early cancer recurrence (HR 1.55; CI95% 1.095-2.205) and in a similar fashion, increased mortality (RER 1.68; CI95% 1.092-2.575).

Tumoral FVII affects relapse-free survival and relative survival in pre-menopausal women- Next we analyzed whether age affects the association between flTF, FVII and survival. We divided the cohort, on the basis of the median age, in women aged <55 years or 55 years and older. We found an association between flTF expression and shorter relapse-free and overall survival in women aged younger than 55 years. Moreover, we found that the associations between FVII expression alone, or combined expression of flTF and FVII, with relapse-free period became more prominent for women younger than 55 years, but were absent in women aged 55 or older. After 10 years, relapse-free rates were 58% for women younger than 55 years with flTF:FVII positive tumors compared to 82% for women > 55 years (figure 3). A similar effect was observed when survival analyses were performed after dividing the cohort between women aged younger than 65 and older than 65 years (data not shown). Moreover, a Cox regression analysis that assessed the interaction between age and presence of both flTF and FVII proved significant (p=0.006). In summary, the effects of combined flTF and FVII expression affects breast cancer in younger age groups and this effect fades out in older aged patients.

Discussion

The notion that flTF mediates signaling events in breast cancer, and thereby influences cancer progression, either via direct PAR2 signaling or indirectly via coagulation activation, is supported by several *in vivo* and clinical studies[1]. Both pathways depend on the presence of blood-borne coagulation factors that are produced in the liver, but it is unclear how tumors have access to coagulation factors when they are still poorly vascularized. Hypoxia-induced expression of ectopic FVII expression in cancer may at least partially answer this conceptual question. In order to examine whether local FVII expression in tumors is related to cancer progression in humans, we investigated the association between FVII or flTF expression and clinical and pathological outcome in breast cancer patients.

We observed that FVII expression is present in only 39% of the investigated tumor specimens, whereas flTF expression is virtually present in all specimens. This suggests that flTF expression is a general phenomenon in breast cancer and that expression of FVII is

associated with cancer progression in a subgroup of breast cancer patients. We found associations between tumoral FVII expression and unfavorable tumor characteristics, such as high histological grade and loss of hormone receptors. Both relapse-free and relative survival were associated with expression of FVII. Moreover, we showed that combined expression of both flTF and FVII further increased the strength of our survival analyses, possibly through an association with triple negative breast cancer, a condition that is associated with aggressive breast cancer at young age.

We found that the effects of combined flTF and FVII expression on outcome were only apparent in patients under 55 years of age. This may suggest that the onset of menopause and related changes in hormonal status is associated with FVII expression in breast cancer. However, a similar association was found when a cut-off of 65 years was applied, which makes menopause a less likely determinant. Future studies in other solid tumors, e.g. colon cancer, will show whether the results that we here report are breast cancer- and gender-specific or rather a general feature of FVII expression in solid tumors.

There are two potential limitations to our study. First, this study is immunohistochemistry-based and potentially blood-borne FVII may have become internalized after binding to flTF. Therefore, a RNA-based follow-up study may take away uncertainties about the origin of FVII that we detected in the tumor samples. The second drawback is a lack of data that confirm that FVII expression in tumor cells indeed triggers flTF:FVII:PAR2 signaling. An earlier study, investigated the role of phosphorylated flTF in relation to clinical outcome and survival[24]. Upon activation of PAR2, the cytoplasmic domain rapidly becomes phosphorylated and detection of phosphorylated flTF can serve as a read-out of PAR2 signaling in these tumor specimens. Indeed, phosphorylation of flTF was associated with poor survival and in future studies it would be interesting to investigate whether flTF phosphorylation is associated with ectopic FVII production.

Regarding other avenues for future studies, it is warranted to assess whether other coagulation proteins that are canonically expressed in the liver become expressed in tumors during oncogenesis as well. Since FXa, thrombin and activated protein C also have effects on cellular processes via either PAR-dependent pathways1 studies on ectopic expression of these proteins in tumors may prove informative in further understanding the relation between coagulation proteins and cancer.

In conclusion, our study describes a strong association between ectopic FVII expression in breast tumors and poor survival in women younger than 55 years. Whether these effects are partially caused by hormonal effects at pre-menopausal age is unclear and remains to be investigated. Future research may also unravel the role of tumor-expressed FVII in flTF-

mediated PAR2 signaling, and potentially also of other ectopically, tumor-expressed coagulation factors.

Reference List

1. van den Berg YW, Osanto S, Reitsma PH, Versteeg HH. The relationship between tissue factor and cancer progression: insights from bench and bedside. Blood 2012;119:924-932.
2. Forster Y, Meye A, Albrecht S et al. Tissue specific expression and serum levels of human tissue factor in patients with urological cancer. Cancer Lett. 2003;193:65-73.
3. Monroe DM, Hoffman M. What does it take to make the perfect clot? Arterioscler.Thromb.Vasc.Biol. 2006;26:41-48.
4. Versteeg HH, Ruf W. Emerging insights in tissue factor-dependent signaling events. Semin.Thromb.Hemost. 2006;32:24-32.
5. Seto S, Onodera H, Kaido T et al. Tissue factor expression in human colorectal carcinoma: correlation with hepatic metastasis and impact on prognosis. Cancer 2000;88:295-301.
6. Yu JL, May L, Lhotak V et al. Oncogenic events regulate tissue factor expression in colorectal cancer cells: implications for tumor progression and angiogenesis. Blood 2005;105:1734-1741.
7. Versteeg HH, Schaffner F, Kerver M et al. Inhibition of tissue factor signaling suppresses tumor growth. Blood 2008;111:190-199.
8. Versteeg HH, Schaffner F, Kerver M et al. Protease-activated receptor (PAR) 2, but not PAR1, signaling promotes the development of mammary adenocarcinoma in polyoma middle T mice. Cancer Res. 2008;68:7219-7227.
9. Hoffman M, Colina CM, McDonald AG et al. Tissue factor around dermal vessels has bound factor VII in the absence of injury. J.Thromb.Haemost. 2007;5:1403-1408.
10. Chapman HA, Jr., Allen CL, Stone OL, Fair DS. Human alveolar macrophages synthesize factor VII in vitro. Possible role in interstitial lung disease. J.Clin.Invest 1985;75:2030-2037.
11. McGee MP, Wallin R, Devlin R, Rothberger H. Identification of mRNA coding for factor VII protein in human alveolar macrophages--coagulant expression may be limited due to deficient postribosomal processing. Thromb.Haemost. 1989;61:170-174.
12. McGee MP, Devlin R, Saluta G, Koren H. Tissue factor and factor VII messenger RNAs in human alveolar macrophages: effects of breathing ozone. Blood 1990;75:122-127.
13. Wilcox JN, Noguchi S, Casanova JR, Rasmussen ME. Extrahepatic synthesis of FVII in human atheroma and smooth muscle cells in vitro. Ann.N.Y.Acad.Sci. 2001;947:433-438.
14. Wilcox JN, Noguchi S, Casanova J. Extrahepatic synthesis of factor VII in human atherosclerotic vessels. Arterioscler.Thromb.Vasc.Biol. 2003;23:136-141.
15. Borissoff JI, Spronk HM, ten CH. The hemostatic system as a modulator of atherosclerosis. N.Engl.J.Med. 2011;364:1746-1760.
16. Koizume S, Jin MS, Miyagi E et al. Activation of cancer cell migration and invasion by ectopic synthesis of coagulation factor VII. Cancer Res. 2006;66:9453-9460.
17. Magnus N, Garnier D, Rak J. Oncogenic epidermal growth factor receptor up-regulates multiple elements of the tissue factor signaling pathway in human glioma cells. Blood 2010;116:815-818.
18. Yokota N, Koizume S, Miyagi E et al. Self-production of tissue factor-coagulation factor VII complex by ovarian cancer cells. Br.J.Cancer 2009;101:2023-2029.
19. Koizume S, Ito S, Miyagi E et al. HIF2alpha-Sp1 interaction mediates a deacetylation-dependent FVII-gene activation under hypoxic conditions in ovarian cancer cells. Nucleic Acids Res. 2012

20. Koizume S, Yokota N, Miyagi E et al. Hepatocyte nuclear factor-4-independent synthesis of coagulation factor VII in breast cancer cells and its inhibition by targeting selective histone acetyltransferases. Mol.Cancer Res. 2009;7:1928-1936.
21. van Nes JG, de Kruijf EM, Faratian D et al. COX2 expression in prognosis and in prediction to endocrine therapy in early breast cancer patients. Breast Cancer Res.Treat. 2011;125:671-685.
22. Putter H, Fiocco M, Geskus RB. Tutorial in biostatistics: competing risks and multi-state models. Stat.Med. 2007;26:2389-2430.
23. Clark GM, McGuire WL. Steroid receptors and other prognostic factors in primary breast cancer. Semin.Oncol. 1988;15:20-25.
24. Ryden L, Grabau D, Schaffner F et al. Evidence for tissue factor phosphorylation and its correlation with protease-activated receptor expression and the prognosis of primary breast cancer. Int.J.Cancer 2010;126:2330-2340.

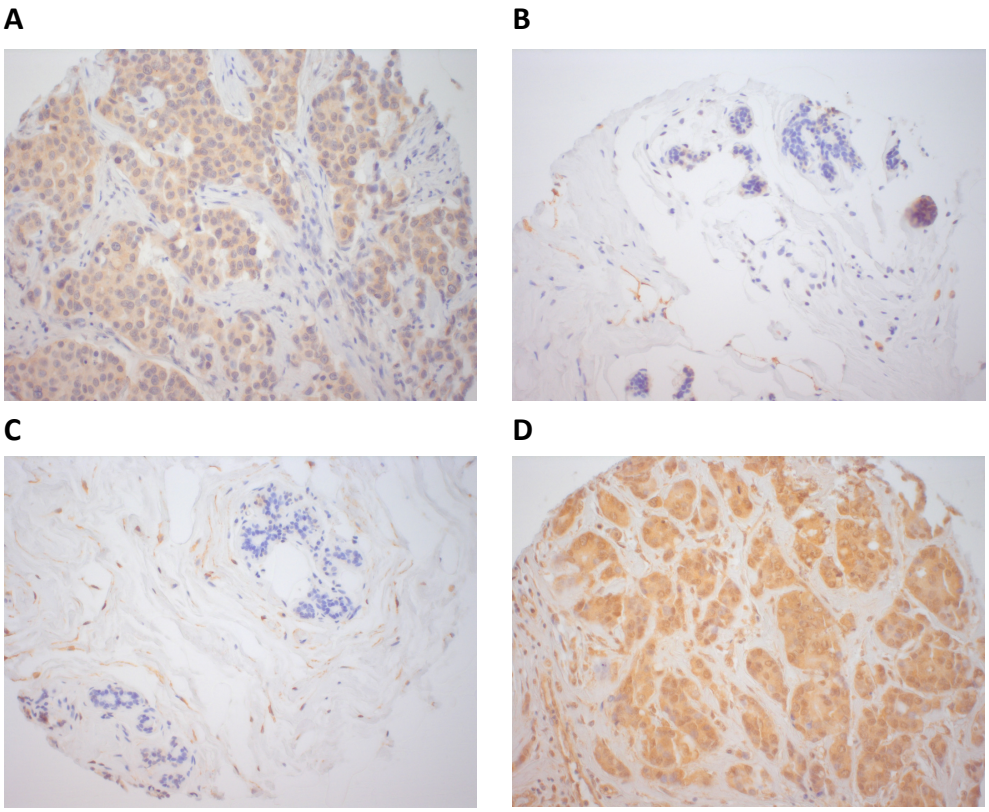

Figure 1. Expression patterns of FVII and flTF in human breast cancer. Breast cancer and matched normal breast specimens were assembled in a TMA fashion and stained with antibodies for either FVII or flTF. Panel A: non-malignant breast tissue, stained for FVII; panel B: FVII positive breast cancer specimen; panel C: non-malignant breast tissue, stained for flTF; panel D: flTF positive breast cancer specimen.

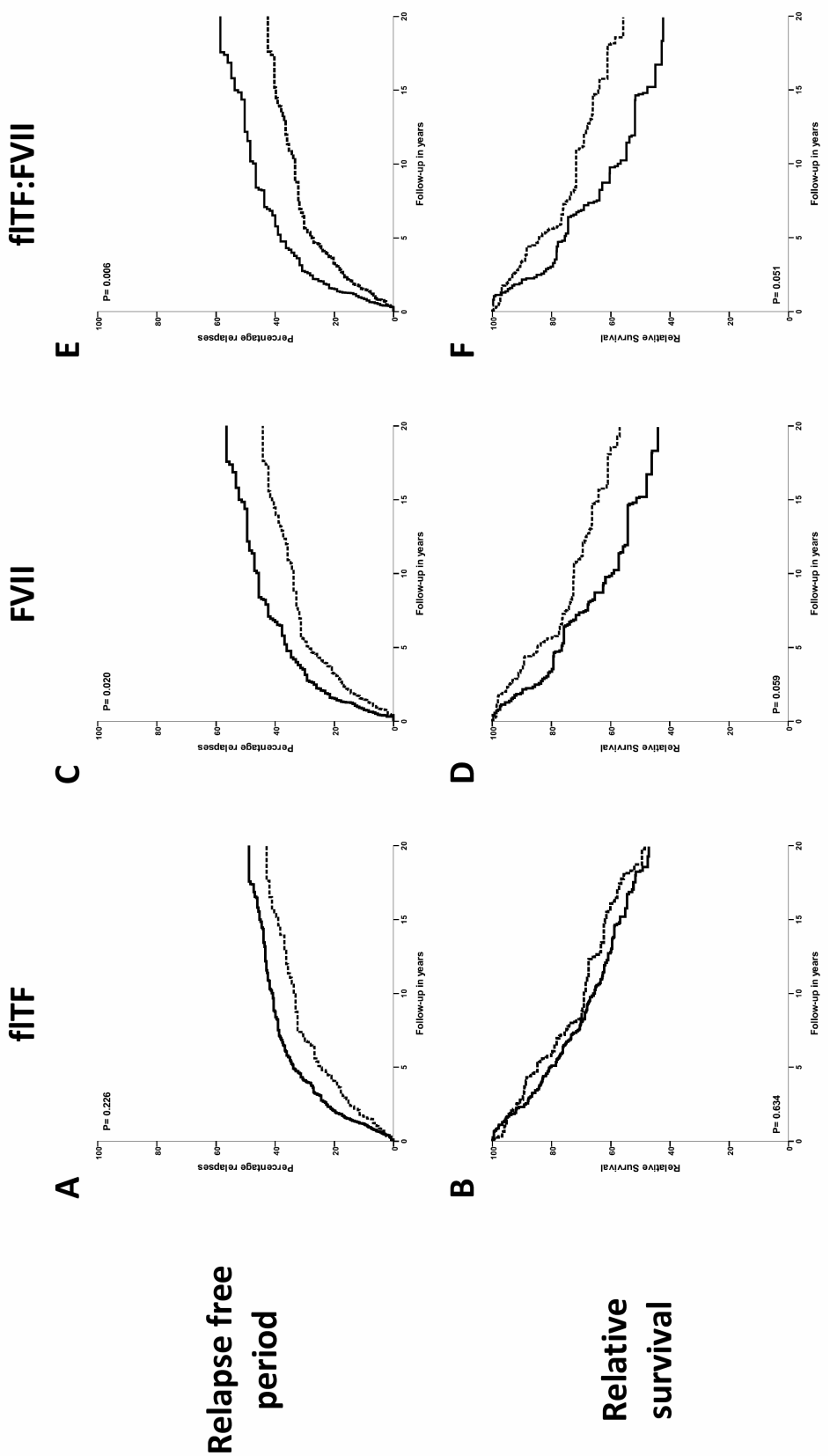

Figure 2. Relapse-free period and relative survival Kaplan-Meier curves. For flTF respectively panel A and B; for FVII respectively panel C and D; for FVII and flTF double expression respectively panel E and F. Continuous lines indicate high expression, dotted lines indicate low or absent expression.

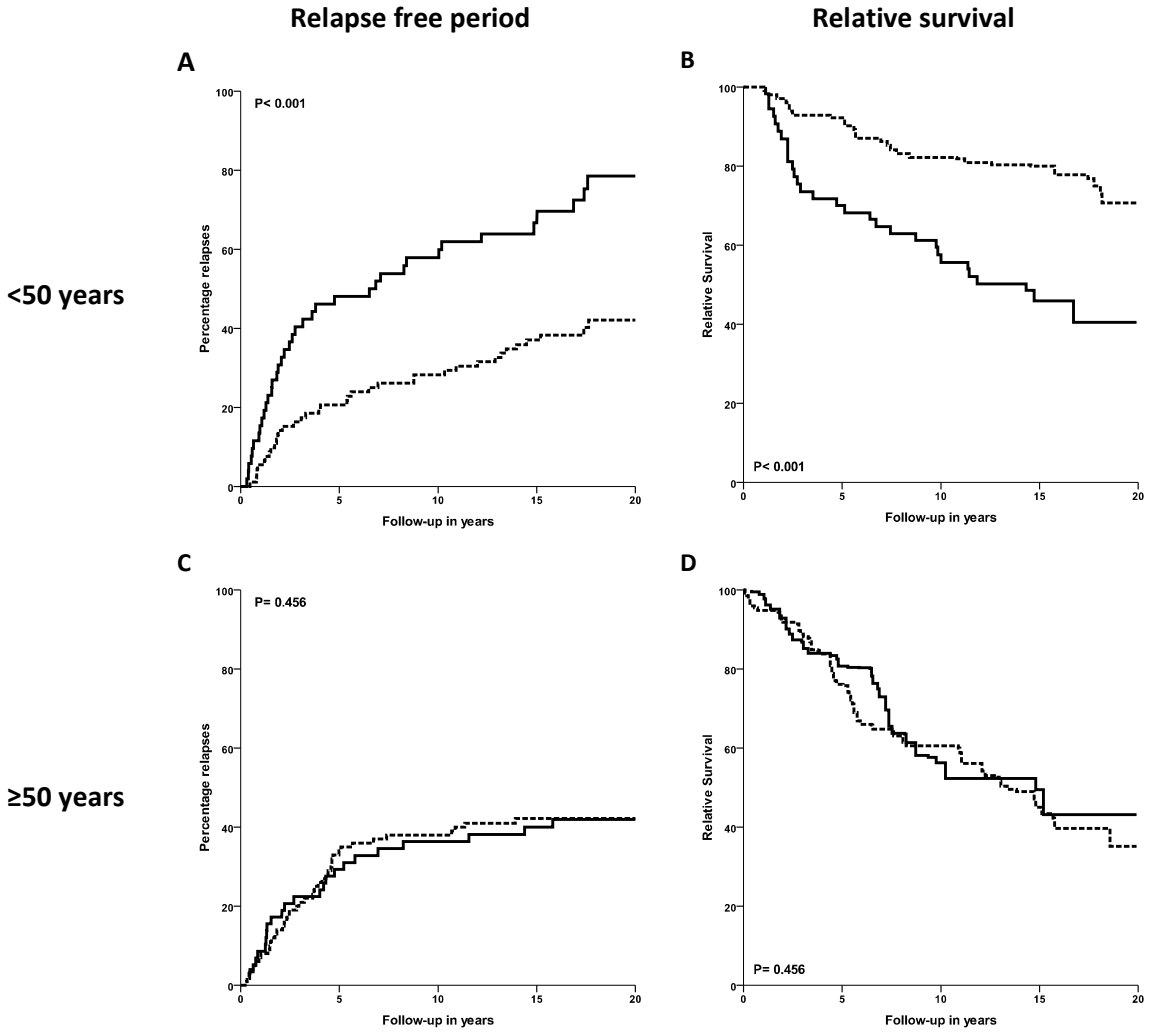

Figure 3. Relapse-free period and relative survival Kaplan-Meier curves for FVII and flTF in patients aged younger than 55 years (repectively panel A and C) or older than 55 years (repectively panel B and D).

	Factor VII					flTF				
	Negative		Positive		p-value	Negative		Positive		p-value
	N	%	N	%		N	%	N	%	
Total	**201**	**100**	**130**	**100**		**156**	**100**	**351**	**100**	
Age										
<40	20	9.9	14	10.8		15	9.6	26	7.4	
40-55	80	39.8	45	34.6		57	36.5	132	37.6	
55-65	36	17.9	32	24.6		30	19.2	77	21.9	
>65	65	32.3	39	30	0.479	54	34.6	116	33.0	0.768
Missing	0	0.0	0	0.0		0	0.0	0	0.0	
Grade										
I	33	16.4	9	6.9		30	19.2	42	12.0	
II	110	54.7	55	42.3		88	56.4	157	44.7	
III	55	27.4	63	48.5	<0.001	36	23.1	148	42.2	<0.001
Missing	3	1.5	3	2.3		2	1.3	4	1.1	
Histological type										
Ductal	179	89.1	121	93.1		134	85.9	320	91.2	
Lobular	20	9.9	6	4.6	0.057	20	12.8	27	7.7	0.065
Missing	2	1.0	3	2.3		2	1.3	4	1.1	
T-status										
T1	87	43.3	39	30.0		63	40.4	120	34.1	
T2	81	40.3	76	58.5		71	45.5	171	48.7	
T3/4	27	13.4	14	10.8	0.009	17	10.9	51	14.5	0.294
Missing	6	3.0	1	0.8		5	3.2	9	2.6	
N-status										
N0	114	56.7	66	50.8		92	59.0	182	51.9	
N1-3	84	41.8	60	46.2	0.359	62	39.7	158	45.0	0.198
Missing	3	1.5	4	3.0		2	1.3	11	3.1	
ER-status										
Negative	63	31.3	60	46.2		57	36.5	124	35.3	
Positive	128	63.7	67	51.5	0.011	96	61.5	217	61.8	0.849
Missing	10	5.0	3	2.3		3	1.9	10	2.9	
PgR-status										
Negative	60	29.9	65	50.0		54	34.6	149	42.5	
Positive	127	63.2	63	48.5	0.001	96	61.5	190	54.1	0.1
Missing	14	6.9	2	1.5		6	3.9	12	3.4	
Her2-status										
Overexpression-	129	64.2	92	70.8		112	71.8	260	74.1	
Overexpression+	13	6.5	16	12.3	0.166	13	8.3	34	9.7	0.730
Missing	59	29.3	22	16.9		31	19.9	57	16.2	
flTF										
low expression	87	43.3	14	10.8						
high expression	91	45.3	110	84.6	<0.001					
Missing	23	11.4	6	4.6						
Factor VII										
Low expression						87	55.8	91	25.9	
High expression						14	9.0	110	31.3	<0.001
Missing						55	35.2	150	42.7	

Table 1. Associations between clinical and pathological parameters, and expression of FVII or flTF. Abbreviations N number of patients; ER estrogen receptor; PR progesterone receptor; HER2 human epidermal growth factor receptor 2.

	Both flTF+/FVII+		flTF-/FVII+		flTF+/FVII-		Both flTF-/FVII-		
	N	%	N	%	N	%	N	%	p-value
Total	**110**	**100**	**14**	**100**	**91**	**100**	**87**	**100**	
Age									
<40	13	11.8	0	0.0	3	3.3	11	12.6	
40-55	39	35.5	5	35.7	37	40.7	36	41.4	0.264
55-65	25	22.7	5	35.7	17	18.7	15	17.2	
>=65	33	30.0	4	28.6	34	37.4	25	28.7	
Missing	0	0.0	0	0.0	0	0.0	0	0.0	
Grade									
I	8	7.3	1	7.1	12	13.2	17	19.5	
II	43	39.0	9	64.3	51	56.0	49	56.3	0.001
III	56	51.0	4	28.6	28	30.8	19	21.8	
Missing	3	2.7	0	0.0	0	0.0	2	2.3	
Histological type									
Ductal	103	93.6	12	85.7	81	89.0	75	86.2	0.142
Lobular	4	3.6	2	14.3	10	11.0	10	11.5	
Missing	3	2.7	0	0.0	0	0.0	2	2.3	
T-status									
T1	34	30.9	3	21.4	35	38.5	40	46.0	0.04
T2	62	56.4	10	71.4	35	38.5	37	42.5	1
T3/4	13	11.8	1	7.1	17	18.7	8	9.2	
Missing	1	0.9	0	0.0	4	4.4	2	2.3	
N-status									
N0	57	51.8	8	57.1	49	53.8	55	63.2	0.741
N1-3	49	44.5	6	42.9	41	45.1	31	35.6	
Missing	4	3.6	0	0.0	1	1.1	1	1.2	
ER-status									
Negative	52	47.2	8	57.1	27	29.7	28	32.2	0.018
Positive	57	51.8	6	42.9	64	70.3	57	65.5	
Missing	1	0.9	0	0.0	0	0.0	2	2.3	
PgR-status									
Negative	54	49.1	9	64.3	32	35.2	25	28.7	
Positive	55	50.0	5	35.7	57	62.6	58	66.7	0.010
Missing	1	0.9	0	0.0	2	2.2	4	4.6	
Her2-status									
expression-	81	73.6	9	64.3	69	75.8	65	74.7	0.050
expression+	14	12.7	4	28.6	8	8.8	4	4.6	
Missing	15	13.6	1	7.1	14	15.4	18	20.7	

Table2. Associations between clinico-pathological parameters and combined expression of FVII and flTF. Abbreviations N number of patients; ER estrogen receptor; PR progesterone receptor; HER2 human epidermal growth factor receptor 2.

Characteristic		Relapse Free Survival							Relative Survival					
		Univariate analysis			Multivariable analysis				Univariate analysis			Multivariable analysis		
	N	HR	95% CI	P	HR	95% CI	P		RER	95% CI	P	RER	95% CI	P
Age														
<40	48	1.00							1.00					
40-50	145	0.97	0.612-1.539	0.422					0.95	0.593-1.527	0.252			
50-60	132	1.17	0.734-1.853						1.32	0.825-2.104				
>60	249	0.90	0.574-1.408						1.28	0.779-2.095				
Grade														
I	80	1.00			1.00				1.00			1.00		
II	282	1.43	0.945-2.172	0.001	1.49	0.728-3.058	0.184		1.57	0.865-2.851	0.011	1.14	0.460-2.811	0.071
III	203	2.02	1.326-3.078		1.88	0.896-3.938			2.22	1.221-4.043		1.91	0.778-4.677	
Histological type														
Ductal	513	1.00		0.291					1.00		0.095	1.00		0.158
Other	53	1.24	0.832-1.846						1.48	0.933-2.348		1.77	0.800-3.936	
Tumor stage														
pT1	211	1.00			1.00				1.00			1.00		
pT2	272	1.59	1.205-2.093	<0.001	1.14	0.763-1.715	0.290		2.46	1.670-3.632	<0.001	1.15	0.680-1.932	0.200
pT3/4	72	2.49	1.706-3.635		1.55	0.896-2.675			4.41	2.782-6.988		1.77	0.923-3.402	
Nodal stage														
Negative	307	1.00		<0.001	1.00		<0.001		1.00		<0.001	1.00		0.001
Positive	250	3.06	2.379-3.945		2.53	1.759-3.629			3.18	2.285-4.416		2.29	1.431-3.663	
ER status														
Negative	203	1.00		0.725					1.00		0.105			
Positive	337	1.05	0.808-1.359						0.77	0.599-1.056				
PgR status														
Negative	223	1.00		0.744					1.00		0.152			
Positive	313	0.96	0.743-1.236						0.79	0.577-1.089				
HER2 status														
Negative	378	1.00		0.401					1.00		0.152			
Positive	44	1.21	0.776-1.883						1.43	0.877-2.324				
TF:FVII														
Either or both low	192	1.00		0.006	1.00		0.014		1.00		0.051	1.00		0.018
Both high	110	1.59	1.141-2.218		1.55	1.095-2.205			1.55	0.998-2.409		1.68	1.092-2.575	

Table 3.Cox univariate and multivariate analysis for recurrence free period and relative survival for TF:FVII. Abbreviations N number of patients; HR hazard ratio; RER relative excess risk; 95%CI 95% Confidence Interval; ER estrogen receptor; PR progesterone receptor; HER2 human epidermal growth factor receptor 2.