VILNIUS UNIVERSITY

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MATRIX METALLOPROTEINASES GENE EXPRESSION AND THEIR POLYMORPHISM IN BREAST AND PROSTATE CANCER

Summary of Doctoral Dissertation
Biomedical Sciences, Medicine (06B)

Vilnius, 2014

The study was carried out and doctoral dissertation was prepared during 2005–2014 at the National Cancer Institute (previous Institute of Oncology, Vilnius University).
The doctoral dissertation to be defended externally.

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The doctoral dissertation will be defended at the open session of the Medical Research Council on the 18th of December 2014, at 12.00 at National Cancer Institute Scientific Research Center, address: P. Baublio Str. 3b, Vilnius, Lithuania.

Summary of doctoral dissertation was sent on November 17, 2014.

The doctoral dissertation is available at Vilnius University Library and Vilnius University website: http://www.vu.lt/naujienos/ivykiu-kalendorius.

VILNIAUS UNIVERSITETAS

DIANA SCHVEIGERT

MATRIKSO METALOPROTEINAZIŲ RAIŠKA IR JŲ POLIMORFIZMAS SERGANT KRŪTIES IR PRIEŠINĖS LIAUKOS VĖŽIU

Daktaro disertacijos santrauka

Biomedicinos mokslai, Medicina (06B)

Vilnius, 2014

Moksliniai tyrimai atlikti ir disertacija rengta 2005–2014 metais Nacionaliniame vėžio institute (buvusiame Vilniaus universiteto Onkologijos institute).
Disertacija ginama eksternu.

Mokslinė konsultantė
prof. dr. Janina Didžiapetrienė (Nacionalinis vėžio institutas; biomedicinos mokslai, medicina – 06B)

Disertacija bus ginama Vilniaus universiteto Medicinos mokslo krypties taryboje:
Pirmininkas – prof. dr. Dainius Characiejus (Vilniaus universitetas; biomedicinos mokslai, medicina – 06B).
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prof. habil. dr. Zita Aušrelė Kučinskienė (Vilniaus universitetas; biomedicinos mokslai, medicina – 06B);
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Disertacija bus ginama viešame Medicinos mokslo krypties tarybos posėdyje 2014 m. gruodžio 18 d. 12.00 val. Nacionalinio vėžio instituto Mokslinių tyrimų centre, 113 auditorijoje. Adresas: P. Baublio 3b, Vilnius, Lietuva.
Disertacijos santrauka išsiuntinėta 2014 m. lapkričio 17 d.
Disertaciją galima peržiūrėti Vilniaus universiteto bibliotekoje ir VU interneto svetainėje adresu: http://www.vu.lt/naujienos/ivykiu-kalendorius.

ABBREVIATIONS

BPH – benign prostate hyperplasia

cDNA – complementary DNA

ER – oestrogens receptors

G1 – well-differentiated tumour

G2 – moderately differentiated tumour

G3 – poorly differentiated tumour

MMP – matrix metalloproteinase

N0 – no metastases in regional lymph nodes are present

N1 – mobile metastases are present in axillar lymph nodes of the same side

N2 – metastases in lymph nodes of the armpit of the same side, the nodes are stuck to each other and surrounding structures

PCR – polymerase chain reaction

PSA – prostate specific antigen

PR – progesterone receptors

RFLP – restriction fragment length polymorphism

RT-PCR – reverse transcription polymerase chain reaction

SNP – single nucleotide polymorphism

TNM – international cancer staging system

1. INTRODUCTION

Matrix metalloproteinases (MMP) are a family of zinc dependent proteolytic enzymes; these enzymes participate in tumour angiogenesis, processes of tumour growth and development of metastases; therefore, it is important to evaluate the changes of expression of these enzymes in patients with malignant tumours, in order to assess the prognosis of the patients with tumours of different localization. Formerly, it was considered that MMP were enzymes that act as stimulators of tumour growth and formation of metastases, only; therefore, it was thought, that blocking of the activity of these enzymes may result in reduction of tumour growth and spread. Consequently, wide range of synthetic MMP inhibitors was developed and clinically studied. The main targets of these inhibitors included MMP-2, MMP-3, MMP-7, MMP-9, MMP-11 and MMP-12. It should be noted, that the majority of the studies were discontinued early, because of absence of clinical effect and presence of marked adverse effects on study subjects. It was concluded that theoretical knowledge concerning expression of MMP in patients with malignant tumours of different localization and relationship of the expression with malignant process was insufficient. During the last several years, a lot of experimental and clinical data was collected, showing that increased activity of MMP may both stimulate and suppress the tumour process [Egeblad M. et al., 2002]. So, the individualization of treatment of a cancer patient requires evaluation of activity of particular MMP, as, in certain cases, MMP stimulates tumour process and may be a target of therapy by means of supressing the activity, in events of tumours of certain localization; on the other hand, in event of tumours of other localization, MMP supresses tumour process; therefore, the expression should not be inhibited [Deryugina E.I. et al., 2006]. As MMP take part in the process of tumour cell metastatic process, the assessment of expression of blood MMP may be a prognostic marker, showing the process of development of micro-metastases. However, no large multicentre studies, supporting the significance of these enzymes in correcting the treatment of the patients and assessing survival, enabling to predict the course of the disease, are performed, yet.

The changes of MMP expression are influenced by mutations of their gene promoter sequences. It should be noted, that single nucleotide polymorphism, detected in MMP gene promoter sequences and possibly influencing MMP expression is associated

with more aggressive tumour growth. The studies of single nucleotide polymorphism become increasingly significant for prognosis of the disease; however, the results of these studies are contradictory, still. For example, in some studies authors show relationship between MMP-9 (-1562 C/T) polymorphism and invasive breast tumour [Przybylowska K. et al., 2006]; in other studies this relationship is not demonstrated [McColgan P. et al., 2009]. So, it is important to evaluate what variants of polymorphism may influence the progression of the disease.

The analysis of studies performed revealed the trend showing that MMP expression and function depend on localization of the tumour. Systemic data concerning the changes of MMP expression regarding localization of the tumour, origin of the cells and rate of progression of the disease are still not present. Therefore, it is important to clarify changes of MMP expression in tumours of certain localization, to link these data with clinical and pathological characteristics of the patients and evaluate the changes regarding prognosis of the disease.

HYPOTHESES STATED

1. Matrix metalloproteinases (MMP) gene expression in cancer patients is associated with clinical and pathological characteristics of the disease.
2. MMP gene expression may be related with survival of cancer patients and it may be an independent prognostic factor of survival rates of cancer patients.
3. Single nucleotide polymorphism (SNP) occurring in promoters of MMP genes may be a factor of predicting breast and prostate cancer.

AIM OF THE STUDY

To assess the expression of matrix metalloproteinases (MMP) genes and single nucleotide polymorphism in promoter sequences of MMP genes in breast and prostate cancer and evaluate parameters investigated regarding prognosis.

TASKS OF THE STUDY

1. To assess matrix metalloproteinases (*MT1-MMP, MMP-1, MMP-2, MMP-3, MMP-9, MMP-11, MMP-13*) gene expression and single nucleotide polymorphism in their genes (MMP-3 -1171 5A/6A; MMP-9 -1562 C/T) in breast cancer patients and evaluate the parameters investigated regarding clinical and pathological characteristics of the disease.
2. To evaluate the relationship between matrix metalloproteinases (*MT1-MMP, MMP-1, MMP-2, MMP-3, MMP-9, MMP-11, MMP-13*) gene expression, single nucleotide polymorphism in these genes (MMP-3 -1171 5A/6A; MMP-9 -1562 C/T) and survival rate of breast cancer patients.
3. To assess matrix metalloproteinases (*MMP-9, MMP-11*) gene expression and single nucleotide polymorphism in their genes (MMP-9 -1562 C/T) in prostate cancer patients and evaluate the parameters investigated regarding clinical and pathological characteristics of the disease.
4. To evaluate the relationship between metalloproteinases (*MMP-9, MMP-11*) gene expression, single nucleotide polymorphism in these genes (MMP-9 -1562 C/T) and survival rate of prostate cancer patients.

Scientific novelty of the study

Breast cancer is the most frequent female oncologic disease and prostate cancer is the most common cancer among the males. The survival rate remains low because of recurrence of the disease, despite development of new diagnostic and therapeutic possibilities. Therefore, it is important to assess the risk of progression of the disease, detect the relapse of the disease earlier and start the treatment as soon as possible.

This study enabled us to compare the informative significance of MMP genes and single nucleotide polymorphism in their genes, as markers investigated insufficiently yet, taking into account clinical and pathological characteristics of cancer patients and survival rate. It was clarified that MMP-3 (-1171 5A/6A) polymorphism was a factor that had influence on survival of stage II breast cancer patients. The relationship between *MMP-11* gene expression and survival rate of prostate cancer patients was also demonstrated.

2. Materials and methods

2.1. Study object

The perspective study was performed at Institute of Oncology, Vilnius University (currently, National Cancer Institute) during the period since 2005 till 2012. The protocol of biomedical research was discussed at the sitting of the Lithuanian Committee of Bioethics, and permission to perform this study was issued (2005-04-22, No.16). All study subjects were informed about the study in details and they confirmed their agreement to take part in it by signing the Informed Consent Form. The blood of patients, drawn prior the operation and tumours removed during surgery, were investigated.

Patient inclusion criteria:

- confirmed primary breast cancer according to International Cancer Staging System (TNM) classification;
- confirmed primary prostate cancer according to clinical TNM classification;
- patients hadn't additional treatment prior diagnosis of the cancer;
- patients had signed Informed Consent Form (the Informed Consent Forms were presented and signed by the physicians in charge).

The exclusion criteria were established, taking into account factors that could interfere the results of tests performed. Patients, meeting any of criteria presented below, were not allowed to take part in the study. These criteria included:

- concomitant central nervous system and psychical diseases;
- mental retardation or speech disorder, impairing communication with researches or ability to understand the essence of tests performed;
- unstable life-threatening cardiovascular, digestive tract, endocrine system, metabolic and haematological diseases, that may affect patient survival (e.g., unstable angina pectoris, acute and chronic renal failure);
- acute severe infectious diseases;
- tumours of other localization;

- autoimmune diseases (rheumatoid arthritis, systemic lupus erythematosus, dermatomyositis, systemic scleroderma, membranoproliferative glomerulonephritis);
- patients who refused to take part in the study.

The sample of the study subjects was calculated, taking into account that during the first two years of the study at former Institute of Oncology, Vilnius University, breast cancer will be diagnosed for 100 patients and prostate cancer – for 100 patients, also. This number was assessed, taking into account patient inclusion criteria (primary cancer; no additional treatment prior diagnosis of the cancer) and that only a small number of patients would disagree to take part in the study.

The number of study subjects sufficient for the study (79 cases) is assessed, taking into account, that MMP expression is detected for approximately 40%, with accuracy of 0.05. Taking account, that MMP gene polymorphism is detected for approximately 30%, the sufficient study subject number (77 cases) is assessed.

EpiTools software was used to determine sample size (*EpiTools epidemiological calculators*. http://epitools.ausvet.com.au).

2.2. Characteristics of study subjects

2.2.1. Breast cancer

The study included 88 female patients with breast cancer, who were treated at the Department of Breast Diseases and Oncology of then Institute of Oncology, Vilnius University. During the study, the mean age of the patients was 57.69 ± 11.99 years (ranging from 31 to 83 years). In accordance with median of the age, the patients were distributed into two groups of age (31 – 56 and 57 – 83 years). According to the stage of the disease, the patients were distributed into three groups. The first group (n=21) included patients with stage I breast cancer, the second group (n=57) included patients with stage II breast cancer and the third group consisted of the patients with stage III and IV, i.e. advanced breast cancer.

The most common diagnosis for the patients, included into the study, was ductal carcinoma (71 cases of 88). The study included 14 patients with lobular carcinoma and only 3 patients with other types (mucinous and medullar) cancer. Histological examination of breast tumours showed that the majority of tumours were moderately differentiated (G2) (54.5 %). The distribution of patients, regarding the changes of lymph nodes was similar: no changes were detected in 51.1% of the patients and 48.9% of the patients had impairment of the nodes. Immune oestrogens receptors (ER) and progesterone receptors (PR) reactions were examined in the tumours of all patients. The patients were distributed into four groups in accordance with receptor expression.

Distribution of the patients, regarding age, clinical and pathological characteristics is shown in Table 1.

Table 1. Distribution of breast cancer patients, in accordance with age, clinical and pathological characteristics

Age, clinical and pathological characteristics	Patients, n (%)
Age group:	
31–56 years	41 (46.6)
57–83 years	47 (53.4)
Stage:	
I	21 (23.9)
II	57 (64.8)
III/IV	10 (11.3)
Histological type of tumour:	
ductal carcinoma	71 (80.7)
lobular carcinoma	14 (15.9)
other types of carcinoma	3 (3.4)
Tumour differentiation grade:	
G1	17 (19.3)
G2	48 (54.5)
G3	23 (26.2)
Lymph node changes:	
N0	45 (51.1)
N1	36 (40.9)
N2	7 (8.0)
ER (score):	
no reaction (0)	34 (38.6)
weak reaction (1-4)	12 (13.6)
moderate reaction (5-9)	19 (21.6)
strong reaction (10-12)	23 (26.2)
PR (score):	
no reaction (0)	33 (37.5)
weak reaction (1-4)	21 (23.9)
moderate reaction (5-9)	25 (28.4)
strong reaction (10-12)	9 (10.2)

G1 – well-differentiated tumour; G2 – moderately differentiated tumour; G3 – poorly differentiated tumour; N0 – no metastases in regional lymph nodes are present; N1 – mobile metastases are present in axillar lymph nodes of the same side; N2 – metastases in lymph nodes of the armpit of the same side, the nodes are stuck to each other and surrounding structures; ER – oestrogen receptors; PR – progesterone receptors.

MMP-9 and *MMP-11* gene expression was examined and MMP-3 (-1171 5A/6A), MMP-9 (-1562 C/T) polymorphism was evaluated in the blood of breast cancer patients; *MT1-MMP*, *MMP-1*, *MMP-2*, *MMP-3*, *MMP-9*, *MMP-11*, *MMP-13* gene expression was examined in the tumours. The samples of breast tumours were taken from surgery material presented to pathologist physician after the pathologist had evaluated the morphology of the material, confirmed the final diagnosis and assessed ER and PR immune reaction.

2.2.2. Prostate cancer

The study included 82 patients, for whom prostate cancer was diagnosed. The patients were treated at the Department of Oncologic Urology of then Institute of Oncology, Vilnius University. The mean age of the patients during the study was 64.61 ± 7.47 years (ranging from 50 to 85 years); according to the median, the patients were distributed into two age groups (50 – 63 and 64 – 85 years). The diagnosis of prostate cancer was confirmed histologically, using material from trans-rectal biopsy of prostate gland or after examination of the sample received on trans-urethral resection of prostate.

The patients were distributed into three groups, in accordance with the stage of the disease. The first group (n=8) included patients with stage I prostate cancer, the second group (n=43) consisted of patients with stage II prostate cancer, the third group (n=31) involved subjects with stage III prostate cancer. The changes in regional lymph nodes (N1) were detected only in one of 82 patients, in remaining subjects changes of the lymph nodes were not identified; therefore, the assessment of lymph nodes status was not included into statistical analysis. For the majority of the patients Gleason score in biopsy was 6 (67.1%). Prostate specific antigen (PSA) blood serum level was examined for the patients and, in accordance with the results, the patients were distributed into 3 groups (Table 2).

The patients were distributed into prognostic groups, in accordance with the guidelines of TNM system 7^{th} edition [Sobin L, et al., 2009]. The patient could be included into prognostic group only when the stage of the disease, Gleason score and PSA level were known. If Gleason score or PSA levels were not known, the patient could not be included into any prognostic group (Table 2).

Table 2. Distribution of prostate cancer patients, in accordance with age, clinical and pathological characteristics

Age, clinical and pathological characteristics	Patients, n (%)
Age group:	
50-63 years	43 (52.4)
64-85 years	39 (47.6)
Stage:	
I	8 (9.8)
II	43 (52.4)
III	31 (37.8)
Gleason's score:	
6	55 (67.1)
7	20 (24.4)
8–10	7 (8.5)
Serum PSA level (ng/ml):	
≤10	65 (79.3)
10–20	11 (13.4)
≥20	6 (7.3)
Prognostic group:	
I	8 (9.8)
IIA+IIB	44 (53.6)
III	30 (36.6)

PSA – prostate specific antigen.

In patients with prostate tumour concomitant inflammation or benign prostate hyperplasia (BPH) may be detected, also. In this study, concomitant inflammation was present in 17 study subjects and 62 patients had BPH (Table 3).

Table 3. Distribution of prostate cancer patients, in accordance with concomitant inflammation and benign prostate hyperplasia

Clinical and pathological characteristics	Patients, n (%)
Inflammation:	
detected	17 (20.7)
not detected	65 (79.3)
BPH:	
detected	62 (75.6)
not detected	20 (24.4)

BPH – benign prostate hyperplasia.

MMP-9, MMP-11 gene expression and MMP-9 (-1562 C/T) polymorphism were examined in the blood of prostate cancer patients.

2.3. Methods of the study

2.3.1. Purification of RNA from blood and tumour tissue

Purification of RNA from blood

RNA was purified from blood using QIAamp RNA Blood Mini Kit (Qiagen, Germany) according to the manufacturer's protocol. 1 ml of blood was mixed with 5 ml of Buffer EL and incubated for 10–15 min on ice. The tube was centrifuged at 400×g for 10 min at 4°C and supernatant was completely removed and discarded. Cell pellet was resuspended in 2 ml of Buffer EL, centrifuged at 400×g for 10 min at 4°C and supernatant was completely removed and discarded. Buffer RLT was added to pelleted leukocytes and mixed. Lysate was directly pipetted to the spin column and centrifuged for 2 min at maximum speed. 1 ml of 70% ethanol was added to lysate, mixture was pipetted into a new spin column and centrifuged for 15 s at 8000×g. Spin column was placed in a new collection tube, 700 µl of Buffer RW1 was added to the spin column and centrifuged for 15 s at 8000×g. Spin column was placed in a new collection tube, 500 µl of Buffer RPE was added to the spin column and centrifuged for 3 min at 20000×g. Spin column was placed in a new collection tube, 40 µl of RNase-free water was added and centrifuged for 1 min at 8000×g. Purified RNA quality and concentration was determined by agarose gel electrophoresis and by spectrophotometric measurement using BioPhotometer (Eppendorf, Germany).

Purification of RNA from tumour tissue

30 mg of fresh frozen tumour tissue was disrupted using homogenizer (Mikro-dismembrator S, France). Homogenized material was collected into microcentrifuge tube, 1 ml of Lysis Buffer was added and mixed. 1 ml of mixture of phenol, chloroform and isoamyl alcohol (components ratio in the mixture 25:24:1) was added to the tube and incubated for 5 min in ice bath. The tube was centrifuged at 12000×g for 5 min at 4ºC. After centrifugation the upper phase was transferred to the new tube. 100 µl of sodium acetate (pH 4.7) and 1 ml of phenol-chloroform solution (pH 4.7) was added and incubated for 15 min in ice bath. The tube was centrifuged at 12000×g for 15 min at 4ºC. After centrifugation the upper phase was transferred to the new tube, 650 µl isopropanol

was added and incubated for 30 min at -20ºC. The tube was centrifuged at 12000×g for 15 min at 4ºC. The RNA pellet was dissolved in RNase-free water. Purified RNA quality and concentration was determined by agarose gel electrophoresis and by spectrophotometric measurement using BioPhotometer (Eppendorf, Germany).

2.3.2. Gene expression analysis

cDNA synthesis

Complementary DNA (cDNA) was synthesized from total RNA using RevertAid First Strand cDNA Synthesis Kit (Fermentas, Lithuania). Negative control reaction (without Reverse Transcriptase) was used for every RNA sample.

Reaction mixture of 0.1–1 μg RNA, 1 μl random hexamer primer and 12 μl water (nuclease-free) was incubated for 5 min at 70°C. Following components were added to the reaction mixture – 4 μl 5x reaction Buffer, 1 μl RNase Inhibitor, 2 μl 10mM dNTP and 1 μl Reverse Transcriptase. Reaction mixture was incubated for 10 min at 25°C followed by 50 min at 42°C.

Reverse transcription polymerase chain reaction (RT-PCR)

PCR mixture was prepared for every primers pair (*MT1-MMP, MMP-1, MMP-2, MMP-3, MMP-9, MMP-11, MMP-13, ß-actin*). PCR mixture consisted of following components – 2 μl cDNA, 5 μl 10x PCR Buffer, 5 μl 2mM dNTP, 3 μl $MgCl_2$, 25 pmol forward primer, 25 pmol reverse primer, 2.5 U *Taq* DNA Polymerase and water (nuclease-free) to 50 μl. The primers sequences, conditions and PCR product size for RT-PCR are summarized in Table 4. PCR products were loaded onto 1% agarose gel and visualized by Ultraviolet (UV) light.

Table 4. Characteristics of RT-PCR reaction

Primers	PCR conditions				Product size
	denaturation	annealing	extension	number of cycles	
MT1-MMP 5'-ctcctgctccccctgctcacg-3' 5'-ctcacccccataaagttgctg-3'	95°–1 min	60°–1 min	72°–1 min	40	828 bp
MMP-1 5'-cgactctagaaacacaagagcaaga-3' 5'-aaggttagcttactgtcacacgctt-3'	95°–50 s	58°–50 s	72°–1 min	35	787 bp
MMP-2 5'-ggcacccatttacacctacaccaa-3' 5'-gcttccaaacttcacgctcttcag-3'	95°–50 s	62°–50 s	72°–1 min	40	694 bp
MMP-3 5'-gaacaatggacaaaggatacaaca-3' 5'-ttcttcaaaaacagcatcaatctt-3'	95°–50 s	58°–50 s	72°–1 min	35	729 bp
MMP-9 5'-accgctatggttacactcgg-3' 5'-gcaggcagagtaggagcg-3'	95°–50 s	62°–50 s	72°–1 min	40	584 bp
MMP-11 5'-caggtggcagcccatgaatt-3' 5'-gtactgagcaccttggaaga-3'	95°–50 s	58°–50 s	72°–1 min	35	456 bp
MMP-13 5'-ctatggtccaggagatgaag-3' 5'-agagtcttgcctgtatcctc-3'	95°–50 s	62°–50 s	72°–1 min	40	390 bp
β-actin 5'-gtggggcgccccagggacca-3' 5'-ctccttaatgtcacgcacgatttc-3'	95°–50s	62°–50s	72°–1 min	35	540 bp

RT-PCR – reverse transcription polymerase chain reaction, bp – base pairs.

2.3.3. Purification of DNA from blood

DNA was purified from blood using Genomic DNA Purification Kit (Fermentas, Lithuania) according to the manufacturer's protocol. 400 µl of Lysis Solution with 20 µl of Proteinase K Solution was added to 200 µl of blood, mixed and incubated for 10 min at 56ºC. 200 µl of 96% ethanol was added to mixture, transferred to purification column and centrifuged for 1 min at 6000xg. Purification column was placed in a new collection tube, 500 µl of Wash Buffer I was added to the column and centrifuged for 1 min at 6000×g. After centrifugation 500 µl of Wash Buffer II was added to the column and centrifuged for 3 min at maximum speed. Purification column was placed in a new collection tube, 100 µl of Elution Buffer was added to the column, incubated for 2 min at room temperature and centrifuged for 1 min at 8000xg. Purified DNA quality and concentration was determined by agarose gel electrophoresis and by spectrophotometric measurement using BioPhotometer (Eppendorf, Germany).

2.3.4. Single nucleotide polymorphism analysis

Polymerase chain reaction (PCR)

PCR mixture was prepared separately for MMP-3 (-1171 5A/6A) and MMP-9 (-1562 C/T) polymorphism analysis. PCR mixture consisted of following components – 2 µl DNA, 5 µl 10x PCR Buffer, 5 µl 2mM dNTP, 3 µl $MgCl_2$, 25 pmol forward primer, 25 pmol reverse primer, 2.5 U *Taq* DNA Polymerase and water (nuclease-free) to 50 µl. The primers sequences and conditions for PCR are summarized in Table 5.

Table 5. Characteristics of PCR reaction

Primers	**PCR conditions**			
	denaturation	annealing	extension	number of cycles
MMP-3 (-1171 5A/6A) 5‘-ggttctccattcctttgatgggggggaaaga-3‘ 5‘-cttcctggaattcacatcactgccaccact-3‘	95° – 30 s	60° – 30 s	72° – 30 s	35
MMP-9 (-1562 C/T) 5‘-gcctggcacatagtaggccc-3‘ 5‘-cttcctagccagccggcatc-3‘	95° – 30 s	58° – 30 s	72° – 30 s	35

Restriction fragment length polymorphism (RFLP) method

PCR-amplified DNA was digested with restriction enzyme. Digestion mixture for *MMP-3* gene polymorphism analysis consisted from following components – 10 µl PCR product, 2 µl 10x Buffer B, 1 µl restriction enzyme *PsyI*, 17 µl nuclease-free water. Restriction mixture was incubated for 2 hours at 37°C. Digested PCR products were loaded onto 8% polyacrylamide gel and visualized by UV light. Variants of *MMP-3* were assigned as follows: 97 bp and 32 bp fragments for 5A/5A variant; 129 bp, 97 bp and 32 bp fragments for 5A/6A variant and 129 bp fragments for 6A/6A variant.

Digestion mixture for *MMP-9* gene polymorphism analysis consisted from following components – 10 µl PCR product, 2 µl 10x Buffer B, 1 µl restriction enzyme *PaeI*, 17 µl nuclease-free water. Restriction mixture was incubated for 90 minutes at 37°C. Digested PCR products were loaded onto 2% agarose gel and visualized by UV light. Variant of *MMP-9* were assigned as follows: 240 bp and 196 bp fragments for T/T variant; 436 bp, 240 bp and 196 bp fragments for C/T variant and 436 bp fragments for C/C variant.

2.3.5. Statistical analysis

Mean values, standard deviations, minimal and maximum values were calculated for quantitative variables; value frequency and percentage of relative frequency were calculated for qualitative variables. Confidence intervals were calculated for binomial variables, distributed according to binomial distribution. Chi-square (χ^2) criterion was used to test interdependence of qualitative variables; in event of small amount of observations, *Fisher* exact test was used. Significance level α=0.05 was chosen, when $p<0.05$, H_0 hypothesis was rejected. In order to assess the strength of relationship between qualitative variables, *Spearman* correlation coefficient (r) was calculated. The correlation was considered to be very weak, when r was less than 0.19; weak, when it was in the range between 0.20 and 0.39; moderate – when it was between 0.40 and 0.69; strong, when it was between 0.70 and 0.89 and very strong, when r ranged from 0.90 and 1.00.

The study subjects were observed for 6 years, they were included into the study at different time points. In the case of breast cancer, the start of observation was the date of operation; in event of prostate cancer, the start was the date of confirmation of diagnosis. The end point event for observation of the patient was patient's death. The data of observation were censored, because the majority of the patients survived the period of observation. The survival rate was analysed using *Kaplan-Meier* method. The *log-rank* test was used to compare the survival curves. The prognostic analysis of survival factors was performed using *Cox* proportional hazards regression model. Multivariate stepwise model was used to analyse the factors that in univariate model showed plausibility equal or less than 0.2.

The statistical analysis was performed using data analysis software package SPSS 20.0 (*Armonk, NY: IBM Corp.,* USA, 2011).

3. RESULTS

3.1. Breast cancer

The majority results of research studies showed the importance of MMP expression in oncology. It is considered that MMP expression could be assessed as prognostic cancer markers, associated with progression of the disease. However, because of lack of systemic results of the studies, evaluation of these enzymes is not applied in clinical practice, still [Hadler-Olsen E. et al., 2013; Köhrmann A. et al., 2009; Mannello F. 2011]. *MMP-9* and *MMP-11* gene expression in blood were chosen for examination of the breast cancer patients. MMP-9 is one of the most investigated MMP in different diseases, including cancer. High expression of MMP in breast cancer patients is associated with poorer prognosis of the disease. It is proposed that MMP, expressed in tumour tissue, are delivered into blood in greater amounts and in this way progression of the disease is stimulated [Stankovic S. et al., 2010]. The results of the studies, performed in different countries, remain contradictory, the results are not systematized; therefore, the studies of MMP gene expression are still important. *MT1-MMP*, *MMP-1*, *MMP-2*, *MMP-3*, *MMP-9*, *MMP-11*, *MMP-13* gene expression in breast tumours and MMP-3 (-1171 5A/6A) (rs3025058) and MMP-9 (-1562 C/T) (rs3918242) gene polymorphism in blood of the patients with breast cancer were investigated in this study, also.

3.1.1. Matrix metalloproteinases gene expression in blood of breast cancer patients

As mentioned above, the study included 88 patients with breast cancer. Positive *MMP-9* gene expression in blood was detected in 40 patients (45.5%, 95% CI 40.15-50.76) and *MMP-11* gene expression – in 30 patients (34.1%, 95% CI 29.04-39.14). The evaluation of MMP gene expression examined regarding age, clinical and pathological characteristics of the disease, statistically significant differences were shown only between *MMP-9* gene expression in blood and the tumour differentiation grade (p=0.02) (Table 6).

The evaluation of relationship between variables using chi-square (χ^2) criterion was not sufficient enough; therefore, the strength of relationship between parameters

examined (*MMP* gene expression and clinical, pathological parameters) was additionally evaluated using correlation analysis. The higher dependency is meant by r value nearer to 1 or – 1; on the other hand, r value nearer to 0 means lower dependency of the variables.

Correlation analysis of strength of relationship of the parameters examined showed a weak, although statistically significant relationship between *MMP-9* gene expression in blood and tumour differentiation grade (r=0.279, p=0.009). The probability to detect *MMP-9* expression in blood increases in event of poorly differentiated tumours. A weak, although statistically significant relationship between *MMP-9* gene expression in blood and ER status (r=–0.260, p=0.01) was detected, also. The probability to detect *MMP-9* gene expression in blood is higher, when ER expression in tumour is absent. No statistically significant relationship between *MMP-11* gene expression in blood and studied clinical and pathological parameters was detected (Table 7).

Table 6. Analysis of MMP gene expression in blood of breast cancer patients, in accordance with age, clinical and pathological characteristics

Variables	***MMP-9* expression**		p	***MMP-11* expression**		p
	Positive, n (%)	Negative, n (%)		Positive, n (%)	Negative, n (%)	
Age group: 31–56 years 57–83 years	 22 (55.0) 18 (45.0)	 19 (39.6) 29 (60.4)	 0.10	 17 (56.7) 13 (43.3)	 24 (41.4) 34 (58.6)	 0.12
Stage: I II III/IV	 5 (12.5) 30 (75.0) 5 (12.5)	 16 (33.3) 27 (56.3) 5 (10.4)	 0.07	 7 (23.3) 19 (63.3) 4 (13.4)	 14 (24.2) 38 (65.5) 6 (10.3)	 0.91
Histological type of tumour: ductal carcinoma lobular carcinoma	 34 (87.2) 5 (12.8)	 37 (80.4) 9 (19.6)	 0,29	 26 (86.7) 4 (13.3)	 45 (81.8) 10 (18.2)	 0.40
Tumour differentiation grade: G1 G2 G3	 3 (7.5) 23 (57.5) 14 (35.0)	 14 (29.1) 25 (52.1) 9 (18.8)	 **0,02**	 6 (20.0) 14 (46.7) 10 (33.3)	 11 (19.0) 34 (58.6) 13 (22.4)	 0.48
Lymph node status: N0 N1 N2	 16 (40.0) 21 (52.0) 3 (8.0)	 29 (60.4) 15 (31.3) 4 (8.3)	 0,12	 15 (50.0) 14 (46.7) 1 (3.3)	 30 (51.8) 22 (37.9) 6 (10.3)	 0.44
ER: negative positive	 21 (52.5) 19 (47.5)	 13 (27.1) 35 (72.9)	 0.13	 15 (50.0) 15 (50.0)	 19 (32.8) 39 (67.2)	 0.09
PR: negative positive	 19 (47.5) 21 (52.5)	 14 (29.2) 34 (70.8)	 0.06	 14 (46.7) 16 (53.3)	 19 (32.8) 39 (67.2)	 0.14

G1 – well-differentiated tumour; G2 – moderately differentiated tumour; G3 – poorly differentiated tumour; N0 – no metastases in regional lymph nodes are present; N1 – mobile metastases are present in axillar lymph nodes of the same side; N2 – metastases in lymph nodes of the armpit of the same side, the nodes are stuck to each other and surrounding structures; ER – oestrogen receptors; PR – progesterone receptors.

Table 7. Correlation analysis between MMP gene expression in blood and clinical, pathological characteristics of breast cancer patients

Variables	***MMP-9 expression***		***MMP-11 expression***	
	r	p	r	p
Age	–0.154	0.15	-0.145	0.17
Stage	0.205	0.05	0.029	0.78
Histological type of tumour	0.100	0.35	0.109	0.31
Tumour differentiation grade	**0.279**	**0.009**	0.073	0.49
Lymph node status	0.178	0.09	-0.017	0.87
ER expression	**–0.260**	**0.01**	-0.168	0.11
PR expression	–0.189	0.07	-0.136	0.20

r – correlation coefficient, ER – oestrogen receptors; PR – progesterone receptors.

Survival probability of 88 patients with breast cancer was evaluated, in accordance with detected *MMP* gene expression in blood.

Survival analysis, performed using *Kaplan–Meier* method, showed that *MMP-9* gene expression was associated with survival of breast cancer patients, depending on age, stage of the disease and PR expression in tumour (Table 8).

Table 8. Analysis of survival probability of breast cancer patients, in accordance with *MMP-9* and *MMP-11* gene expression in blood

Variables	***MMP-9 expression***	***MMP-11 expression***
	p (*log-rank*)	
Age group:		
31–56 years	0.49	0.90
57–83 years	**0.01**	0.46
Stage:		
I	0.57	0.48
II	0.26	0.57
III/IV	<**0.001**	0.81
Histological type of tumour:		
ductal carcinoma	0.08	0.49
lobular carcinoma	0.66	0.90
Tumour differentiation grade:		
G1	0.50	0.68
G2	0.34	0.81
G3	0.63	0.90
Lymph node status:		
N0	0.80	0.16
N1	0.65	0.70
N2	0.24	0.68
ER:		
negative	0.46	0.22
positive	0.65	0.39
PR:		
negative	**0.02**	0.16
positive	0.71	0.64

G1 – well-differentiated tumour; G2 – moderately differentiated tumour; G3 – poorly differentiated tumour; N0 – no metastases in regional lymph nodes are present; N1 – mobile metastases are present in axillar lymph nodes of the same side; N2 – metastases in lymph nodes of the armpit of the same side, the nodes are stuck to each other and surrounding structures; ER – oestrogen receptors; PR – progesterone receptors.

The elder breast cancer patients (57 – 83 years) without *MMP-9* gene expression in blood survived for a longer time, in comparison with the patients of the same age, in whom *MMP-9* expression in blood was detected (*log-rank* p=0,01) (Fig. 1).

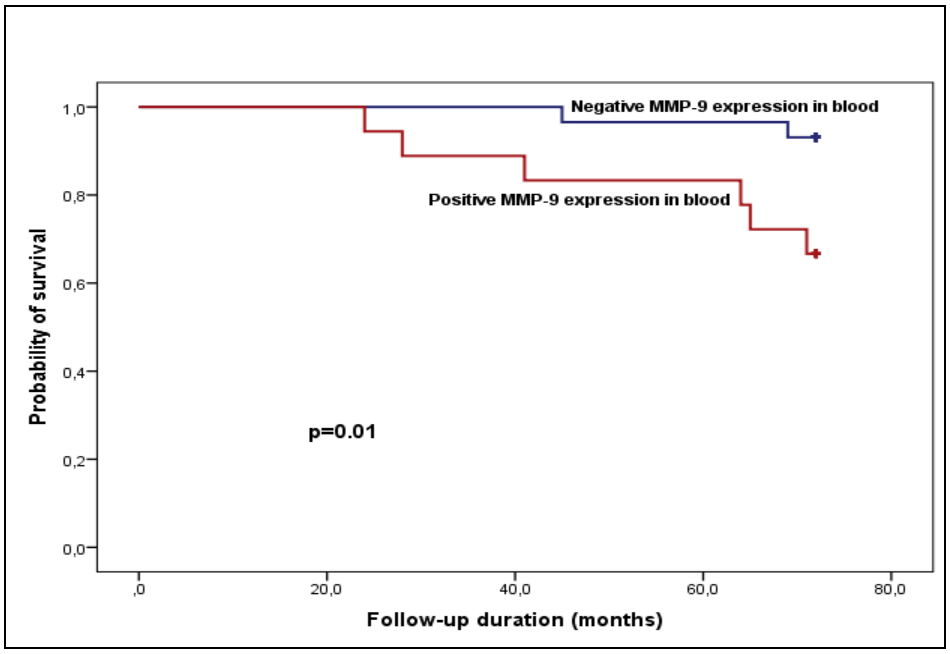

Figure 1. Survival curves of elderly (57–83 years) breast cancer patients according to *MMP-9* expression in blood

After distribution of the patients regarding the stage of the disease, in the group studied, it was revealed that breast cancer patients for whom advanced stage of the disease was diagnosed and for whom *MMP-9* expression in blood was not detected, had a better survival rate in comparison with those advanced stage patients, in whom *MMP-9* expression was identified ($p<0.001$). Similar results were present after distribution of breast cancer patients according PR expression in tumours. The patients for whom PR expression in tumours and *MMP-9* expression in blood were not detected, survived longer in comparison with those for whom *MMP-9* gene expression in blood was identified (*log-rank* p=0.02) (Table 8). *MMP-11* expression in blood was not associated with survival of breast cancer patients.

3.1.2. Polymorphism in matrix metalloproteinases genes in breast cancer patients

MMP-3 (-1171 5A/6A) (rs3025058) polymorphism was chosen for examination, because in the group of study subjects with breast cancer relationship between *MMP-3* expression in breast tumour, clinical and pathological characteristics was detected (Chapter 3.1.3.). MMP-9 (-1562 C/T) (rs3918242) gene polymorphism is the most common object of investigations in different diseases, including breast cancer.

MMP-3 (-1171 5A/6A) and MMP-9 (-1562 C/T) polymorphism was detected in all study participants. *MMP-3* gene polymorphism 5A/5A, 5A/6A and 6A/6A variants were detected in 15 (17.0%, 95% CI 13.04-21.05), 57 (64.8 %, 95% CI 59.68-69.86) and 16 (18.2 %, 95% CI 14.07-22.29) cases, respectively. *MMP-9* gene polymorphism C/C, C/T and T/T variants were detected in 39 (44.3 %, 95% CI 39.02-49.61), 38 (43.2 %, 95% CI 37.90-48.46) and 11 (12.5 %, 95% CI 8.97-16.03) cases. Analysis of variants distribution regarding age, clinical and pathological parameters, showed no statistically significant difference of MMP-3 (-1171 5A/6A) gene polymorphism variant distribution between the groups. Statistically significant distributions were detected between MMP-9 (-1562 C/T) gene polymorphism variants and stage of the disease (p=0.02), histological type of the tumour (p=0.05) and lymph nodes status (p=0.002) (Table 9).

Table 9. Analysis of MMP genes polymorphism in blood of breast cancer patients, in accordance with age, clinical and pathological characteristics

Variables	MMP-3 -1171 polymorphism			p	MMP-9 -1562 polymorphism			p
	5A/5A, n (%)	5A/6A, n (%)	6A/6A, n (%)		C/C, n (%)	C/T, n (%)	T/T, n (%)	
Age group:								
31–56 years	7 (46.7)	26 (45.6)	8 (50.0)	0.95	20 (51.3)	15 (39.5)	6 (54.5)	0.49
57–83 years	8 (53.3)	31 (54.4)	8 (50.0)		19 (48.7)	23 (60.5)	5 (45.5)	
Stage:								
I	1 (6.7)	14 (24.6)	6 (37.5)		12 (30.8)	9 (23.7)	0	
II	10 (66.7)	39 (68.4)	8 (50.0)	0.09	25 (64.1)	25 (65.8)	7 (63.6)	**0.02**
III/IV	4 (26.7)	4 (7.0)	2 (12.5)		2 (5.1)	4 (10.5)	4 (36,4)	
Histological type of tumour:								
ductal carcinoma	13 (86.7)	47 (85.5)	11 (73.3)	0.49	34 (94.4)	28 (73.7)	9 (81.8)	0.05
lobular carcinoma	2 (13.3)	8 (14.5)	4 (26.7)		2 (5.6)	10 (26.3)	2 (18.2)	
Tumour differentiation grade:								
G1	3 (20.0)	11 (19.3)	3 (18.8)		11 (28.2)	5 (13.2)	1 (9.1)	
G2	7 (46.7)	32 (56.1)	9 (56.2)	0.96	18 (46.2)	22 (57.9)	8 (72.7)	0.33
G3	5 (5.7)	14 (24.6)	4 (25.0)		10 (25,6)	11 (28,9)	2 (18.2)	
Lymph node status:								
N0	7 (46.7)	29 (50.9)	9 (56.3)		24 (61.5)	19 (50.0)	2 (18.2)	
N1	6 (40.0)	24 (42.1)	6 (37.5)	0.92	14 (35.9)	17 (44.7)	5 (45.5)	**0.00**
N2	2 (13.3)	4 (7.0)	1 (6.2)		1 (2.6)	2 (5.3)	4 (36.4)	**2**
ER:								
negative	7 (46.7)	23 (40.4)	4 (25.0)	0.42	14 (35.9)	13 (34.2)	7 (63.6)	0.18
positive	8 (53.3)	34 (59.6)	12 (75.0)		25 (64.1)	25 (65.8)	4 (36.4)	
PR:								
negative	6 (40.0)	21 (36.8)	6 (37.5)	0.97	13 (33.3)	14 (36.8)	6 (54.5)	0.43
positive	9 (60.0)	36 (63.2)	10 (62.5)		26 (66.7)	24 (63.2)	5 (45.5)	

G1 – well-differentiated tumour; G2 – moderately differentiated tumour; G3 – poorly differentiated tumour; N0 – no metastases in regional lymph nodes are present; N1 – mobile metastases are present in axillar lymph nodes of the same side; N2 – metastases in lymph nodes of the armpit of the same side, the nodes are stuck to each other and surrounding structures; ER – oestrogen receptors; PR – progesterone receptors.

The strength of relationship of parameters examined was also analysed, using the method of correlative analysis. The results of correlative analysis, regarding MMP gene polymorphism in the blood of breast cancer patients, clinical and pathological characteristics are presented in Table 10.

Table 10. Correlation analysis between MMP gene polymorphism, clinical and pathological characteristics of breast cancer patients

Variables	MMP gene polymorphism			
	MMP-3 (-1171 5A/6A)		MMP-9 (-1562 C/T)	
	r	p	r	p
Age	–0.021	0.84	0.048	0.65
Stage	**0.228**	**0.03**	**0.273**	**0.01**
Histological type of tumour	0.140	0.19	0.096	0.37
Tumour differentiation grade	–0.032	0.76	0.102	0.34
Lymph node status	0.070	0.51	**0,288**	**0.007**
ER expression	0.133	0.21	–0.109	0.31
PR expression	0.015	0.89	–0.110	0.30

r – correlation coefficient, ER – oestrogen receptors; PR – progesterone receptors.

The data presented in Table 10 demonstrate that weak, although statistically significant relationship between MMP-3 (-1171 5A/6A) gene polymorphism and the stage of the disease was detected. In event of advanced stages of the disease, 5A/5A or 5A/6A variants in blood were more common; these polymorphism variants are associated with poorer prognosis of the disease. Similarly, it was found out that MMP-9 (-1562 C/T) gene polymorphism had weak, although statistically significant relationship with a stage of the disease and lymph node status. In event of advanced stages of the disease and development of lymph node changes, T/T variant was more common; this variant is associated with poorer prognosis.

Survival analysis using *Kaplan-Meier* method was performed and showed that MMP-3 gene (-1171 5A/6A) polymorphism was associated with survival of breast cancer patients, according to the stage of the disease, histology of the tumour, differentiation grade, lymph node status and PR expression in the tumour (Table 11).

Table 11. Analysis of probability of survival of breast cancer patients, in accordance with MMP-3 (-1171 5A/6A) gene polymorphism

Variables	**MMP-3 (-1171 5A/6A) gene polymorphism**
	p (*log-rank*)
Age group:	
31–56 years	0.13
57–83 years	0.14
Stage:	
I	0.77
II	**0.04**
III/IV	0.49
Histological type of tumour:	
ductal carcinoma	**0.01**
lobular carcinoma	0.32
Tumour differentiation grade:	
G1	**0.003**
G2	0.47
G3	0.09
Lymph node status:	
N0	**<0.001**
N1	0.68
N2	0.68
ER:	
negative	0.29
positive	0.06
PR:	
negative	0.49
positive	**0.02**

G1 – well-differentiated tumour; G2 – moderately differentiated tumour; G3 – poorly differentiated tumour; N0 – no metastases in regional lymph nodes are present; N1 – mobile metastases are present in axillar lymph nodes of the same side; N2 – metastases in lymph nodes of the armpit of the same side, the nodes are stuck to each other and surrounding structures; ER – oestrogen receptors; PR – progesterone receptors.

The patients for whom stage II of the disease was diagnosed and who had MMP-3 -1171 5A/6A or 6A/6A variants in blood, had a better survival rate, in comparison with the patients of the same stage of the disease with 5A/5A variant detected (*log-rank* p=0.04) (Fig. 2).

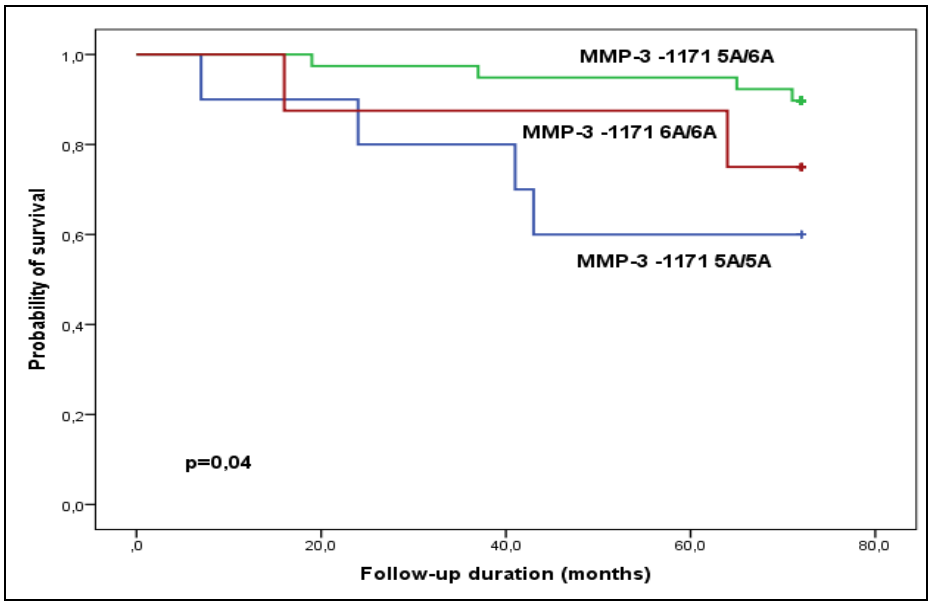

Figure 2. Survival curves of stage II breast cancer patients, in accordance with MMP-3 (-1171 5A/6A) polymorphism in blood

The patients who had ductal carcinoma and MMP-3 -1171 5A/6A or 6A/6A variants in blood, had a better survival rate, in comparison with patients who had 5A/5A variant in blood (*log-rank* p=0.01) (Fig. 3).

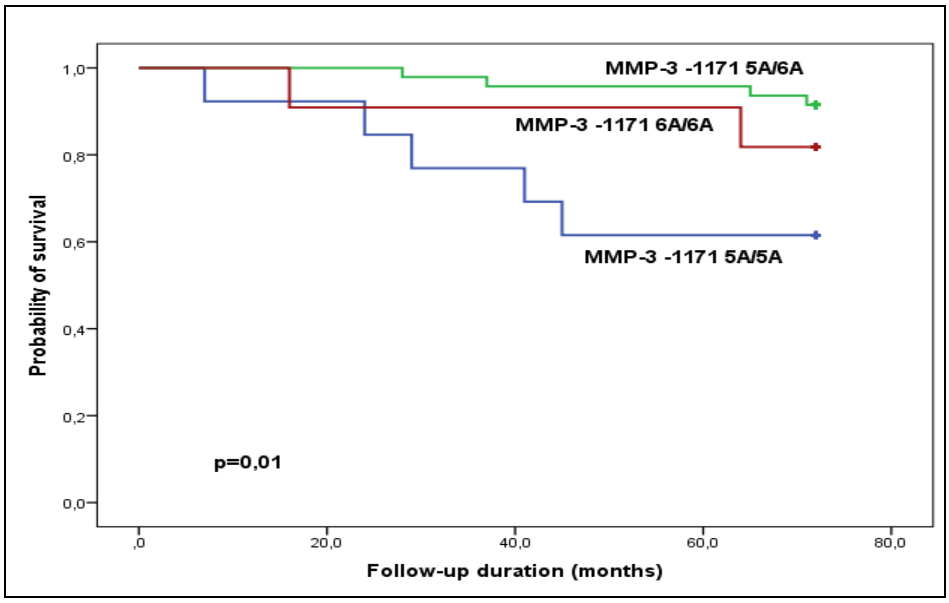

Figure 3. Survival curves of breast cancer patients for whom ductal cancer was diagnosed, in accordance with MMP-3 (-1171 5A/6A) polymorphism in blood

Similar results were obtained after evaluation of other clinical and pathological parameters regarding MMP-3 gene polymorphism investigated. In different groups, in patients for whom tumour was diagnosed as well-differentiated and who had no lymph node changes and in whom positive tumour PR expression was found out, the survival rate was better in event of detection of MMP-3 -1171 5A/6A or 6A/6A variants, in comparison with those for whom 5A/5A variant was identified (respectively, *log-rank* p=0.003, p<0.001, p=0.02) (Table 11).

Kaplan-Meier analysis of survival demonstrated that MMP-9 (-1562 C/T) polymorphism was associated with breast cancer patient survival, in accordance with the tumour differentiation grade and lymph nodes changes (Table 12).

Table 12. Analysis of probability of survival of breast cancer patients, in accordance with MMP-9 (-1562 C/T) gene polymorphism in blood

Variables	**MMP-9 (-1562 C/T) gene polymorphism**
	p (*log-rank*)
Age group:	
31–56 years	0.33
57–83 years	0.78
Stage:	
I	0.24
II	0.24
III/IV	0.15
Histological type of tumour:	
ductal carcinoma	0.16
lobular carcinoma	0.50
Tumour differentiation grade:	
G1	0.80
G2	0.95
G3	**0.001**
Lymph node status:	
N0	**<0.001**
N1	0.61
N2	0.28
ER:	
negative	0.29
positive	0.67
PR:	
negative	0.29
positive	0.62

G1 – well-differentiated tumour; G2 – moderately differentiated tumour; G3 – poorly differentiated tumour; N0 – no metastases in regional lymph nodes are present; N1 – mobile metastases are present in axillar lymph nodes of the same side; N2 – metastases in lymph nodes of the armpit of the same side, the nodes are stuck to each other and surrounding structures; ER – oestrogen receptors; PR – progesterone receptors.

The patients who had poorly differentiated breast tumour and MMP-9 -1562 C/T or C/C variant, had a better survival rate, in comparison with those in whom T/T variant was detected (*log-rank* p=0.001). Similar results were found out after distribution of the patients, in accordance with lymph node changes. In event of absence of lymph node changes (N0), the duration of survival was longer in patients who had MMP-9 – 1562 C/T or C/C variant, in comparison with those, who had T/T variant (*log-rank* p<0.001).

3.1.3. Matrix metalloproteinases gene expression in breast tumours

As mentioned above, *MT1-MMP*, *MMP-1*, *MMP-2*, *MMP-3*, *MMP-9*, *MMP-11* and *MMP-13* gene expression in breast tumours were examined.

In tumours of breast cancer patients *MT1-MMP* expression was detected in 82 cases (93.2 %, 95% CI 90.49-95.87), *MMP-1* – 37 cases (42 %, 95% CI 36.78-47.31), MMP-2 – 82 cases (93.2 %, 95% CI 90.49-95.87), *MMP-3* – 53 cases (60.2 %, 95% CI 55.01-65.44), *MMP-9* – 82 cases (93.2 %, 95% CI 90.49-95.87), *MMP-11* – 87 cases (98.8 %, 95% CI 97.73-99.99), *MMP-13* – 62 cases (70.5 %, 95% CI 65.59-75.32).

After patients distribution in accordance with age and evaluation whether it was acceptable to relate MMP gene expression to the age, conclusion was made that *MMP-3* was more common in tumours of younger patients (31 – 56 years of age) (p=0.04) (Table 13, Appendices 1 and 2).

The patients were distributed, in accordance with the stage of the disease, and it was concluded that expression of four MMP genes investigated, including *MT1-MMP* (p=0.002), *MMP-1* (p=0.02), *MMP-3* (p=0.005) and *MMP-13* (p=0.006), was related to the stage of the disease (Table 13). The evaluation of MMP gene expression regarding tumour differentiation grade and lymph nodes status, showed statistically significant differences between *MMP-13* gene expression and tumour differentiation grade (p=0.02), *MT1-MMP* gene expression and lymph nodes status (p=0.04) (Table 13). No other statistically significant differences between MMP gene expression and clinical or pathological characteristics (e.g. histological type of the tumour, ER, PR expression) were found out.

The strength of relationship of parameters investigated was evaluated using the method of correlation analysis. The data presented in Table 14 (Appendix 3), show a

weak, although statistically significant relationship between expression of *MT1-MMP*, *MMP-3* and *MMP-13* genes in a tumour and the stage of the diseases. The probability to detect expression of these genes in a tumour is greater, in event of advanced stage disease. A weak, although statistically significant, relationship between *MT1-MMP* and *MMP-11* gene expression in a tumour and tumour's histological type was detected, also. The probability to detect *MMP-13* gene expression in a tumour is higher, in event of poorly differentiated tumour. Furthermore, in event of changes in regional lymph nodes, the probability to detect *MT1-MMP* and *MMP-3* gene expression in a breast tumour also increases, as weak, although statistically reliable relationship is found out (Table 14, Appendix 3).

The probability of survival of breast cancer patients was evaluated in accordance with MMP gene expression in breast tumour detected. As with survival analysis of MMP gene expression in blood, the analysis was performed for all 88 patients; however, survival analysis regarding MMP gene expression in a tumour and lymph nodes status was not performed for the patients for whom changes were found to be present in more than one regional lymph node, because the number of patients was insufficient in this group (n=7).

Univariate *Kaplan-Meier* analysis demonstrated that among the range of seven MMP investigated, expression of *MMP-1*, *MMP-3* and *MMP-13* genes in breast tumours was associated with breast cancer patients survival (Table 15).

Table 15. Analysis of probability of survival of breast cancer patients, in accordance with MMP gene expression in tumours

Variables	***MT1-MMP***	***MMP-1***	***MMP-2***	***MMP-3***	***MMP-9***	***MMP-11***	***MMP-13***
	p (*log-rank*)						
Age group:							
31–56 years	0.66	0.61	0.53	0.07	0.53	0.66	0.07
57–83 years	0.30	0.38	0.36	0.50	0.36	--	0.78
Stage:							
I	0.57	0.07	--	0.15	0.82	0.82	0.24
II	0.65	0.89	0.25	0.26	0.30	--	0.08
III/IV	--	**0.04**	--	0.06	--	--	0.08
Histological type of tumor:							
Ductal carcinoma	0.46	0.91	0.39	0.43	0.29	--	0.32
Lobular carcinoma	0.32	0.05	0.44	**0.03**	--	0.59	0.23
Tumour differentiation grade:							
G1	0.71	0.82	--	0.17	0.71	0.71	0.89
G2	0.51	0.98	0.33	0.64	0.38	--	0.49
G3	0.37	0.58	--	0.29	--	--	0.26
Lymph node status:							
N0	0.36	0.31	0.54	0.15	0.54	0.73	0.43
N1	--	0.73	0.43	0.13	0.33	--	0.05
ER:							
negative	0.45	0.85	0.60	0.14	0.45	0.60	**0.04**
positive	0.44	0.28	0.38	0.41	0.44	--	0.96
PR:							
negative	0.51	0.92	0.65	0.18	0.41	0.65	0.06
positive	0.38	0.24	0.32	0.27	0.45	--	0.70

G1 – well-differentiated tumour; G2 – moderately differentiated tumour; G3 – poorly differentiated tumour; N0 – no metastases in regional lymph nodes are present; N1 – mobile metastases are present in axillar lymph nodes of the same side; N2 – metastases in lymph nodes of the armpit of the same side, the nodes are stuck to each other and surrounding structures; ER – oestrogen receptors; PR – progesterone receptors.

The comparison of survival curves demonstrated that the patients for whom breast cancer was diagnosed in advanced stages and no *MMP-1* gene expression was detected in tumour, had a better survival rate, in comparison with these of the same disease stage for whom *MMP-1* gene expression was detected (p=0.04) (Fig. 4).

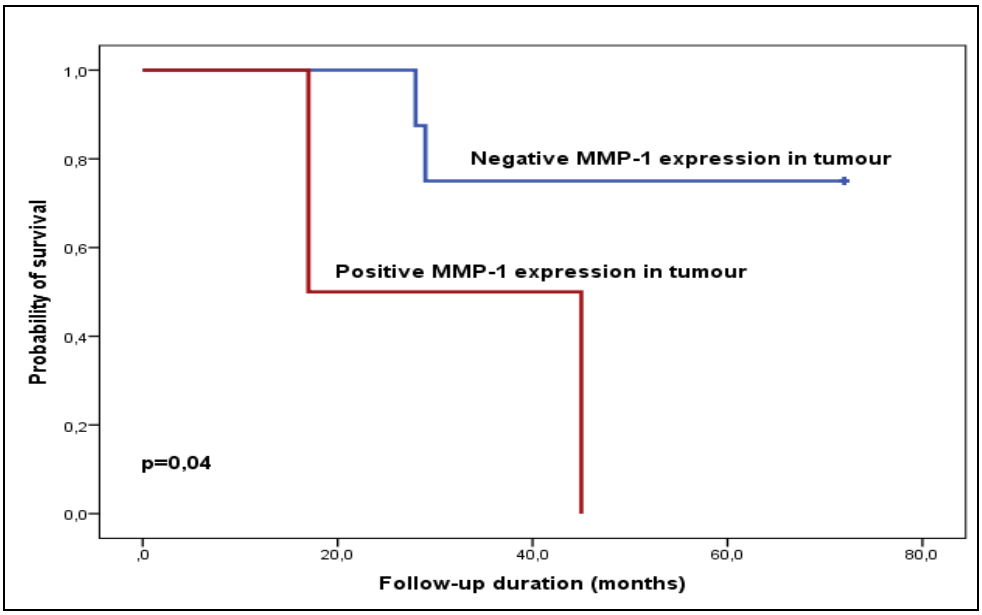

Figure 4. Survival curves of breast cancer patients for whom advanced stages of the disease were diagnosed, in accordance with *MMP-1* gene expression in tumour

Similar results were found out in the group of breast cancer patients, in whom histology examination revealed lobular carcinoma. The patients of this group, in whom *MMP-3* gene expression in tumour was not detected, survived for a longer time, in comparison with these, for whom *MMP-3* gene expression was detected (p=0.03). The survival rate of breast cancer patients, free of ER and *MMP-13* expression, was better, in comparison with these, who had *MMP-13* expression in a tumour, but in whom ER expression was absent (p=0.04).

3.1.4. Factors influencing survival rate of breast cancer patients

The results presented in above chapters, show that a large range of factors examined had an influence on survival rate of women with breast cancer. Therefore, multivariate analysis of the factors examined was performed, using *Cox* proportional hazards regression model, in order to make clear the most important factors for survival rate of breast cancer patients.

Prognostic analysis included the following factors: age, stage of the disease, histological type of the tumour and differentiation grade, lymph node status, ER and PR expression in a tumour, MMP-3 (-1171 5A/6A) and MMP-9 (-1562 C/T) polymorphism,

MMP-9, *MMP-11* gene expression in blood, *MMP-1*, *MMP-3* and *MMP-13* gene expression in tumour (Table 16).

Table 16. Multivariate analysis of factors influencing survival rate of breast cancer patients

Variables	Hazard ratio	95% CI	p
Age	0.433	0.117-1.600	0.20
Stage of the disease	0.132	0.031-0.569	**0.007**
Histological type of tumour	3.419	0.743-15.743	0.11
Tumour differentiation grade	0.107	0.020-0.570	**0.009**
Lymph node status	12.014	0.987-146.294	0.05
ER expression	9.646	1.564-59.476	**0.01**
PR expression	0.076	0.011-0.542	**0.01**
MMP-3 (-1171 5A/6A) polymorphism	0.071	0.006-0.806	**0.03**
MMP-9 (-1562 C/T) polymorphism	1.003	0.127-7.950	0.99
MMP-9 expression in blood	0.998	0.215-4.634	0.99
MMP-11 expression in blood	0.630	0.181-2.192	0.46
MMP-1 expression in tumour	1.014	0.279-3.692	0.98
MMP-3 expression in tumour	0.359	0.079-1.643	0.18
MMP-13 expression in tumour	0.816	0.139-4.780	0.82

CI – confidence interval; ER – oestrogen receptors; PR – progesterone receptors.

Multivariate analysis showed that the stage of the disease, tumour differentiation grade, ER, as well as PR expression in a tumour and MMP-3 (-1171 5A/6A) polymorphism are independent prognostic factors of survival in breast cancer patients. Age, histological type of tumour, lymph node status, MMP-9 (-1562 C/T) polymorphism, *MMP-9*, *MMP-11* gene expression in blood, *MMP-1*, *MMP-3* and *MMP-13* gene expression in tumour had no influence on survival of breast cancer patients.

The stage II breast cancer was diagnosed for more than a half of female patients (n=57) who had participated in this study. Therefore, the analysis of factors influencing the survival rate of the patients with stage II breast cancer was performed. Multivariate analysis of prognostic factors demonstrated that tumour differentiation grade and MMP-3 (-1171 5A/6A) polymorphism were independent prognostic factors of survival of the

patients (Table 17). Breast cancer patients with moderately or well-differentiated tumours had a better survival rate, in comparison with the patients for whom poorly differentiated tumour was identified (*log-rank* p=0.006). Similarly, the patients for whom MMP-3 – 1171 5A/6A or 6A/6A variants were detected had a better survival rate, in comparison with those for whom MMP-3-1171 5A/5A was detected (*log-rank* p=0.04).

Table 17. Multivariate analysis of factors influencing survival rate of stage II breast cancer patients

Variables	Hazard ratio	95% CI	p
Tumour histology	0.005	0.001-4.335	0.12
Tumour differentiation grade	0.002	0.000-0.072	**0.01**
Lymph node status	0.001	0.000-4.921	0.97
Oestrogen receptor expression	0.334	0.008-14.347	0.56
MMP-3 (-1171 5A6A) polymorphism	1.813	0.331-9.914	**0.02**
MMP-9 (-1562 CT) polymorphism	0.052	0.000-7.714	0.24
MMP-9 expression in blood	3.329	0.170-65.047	0.42
MMP-11 expression in blood	0.101	0.003-3.568	0.20
MMP-1 expression in tumour	10.559	0.798-139.778	0.07
MMP-13 expression in tumour	0.001	0.000-1.366	0.83

CI – confidence interval; ER – oestrogen receptors; PR – progesterone receptors.

The data show that expression of MMP examined is related to clinical and pathological characteristics of the disease, and that MMP-3 (-1171 5A/6A) polymorphism is an independent factor for cancer prognosis.

3.2. Prostate cancer

As mentioned above, relatively frequently, after evaluation of probability of progression of the disease, it is sufficient only to observe the prostate cancer patients; therefore, MMP participating in the tumour progression may be useful for evaluation of the prognosis of prostate cancer.

3.2.1. Matrix metalloproteinases gene expression in blood of prostate cancer patients

MMP-9 and *MMP-11* gene expression in blood was examined in prostate cancer patients.

The group of patients with prostate cancer consisted of 82 subjects. Expression of *MMP-9* gene in blood was detected in 63 patients (76.8 %, 95% CI 72.17-81.49) and expression of *MMP-11* gene in 26 patients (31.7 %, 95% CI 26.57-36.85).

After distribution of the patients regarding age and evaluation whether MMP gene expression of the study subjects could be related to the age of the patients, it was stated, that expression of *MMP-9* ($p=0.001$) and expression of *MMP-11* ($p=0.02$) in blood was significantly more frequently detected in elderly (64-85 years old) prostate cancer patients (Table 18).

Evaluation of *MMP-9* and *MMP-11* gene expression in blood, in accordance with clinical and pathological characteristics, showed statistically significant differences between stage of the disease and *MMP-9* ($p=0.02$), *MMP-11* ($p<0.001$) gene expression, between prognostic group and *MMP-9* gene expression ($p=0.005$), as well as between *MMP-11* gene expression in blood ($p<0.001$). Statistically significant differences between *MMP-11* gene expression and PSA blood levels ($p=0.001$) were found out, also. Other statistically significant differences between *MMP* gene expression in blood and clinical, pathological parameters were not revealed (Table 18).

Table 18. Analysis of MMP gene expression in blood of prostate cancer patients, in accordance with age, clinical and pathological characteristics

Variables	***MMP-9* expression**		**p**	***MMP-11* expression**		**p**
	Positive, n (%)	Negative, n (%)		Positive, n (%)	Negative, n (%)	
Age group: 50-63 years 64-85 years	 27 (42.9) 36 (57.1)	 16 (84.2) 3 (15.8)	**0.001**	 9 (34.6) 17 (65.4)	 34 (60.7) 22 (39.3)	**0.02**
Stage: I II III	 4 (6.3) 29 (46.1) 30 (47.6)	 4 (21.1) 14 (73.7) 1 (5.2)	**0.02**	 0 5 (19.2) 21 (80.8)	 8 (14.2) 38 (67.9) 10 (17.9)	**<0.001**
Gleason's score: 6 7 8-10	 40 (63.5) 16 (25.4) 7 (11.1)	 15 (78.9) 4 (21.1) 0	0.25	 16 (61.5) 6 (23.1) 4 (15.4)	 39 (69,6) 14 (25.0) 3 (5.4)	0.31
PSA serum level (ng/ml): < 10 10–20 > 20	 49 (77.8) 8 (12.7) 6 (9.5)	 16 (84.2) 3 (15.8) 0	0.37	 15 (57.7) 6 (23.1) 5 (19.2)	 50 (89.3) 5 (8.9) 1 (1.8)	**0.002**
Prognostic level: I II III	 5 (8.0) 29 (46.0) 29 (46.0)	 3 (15.8) 15 (78.9) 1 (5.3)	**0.005**	 0 6 (23.1) 20 (76.9)	 8 (14.2) 38 (67.9) 10 (17.9)	**<0.001**
Inflammation: detected not detected	 15 (23.8) 48 (76.2)	 2 (10.5) 17 (89.5)	0.17	 19 (73.1) 7 (26.9)	 46 (82.1) 10 (17.9)	0.25
BPH: detected not detected	 48 (76.2) 15 (23.8)	 14 (73.7) 5 (26.3)	0.52	 12 (21.4) 44 (78.6)	 8 (30.8) 18 (69.2)	0.25

PSA – prostate specific antigen, BPH – benign prostate hyperplasia.

The strength of relationship between parameters examined was additionally tested using method of correlation analysis. The results of correlation analysis regarding investigated MMP gene expression in blood, patient age, clinical and pathological characteristics are presented in Table 19.

Table 19. Correlation analysis between MMP gene expression in blood, patient age, clinical and pathological characteristics

Variables	***MMP-9 expression***		***MMP-11 expression***	
	r	p	r	p
Age	**0.349**	**0.001**	**0.243**	**0.02**
Stage	**0.387**	**<0.001**	**0.591**	**<0.001**
Gleason's score	0.157	0.15	0.104	0.35
Serum PSA level	0.082	0.46	**0.375**	**0.001**
Prognostic group	**0.342**	**0.002**	**0.564**	**<0,001**
Inflammation	0.138	0.21	0.104	0.35
BPH	0.025	0.82	–0.101	0.36

r – correlation coefficient, PSA – prostate specific antigen, BPH – benign prostate hyperplasia.

The data presented in Table 19 show that there is a weak, although statistically significant relationship between *MMP-9* expression in blood and patient age, stage and prognostic group. A weak statistically significant relationship between *MMP-11* expression in blood, patient age and serum PSA levels was detected, also, as well as relationship of moderate strength between *MMP-11* expression in blood, stage of the disease (r=0.591) and prognostic group (r=0.564).

The probability to detect *MMP-9* and *MMP-11* expression in blood is higher in elderly prostate cancer patients (64 – 85 years), in patients with confirmed stage III disease or belonging to prognostic group III. The probability to detect *MMP-11* expression in blood is also higher in event PSA serum levels are higher.

The probability of survival of prostate cancer patients was evaluated in accordance with MMP expression in blood. The analysis was performed for all 82 patients; however, because of insufficient number of subjects in certain study groups, survival analysis was not performed in patients of groups where Gleason's score in biopsy material was between 8 and 10 (n=7) or serum PSA level was higher than 20 ng/ml (n=6). Univariate *Kaplan-Meier* analysis demonstrated that *MMP-9* and *MMP-11* gene expression in blood was associated with survival of prostate cancer patients (Table 20).

Table 20. Analysis of survival probability of prostate cancer patients, in accordance with MMP gene expression in blood

Variables	***MMP-9***	***MMP-11***
	p (*log-rank*)	
Age group:		
50-63 years	0.90	0.55
64-85 years	0.65	0.15
Stage:		
I	0.38	--
II	0.17	0.51
III	**0.04**	0.49
Gleason's score:		
6	0.24	0.43
7	0.80	0.06
Serum PSA level (ng/ml):		
≤ 10	0.21	0.24
10–20	0.36	0.20
Prognostic group:		
I	0.24	--
II	**0.03**	0.65
III	**0.04**	0.56
Inflammation:		
detected	0.27	0.15
not detected	0.18	0.05
BPH:		
detected	0.08	0.43
not detected	0.27	**0.001**

PSA – prostate specific antigen, BPH – benign prostate hyperplasia..

The data presented in Table 20 show that survival rate of prostate cancer patients is not associated with *MMP-9* and *MMP-11* gene expression in blood, depending on age, Gleason's score and serum PSA levels.

While evaluating concomitant conditions of prostate cancer, it should be noted, that the prostate cancer patients who had no BPH and for whom *MMP-11* gene expression in blood was not detected, had a better survival rate, in comparison with the patients of the same group for whom *MMP-11* expression in blood was detected (*log-rank* p=0.001) (Fig. 5).

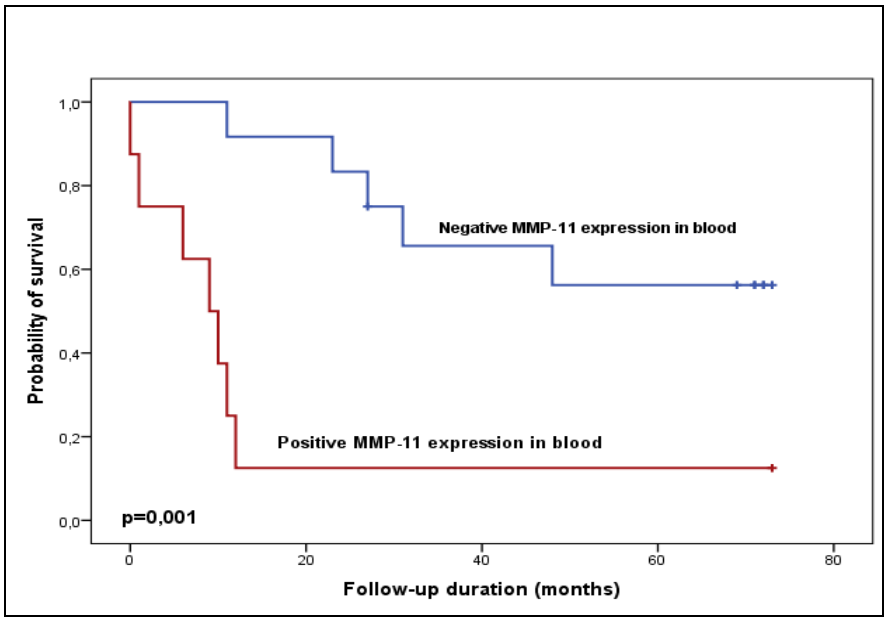

Figure 5. Survival curves of prostate cancer patients without benign prostate hyperplasia (BPH), in accordance with *MMP-11* expression in blood

3.2.2. Matrix metalloproteinase 9 gene polymorphism in blood of prostate cancer patients

MMP-9 (-1562 C/T) polymorphism was detected in all study participants. C/C, C/T and T/T variants of MMP-9 gene polymorphism were detected in 53 (64.6 %, 95% CI 59.35-69.91), 15 (18.3 %, 95% CI 14.02-22.56) and 14 (17.1 %, 95% CI 12.92-21.23) of the cases, respectively. The analysis of distribution of variants of polymorphism regarding age, clinical and pathological characteristics showed statistically significant distributions between variants of MMP-9 (-1562 C/T) gene polymorphism and the stage of the disease ($p<0.0001$), prognostic group ($p<0.0001$) and presence of concomitant BPH ($p=0.03$) (Table 21).

Table 21. Analysis of MMP-9 (-1562 C/T) gene polymorphism in blood of prostate cancer patients, in accordance with age, clinical and pathological characteristics

Variables	MMP-9 -1562 polymorphism			p
	CC, n (%)	CT, n (%)	TT, n (%)	
Age group:				
50-63 years	31 (58.5)	8 (53.3)	4 (28.6)	0.13
64-85 years	22 (41.5)	7 (46.7)	10 (71.4)	
Stage:				
I	5 (9.4)	3 (20.0)	0	
II	32 (60.4)	11 (73.3)	0	**<0.0001**
III	16 (30.2)	1 (6.7)	14 (100.0)	
Gleason's score:				
6	36 (72.0)	11 (78.6)	5 (45.5)	0.10
7	11 (22.0)	3 (21.4)	3 (27.3)	
8-10	3 (6.0)	0	3 (27.3)	
Serum PSA level (ng/ml):				
≤ 10	41 (83.7)	10 (83.3)	7 (58.3)	0.15
10–20	5 (10.2)	2 (16.7)	2 (16.7)	
> 20	3 (6.1)	0	3 (25.0)	
Prognostic group:				
I	6 (11.3)	2 (13.3)	0	
II	31 (58.5)	13 (86.7)	0	**<0.0001**
III	16 (30.2)	0	14 (100.0)	
Inflammation:				
detected	9 (17.0)	4 (26.7)	4 (28.6)	0.52
not detected	44 (83.0)	11 (73.3)	10 (71.4)	
BPH:				
detected	44 (83.0)	11 (73.3)	7 (50.0)	**0.03**
not detected	9 (17.0)	4 (26.7)	7 (50.0)	

PSA – prostate specific antigen, BPH – benign prostate hyperplasia.

The strength of parameters examined was evaluated by means of correlation analysis. Statistically significant results of correlation analysis of examined MMP-9 (-1562 C/T) gene polymorphism, patient clinical and pathological characteristics are presented in Table 22.

Table 22. Correlation analysis between MMP-9 (-1562 C/T) gene polymorphism in blood of prostate cancer patients, in accordance with clinical and pathological characteristics

Variables	MMP-9 (-1562 C/T)	
	r	p
Age	0.164	0.14
Stage	**0.300**	**0.006**
Gleason's score	0.098	0.38
PSA serum level	0.127	0.25
Prognostic group	**0.343**	**0.002**
Inflammation	0.125	0.26
BPH	0.80	0.47

r – correlation coefficient, PSA – prostate specific antigen, BPH benign prostate hyperplasia.

The data, presented in Table 22, show that there is a weak, although statistically significant relationship between distribution of MMP-9 (-1562 C/T) polymorphism variants and the stage of the disease, as well as with the prognostic group of the patients. The probability to detect MMP-9 -1562 T/T variant, associated with poorer prognosis, is greater in event of advanced disease and for prostate cancer patients included into the prognostic group III.

The probability of survival of study participants was evaluated regarding MMP-9 (-1562 C/T) gene polymorphism variants detected in blood. The analysis was performed for all 82 subjects; however, because of insufficient number of participants, the analysis of survival was not performed for groups that included patients with Gleason's score in biopsy material ranging from 8 to 10 (n=6) or patients with serum PSA higher than 20 ng/ml (n=6).

Univariate *Kaplan-Meier* analysis demonstrated that MMP-9 gene (-1562 C/T) polymorphism was associated with prostate cancer patients' survival rate, depending on age, serum PSA level and diagnosed concomitant pathological conditions, including inflammation or BPH (Table 23).

Table 23. Analysis of probability of survival of prostate cancer patients, in accordance with MMP-9 gene (-1562 C/T) polymorphism in blood

Variables	**MMP-9 -1562 C/T polymorphism**
	p (*log-rank*)
Age group:	
50-63 years	0.21
64-85 years	**0.02**
Stage:	
I	0.24
II	0.84
III	0.20
Gleason's score:	
6	**0.04**
7	0.37
Serum PSA level (ng/ml):	
≤ 10	0.23
10-20	**0.001**
Prognostic group:	
I	0.11
II	0.70
III	0.09
Inflammation:	
detected	**0.02**
no detected	0.30
BPH:	
detected	**0.04**
not detected	0.31

PSA – prostate gland specific antigen; BPH – benign prostate hyperplasia.

The elderly prostate cancer patients (64–85 years), in whom MMP-9 -1562 C/C variant was detected, had a better survival rate, in comparison with the patients of the same age with T/T variant (*log-rank* p=0.02) (Fig. 6).

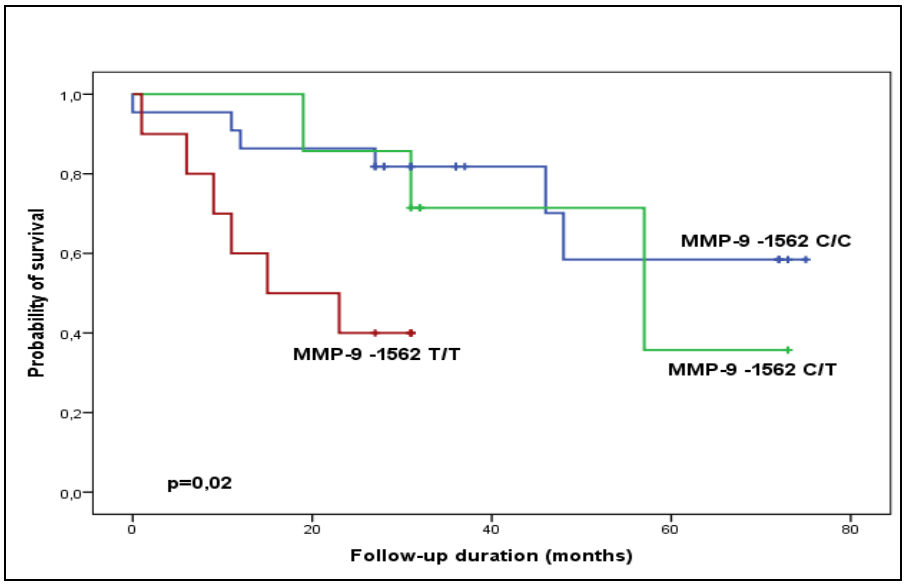

Figure 6. Survival curves of elderly patients (64–85 years) with prostate cancer, in accordance with MMP-9 (-1562 C/T) polymorphism in blood

The evaluation of survival rate, in accordance with PSA levels and MMP-9 (-1562 C/T) polymorphism, showed that the patients, who had PSA level ranging from 10 to 20 ng/ml and in whom MMP-9 1562 C/C variant was detected in blood, survived better, in comparison with the patients of the same group, in whom T/T variant was detected (*log-rank* p=0.001) (Table 23).

As it was mentioned above, other pathologic conditions, including inflammation and BPH, were evaluated together with prostate tumour characteristics. In 65 patients inflammation of prostate gland was not revealed and 17 patients had concomitant inflammation of prostate gland. The patients who had MMP-9 -1562 C/C or C/T variant in blood and concomitant inflammation in prostate gland, survived better, in comparison with the patients who had T/T variant. Survival curves of the patients who had prostate gland inflammation, in accordance with MMP-9 polymorphism (*log-rank* p=0.02), are presented in Figure 7.

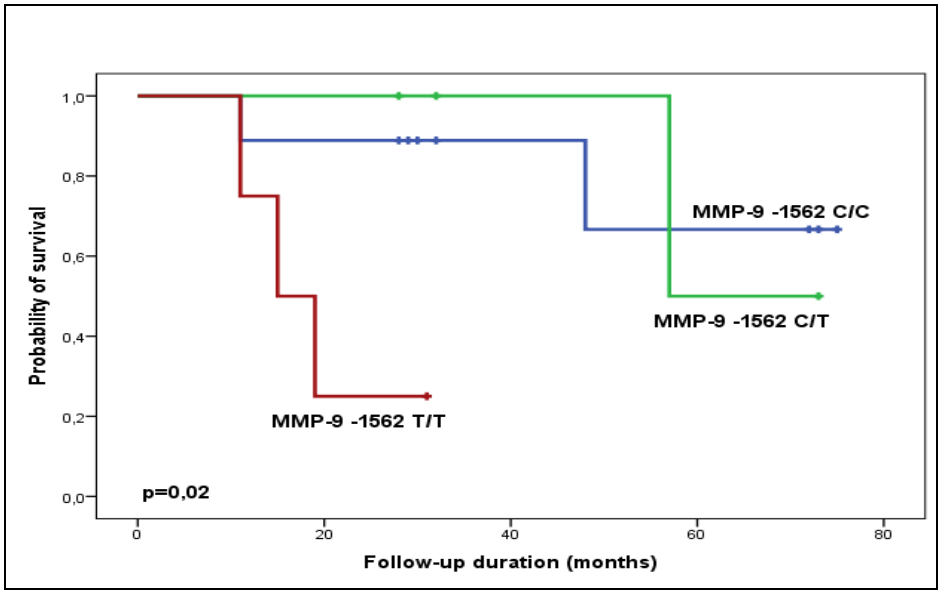

Figure 7. Survival curves of prostate cancer patients with detected concomitant inflammation of prostate gland, in accordance with MMP-9 (-1562 C/T) polymorphism in blood

Concomitant BPH was diagnosed for 62 prostate cancer patients. The patients of this group, for whom MMP-1562 C/C or C/T variants in blood were detected, had a better survival rate, in comparison with patients, in whom T/T variant was present (*log-rank* p=0.04) (Fig. 8).

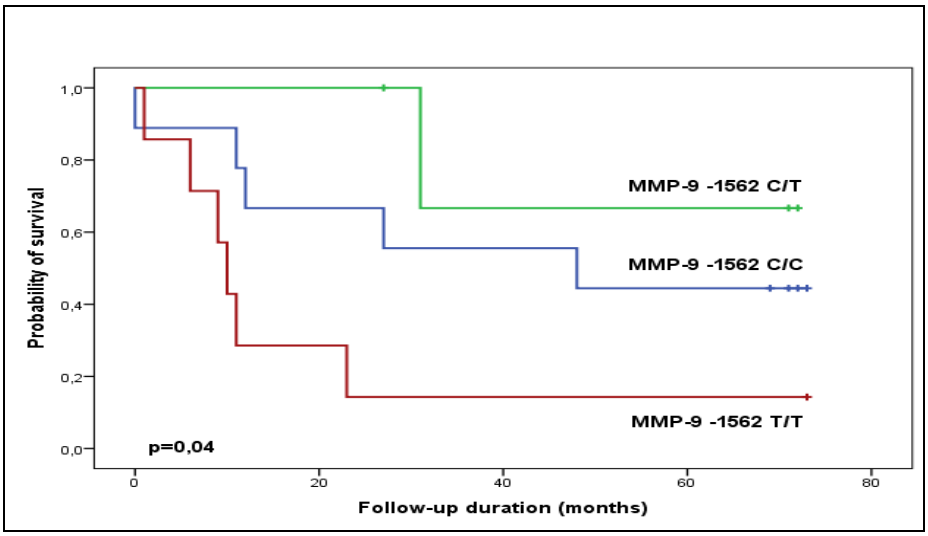

Figure 8. Survival curves of prostate cancer patients with diagnosed benign prostate hyperplasia (BPH), in accordance with MMP-9 (-1562 C/T) polymorphism in blood

3.2.3. Factors influencing survival rate of prostate cancer patients

The results presented above show that a lot of factors investigated have influence on survival rate of prostate cancer patients. Therefore, multivariate analysis of the factors investigated was performed using *Cox* proportional hazards regression model, in order to evaluate the main factors that had the greatest impact on patients' survival rate.

Prognostic analysis included the following factors: age, stage of the disease, Gleason's grade of tumour differentiation, presence of concomitant pathologic conditions of prostate gland (inflammation, BPH), MMP-9 (-1562 C/T) polymorphism, *MMP-9* and *MMP-11* gene expression in blood (Table 24).

Table 24. Multivariate analysis of factors influencing survival rate of prostate cancer patients

Variables	Hazard ratio	95% CI	p
Age	0.270	0.071-1.028	0.05
Stage	0.321	0.019-5.468	0.43
Gleason's score	0.134	0.035-0.515	**0.003**
Serum PSA level (ng/ml)	1.428	0.325-6.279	0.63
Inflammation	1.027	0.297-3.550	0.96
BPH	5.177	1.916-13.989	**0.001**
MMP-9 (-1562 C/T) polymorphism	0.855	0.142-5.149	0.86
MMP-9 expression in blood	0.816	0.096-6.906	0.85
MMP-11 expression in blood	0.340	0.119-0.975	**0.04**

CI – confidence intervals; PSA – prostate specific antigen; BPH – benign prostate hyperplasia

The results showed that Gleason's score of tumour differentiation, BPH and *MMP-11* gene expression were independent prognostic factors of prostate cancer patients' survival. On the other hand, age, serum PSA level, prostate gland inflammation, MMP-9 (-1562 C/T) polymorphism and *MMP-9* gene expression in blood had no influence on survival rate of prostate cancer patients.

The data presented show that MMP gene expression in prostate cancer patients is related to clinical and pathological characteristics of the disease and may be an independent factor for cancer prognosis.

6. CONCLUSIONS

1. It was found out that in event of breast cancer:

- MMP expression in a tumour was associated with younger age of the patient *(MMP-3)*, histology type of the tumour – ductal carcinoma (*MT1-MMP*, *MMP-11*), poorly differentiated tumour (*MMP-13*) and status of regional lymph nodes – axillary metastases on the same side (*MT1-MMP*, *MMP-3*);
- only *MMP-9* gene expression in blood was associated with poorly differentiated tumour among MMP investigated;
- MMP-3 (-1171) polymorphism variants 5A/5A and 5A/6A variants, as well as MMP-9 (-1562) polymorphism variant C/C and C/T were identified statistically significantly more often.

2. The better survival rate of breast cancer patients is influenced not only by stage of the disease, tumour differentiation grade, status of oestrogen and progesterone receptors, but also by MMP-3 (-1171 5A/6A) polymorphism.

3. It was found out in event of prostate cancer:

- *MMP-9* and *MMP-11* expression in blood was associated with the elderly age of the patients, stage of the disease and prognostic group; furthermore, *MMP-11* expression was related to serum PSA level, also;
- MMP-9 (-1562) C/C variant was detected in patients statistically significantly more often, in comparison with C/T and T/T variants.

4. Gleason's tumour differentiation score, benign prostate hyperplasia and *MMP-11* gene expression in blood were independent prognostics factors of survival of prostate cancer patients.

7. LIST OF DISSERTATION-RELATED SCIENTIFIC PUBLICATIONS AND PRESENTATIONS

Articles:

1. **D. Stančiūtė (Schveigert)**, J. Didžiapetrienė, J Kadziauskas. Matrikso metaloproteinazės sergant piktybiniais navikais. Medicina. 2004; 40(12): 1143-1150.

2. **D. Stančiūtė (Schveigert)**, R. Aškinis, J. Didžiapetrienė. Matrikso metaloproteinazių sintetiniai inhibitoriai (ikiklinikinių ir klinikinių tyrimų apžvalga). Sveikatos mokslai. 2006; 6: 622-628.

3. **D. Schveigert**, S. Bružas, I. Nedzelskienė, G. Chvatovič, R. Meškauskas, J. Didžiapetrienė. Matrikso metaloproteinazės sergant krūties vėžiu. Laboratorinė medicina. 2011; 4(52): 179-184.

4. **D. Schveigert**, S. Cicenas, S. Bruzas, N. E. Samalavicius, Z. Gudleviciene, J. Didziapetriene. The value of MMP-9 for breast and non-small cell lung cancer patients' survival. Adv Med Sci. 2013; 58(1): 73-82.

5. **D. Schveigert**, K. P. Valuckas, V. Kovalcis, A. Ulys, G. Chvatovic, J. Didziapetriene. Significance of MMP-9 expression and MMP-9 polymorphism in prostate cancer. Tumori. 2013; 99(4): 537-543.

Abstracts:

1. **D. Stanciute (Schveigert)**, J. Didziapetriene, F. Jankevicius. Matrix metalloproteinase 9 single nucleotide polymorphism analysis in blood of urological cancer patients. EJC Suppl. 2006, 4(6):36.

2. **D. Schveigert**, J. Didziapetriene. Matrix Metalloproteinase-9 as Prognostic Marker of Cancer. Eur J Hum Genet. 2012, 20(1): 187.

Presentation:

D. Schveigert, J. Didžiapetrienė, S. Bružas, A. Ulys. Prognostic significance of matrix metalloproteinases for patients with hormone-dependent cancer.

The 11th Baltic Congress of Laboratory Medicine, 9-11 May 2012, Vilnius, Lithuania.

Appendix 1

Table 13. Analysis of MMP gene expression in breast tumours, in accordance with age, clinical and pathological characteristics

Characteristic / MMP expression	**Age**		p	**Stage**			p	**Histological type of tumour**		p	**Tumour differentiation grade**			p
	31–56 years	57–83 years		I	II	III/IV		DCa	LCa		G1	G2	G3	
MT1-MMP														
Positive, n (%)	40 (48.8)	42 (51.2)	0.13	16 (19.5)	56 (68.3)	10 (12.2)	**0.002**	68 (84.0)	13 (16.0)	0.52	16 (19.5)	45 (54.9)	21 (25.6)	0.91
Negative, n (%)	1 (16.7)	5 (83.3)		5 (83.3)	1 (16.7)	0		3 (75.0)	1 (25.0)		1 (16.7)	3 (50.0)	2 (33.3)	
MMP-1														
Positive, n (%)	20 (54.1)	17 (45.9)	0.16	5 (13.5)	30 (81.1)	2 (5.4)	**0.02**	31 (83.8)	6 (16.2)	0.59	7 (18.9)	16 (43.3)	14 (37.8)	0.08
Negative, n (%)	21 (41.2)	30 (58.8)		16 (31.4)	27 (52.9)	8 (15.7)		40 (83.3)	8 (16.7)		10 (19.6)	32 (62.8)	9 (17,6)	
MMP-2														
Positive, n (%)	39 (47.6)	43 (52.4)	0.40	21 (25.6)	51 (62.2)	10 (12.2)	0.17	67 (84.8)	12 (15.2)	0.25	17 (20.7)	42 (51.2)	23 (28.1)	0.06
Negative, n (%)	2 (33.3)	4 (66.7)		0	6 (100.0)	0		4 (66.7)	2 (33.3)		0	6 (100.0)	0	
MMP-3														
Positive, n (%)	29 (54.7)	24 (45.3)	**0.04**	7 (13.2)	37 (69.8)	9 (17.0)	**0.005**	44 (83.0)	9 (17.0)	0.56	9 (17.0)	28 (52.8)	16 (30.2)	0.52
Negative, n (%)	12 (34.3)	23 (65.7)		14 (40.0)	20 (57.1)	1 (2.9)		27 (84.4)	5 (15.6)		8 (22.9)	20 (57.1)	7 (20.0)	
MMP-9														
Positive, n (%)	39 (47.6)	43 (52.4)	0.40	20 (23.9)	52 (64.7)	10 (11.4)	0.54	65 (82.3)	14 (17.7)	0.32	16 (19.5)	43 (52.4)	23 (28.1)	0.26
Negative, n (%)	2 (33.3)	4 (66.7)		1 (16.7)	5 (83.3)	0		6 (100.0)	0		1 (16.7)	5 (83.3)	0	
MMP-11														
Positive, n (%)	40 (46.0)	47 (54.0)	0.46	20 (23.0)	57 (65.5)	10 (11.5)	0.19	71 (81.6)	16 (18.4)	0.19	16 (18.4)	48 (55.2)	23 (26.4)	0.12
Negative, n (%)	1 (100.0)	0		1 (100.0)	0	0		0	1 (100.0)		1 (100.0)	0	0	
MMP-13														
Positive, n (%)	29 (46.8)	33 (53.2)	0.57	9 (14.5)	45 (72.6)	8 (12.9)	**0.006**	49 (80.3)	12 (19.7)	0.17	8 (12.9)	34 (54.8)	20 (32.3)	**0.02**
Negative, n (%)	12 (46.2)	14 (53.8)		12 (46.2)	12 (46.2)	2 (7.6)		22 (91.7)	2 (8.3)		9 (34.6)	14 (53.9)	3 (11.5)	

DCa – ductal carcinoma; LCa – lobular carcinoma, G1 – well-differentiated tumour; G2 – moderately differentiated tumour; G3 – poorly differentiated tumour.

Appendix 2

Table 13 (continued). Analysis of MMP gene expression in breast tumours, in accordance with age, clinical and pathological characteristics

Characteristic / MMP expression	Lymph nodes status			p	ER		p	PR		p
	N0	N1	N2		Negative	Positive		Negative	Positive	
MT1-MMP										
Positive, n (%)	39 (47.6)	36 (43.9)	7 (8.5)	**0.04**	32 (39.0)	50 (61.0)	0.57	31 (37,8)	51 (62.2)	0.59
Negative, n (%)	6 (100.0)	0	0		2 (33.3)	4 (66.7)		2 (33.3)	4 (66.7)	
MMP-1										
Positive, n (%)	17 (45.9)	19 (51.4)	1 (2.7)	0.11	16 (43.2)	21 (56.8)	0.29	16 (43.2)	21 (56.8)	0.23
Negative, n (%)	28 (54.9)	17 (33.3)	6 (11.8)		18 (35.3)	33 (64.7)		17 (33.3)	34 (66.7)	
MMP-2										
Positive, n (%)	42 (51.2)	34 (41.5)	6 (7.3)	0.70	33 (40.2)	49 (59.8)	0.24	32 (39.0)	50 (61.0)	0.26
Negative, n (%)	3 (50.0)	2 (33.3)	1 (16.7)		1 (16.7)	5 (83.3)		1 (16.7)	5 (83.3)	
MMP-3										
Positive, n (%)	22 (41.5)	25 (47.2)	6 (11.3)	0.06	22 (41.5)	31 (58.5)	0.32	19 (35.8)	34 (64.2)	0.43
Negative, n (%)	23 (65.7)	11 (31.4)	1 (2.9)		12 (34.3)	23 (65.7)		14 (40.0)	21 (60.0)	
MMP-9										
Positive, n (%)	42 (51.3)	33 (40.2)	7 (8.5)	0.72	32 (39.0)	50 (61.0)	0.57	30 (36.6)	52 (63.4)	0.40
Negative, n (%)	3 (50.0)	3 (50.0)	0		2 (33.3)	4 (66.7)		3 (50.0)	3 (50.0)	
MMP-11										
Positive, n (%)	44 (50.6)	36 (41.4)	7 (8.0)	0.61	33 (37.9)	54 (62.1)	0.38	32 (36.8)	55 (63.2)	0.37
Negative, n (%)	1 (100.0)	0	0		1 (100.0)	0		1 (100.0)	0	
MMP-13										
Positive, n (%)	29 (46.8)	27 (43.5)	6 (9.7)	0.38	24 (38.7)	38 (61.3)	0.58	22 (35.5)	40 (64.5)	0.35
Negative, n (%)	16 (61.6)	9 (34.6)	1 (3.8)		10 (38.5)	16 (61.5)		11 (42.3)	15 (57.7)	

N0 – no metastases in regional lymph nodes are present; N1 – mobile metastases are present in axillar lymph nodes of the same side; N2 – metastases in lymph nodes of the armpit of the same side, the nodes are stuck to each other and surrounding structures; ER – oestrogen receptors; PR – progesterone receptors.

Appendix 3

Table 14. Correlation analysis between MMP gene expression in breast tumours, clinical and pathological characteristics

Variables	***MT1-MMP***		***MMP-1***		***MMP-2***		***MMP-3***		***MMP-9***		***MMP-11***		***MMP-13***	
	r	p	r	p	r	p	r	p	r	p	r	p	r	p
Age	–0.162	0.13	–0.127	0.23	–0.072	0.50	–0.200	0.06	–0.072	0.50	0.115	0.28	–0.006	0.95
Stage	**0.340**	**0.01**	0.081	0.45	–0.069	0.52	**0.346**	**0.001**	0.013	0.90	0.167	0.11	**0.300**	**0.005**
Histological type of tumour	**0.210**	**0.04**	0.067	0.53	0.096	0.37	0.073	0.50	–0.132	0.21	**0.219**	**0.04**	–0.065	0.55
Tumour differentiation grade	–0.040	0.70	0.159	0.13	0.035	0.74	0.118	0.27	0.099	0.35	0.166	0.12	**0.287**	**0.007**
Lymph node status	**0.256**	**0.01**	0.037	0.73	–0.028	0.79	**0.251**	**0.01**	0.015	0.89	0.102	0.34	0.145	0.17
ER expression	–0.029	0.78	–0.081	0.45	–0.122	0.25	–0.073	0.50	–0.029	0.78	0.135	0.20	–0.002	0.98
PR expression	–0.023	0.83	–0.101	0.34	–0.116	0.28	0.042	0.69	0.070	0.51	0.138	0.19	0.064	0.55

r – correlation coefficient, ER – oestrogen receptors; PR – progesterone receptors.

8. SUMMARY IN LITHUANIAN

Tiriamoji problema ir jos aktualumas

Matrikso metaloproteinazės (MMP) yra šeima nuo cinko priklausomų proteolitinių fermentų, dalyvaujančių naviko angiogenezėje, jo augimo bei metastazavimo procesuose, todėl, siekiant įvertinti sergančiųjų įvairių lokalizacijų vėžiu prognozę, svarbu nustatyti šių fermentų raiškos pokyčius sergant piktybiniais navikais. Buvo manyta, kad MMP yra fermentai, kurie veikia tik kaip naviko augimo ir metastazavimo procesų stimuliatoriai, ir todėl laikytasi nuomonės, kad užblokavus šių fermentų aktyvumą būtų galima slopinti naviko augimo ir plitimo procesus. Todėl buvo sukurti ir tirti klinikoje plataus veikimo spektro sintetiniai MMP slopikliai. Pagrindiniai šių slopiklių taikiniai buvo MMP-2, MMP-3, MMP-7, MMP-9, MMP-11 ir MMP-12. Pažymėtina, kad dauguma su šiais slopikliais susijusių tyrimų buvo nutraukti anksčiau laiko, nes negauta klinikinio efekto arba pasireiškė ženklus nepageidaujamas poveikis tiriamųjų organizmui. Tai parodė, kad teorinės žinios apie MMP raišką sergant įvairių lokalizacijų piktybiniais navikais ir šios raiškos sąsają su navikiniu procesu nepakankamos. Pastaraisiais metais daugėja eksperimentinių ir klinikinių duomenų, iš kurių matyti, kad padidėjęs MMP aktyvumas gali tiek skatinti, tiek slopinti navikinį procesą [Egeblad M. ir kt. 2002]. Taigi, individualizuojant onkologinio paciento gydymą, reikia atsižvelgti į konkrečios MMP aktyvumą, nes vienu atveju MMP skatina navikinį procesą ir gali būti tam tikrų lokalizacijų navikų terapijos taikiniu, tad jų raišką reikia slopinti, o kitu atveju, esant tam tikrų lokalizacijų navikui, MMP slopina navikinį procesą, ir jų raiškos nereikėtų slopinti [Deryugina E.I. ir kt. 2006]. Kadangi MMP dalyvauja navikinių ląstelių metastazavimo procese, jų raiškos kraujyje įvertinimas galėtų būti prognozinis žymuo, rodantis mikrometastazių formavimosi procesą. Tačiau vis dar nėra atlikta didelių daugiacentrių tyrimų, patvirtinančių šių fermentų svarbą koreguojant pacientų gydymo taktiką bei vertinant pacientų išgyvenamumą, kas leistų prognozuoti ligos eigą.

MMP raiškos pokyčiams įtakos turi mutacijos jų genų promotorinėse sekose. Pažymėtina, kad vieno nukleotido polimorfizmas, nustatomas MMP genų promotorinėse sekose ir galintis turėti įtakos MMP raiškai, siejamas su agresyvesne navikinio proceso eiga. Vieno nukleotido polimorfizmo tyrimai įgyja vis daugiau reikšmės prognozuojant

ligą, tačiau iki šiol tyrimų rezultatai nevienareikšmiai. Pavyzdžiui, MMP-9 (-1562 C/T) polimorfizmas vienų autorių darbuose siejamas su invaziniu krūties naviku [Przybylowska K. ir kt. 2006], o kiti autoriai šios sąsajos nekonstatuoja [McColgan P. ir kt. 2009]. Vadinasi, svarbu nustatyti bei įvertinti, kokie polimorfizmo variantai gali turėti įtakos ligos eigai.

Analizuojant atliktus tyrimus matoma tendencija, kad MMP raiška ir funkcijos priklauso nuo naviko lokalizacijos. Susistemintų duomenų apie MMP raiškos pokyčius atsižvelgiant į naviko lokalizaciją, ląstelių kilmę, ligos vystymosi greitį vis dar nėra. Todėl svarbu išsiaiškinti MMP raiškos pokyčius esant tam tikros lokalizacijos navikams, susieti juos su pacientų klinikinėmis-patologinėmis charakteristikomis bei įvertinti prognoziniu aspektu.

SUFORMULUOTOS HIPOTEZĖS

1. Matrikso metaloproteinazių (MMP) genų raiška sergant vėžiu yra susijusi su klinikinėmis-patologinėmis ligos charakteristikomis.
2. Matrikso metaloproteinazių (MMP) genų raišką galima sieti su sergančių vėžiu pacientų išgyvenamumu ir ši raiška gali būti nepriklausomas prognozinis veiksnys vertinant sergančių vėžiu pacientų išgyvenamumą.
3. Matrikso metaloproteinazių (MMP) genų promotoriuose pasitaikantis vieno nukleotido polimorfizmas (VNP) gali būti prognozinis veiksnys numatant krūties ir priešinės liaukos vėžio eigą.

DARBO TIKSLAS

Nustatyti matrikso metaloproteinazių genų (MMP) raišką ir vieno nukleotido polimorfizmą MMP genų promotorinėse sekose sergant krūties ir priešinės liaukos vėžiui bei įvertinti tirtuosius parametrus prognoziniu aspektu.

DARBO UŽDAVINIAI

1. Sergančioms krūties vėžiu pacientėms nustatyti matrikso metaloproteinazių (*MT1-MMP, MMP-1, MMP-2, MMP-3, MMP-9, MMP-11, MMP-13*) genų raišką ir vieno nukleotido polimorfizmą jų genuose (MMP-3 -1171 5A/6A; MMP-9 -1562 C/T) bei įvertinti tirtuosius parametrus priklausomai nuo klinikinių-patologinių ligos charakteristikų.
2. Įvertinti sąsają tarp matrikso metaloproteinazių (*MT1-MMP, MMP-1, MMP-2, MMP-3, MMP-9, MMP-11, MMP-13*) genų raiškos bei vieno nukleotido polimorfizmo jų genuose (MMP-3 -1171 5A/6A; MMP-9 -1562 C/T) ir sergančiųjų krūties vėžiu išgyvenamumo.
3. Sergantiems priešinės liaukos vėžiu pacientams nustatyti matrikso metaloproteinazių (*MMP-9, MMP-11*) genų raišką ir vieno nukleotido polimorfizmą jų genuose (MMP-9 -1562 C/T) bei įvertinti tirtuosius parametrus priklausomai nuo klinikinių-patologinių ligos charakteristikų.
4. Įvertinti sąsają tarp matrikso metaloproteinazių (*MMP-9, MMP-11*) genų raiškos, vieno nukleotido polimorfizmo jų genuose (MMP-9 -1562 C/T) ir sergančiųjų priešinės liaukos vėžiu išgyvenamumo.

Darbo mokslinis naujumas

Krūties vėžys yra dažniausiai diagnozuojama onkologinė liga tarp moterų, o priešinės liaukos vėžys – tarp vyrų. Nepaisant diagnostinių ir terapinių galimybių tobulėjimo, sergančių vėžiu pacientų išgyvenamumas išlieka vis dar mažas dėl ligos atsinaujinimo. Todėl svarbu nustatyti ligos progresavimo riziką, kas leistų anksčiau pastebėti atsinaujinusią ligą ir kuo greičiau pradėti gydymą.
Mūsų atliktas tyrimas leido palyginti vis dar nepakankamai ištirtų MMP ir jų genų vieno nukleotido polimorfizmo kaip žymenų informatyvumą priklausomai nuo pacientų klinikinių-patologinių charakteristikų ir išgyvenamumo. Išryškinta MMP-3 (-1171 5A/6A) polimorfizmo, kaip veiksnio, turinčio įtakos pacienčių išgyvenamumui, svarba sergant II stadijos krūties vėžiu. Parodyta *MMP-11* geno raiškos sąsaja su ilgesne pacientų, sergančių priešinės liaukos vėžiu, gyvenimo trukme.

IŠVADOS

1. Sergant krūties vėžiu nustatyta:
   - MMP raiška navike susijusi su jaunesniu pacientės amžiumi (*MMP-3*), naviko histologiniu tipu – duktaline karcinoma (*MT1-MMP*, *MMP-11*), blogai diferencijuotu naviku (*MMP-13*) bei sritinių limfmazgių būkle – metastazėmis tos pačios pusės pažasties limfmazgiuose (*MT1-MMP*, *MMP-3*);
   - tik *MMP-9* geno raiška kraujyje susijusi su blogai diferencijuotu naviku;
   - statistiškai reikšmingai dažniau nustatyti MMP-3 polimorfizmo 5A/5A ir 5A/6A variantai bei MMP-9 polimorfizmo C/C ir C/T variantai.

2. Sergančiųjų krūties vėžiu ilgesnei gyvenimo trukmei daro įtaką ne tik ligos stadija, naviko diferenciacijos laipsnis, estrogenų ir progesterono receptorių būklė, bet ir MMP-3 polimorfizmas.

3. Sergant priešinės liaukos vėžiu nustatyta:
   - *MMP-9* ir *MMP-11* raiška kraujyje susijusi su vyresniu pacientų amžiumi, ligos stadija ir prognozine grupe; o *MMP-11* raiška susijusi dar ir su PSA koncentracija serume;
   - statistiškai reikšmingai dažniau nustatytas MMP-9 -1562 C/C variantas, palyginti su C/T ar T/T variantais.

4. Naviko diferenciacijos laipsnis pagal Glisoną, gerybinė priešinės liaukos hiperplazija ir *MMP-11* geno raiška kraujyje yra nepriklausomi sergančiųjų priešinės liaukos vėžiu išgyvenamumo prognoziniai veiksniai.

PADĖKA

Nuoširdžiai dėkoju buvusiam Vilniaus universiteto Onkologijos instituto direktoriui prof. habil. dr. Konstantinui Povilui Valuckui ir dabartiniam Nacionalinio vėžio instituto direktoriui prof. dr. Narimantui Evaldui Samalavičiui už suteiktą galimybę ir paramą vykdant šį mokslinį darbą.

Esu ypač dėkinga mokslinio darbo konsultantei prof. dr. (HP) Janinai Didžiapetrienei, kuri uždegė žingeidumą moksliniam darbui. Nuoširdžiai dėkoju už visapusišką globą, padrąsinimus ir patarimus, reiklumą bei skatinimą siekti kuo geresnių darbo rezultatų.

Labai ačiū Instituto kolegoms prof. dr. (HP) Sauliui Cicėnui, dr. Albertui Uliui, dr. Sauliui Bružui už bendradarbiavimą ir pagalbą atliekant klinikinę mokslinio darbo dalį.

Noriu padėkoti dr. Giedrei Smailytei ir dr. Irenai Kuzmickienei už konsultacijas statistiškai apdorojant disertacijos duomenis.

Nuoširdus ačiū doc. dr. Simonai Rūtai Letautienei už palaikymą ir nuolatinį skatinimą.

Dėkoju Valstybinio patologijos centro darbuotojui gydytojui patologui konsultantui Raimundui Meškauskui už histologinių tyrimų vertinimą.

Esu labai dėkinga savo mamai ir tėčiui už nuolatinį rūpestį, tikėjimą bei palaikymą, taip pat savo vyrui už visapusišką supratimą.

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