

## Clinical impact of PAI 1 4G/5G gene polymorphism in colorectal carcinoma patients

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Plasminogen activator inhibitor (PAI 1) belongs to the plasminogen activator system, which is part of the metastatic cascade and significantly contributes to invasive growth and angiogenesis of malignant tumors. Its plasma level is normally low but 4G/4G homozygotes have higher concentrations of PAI 1. This genotype may be associated with worse prognosis and proximal location of colorectal cancer than 5G/5G homozygotes. In our prospective evaluation we examined plasma level PAI 1 (using photometric microplate method ELISA) pre-surgery and, subsequently, 6-8 weeks later, from 80 patients. For the PAI 1 rs1799889 -675 4G/5G polymorphism test the PCR amplification was used.

Analysis of collected data was confirmed that significantly higher plasma levels of PAI 1 were found in patients before starting therapy, which decreased ( $p=0.004$ ) after initiation of treatment. Patients with higher plasma level PAI 1 before ( $p=0.013$ ) and after therapy ( $p=0.004$ ) had significantly shorter survival. We found no relationship between polymorphisms of PAI 1 (-675 4G/5G) in relation to stage, survival or tumor location. PAI 1 is useful as a negative marker of prognosis and could be advantageous when planning adjuvant treatment of patients with colorectal carcinoma. Although opinions on the importance of polymorphisms of PAI 1 in relation to the prognosis are not uniform, it does seem that their role in the prognosis of patients with colorectal cancer is not essential.

*Key words: plasminogen activator system, gastrointestinal cancer, plasminogen activator inhibitor 1, -675 4G/5G gene polymorphism*

Fibrinolysis is a process, which leads to the degradation of fibrin to fibrin monomers and helps to regulate hemostasis and prevents the creation of an inappropriately large thrombus, which could reduce blood flow in the bloodstream. The main enzyme involved in fibrinolysis is plasmin, which is produced by the liver in inactive form as plasminogen, its half-life is about 2 days, but depending on the activation of the fibrinolytic system, may be significantly shorter [1]. Tissue plasminogen activator (tPA) and urokinase (uPA) are agents converting plasminogen into active plasmin. Tissue plasminogen activator (tPA) together with its receptor (uPAR), tissue activator (tPA,

which is synthesized from proenzymes pro-tPA or sc-tPA) and urokinase inhibitors (PAI 1, PAI 2, and PAI 3 – which is identical to the protein C inhibitor and protease nexin) [1] forms the plasminogen activator system (PAS), which is part of the metastatic cascade and significantly contributes to invasive growth and angiogenesis of malignant tumors [2]. PAI 1 and PAI 2, both belonging to the serpin family (serine protease inhibitor super-family), are its most important specific inhibitors [3].

PAI 1 is a single-chained glycoprotein consisting of 379 amino acids and is predominantly found in vascular smooth

muscle, in megakaryocytes, endothelium, granulocytes, monocytes, macrophages and tumor cells. It is a serpin, present in plasma and  $\alpha$ -granules of platelets and has the ability to bind to vitronectin [4-6]. Complex vitronectin PAI 1 has a longer elimination half-life and inhibits the migration of smooth muscle tissue cells by blocking the bond of  $\alpha_v\beta_3$  integrin to vitronectin [7, 8]. Apart from  $\alpha_v\beta_3$  integrin, it also binds to other groups of integrins, including  $\alpha_v\beta_1$ ,  $\alpha_v\beta_5$ ,  $\alpha_{IIb}\beta_3$  and  $\alpha_8\beta_1$  [9]. The interaction of plasminogen activator inhibitor 1 with the uPA/uPAR complex leads to internalization of the triplet, which stimulates cell proliferation [10]. PAI 1 is the most effective inhibitor of uPA and tPA. The binding of PAI 1 to the uPA/uPAR complex leads to internalization of the complex through endocytic receptors low density lipoprotein (LDL) receptor family as a protein related to the LDL receptor (LDL receptor related protein – LRP). The binding of uPA to PAI 1 interrupts the normal PAI 1-vitronectin interaction and initiates conformational changes in plasminogen activator inhibitor 1, which allows the exposure of a hidden high affinity binding place on LRP [11]. PAI 1 plasma levels are normally low and high plasma levels are associated with various diseases, e.g. vascular diseases [8, 9] or malignant tumors.

An increased expression of plasminogen activator inhibitor 1 was found on malignant cells. PAI 1-positive malignant cells have been identified mainly in central parts of tumors, peripheries showed high expression of uPA together with signs of tissue destruction; this supports the theory that an inhibitor could protect tissue from the effects of uPA [3]. It has been shown that PAI 1 inhibits uPA-dependent invasiveness of the lung carcinoma, ovarian carcinoma, choriocarcinoma and fibrosarcoma cell lines [12-14]. Paradoxically, however, increasing PAI 1 expression in tumor tissue of oncology patients is associated with poorer prognosis. Therefore, its role in the entire process of tumor growth remains, for the time being, unclear [15, 16] and it is not known why the increasing level of proteolytic inhibitor should be associated with poorer prognosis. Nevertheless, studies with PAI 1-deficient transgenic mice showed that local invasion and tumor-associated angiogenesis are dependent on PAI 1 presence [17]. Therefore, what is PAI 1's involvement in tumor progression? The explanation could be as follows: uPAR induction results in a modification of integrin-mediated cell adhesion as the result of interaction with vitronectin. The binding of uPA to uPAR brings about increased affinity to vitronectin. It is, therefore, likely that a PAI 1 increase decreases cell adhesion to the extracellular matrix caused by interference with vitronectin-bound uPAR. This, consequently, facilitates cell invasion, migration, apoptosis and angiogenesis [8, 18-22].

PAI 1 is a glycoprotein whose gene is located on chromosome 7. Up to date at least 28 non-synonymous coding from totally 334 variants has been published [23]. The most commonly studied is a single nucleotide insertion/deletion of guanosine at position -675. 4G/4G genotype leads to increased gene transcription and thereby increases the plasminogen activator inhibitor 1 protein in plasma. A higher plasmatic

level of plasminogen activator inhibitor 1 is associated with reduced fibrinolytic activity. 4G/4G homozygotes have higher concentrations of PAI 1 than 5G/5G homozygotes. The level of PAI 1 in plasma of 4G/5G heterozygotes is intermediate. The presence of 4G/4G genotype in the promoter of the PAI 1 gene increases the risk of thrombosis. This polymorphism is also often associated with other diseases such as heart attack [24] or diabetes mellitus [25], and with other types of cancers such as endometrial cancer or breast cancer [26, 27]. The distribution of each variant is different in different populations, the 4G/4G genotype is more common among Asians (59%) [28] and Caucasian populations (51%) [29], Spaniards (47%) [30] and Indians (54%) [31], than for Mexicans (34%) [32] and African-Americans (24%) [33].

In patients with breast cancer and 4G/4G genotype, a greater probability of the involvement of lymph nodes has been shown, thus making prognosis worse [27]. Some published studies also suggest that the 4G/4G genotype may be associated with advanced colorectal cancer and 5G/5G genotype with less advanced tumors [34], while the 4G/4G genotype may be associated with proximal cancer of the colon [35].

There were also published articles, according to which the other polymorphism 1334G/A should be associated with a higher incidence of colorectal cancer, so far it has been tested only a small group of patients and these findings should be corroborated by others studies [36]. Generally, however, it were published a very few papers focused on the topic of colorectal cancer.

## Patients and methods

**Patients.** Prospective evaluation involved a total of 80 patients treated for adenocarcinoma of the colon or rectum at the Department of Clinical Oncology, University Hospital Brno, comprising of 55 men and 25 women, aged 39-80 years with a median of 62.5 years. The group consisted of 25 (31.3%) patients of TNM classification stage I, 19 (23.8%) stage II, 17 (21.3%) stage III patients and 19 (23.8%) stage IV patients. Sixteen patients died during the evaluation period, the death of 3 patients were not associated with the primary disease, 13 patients died due to the progression or relapse of the colorectal cancer. Of the total of 80 patients, there were 22 (27.5%) with cancer of colon descendens and 14 (17.5%) patients suffered from carcinoma of the colon ascendens, and 44 patients (55%) were diagnosed with adenocarcinoma of the rectum.

To simplify categorization, transverse carcinomas were evaluated according to their direct location: if the tumor was in the splenic flexure, it was assigned to the group of descending colon cancer, along with tumors of the sigmoid colon. If the tumor was located in the hepatic flexure, then it was placed in the group of ascending colon cancer. The main patient characteristics are summarized in Table 1.

For the project the tumor tissue of patients was used after resection for colorectal cancer (primary tumor or metastases) conducted in the Surgical Clinic of the University Hospital

Brno. Patients were included in the project after giving their informed consent. Participation in the study was represented only by a sampling of tumor tissue, which was not carried beyond the standard treatment and diagnostic procedures and collection of peripheral blood at the same time, also as part of standard tests. Patients, in addition to the above characteristics (plasminogen activator inhibitor 1 in peripheral blood before treatment and 6-8 weeks after initiation of therapy {for stages I and II after surgery, for stage III after the initiation adjuvant chemotherapy, for stage IV after start of palliative chemotherapy}, rs1799889 -675 4G/5G gene polymorphism of PAI 1) were assessed for age, gender, type, grade, TNM classification, Dukes classification, response to treatment (by RECIST criteria [37]), overall survival.

As the adjuvant chemotherapy, the protocol based on FUFA (5-Fluorouracil/Leucovorin) was administered. In the scope of palliative treatment only the effect the first line chemotherapy based on irinotecan or oxaliplatin, possibly in combination with bevacizumab was evaluated. During the monitoring 2 patients were treated with acetyl salicylic acid and 1 patient with low molecular weight heparin, no patient was treated with warfarin or other oral anticoagulation therapy.

**Material sampling and processing.** Peripheral blood for plasma PAI 1 analysis was sampled pre-surgery or, in TNM stage IV patients, before the initiation of chemotherapy and, subsequently, 6-8 weeks later. The evaluation of functional activity of PAI 1 was performed using the photometric microplate method on the ELISA reader Expert Plus (ASYS Hitech) with Spectrolyse/(pLPAI-1) set by Biopool (A Trinity Biotech Company). Plasma samples were prepared from blood samples collected into 1:10 diluted 0.109M sodium citrate and centrifuged for 15 minutes at 2500g in a cooled centrifuge (15°C) and stored at -70°C. The reference range of 0-10.0 AU/ml was used to assess PAI 1 plasma levels.

For the determination of gene polymorphisms of plasminogen activator inhibitor 1 rs1799889 -675 4G/5G a DNA sample from peripheral blood sample was isolated using a commercially available isolation kit (QIA Blood Mini Kit, Qiagen), allowing rapid isolation of DNA from small quantities of peripheral blood (200 µl) collected into collection tubes with K3 EDTA. For the 4G/5G polymorphism test the PCR amplification method was used followed by the cleavage product, using primers 5' CAC AGA GAG AGT CTG GCC ACG T-3' and 5' CCA ACA GAG GAC TCT TGG TCT 3' (East-Port Prague, Ltd., UK) and restriction enzyme BSL I (Biotech, R0555S). PCR was carried out in a thermal cycler (Mastercycler gradient, Eppendorf), each sample contained 0.5 µg genomic DNA, 15pmol of each primer, dNTP 100µmol (Promega U1240, U.S.), 5 Green GoTaq buffer and 1U GoTaq polymerase (Promega M3171, U.S.)

PCR was carried out in 35 cycles at temperatures of 95°C for 20s, 60°C for 25s and 20s at 72°C. Subsequently, amplicons were incubated with a volume of 20µl for at least 3 hours at 55°C with 1U of restriction enzyme BSL I (R0555S Biotech). PCR fragments were electrophoretically separated on 4.5%

**Table 1. Description of patients**

Age (mean, median (min - max))	62.5, 62.5 (39.0 - 80.0)
Men - N (%)	55 (68.8)
Follow-up median (min - max) - months	18.5 (3.0 - 42.0)
Stage (TNM classification)	N (%)
1	25 (31.3)
2	19 (23.8)
3	17 (21.3)
4	19 (23.8)
Stage (Dukes classification)	N (%)
A	25 (31.3)
B	19 (23.8)
C	17 (21.3)
Grade	N (%)
1	25 (31.3)
2	39 (48.8)
3	14 (17.5)
4	1 (1.3)
unknown	1 (1.3)
Gene polymorphism PAI 1 rs1799889 -675 4G/5G	N (%)
4G/4G	26 (32,5)
4G/5G	32 (40,0)
5G/5G	22 (27,5)

agarose gel (LE Agarose, Promega) containing ethidium bromide and visualized under UV light (Transilluminator, Vilber Lourmat TFX 20M). Each sample was classified into one of the three variants of possible genotypes 4G/4G, 4G/5G or 5G/5G.

**Clinical evaluation.** All patients were monitored as part of their routine follow-up; patients in complete remission were, after the post-surgery sampling, evaluated at 3-monthly intervals using imaging methods. Chemotherapy patients were evaluated at monthly intervals, using imaging methods every 3 months. Suspicion of progressive disease was verified by CT abdomen/thorax or chest X-ray. Treatment response was assessed according to RECIST criteria [37].

**Ethical aspects.** The study patients were enrolled after signing an informed consent form that was approved by the Ethical Committee of the University Hospital Brno.

**Statistical analysis.** Descriptive statistics (N, median, minimum and maximum, 5 and 95% percentile) were calculated for continuous variables and absolute and relative numbers of patients in each category for categorical variables. Contingency tables were tested with a Chi-square test and Fisher's exact test. A Paired t-test was employed to assess the difference between PAI 1 before and after treatment. The relationship between continuous variables was calculated using Spearman's Non-parametric Correlation. The association between categorical variables and survival was projected into the Kaplan-Meier survival curve and tested with the log-rank test. A P value of less than 0.05 was considered as statistically significant. The analysis was performed with the support of Statistica for Windows 8 and SPSS 17.

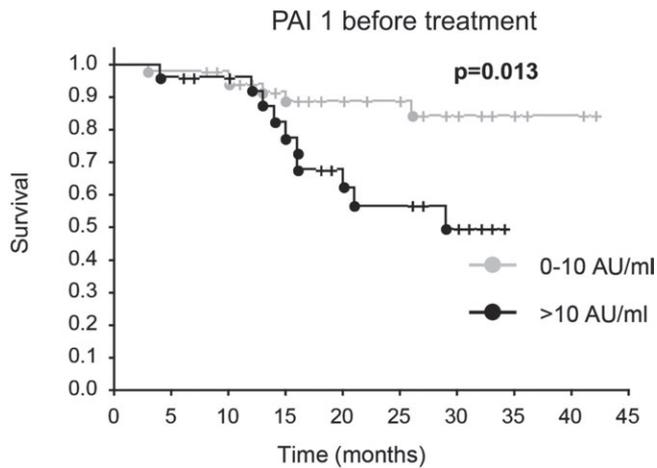


Fig. 1. Correlation between plasma level PAI 1 before therapy and survival

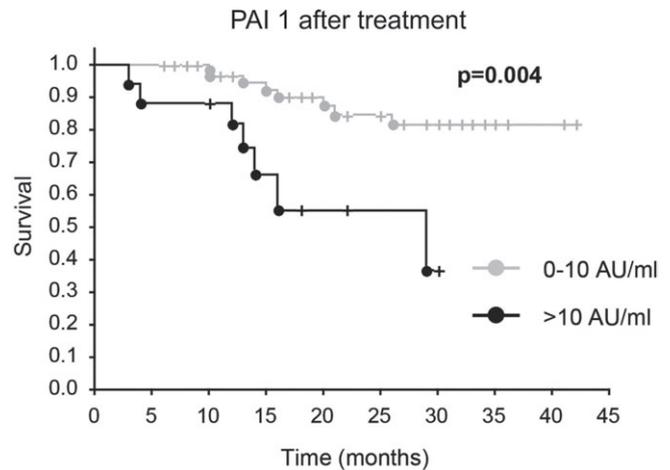


Fig. 2. Correlation between plasma level PAI 1 after initiation of therapy and survival

## Results

Tab. 2 shows that plasma PAI 1 levels decreased significantly after the operation or start of treatment. This difference in plasma PAI 1 levels before and after operation or treatment initiation was statistically significant ( $p=0.004$ ). It follows that the plasma levels of PAI 1 is affected by the surgery and initiation of treatment and after several weeks decreases. This could

Table 2. Relationship of plasma levels of PAI 1 before and after initiation of therapy

	PAI 1 (AU/ml)		Difference of PAI 1
	before therapy	after therapy	
N	80	80	80
Median	6.7	3.5	-2.8
Min - max	0.0 - 42.2	0.0 - 33.0	-31.4 - 25.4
Percentil 5 - 95%	0.0 - 31.1	0.0 - 23.8	-15.8 - 15.6
$p^1$			<b>0.004</b>

<sup>1</sup> Tested with paired t-test.

Table 3. Relationship between gene polymorphism PAI 1 -675 4G/5G and stage of disease

	Stage				$p^1$
	I	II	III	IV	
4G/4G genotype N (%)	8 (32.0)	3 (15.8)	7 (41.2)	8 (42.1)	0.321
4G/5G genotype N (%)	10 (40.0)	7 (36.8)	7 (41.2)	8 (42.1)	
5G/5G genotype N (%)	7 (28.0)	9 (47.4)	3 (17.6)	3 (15.8)	
$p^1$ (group vs. others)	0.997	0.057	0.516	0.350	

<sup>1</sup> Tested with maximum likelihood Chi-square test (more than two categories) or Fisher's exact test (two categories)

mean, that PAI 1 occurs in the organism in close association with the malignant process, and after its removal the level of PAI 1 in peripheral blood decreases.

Fig. 1 and fig. 2 also suggest that there is a statistically significant association between soluble PAI 1 levels and survival, i.e. that survival is significantly lower in patients with pathological plasma PAI 1 levels than in patients with plasma levels within the reference range. Statistically, survival was significantly shorter in patients with pathological PAI 1 plasma levels measured before the initiation of treatment as well as 6-8 weeks later [38].

Another part of our study was to evaluate the impact of genetic polymorphisms of plasminogen activator inhibitor 1 in relation to the stage of disease. Thus, as suggested by some authors, the 4G/4G genotype should be associated with more advanced colorectal cancers and 5G/5G genotype with less advanced colorectal cancer tumors. In our analysis, we did not find any statistical evidence to confirm these findings (Table 3).

We analyzed also whether individual polymorphisms may be associated with specific tumor location in the colon and rectum. The 4G/4G genotype should be, according to some publications, associated with proximal colon cancers [35].

Even in this case we have not found a statistically significant relationship (Table 4).

As shown in fig. 3. we did not find a relationship between the gene polymorphisms of PAI 1 and patient survival (4G/4G  $p=0.718$ ; 4G/5G  $p=0.928$ ; 5G/5G  $p=0.633$ ).

## Discussion

A number of studies explored in depth the importance of the individual plasminogen activator system elements for oncology patients' survival. An increased uPA, uPAR, PAI 1 and PAI 2 expression in tumor tissue was confirmed in a number of tumors, such as breast, ovarian, kidney and stomach [39-47] and it is certain that these factors play an important role in tumor invasiveness and metastasizing. Low PAI 1 expression in tumor tissue in early stages of the disease is in accordance with the theory that, by releasing tumor cells from the matrix, PAI 1 facilitates the invasion of tumors [48, 18].

uPA as well as PAI 1 expression in other tumors, e.g. in breast cancer, was perceived as an independent prognostic factor for shorter survival [49]. Knoop et al. demonstrated that, in these patients, PAI 1 itself is a predictor of distant rather than local relapse. This verifies the important role of PAS in the metastasizing of malignant tumors [50]. Even though Ito et al. [51] states that PAI 1-negative malignant stomach tumors show higher incidence of liver metastases and thus poorer prognosis.

At the beginning of the study we assumed the correlation between plasma levels of PAI 1 before treatment and its decrease 6-8 weeks after the start of therapy (after surgery or initiation of chemotherapy with or without radiotherapy). We have shown a statistically significant reduction in plasmatic PAI 1 levels after surgery or treatment initiation. This suggests a possible link between soluble PAI 1 levels and tumor viability and could serve as a marker of surgical procedure curativeness or, alternatively, tumor treatment effectiveness [52]. Our model of patients' survival associated with soluble PAI 1 levels, on a Kaplan-Meier curve, in line with the literature [53], showed statistically significantly lower survival of patients with pathological levels of soluble PAI 1 prior to treatment, even when PAI 1 values did not decline to the reference range after treatment initiation [38].

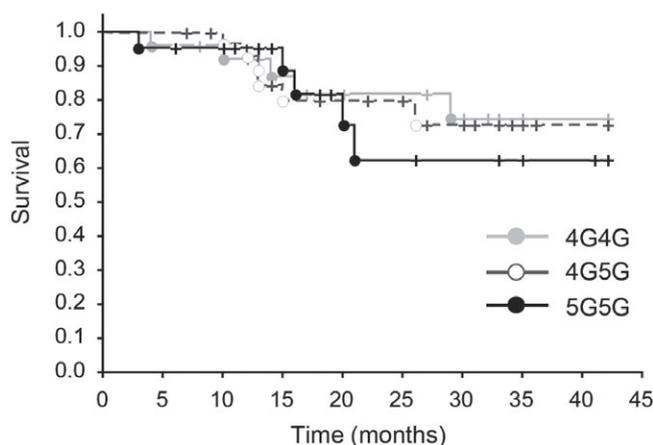


Fig. 3. Relationship between gene polymorphism 4G/4G PAI 1 and survival

In their group of patients Herszényi et al. demonstrated a relationship between patient survival and plasmatic PAI 1 levels as well as the importance of plasmatic PAI 1 in invasively growing colorectal carcinomas and the progression of colorectal non-cancerogenic lesions into carcinomas [54]. But in our study we show that a decrease of plasmatic PAI 1 level after surgery or treatment initiation is very important for prognosis.

In the other part of our analysis, we focused on the research of -675 4G/5G (rs1799889) polymorphisms of PAI 1 for patients with colorectal carcinoma. Castelló et al. and Zhang et al. in their group of patients showed a greater likelihood of lymph node involvement in patients with breast cancer with the 4G/4G genotype, and thus worse prognosis [27, 55]. Lei et al. on the contrary, in his research showed a worse prognosis in patients with 5G/5G genotype [56]. Neither of these hypotheses were confirmed by Blasiak et al. in his study [57]. The results of the current research can be regarded as ambiguous. This type of polymorphism has been studied in the context of tumor aggressiveness in patients with prostate cancer, where its relationship to prognosis was also not confirmed [58]. And results of study of Vossen et al. did not support an effect of PAI 1 4G/5G on colorectal cancer risk [59]. Palmirotta et al.

Table 4. Relationship between gene polymorphism PAI 1 -675 4G/5G and tumor location (Due to discrepancies in the source documents, 3 patients were not included in this analysis).

	Tumor location			p <sup>1</sup>
	colon ascendens	colon descendens	rectum	
4G/4G genotype N (%)	4 (28.6)	7 (35.0)	12 (27.9)	0.943
4G/5G genotype N (%)	5 (35.7)	8 (40.0)	19 (44.2)	
5G/5G genotype N (%)	5 (35.7)	5 (25.0)	12 (27.9)	
p <sup>1</sup> (group vs. others)	0.801	0.831	0.859	

<sup>1</sup> Tested with maximum likelihood Chi-square test (more than two categories) or Fisher's exact test (two categories)

in their research concluded that plasma PAI 1 levels in breast cancer patients could represent a useful prognostic variable for relapse, although PAI 1 polymorphism might not represent a genetic susceptibility factor [60]. Tee et al. showed no significant association between cervical neoplasia cases and gene polymorphisms of uPA, uPAR and PAI 1. However, they found a mutual interaction between uPA/PAI 1 genes, which women carrying the uPA/PAI 1 CC/4G4G allele had a 1.70-fold higher risk of cervical neoplasia compared with those carrying the CC/4G5G allele. Individuals with uPA/PAI 1 CC/4G5G allele were in high susceptibility for cervical neoplasia. The combined polymorphism of uPA/PAI 1 might diminish the ability of PAI 1 to inhibiting cervical cancer carcinogenesis when PAI 1 alone as the role of inhibitor [61]. Contrary to this study Su et al. and Gilabert-Estellés et al. published that patients with 4G/4G genotype were at significantly higher risk of endometrial cancer [62,63].

For colorectal cancer Försti et al. in their work demonstrates that the 4G/4G genotype is associated with shorter survival because it is linked more to Dukes C tumors, in contrast to the 5G/5G genotype, which is associated more with tumors stage A and B [34]. But in our group of patients we have not seen in either of the examined polymorphisms a significant relation to survival. In relation to the stages, the distribution of polymorphisms was entirely homogeneous and hence in this case a statistically significant difference wasn't proven. These results are consistent with other studies, Smolarz et al., in which the relationship of PAI 1 4G/5G polymorphism to the prognosis of patients with colorectal cancer, was not confirmed [64]. Nor did we prove the association of these polymorphisms to localization of colorectal cancer, as suggested by Loktionov et al. in his article (4G/4G genotype should be associated with proximal colon cancers) [35]. This is so far the only published work focused on monitoring the 4G/5G polymorphism in relation to the localization of colorectal carcinoma.

We have shown a statistically significant reduction in plas-matic PAI 1 levels after surgery or treatment initiation. Our model of patients' survival associated with soluble PAI 1 levels, on a Kaplan-Meier curve showed statistically significantly lower survival of patients with pathological levels of soluble PAI 1 prior to treatment, even when PAI 1 values did not decline to the reference range after treatment initiation. Plasma PAI 1 could be advantageous when planning adjuvant treatment of patients with colorectal carcinoma. Although opinions on the importance of polymorphisms of PAI 1 in relation to the prognosis of cancer are not uniform, it does seem, and our results confirm, that their role in the prognosis of patients with colorectal cancer is not essential but it must be admitted that the analysis based upon a small number of enrolled patients could potentially lead to false-negative results. Whatever the case, this issue deserves further exploration.

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