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The Role of PAR1 Autoantibodies in Patients with Primary Epithelial Ovarian Cancer

KATRIN KREIENBRING[1], ANNIKA FRANZ[1], ROLF RICHTER[1], DUSKA DRAGUN[2], HARALD HEIDECKE[3], DOMINIK MÜLLER[4], MONIKA MENTZE[1], RALF DECHEND[4], JALID SEHOULI[1] and ELENA IOANA BRAICU[1]

Departments of [1]Gynecology, and [2]Nephrology and Critical Care Medicine, Charitè University Berlin, Berlin, Germany;
[3]CellTrend, Luckenwalde, Germany;
[4]Experimental and Clinical Research Center, Berlin, Germany

Abstract. *Aim: This study aimed to analyze the predictive, prognostic and diagnostic value of autoantibodies to coagulation factor II thrombin receptor (F2R; protease-activated receptor 1, PAR1) (PAR1-AB) in patients with primary epithelial ovarian cancer (EOC). Materials and Methods: A total of 197 patients with primary EOC and 200 healthy female blood donors were included in the study. Enzyme-linked immunosorbent assay was applied to determine PAR1-AB levels in blood sera taken preoperatively. Correlation of PAR1-AB with clinicopathological outcome, progression-free (PFS) and overall (OS) survival was analyzed and patients were compared with controls. Results: PAR1-AB was significantly negatively correlated with histological grading (p=0.008) and was significantly lower in the patient group compared to healthy controls (p<0.001). There was no significant correlation of PAR1-AB level with PFS or OS. Conclusion: This study showed PAR1-AB to significantly decrease in patients with primary EOC and with histological high-grade carcinoma. The relevance of PAR1-AB in early detection of ovarian cancer and follow-up for EOC should be further investigated.*

With a 5-year-survival of 92% in patients with early Fédération Internationale de Gynécologie et d'Obstétrique (FIGO) stages to 6% in those with advanced-stage disease, epithelial ovarian cancer (EOC) is the most lethal disease among gynaecological cancers. However, only about 15% of all cases are diagnosed in FIGO stage I (1). The rapid lymphatic and peritoneal dissemination combined with late and non-specific symptoms makes the development of sufficient screening methods even more important. Currently, there are no early detection methods, such as transvaginal ultrasound or cancer antigen 125 (CA-125) level, which have been shown to be clinically useful. Standard-of-care for EOC is surgery followed by platinum taxane-based chemotherapy. Even though only 20% of EOCs are resistant to chemotherapy, more than 60% of patients will experience relapse (2). This is based on the advanced FIGO stage at diagnosis and molecular complexity of EOC that leads to development of chemotherapy-resistant clones. Additional approaches are targeted therapies which seem to improve progression-free survival (PFS), but unfortunately have no impact on overall survival (OS) (3). Therefore, the knowledge of EOC biology, for example signal transduction pathways, tumour-suppressor genes and proliferation factors, is essential. Simultaneously powerful predictive markers to identify specific targeted therapies for patients are needed. Random tumour markers and markers that have been found to be up-regulated in EOC are a focus of research.

Many proteases that are secreted or produced during tissue damage are able to activate PARs. Coagulation factor II thrombin receptor F2R [protease-activated receptor 1 (PAR1)] is a G-protein-coupled receptor (4) found to be overexpressed in various epithelial neoplasms, such as laryngeal (5), pancreatic (6), renal cell (7), prostatic (8), breast (9-11), colonic (12, 13), lung (14) and ovarian cancer (15). PAR1 is also known to be expressed in acute lymphoblastic leukaemia (16), as well as malignant melanoma, and correlates with its metastatic activity (17) and angiogenesis (18). Wojtukiewicz *et al.* were the first to identify this receptor on rat sarcoma and mice melanoma cells in 1995 (19).

Many proteases that are secreted or produced during tissue damage are able to activate PARs. The tumour microenvironment is replete of such proteases, while tumour cells themselves secrete PAR1 on their surface. Such proteolytic enzymes are metalloproteinases (MMP) such as MMP1, which is a collagenase and agonist of PAR1. The

Correspondence to: Katrin Kreienbring, Charité Virchow Klinikum, Frauenklinik, TOC-Labor, Augustenburger Platz 1, 13353 Berlin, Germany. Tel: +49 1775501655, e-mail: katrin.kreienbring@gmx.de

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proteolytic properties of MMP1 can reduce adhesion between cells and facilitate cell motility. This is suggested to explain the association of PAR1 with increased metastasis (20).

The most frequent agonists of PAR1 are thrombin and trypsin (21), as well as plasmin (22), factor Xa (23) and activated protein C (24). Recently our group showed that regulators of the plasminogen activator system, plasminogen activator inhibitor type 1 (PAI1) and PAI-mRNA binding protein type 1 (PAI-RBP1), are predictive markers of outcome in EOC (25). Many carcinomas are known to secrete thrombin, including EOC (26). Binding of thrombin to PAR1 (PAR1-thrombin) has been shown to release growth factors in EOC (27), which promote angiogenesis, pro-survival signalling and metastasis, lead to poor survival (28) and prevent apoptosis in patients with EOC (29). In a study by Karabulut et al., no predictive or prognostic value of PAR1 was found in a group of 44 patients with EOC but it was significantly up-regulated in the serum of the patient group compared with controls (p=0.03) (30).

There is increasing evidence that autoantibodies against PAR1 (PAR1-AB) inhibit PAR1 and thereby reduce migration and invasion of cancer cells. Shi *et al*. showed that binding between PAR1-AB and PAR1 averts cleavage and activation of the receptor by MMP1 in HER2-negative breast cancer tissue and reduces velocity of the affected cells, as well as the number of invading cells (31). In another study, Shi *et al*. showed that blocking PAR-1 with PAR1-AB and a monoclonal antibody against the thrombin-binding region on human metastatic melanoma cells significantly inhibited migration (32). As those studies suggest PAR1-AB products to be potential anticancer drugs, the intention of this study was to analyse the level of PAR1-AB in patients with EOC and examine its clinical significance. We anticipated decreased PAR-1 AB level in patients compared with healthy controls and patients with advanced disease.

Materials and Methods

Patients. The serum samples of 197 patients with primary EOC, median age at diagnosis was 60 years (range=28-92 years), were taken before cytoreductive surgery. These were collected between 2000 and 2011 and the data of the patients was updated by the Tumor Bank of Ovarian Cancer (TOC) (http://www.toc-network.de/). TOC is a multicentre project that started in 2000 at the Department of Gynaecology at Campus Virchow Clinics Berlin, Germany. For more than 15 years, the clinics have specialised in multimodal therapy of ovarian cancer. The follow-up ended 2013 and was a mean of 44.8 months.

For comparison, serum samples from 200 healthy asymptomatic women with a median age of 57.5 years (range=42-83 years) were collected at the University Medical Center Göttingen, and processed using the same protocol.

The serum levels of PAR1-AB were assayed by CellTrend GmbH (http://www.celltrend.de/) using an enzyme-linked immunosorbent assay (ELISA).

PAR1-AB ELISA. PAR1-ABs were measured in serum samples using a sandwich ELISA kit (CellTrend GmbH, Luckenwalde, Brandenburg, Germany). The microtitre 96-well plates were coated with chemically synthesized human PAR1 isoform 1 (SEQ ID NO:1, Figure 1) (CellTrend GmbH, Luckenwalde, Brandenburg, Germany). To maintain the conformational epitopes of the receptor, 1 mM calcium chloride was added to every buffer. Duplicate samples of a 1:100 serum dilution were incubated at 4°C for 2 hours. After washing steps, plates were incubated for 60 minutes with a 1:20.000 dilution of horseradish-peroxidase–labeled goat anti-human IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) used for detection. In order to obtain a standard curve plates were incubated with test sera from a PAR1-AB-positive index patient. The ELISA was validated according to the FDA's "Guidance for Industry: Bioanalytical Method Validation" (33). To set a standard for the concentration of PAR1-AB, a standard curve was generated. A serum sample of a patient with systemic sclerosis was diluted 1:100, 1:200, 1:400, 1:800 and 1:1,600 for standards of 40, 20,10, 5 and 2.5 Units/ml, respectively. The optical density was then determined. Each standard was determined in duplicates.

Statistical methods. For statistical evaluation, IBM SPSS Statistics ver. 22 (IBM Corp., Armonk, NY, USA) was used. Mann–Whitney U-test and Kruskal–Wallis test were applied for the association of PAR1-AB level with clinical and pathological factors, PFS and OS, patients and healthy controls. The α-level was assumed at less than 0.05 and all tests were two-sided. The survival distribution was assessed with Kaplan–Meier estimator and the equality of survival distribution for the different serum levels was tested with log-rank test (Mantel–Cox). Any clinically or histologically confirmed cancer recurrence was defined as an event for the calculation of PFS. The duration of OS was defined as the interval between the date of diagnosis and the death of the patient.

Ethics statement. The Ethical Committee of Charité Medical University, Berlin (no. 207/2003) and University Medical Center Göttingen (No. EK 22/2/04) grated ethical approval for these investigations. The patients gave their written informed consent to participation before enrolment and sample collection.

Results

In this study, serum samples of 197 patients with primary EOC were analysed for PAR1-AB. Table I shows patient characteristics and distribution of their age, FIGO stage, histology, clinical factors and response to platinum-based chemotherapy. Nearly 92% of patients already had an advanced tumour disease (FIGO III/IV) at diagnosis. Two patients (1.02%) were treated with neoadjuvant chemotherapy. After primary surgery, 35 patients (17.8%) needed completion surgery and two patients (1.02%) interval debulking after chemotherapy. Chemotherapy was administered to 170 patients (86.3%) over six cycles on average (range=1-16). Most of the patients received paclitaxel and carboplatin (N=154; 78.2%), 11 (5.6%) of them were treated with other platinum-containing combinations and five patients (2.5%) received immunotherapy. Platinum sensitivity was defined according to Gyneacologic Cancer InterGroup criteria (34) as

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1    MGPRRLLLVA ACFSLCGPLL SARTRARRPE SKATNATLDP RSFLLRNPND
51   KYEPFWEDEE KNESGLTEYR LVSINKSSPL QKQLPAFISE DASGYLTSSW
101  LTLFVPSVYT GVFVVSLPLN IMAIVVFILK MKVKKPAVVY MLHLATADVL
151  FVSVLPFKIS YYFSGSDWQF GSELCRFVTA AFYCNMYASI LLMTVISIDR
201  FLAVVYPMQS LSWRTLGRAS FTCLAIWALA IAGVVPLLLK EQTIQVPGLN
251  ITTCHDVLNE TLLEGYYAYY FSAFSAVFFF VPLIISTVCY VSIIRCLSSS
301  AVANRSKKSR ALFLSAAVFC IFIICFGPTN VLLIVHYSFL SHTSTTEAAY
351  FAYLLCVCVS SISCCIDPLI YYYASSECQR YVYSILCCKE SSDPSSYNSS
401  GQLMASKMDT CSSNLNNSIY KKLLT
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Figure 1. *Amino acid sequence of the human protease-activated receptor-1 (PAR1) (isoform 1) [SEQ ID NO:1].*

no relapse within 6 months of platinum-based chemotherapy. After surgery eight patients (4.1%) died from complications and 128 patients (65%) had died by the end of the follow-up.

The mean and median serum levels of PAR1-AB in the patient and control groups are presented in Table II. Serum PAR1-AB level was significantly negatively correlated ($p<0.001$) with the presence of primary EOC (Figure 2).

The correlation of serum PAR1-AB with clinicopathological factors is shown in Table III. PAR1-AB was significantly inversely correlated with histological grading ($p<0.007$) but with none of the other listed factors. The median PAR1-AB of the low-grade group was 2.2 U/ml compared with 1.0 U/ml for the high-grade group (Figure 3).

The mean PFS of the whole patient group was 20.7 months (range=0-114 months), with a median of 14 months. The OS of the patient group was a mean of 34.9 months (range=0.5-114 months with a median of 40 months. The patients were classified into three groups using the upper and lower quartiles of PAR1-AB distribution; PFS and OS were analysed according to these PAR1-AB groups (Table IV). Log-rank tests showed no significant relation of OS ($p=0.110$) or PFS ($p=0.148$) with PAR1-AB level (Figures 4 and 5).

Discussion

This investigation found the serum level of PAR1-AB to be significantly lower in patients with EOC *versus* healthy controls. Furthermore, PAR1-AB was significantly inversely correlated with histological grading. The prognosis of patients with EOC seems not to be predictable by PAR1-AB level.

It is suggested that serum antibodies are more reliable tumour markers than antigens because of their less inconsistent and more distinct serum levels, especially in early stages of carcinoma (35). In this study, the serum

Table I. *Patient clinical and pathological characteristics (n=197).*

Parameter	Value
Age at diagnosis, years	
Median (range)	60 (28-92)
Follow up period, months	
Mean (range)	44.8 (3-114)
Histology, n (%)	
Serous	183 (92.9)
Endometrioid	1 (0.5)
Clear cell tumour	1 (0.5)
Mixed	4 (2.0)
Other	8 (4.1)
FIGO, n (%)	
I	5 (2.5)
II	11 (5.6)
III	135 (68.5)
IV	46 (23.4)
Histological grading, n (%)	
I	8 (4.1)
II	49 (24.9)
III	140 (71.0)
Volume of ascites, n (%)	
None	47 (23.9)
<500 ml	77 (39.1)
>500 ml	73 (37.0)
Peritoneal carcinomatosis, n (%)	
Present	174 (88.3)
Absent	23 (11.7)
Residual tumour mass, n (%)	
None	105 (53.3)
<0.5 cm	34 (17.3)
<1 cm	30 (15.2)
1-2 cm	6 (3.0)
>2 cm	22 (11.2)
Response to platinum-based chemotherapy, n (%)	
Sensitive	116 (58.9)
Resistant	55 (27.9)
No such therapy	26 (13.2)

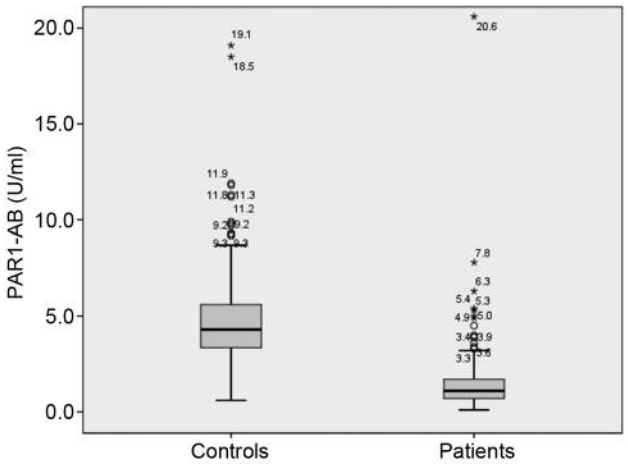

Figure 2. *Serum protease-activated receptor-1 antibody (PAR1-AB) level in patients with epithelial ovarian cancer and controls. The level in patients was significantly lower (p<0.001).* □ *Interquartile range (25th-75th percentile); – median;* ⊥ - ┬ *min-max except outliers;* ○ *outliers (>1.5 times interquartile ranges); *extreme outliers (>3 times interquartile ranges).*

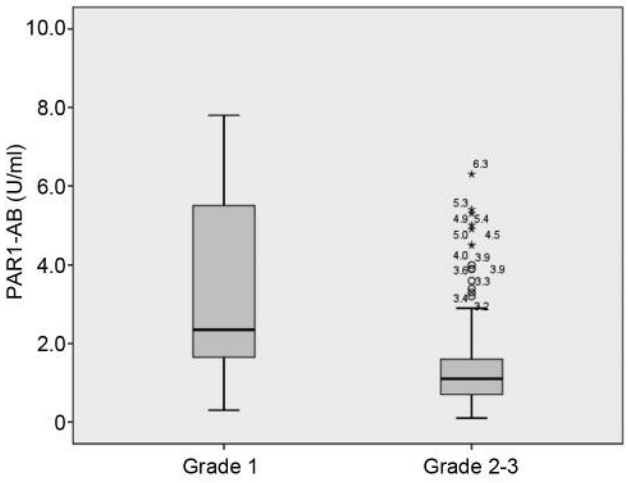

Figure 3. *Serum protease-activated receptor-1 antibody (PAR1-AB) level in patients with epithelial ovarian cancer according to histological grading. The level of PAR1-AB was significantly lower in patients with grade 2 or 3 disease (p=0.007).* □ *Interquartile range (25th-75th percentile); – median;* ⊥ - ┬ *min-max except outliers;* ○ *outliers (>1.5 times interquartile ranges); *extreme outliers (>3 times interquartile ranges).*

Table II. *Mean and median serum protease-activated receptor-1 antibody (PAR1-AB) levels in patients and controls.*

	Patients (N=197)		Controls (N=200)		
	Mean (range)	Median	Mean	Median	*p*-Value
PAR1-AB, U/ml	1.508 (0.1-20.6)	1.1	4.989 (0.6-36.2)	4.3	<0.001

PAR1-AB level has lower levels in patients with EOC than in healthy controls. These results imply that unbound antibodies seem to decrease with the development of EOC, while expression of the receptor increases on the cell surface (15, 20) and in the blood (30).

To the best of our knowledge, there has been no study that analysed PAR1-AB concentrations in the serum of patients with ovarian cancer. There is one publication by Karabulut *et al.* which investigated PAR1 level in serum samples of patients with EOC by ELISA in 2014. The study encompassed 44 patients and 25 controls. The serum level of PAR1 was significantly higher in the patient group (p=0.03) (30). Studies that measured PAR1 expression in cancer tissue also showed an increased level compared with normal ovarian tissue (15, 20). Grisaru-Granovsky *et al.* isolated RNA from 51 paraffin blocks of invasive ovarian cancer, 11 of low invasive potential and 26 of normal ovarian tissue, and used polymerase chain reaction, *in situ* hybridization and immunohistochemical localization for the PAR1 protein. They showed that there was no expression of the receptor on normal ovarian tissue, while it was overexpressed in all samples of low invasive and carcinoma tissue. Furthermore, they described PAR1 as an activator of the major focal contact protein focal adhesion kinase (FAK) (15). As well as enabling integrin signalling, FAK is known to control cell spreading, survival and migration (36), which increase ovarian cancer malignancy. In another study, Wang *et al.* identified the MMP1–PAR1 axis as a mediator of EOC invasiveness and a potential therapeutic target.in 96 ovarian cancer tissues (20). Agarwal *et al.* showed that giving PAR1 pepducins intraperitoneally over 6 weeks reduced ascites and angiogenesis in mice with ovarian serous carcinoma and prolonged survival significantly (37).

In other types of cancer, the serum levels of PAR1 vary. Tas *et al.* showed no effect on the receptor level in blood in patients with cutaneous melanoma (38) even though increased expression in melanoma cells has been reported (39). In gastric cancer, there was no effect on serum PAR1 level (40), while it was overexpressed in cancer tissue (41). In addition, Eturk *et al.* showed that the serum PAR1 level was significantly higher in a group of 80 patients with lung cancer than control group (p<0.001) (42).

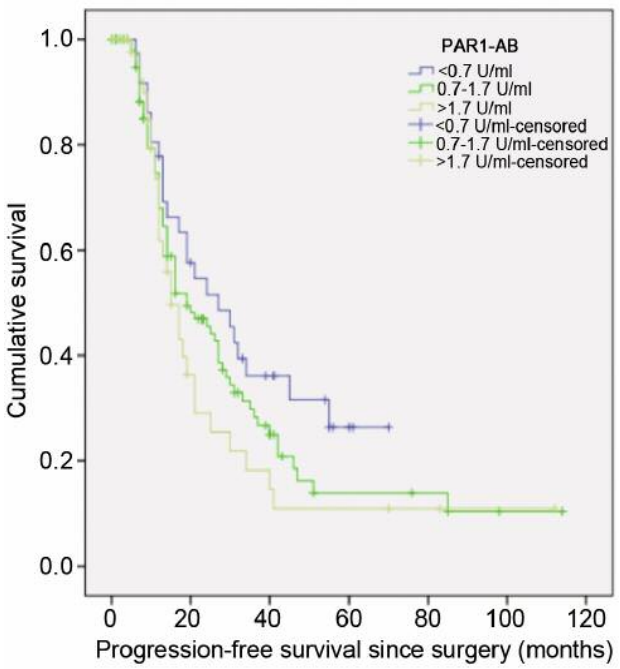

Figure 4. *Progression-free survival curves for patients with epithelial ovarian cancer according to serum protease-activated receptor-1 antibody (PAR1-AB) level (p=0.148).*

Table III. *Median (range) serum protease-activated receptor-1 antibody (PAR1-AB) levels according to clinicopathological parameters.*

Clinicopathological parameter	N	PAR1-AB, U/ml	*p*-Value
Ascites			
None	47	1.3 (0.2-20.6)	0.177[a]
<500 ml	77	1.1 (0.1-4.5)	
>500 ml	73	0.9 (0.2-4.9)	
FIGO classification			
I	5	1.6 (0.6-2.3)	0.288[a]
II	11	1.0 (0.3-7.8)	
III	135	1.1 (0.1-20.6)	
IV	46	1.45 (0.2-6.3)	
Age at diagnosis			
<50 Years	38	1.1 (0.4-20.6)	0.464[a]
50-65 Years	100	1.1 (0.1-5.4)	
>65 Years	59	1.1 (0.1-4.5)	
Residual tumour mass after surgery			
None	105	1.1 (0.1-20.6)	0.973[b]
Present	92	1.05 (0.1-6.3)	
Histological grade			
I	8	2.35 (0.3-20.6)	0.008[a]
II	49	1.0 (0.1-5.3)	
III	140	1.1 (0.1-6.3)	
Histopathology			
Serous	183	1.1 (0.1-20.6)	0.325[b]
Non-serous	14	0.9 (0.2-5.0)	
Platinum response			
Sensitive	108	1.05 (0.1-20.6)	0.608[b]
Resistant	50	1.1 (0.2-4.9)	

Statistical analysis: [a]Kruskal–Wallis test; [b]Mann–Whitney *U*-test.

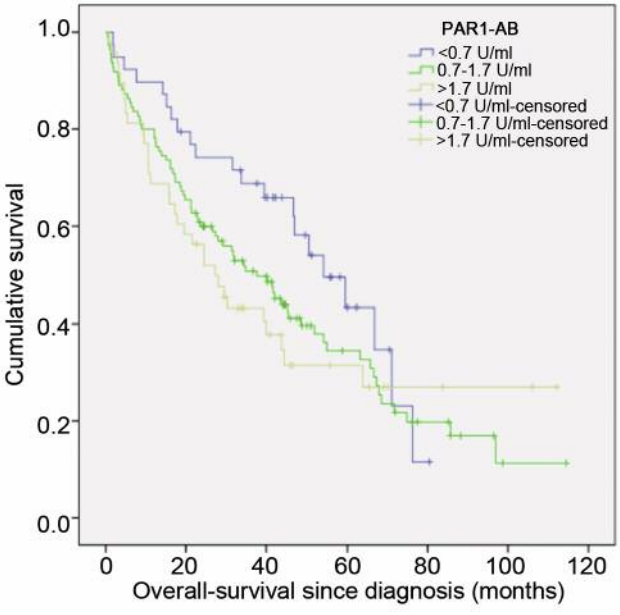

Figure 5. *Overall survival curves for patients with epithelial ovarian cancer according to serum protease-activated receptor-1 antibody (PAR1-AB) level (p=0.110).*

The decrease found here in autoantibodies against PAR1 in patients with ovarian cancer suggests any diagnostic relevance seems improbable. Nevertheless, there is evidence that targeting PAR1 might be a promising treatment strategy. Recently Zhong *et al*. showed that doxycycline is a direct inhibitor of PAR1-thrombin activation, with subsequent down-regulation of tumour cell migration and tumour growth (43). Furthermore Agarwal *et al*. showed inhibition of angiogenesis, ascites formation and metastasis in xenograft models of peritoneal EOC by giving intracellular pepducins to disturb PAR1–MMP signalling systems (37). Such findings may open a new way for future targeted chemotherapy in patients with EOC.

Conclusion

In the current study, autoantibodies directed against PAR1 were found at significantly lower levels in patients with EOC and particularly in those with histological high-grade carcinoma. There was no predictive or prognostic value in the survival analyses. Further studies with focus on the diagnostic value of the PAR1 thrombin receptor in a prospective cohort are needed.

Table IV. *Overall (OS) and progression-free (PFS) survival according to protease-activated receptor-1 antibodies (PAR1-AB) expression.*

		PFS		OS	
PAR1-AB, U/ml	Total patients, n (%)	Mean/median, months	Progression (censored), n (%)	Mean/median, months	Deaths (censored), n (%)
<0.7	39 (19.8)	34.6/27	24 (15)	50/54.1	21 (18)
0.7 –1.7	110 (55.8)	32.8/19	68 (42)	45.6/37.7	75 (35)
>1.7	48 (24.4)	27.8/15	29 (19)	45.8/40.3	32 (16)
Overall	197 (100)	35.0/19	121 (76)	47.3/40.3	128 (69)

Targeting PAR1-thrombin and MMP-1 axis is being investigated and might improve survival in patients with ovarian cancer.

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