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Matrix metalloproteinase-2 and its correlation with basal membrane components laminin-5 and collagen type IV in paediatric burn patients measured with Surface Plasmon Resonance Imaging (SPRI) biosensors

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ABSTRACT

The purpose of this study was the determination of matrix metalloproteinase-2 and its correlation with basal membrane components laminin-5 and collagen type IV in the blood plasma of burn patients measured with Surface Plasmon Resonance Imaging (SPRI) biosensors.
Material and methods: 31 children scalded by hot water who were managed at the Department of Paediatric Surgery between 2014–2015, after primarily presenting with burns in 4–20% TBSA were included into the study (age 9 months up to 14 years, mean age 2,5 + 1 years). There were 10 girls and 21 boys. Venous blood samples were drawn 2–6 h, and 12–16 h after the thermal injury, and on the subsequent days 3, 5 and 7. The matrix metalloproteinase-2, collagen type IV and laminin-5 concentrations were assessed using Surface Plasmon Resonance Imaging by the investigators blinded to the other data.
Results: The MMP-2, laminin-5 and collagen type IV concentrations in the blood plasma of patients with burns, were highest 12–16 h after thermal injury, the difference was statistically significant. The MMP-2, laminin-5 and collagen type IV concentrations measured 3 days, 5 days and 7 days after the thermal injury, slowly decreased over time, and on the 7th day reached the normal range, when compared with the concentration measured in controls.
Conclusion: Current work is the first follow-up study regarding MMP-2 in burns. MMP-2, laminin-5 and collagen type IV levels were elevated early after burn injury in the plasma of studied patients, and were highest 12–16 h after the injury. MMP-2, laminin-5 and collagen type IV levels were not proportional to the severity of the burn. We believe in the possibility that the gradual decrease of MMP-2, collagen type IV and laminin-5 concentrations could be connected with the process of healing, but to prove it, more investigation is needed in this area. The SPR imaging biosensor is a good diagnostic tool for determination of MMP-2, laminin-5 and collagen type IV in blood plasma of patients with burns.

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1. Introduction

Thermal injury is an extremely common, but very complex, clinical challenge, and still remains a leading cause of childhood death [1]. Severe burns are associated with systemic inflammatory response syndrome (SIRS) which in the early post-burn period alter proinflammatory and anti-inflammatory cytokines [2]. One of the post-burn changes is upregulation of metalloproteinases (MMPs), a major group of zinc-dependent endopeptidases, that contribute to remodelling of the extracellular matrix (ECM) by degrading proteins and polysaccharides including basement membrane components (i.e. collagen type IV and laminin) [1,3]. Wound extracellular matrix is a regulator of cell adhesion, migration, proliferation and differentiation during cutaneous repair. The structure of normal wound ECM is determined by a dynamic balance among matrix synthesis, deposition, and degradation [4].

The matrix metalloproteinases are produced by a number of cell types involved in wound repair, including fibroblasts, macrophages, endothelial cells, and keratinocytes [2,5]. MMPs function is not only reduced to degradation of the ECM, they have a key role in the wound healing process [6]. MMPs also modulate the inflammatory response, due to degradation of basal membrane, they are responsible for migration and extravasation of leukocytes [7]. MMPs are also considered as signalling proteases — tissue inhibitors of metalloproteinases (TIMPs) can activate granulocytes and are able to protect inflammatory cells from apoptosis [2,8].

In animal models, Schaffer et al. reported the presence of mRNA for MMP-2 and MMP-9 during the healing of wounds caused by lasers, excisions or burns [9]. MMP-2 degrades not only extracellular matrix proteins (collagen type I, III, IV, V, VII, X, XI, gelatin, elastin, ecorin, aggrecan, fibronectin, laminin, tenascin and vitronectin) but also several nonmatrix proteins such as interleukin 1 beta, protransforming growth factor beta (pro-TGFβ), pro-tumor necrosis factor (pro-TNF) and others [6]. Under physiological conditions, MMP-2 is involved in remodelling of the vasculature, tissue repair, angiogenesis, inflammation, tumor invasion, and atherosclerotic plaque rupture. Other researchers have also shown significant elevation of MMP-2 and MMP-9 mRNA during healing of burn wound, but still did not fully elucidate their association with thermal injury [1,10].

To determine the MMP-2 concentration in body fluids the immunohistochemistry (ELISA) method can be used [11]. An alternative method is Surface Plasmon Resonance Imaging (SPRI). So far several SPRI biosensors were used for clinical research to determinate e.g. lysosomal proteases, 20S proteasome, ubiquitin carboxyl-terminal hydrolase L1 (UCHL1) and immunoproteasome [12–14]. SPRI is based on the dependence of light reflectivity on the number of molecules adsorbed on the surface. Surface plasmon resonance (SPR) is an optical detection process that occurs when a polarized light hits a prism covered by a thin (gold) metal layer. Under certain conditions (wavelength, polarization and incidence angle) free electrons at the surface of the biochip absorb incident light photons and convert them into surface plasmon waves [6]. A dip in reflectivity of the light is seen under these SPR conditions. Perturbations at the gold surface of the biochip, such as an interaction between probe molecules immobilized on the chip and captured target molecules, induce a modification of resonance conditions which are in turn seen as a change in reflectivity and which can be measured. This technique uses two types of very specific interactions: antibody-antigen or inhibitor-enzyme [6].

Therefore the purpose of our study was to investigate the relationship between MMP-2 and thermal injury severity, and its correlation with basal membrane components — laminin-5 and collagen type IV, using novel technique Surface Plasmon Resonance Imaging (SPRI).

2. Methods

2.1. Patients

We included into the study 31 consecutive paediatric patients, with peripheral burn wounds, caused by hot water, who were admitted to the Department of Paediatric Surgery between 2014–2015, because of burns in 4–20% TBSA (age 9 months up to 14 years, mean age 2,5+1 years) (Table 1). Among them were 10 girls and 21 boys. Our patients were divided into three groups depending on the severity of the burn according to American Burns Association: children with minor burns n=8 (<5% TBSA burn, <2% full thickness burn), patients with moderate burns n=13 (5–10% TBSA burn, 2–5% full-thickness burn), and patients with severe burns n=10 (>10% TBSA burn, >5% full-thickness burn). The control group represented 18 healthy, age matched subjects, admitted for planned herniotomy between 2014 and 2015 (for this type of surgery, children are admitted to the department after paediatric examination and after excluding infections or prolonged medication). Exclusion criteria were: admission to the hospital later than 6h after thermal injury, immunological or cardiovascular diseases that required long-term medication, and severe preexisting infections. All parents of our patients, gave written informed consent for both clinical and biochemical follow-up. The study had the Ethics Committee of University of Bialystok approval R-I-002/19/2011. The patients received standard routine care according to accepted guidelines.

2.2. Materials

As a standard, Recombinant Matrix Metalloproteinase-2 (Sigma Steinheim, Germany), collagen type IV (Sigma, Steinheim, Germany) and recombinant human protein laminin-5 (Abcam, USA) was used. ARP 101 — selective MMP-2 inhibitor ((2R)-2-[([1,10-biphenyl]-4-ylsulfonyl)(1-methylethoxy)amino]-*N*-hydroxy-3-methyl-butanamide, M1/4 405.50kDa, Sigma Steinheim, Germany), purified monoclonal mouse anti-human collagen type IV (Tebubio, France), rabbit polyclonal antibody specific to human laminin-5 (Abcam, USA), 1-octadecanothiol (ODM), 2-amino-ethanethiol hydrochloride (cysteamine hydrochloride), *N*-ethyl-N0-(3-dimethylaminopropyl) carbodiimide (EDC), *N*-hydroxysuccinimide (NHS) and 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid sodium salt (HEPES) (all Aldrich, Steinheim, Germany), photopolymer ELPEMER SD 2054, and

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Table 1 – Characteristic of patients with thermal injuries.

	Patients	
	N=31 (burned children)	
Gender (F/M)	Male Female	N=21 (67.74%) N=10 (32.26%)
Age	0.75–14 year (mean age 2,5+1 year)	
Type of burns	Thermal	N=31 (100%)
Burn severity	Minor Moderate Severe	N=8 (25.81%) N=13 (41.94%) N=10 (32.26%)
Total body surface area burned [%]	<5% 5–10% >10%	N=8 (25.81%) N=13 (41.94%) N=10 (32.26%)
Complications and coexisting disease which required long-term medication	None	
Severe preexisting infections	None	
Length of hospital stay	>5 days 5–7 days	N=10 (32.36%) N=21 (67.74%)

hydrophobic protective paint SD 2368 UV SG-DG (PETERS, Kempen, Germany) were used, as well as absolute ethanol, acetic acid, sodium chloride, sodium acetate (all POCh, Gliwice, Poland), HBS-ES buffer pH 7.4 (0.01M HEPES, 0.15M sodium chloride, 0.005% Tween 20, 3mM EDTA), and Phosphate Buffered Saline (PBS), (BIOMED, Lublin, Poland) were used as received. Aqueous solutions were prepared with Milli-Q water (Simplicity® MILLIPORE). High-purity (99.999%) argon N 5.0 (AIR LIQUIDE Polska Sp.z o.o., Poland) were used.

2.3. *Biological samples*

Venous blood samples (1–2mL) were drawn 2–6h (on the admission), and 12–16h after the thermal injury, and on the subsequent days 3, 5 and 7. Blood samples were collected in EDTA tubes, plasma prepared according to the standard protocols and stored at −80°C. After all blood samples were collected and patient data recorded, the collagen type IV and laminin-5 concentrations were assessed using Surface Plasmon Resonance Imaging by the investigators blinded to the other data. 2mL of blood was centrifuged (1000 × *g*) for over 15min and filtered 3 times for the separation of blood plasma from the cells. The supernatant was used for the analysis. The blood plasma samples were frozen and stored at −80°C until their further use. Blood plasma samples were diluted 10 times with PBS buffer prior to measurement.

2.4. *Procedure of MMP-2, collagen type IV and lamini-5 determination*

MMM-P, collagen type IV and laminin-5 was determination by Surface Plasmon Resonance Imaging (SPRI) biosensors. The biosensors and procedures are described in previous articles [6,16,17].

2.5. *Chip preparation*

The gold chips were manufactured as described in the previous paper [6]. The gold surface of the chip was covered with photopolymer and hydrophobic paint as described previously [6,16] The chip contained nine places; each place had twelve free gold surfaces (twelve active, reaction spots). The use of such a chip gave the possibility to measure nine different deposited samples (solutions) simultaneously without their intermixing and to record twelve single SPRI signals from one sample (twelve repetition of SPRI signals from a single sample).

2.6. *ARP 101 immobilization*

In order to bind MMP-2 from the sample it was necessary to immobilize a suitable biological receptor layer, which is the selective MMP-2 inhibitor — ARP 101 (described above). The hydrophobic type of immobilization was used for this purpose. ARP 101 immobilization consisted of two steps. The first one was the formation of a 1-octadecanothiol monolayer by immersion of the chip in 20mM 1-octadecanothiol ethanolic solution for at least 24h at room temperature. After this time, the chip was rinsed with ethanol and water and dried with a stream of argon. The second step was the binding of ARP 101 with ODM by formation of hydrophobic bonds (hydrophobic immobilization). On each of the nine active places (each has 12 reaction spots) modified by ODM, 2mL of nine different ARP 101 water solutions were placed on the chip and incubated at 37°C for 24h. Next, the surface of the biosensor was washed twice with HBS-ES buffer and then at least ten times with

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distilled water. The biosensor prepared in this way was ready for the quantitative determination of MMP-2 in various types of samples [6].

2.7. Antibody immobilization and collagen type IV and laminin-5 entrapment

The first stage was the formation of thiol monolayer on the gold. For this purpose the chips were rinsed with ethanol and water, dried under a stream of nitrogen and were then immersed in 20 mM of cysteamine ethanolic solutions. After two hours the chips were rinsed and dried again under a stream of nitrogen. Next 50 mL of antibody solution (6 $\mu g mL^{-1}$ for antibody of collagen type IV and 5 $ng mL^{-1}$ for antibody of laminin-5) was mixed with 250 mL of 50 mM *N*-hydroxysuccinimide (NHS), 250 mL of 200 mM *N*-ethyl-*N*′-(3-dimethylaminopropyl) carbodiimide (EDC) and 100 mL of carbonate buffer, was placed on the amine-modified surface, and incubated at 37 °C for 1 h. After this time the surface of the biosensors were washed five times with distilled water. The biosensors prepared in this way were ready for the quantitative determination of collagen IV and laminin-5. Next, the chip was then treated with the analyte solution (containing collagen type IV or laminin-5) for 10 min, rinsed with the buffer and dried, and the SPRI measurement was performed [16,17].

2.8. AFM measurements

AFM measurements were performed to confirm the creation of subsequent layers. As described in the previous paper by Tokarzewicz et al.: "Atomic Force Microscopy (AFM) measurements were performed with a commercial Ntegra Prima scanning probe microscope (NT-MDT, Russia) in a tapping mode and under ambient conditions" [6].

2.9. SPRI measurements

SPRI measurements were performed on a home-made apparatus which has been successfully used and described previously [6,15–17]. The schematic diagram of the apparatus and the principles of SPRI measurements were presented in the previous paper [17]. The SPRI signal was proportional to the protein amount bound with the immobilized receptor's layer (biosensor surface), in the linear range of the calibration curves. At the beginning of the creation of the biosensor it was necessary to select a correct SPR angle. It was done for the chip after the immobilization of the receptor layer. The selection of the correct angle was based on the image, by the choice of the angle where the contrast between the active sites, which consisted of gold, thiol, and receptor, and the background was the largest one. In the second step, two SPRI images were acquired. The first one was a photo of the biosensor with the immobilized receptor layer, before adding the sample with analyte. The second one was the photo taken after the interaction of receptor with the analyte. The SPRI signal was measured at the fixed, correct incident angle on the basis of images registered by a CCD camera and it was calculated by comparing the two intensities of the light reflected from the biosensor surface before and after the biosensor interaction with the analyte, for each spot separately. The contrast values obtained for all pixels across a single spot of the particular sample were integrated. Then, the SPRI signal was integrated over the whole spot area. Digital image processing software ImageJ19 version 1.32 was used to evaluate the SPRI images in 2D form and to digitize the signal intensity (in A.U.).

2.10. Preparation of the calibration curve

Preparation of a calibration curve i.e. the SPRI signal dependence on the MMP-2, collagen IV or laminin-5 concentration was necessary for the quantitative determination of this protein in the biological samples. The standard solution of MMP-2, collagen IV or laminin-5 in PBS buffer (pH 7.4) was used for preparation calibration curves. The procedures were described previously in the paper [6,16,17].

2.11. Determination MMP-2, collagen IV and laminin-5 in plasma samples.

After equilibration to room temperature, the blood plasma samples were transferred onto the sensor surface for 10 min to interact with suitable receptor. After the interaction, the surface of the biosensor was washed twice with HBS-ES buffer and then ten times with distilled water. Immediately, the SPRI signal was measured. The MMP 2, collagen IV and laminin-5 plasma concentration in the samples were calculated from the calibration curves. A fixed angle of incident light was maintained during the measurements, and the reflectivity was simultaneously measured across the entire chip surface. The contrast values obtained for all pixels across a particular sample single spot were integrated. Thus the SPRI signal was integrated over the single spot area. A background correction was applied (it is the signal difference between the place of the receptor-analyte complex and the receptor-free space treated only with PBS buffer). Non-specific binding was monitored by measuring the signal SPRI in place on the chip without the receptor (ligand). Minimization of the non-specific binding obtained by preparing samples in PBS buffer (NaCl an KCl concentration ~200 mM) at pH 7.4 near the isoelectric point of the protein. Non-specific binding in the case of natural samples is eliminated by a background correction. It is the signal difference between the place of the receptor-analyte complex and the receptor-free space treated with natural sample [6,16,17].

The signal was measured twice based on the registered images, after immobilization of the receptor, and then after interaction with the sample containing MMP-2,collagen type IV or laminin-5. The SPRI signal, which is proportional to the mass of entrapped protein was obtained as the difference between the signals before and after interaction with the analyzed sample, for each spot separately [6,16,17].

MMP-2, laminin-5 and collagen type IV levels at hours from injury, their maximal levels, and their time-adjusted means were compared between groups. Correlations between MMP-2, laminin-5 and collagen type IV and also the extent of burn were analysed.

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2.12. *ELISA measurements*

During development of new methods for determination of MMP-2, laminin-5 and collagen IV, validation of new biosensor was performed. For this purpose, comparative determination of analyte concentration by the SPRI biosensor and commercial ELISA kit was performed previously [6,16,17].

2.13. *Statistical analysis*

Statistical analysis was performed using the STATISTICA PL release 12.5 Program. All the results are presented as median with 25th and 75th percentiles. Because tested parameters in plasma of patients did not pass the normality Shapiro-Wilk test continuous variables were compared by the nonparametric Kruskal-Wallis test with the Mann-Whitney U test for post hoc comparisons. For multiple comparisons experiment-wise error rate was reduced by using Bonferroni correction ($p<0.0125$) Correlations were studied by using the Spearman Correlation Test. A p value of $p<0.05$ indicated a statistically significant difference.

3. Results

The age and sex composition in each group of patients with minor, moderate and severe burns was comparable. Median time from burn injury to admission to the clinic was similar — between 2 h and 6 h.

Plasma MMP-2, laminin-5 and collagen type IV levels after burns, were higher than in healthy controls. Statistically significant differences in concentration of metalloproteinase-2, collagen type IV and laminin-5 between burned and healthy children, were observed during the first five-seven days after thermal injury (Figs. 1–3). All children with minor burns were discharged home on the fifth day, when wound dressing was no longer needed, and they were treated at home with topical creams or ointments. The healing of burns was defined by our standard clinical criteria. Children with moderate burns were usually discharged home on the seventh day, when they wounds were cleaned out of necrotic tissues, and later their wounds were treated in outpatient service. The hospitalization of children with severe burns usually took longer, but we stopped our observation of plasma levels of laminin-5, collagen type IV and MMP-2 on the seventh day, because we could not compare the results from all groups. We observed sudden increase in the plasma concentrations of MMP-2, collagen type IV and laminin-5, 12–16 h after thermal injury. The MMP-2, laminin-5 and collagen type IV concentrations in the blood plasma of patients with burns, were highest 12–16 h after thermal injury. The MMP-2, laminin-5 and collagen type IV concentrations measured 3 days, 5 days and 7 days after the thermal injury, slowly decreased over time, and on the 7th day reached the normal range, when compared with the concentration measured in controls (Table 2).

We found no correlation between matrix metalloproteinase-2 and laminin-5 concentrations. There was negative, moderately strong correlation, between matrix metaloproteinase-2 and collagen type IV concentrations, 3 days after injury ($r=-0.530$, $p=0.009$) (Fig. 4). There was no correlation between matrix metalloproteinase-2, collagen type IV or laminin-5 concentration and the severity of the thermal injury, but there was tendency, that their median values were higher in children with severe burns, without statistical significance ($p>0.017$). The matrix metalloproteinase-2, collagen type IV and laminin-5 concentrations in the plasma of our patients did not correlate with age, or sex. We did not find statistically significant differences between matrix metalloproteinase-2, collagen IV and laminin-5 concentrations in the plasma in the group of children aged 1 year old and below 1 year old, and in the group of

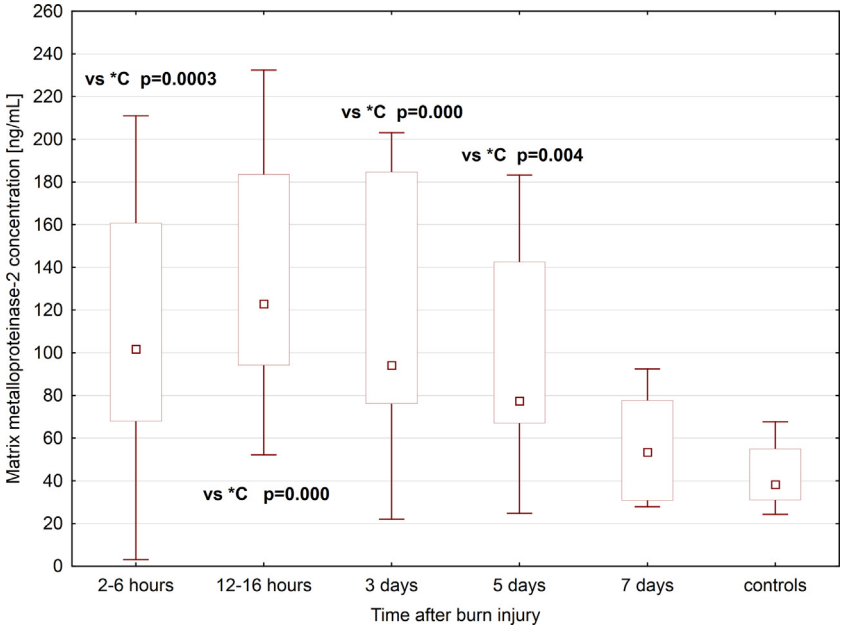

Fig. 1 – Matrix metalloproteinase-2 in the plasma of children 2–6 h (N = 31), 12–16 h (N = 31), 3 days (N = 23), 5 days (N = 20) and 7 days (N = 18) after thermal injury and in control group-C.

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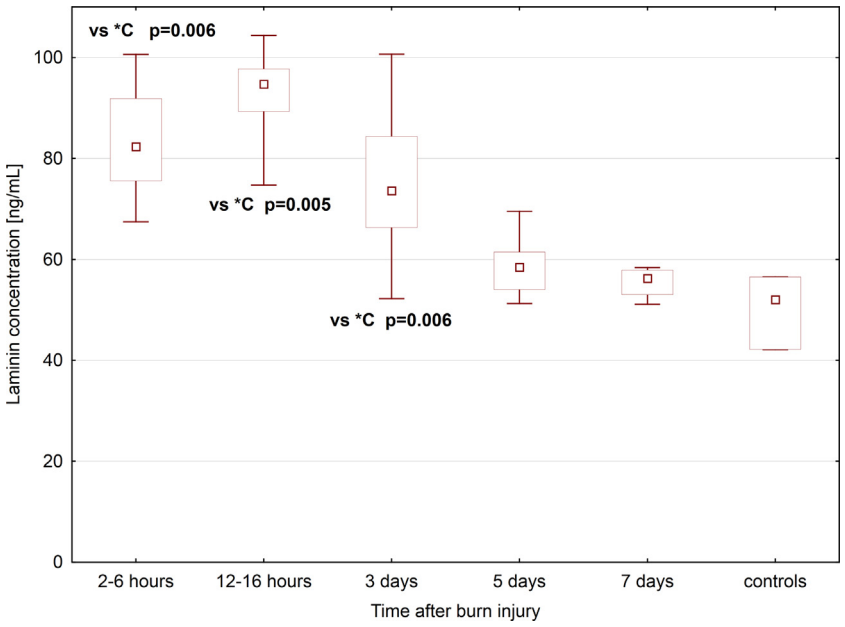

Fig. 2 – Laminin concentration in the plasma of children 2–6 h (N = 31), 12–16 h (N = 31), 3 days (N = 23), 5 days (N = 20) and 7 days (N = 18) after thermal injury and in control group-C.

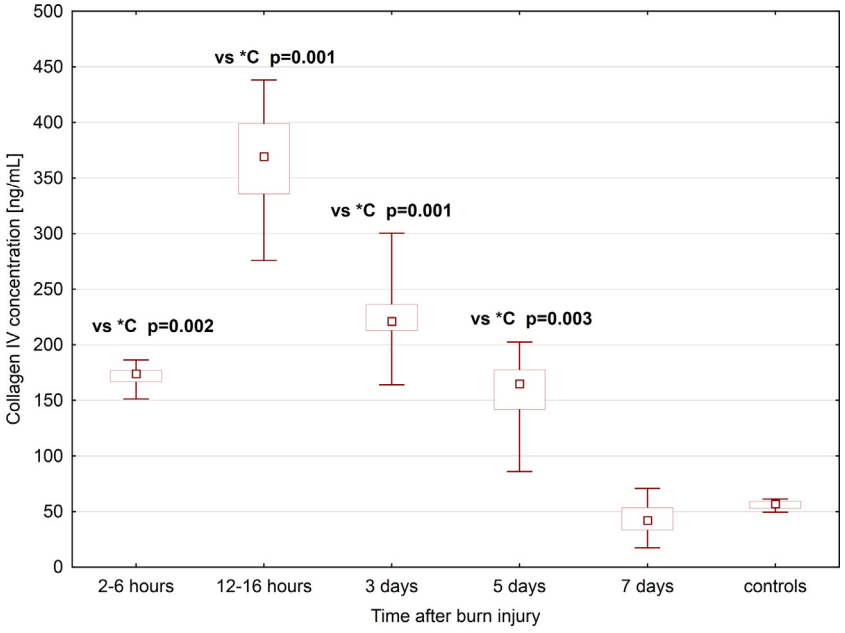

Fig. 3 – Collagen IV concentration in the plasma of children 2–6 h (N = 31), 12–16 h (N = 31), 3 days (N = 23), 5 days (N = 20) and 7 days (N = 18) after thermal injury and in control group-C.

children aged above 1 year old (data not shown). None of the patients developed sepsis, septic shock, or multiple organ failure (MOF), we all patients survived until discharge from the hospital. Patients were discharged home in good general state, on the final stage of healing of the burn wound.

4. Discussion

Thermal injury induce a wide variety of systemic inflammatory responses [3]. According to Hästbacka et al. in burns larger than 20% of the total body surface, generalized inflammatory response leads to "increased capillary permeability, fluid shifts, increased risk of sepsis, and multiple organ dysfunction" [18]. Changes of permeability, which are associated with thermal injury, are caused among other mediators, also by matrix metalloproteinases [18,19]. Matrix metalloproteinases represent a major group of enzymes that regulate cell-matrix composition [20]. Upregulation of MMPs due to thermal injury is causing the degradation of the basal lamina, leading to detrimental swelling of surrounding tissues [21]. The MMP-2, gelatinase belonging to MMPs family, is implicated in various

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Table 2 – Biomarkers levels 12–16 h after thermal injury in the plasma of children with minor (n = 8), moderate (n = 13) and severe burns (n = 10).

Statistic parameters	Laminin concentration [ng/mL]			Collagen IV concentration [ng/mL]			Matrix metalloproteinase-2 concentration [ng/mL]		
	Minor	Moderate	Severe	Minor	Moderate	Severe	Minor	Moderate	Severe
Median	**94.875**	**95.484**	**91.671**	**366.957**	**362.602**	**382.255**	**107.755**	**122.937**	**127.471**
Minimum	76.546	89.753	74.743	300.074	312.342	298.466	52.236	57.959	76.031
Maximum	99.001	104.379	102.859	379.534	408.084	438.329	150.229	207.496	232.512
Percentiles (25%–75%)	85.509–96.943	93.897–100.380	89.066–99.153	325.156–379.272	335.667–379.272	364.734–416.212	87.733–128.658	97.924–186.547	94.240–198.279
CV [%]	8.635	5.743	8.506	9.309	8.633	9.282	19.613	24.652	23.208
* p value	**p > 0.017**			**p > 0.017**			**p > 0.017**		

* A p value > 0.017 for threefold pairwise testing is considered to show a non significant difference between groups (accordingly to Bonferroni correction).

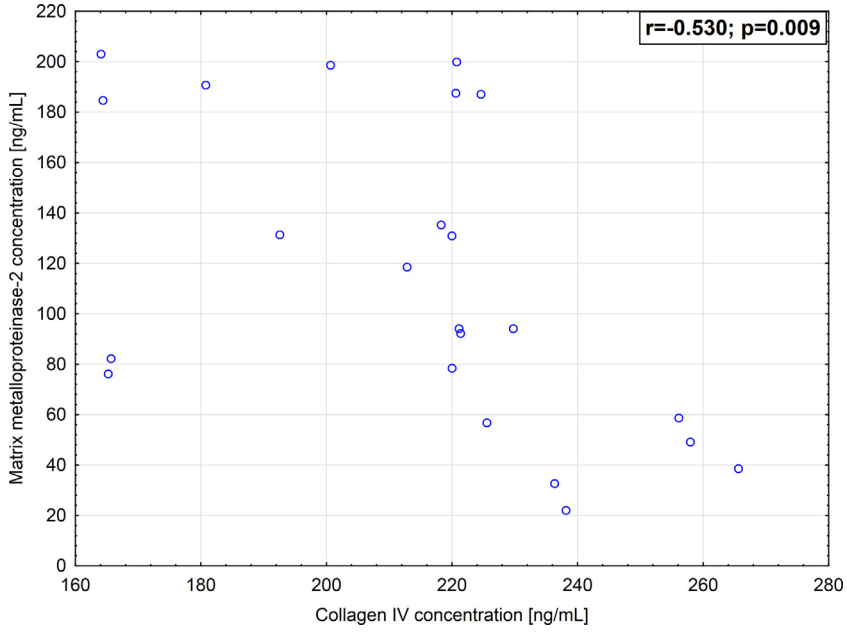

Fig. 4 – Correlation between matrix metalloproteinase-2 and collagen IV concentration (r = −0.530) in children plasma 3 days after burn injury (N = 23).

aspects of inflammation including attracting of inflammatory cells, tissue injury healing, and remodelling [22]. Our focus was to establish a direct relationship between thermal injury and circulating MMP-2 and its correlation with basal membrane components, laminin-5 and collagen type IV, which influence wound healing. The novelty of our study is the determination of the time course of circulating MMP-2 after burns. The limitations of our study was the relatively small study population.

The expression of matrix metalloproteinases after thermal injury and wound healing is regulated by inflammatory cytokines, cellular transformation, hormones and growth factors [5,20]. Tumor necrosis factor-α (TNF-α) is released by macrophages almost immediately after burn and affect metabolism and immunological reactions [5,23]. Han et al. in their study showed that TNF-α mediated and started activation of MMP-9 and pro-MMP-2 in human skin during the inflammatory phase of wound healing [24,25].

MMPs and tissue inhibitors of metalloproteinase (TIMPs) have been evaluated extensively in sepsis, a potentially fatal complication in burned patients [22,26]. Nagy et al. found a 1,4-fold increase of serum TIMP-1 levels after burn injury [2]. Mühl et al. hypothesized that metalloproteinases may represent a new class of biomarkers for the prognosis of septic patients, but in patients with sepsis, did not prove changes in the levels of MMP-2, which were worthy of attention [22]. Contrary to that, Lalu et al. presented a steady increase of MMP-2 until the 24th hour in a septic rat myocardium model, hypothesizing, that the MMP-2 decrease may have a cardioprotective effect [27].

Also patients with extended hypertrophic scars after burns had a significantly higher TIMP-1 levels in the serum [2].

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During the repair phase, the wound matrix is remodelled and replaced with scar tissue, consisting of new collagen fibres, proteoglycans and elastin fibres, in order to re-establish the structure of the tissue and bring back its function. MMP-2 and MMP-9 remove collagen and other ECM components that were denatured during the injury [2].

Reyes et al. in their animal study demonstrated that thermal injury causes increased blood–brain barrier permeability and brain oedema associated with increased MMP-9 expression and a decrease in basal laminar proteins (collagen IV, fibronectin, and laminin) [3]. Collagen IV, laminin and fibronectin are the target proteins degraded by MMPs, and major constituents of all basement membranes (BM) [3].

Basement membranes are specialized structures of the cellular matrix. Their composition is important for maintenance of tissue structure, control of fluid and substrate exchange, and regulation of cell growth and differentiation [28]. Collagen IV primarily stabilizes the BM, providing mechanical strength, and laminin-5 is a component of anchoring filaments localized in the BM of skin, mucosa, lung, and intestine [27]. The laminin-5 gene can be activated by TGF-β present in the wound fluids [29]. Study by Kainulainen et al. have shown that laminin-5 is expressed by migrating keratinocytes during reepithelialization of experimental human full-thickness wounds [29]. Regeneration of surface epithelium during wound healing takes place in a controlled fashion through migration, proliferation, and differentiation [29]. Keratinocytes must be able to interact with the BM zone matrix, the blood clot matrix, or with the connective tissue matrix, which contains various collagen types and proteoglycans. Laminin-5 expression appears to be critical during reepithelialisation — laminin-5 inhibit cell migration and promote the formation of stable contacts with BM [29].

The initial response of the body to the thermal injury is acute inflammation which helps to remove devitalized tissue, and prevents infection [3]. After an injury, adjacent keratinocytes are exposed to dermal collagens, within a few hours these cells secrete large amounts of laminin-5, probably as a response to activation by transforming growth factor-β, interferon-γ and other inflammatory cytokines, which is followed by disassembly of cell junctions [4]. Collagen type IV, is primarily located in basement membranes of heart, lung, vessels, liver and kidney tissue [28]. It can be assumed, that the only source of laminin-5 and collagen type IV in plasma of patients after thermal injuries, was the burned skin. As to MMPs — they are ubiquitous in tissues and biological fluids and are produced by a wide variety of cells. During burn injury, the MMPs are produced by cells involved in wound repair: fibroblasts, macrophages, endothelial cells, and keratinocytes [1–3,5,30].

We observed statistically significant differences in concentration of metalloproteinase-2, collagen type IV and laminin-5, between burned and healthy children, during the first five days after thermal injury. In our study, we demonstrate that plasma levels of MMP-2, laminin-5 and collagen type IV are elevated in the early after thermal injury. Hästbacka et al. also observed an early increase in plasma levels of MMP-9 in human patients after thermal injuries [2,18].

We found no correlation between matrix metalloproteinase-2 and laminin-5 concentrations. Laminin-5 is rather degraded by different metalloproteinases, as in the study by Gilles et al. [31]. This is consistent with the observation by Misko et al. that laminin remains intact in the presence of elevated MMP-2 and MMP-9 in TrJ neuropathy nerves [32].

In our group of burned patients, there was negative, moderately strong correlation, between matrix metaloproteinase-2 and collagen type IV concentrations, 3 days after burn injury. This observation is in common with the study by Sand et al., according to which, collagen IV is the subject to MMP proteolysis [33]. It might be connected with the fact that proliferative phase during which collagen IV was degraded, started on the third day of wound healing processes.

During the repair phase, the provisional wound matrix is remodelled and replaced with scar tissue, consisting of new collagen fibres, proteoglycans and elastin fibres, which partially restore the structure and function of the tissue.

The matrix metalloproteinase-2, collagen type IV and laminin-5 concentrations in the plasma of our patients did not correlate with age, or sex. Similar observations were made by Tayebjee et al. who found that, age and exercise have modest effects on circulating concentrations of MMP-2 [34].

The ECM consists of many components: collagens, and laminins, elastins and fibronectins which provide flexibility; proteoglycans and hyaluronan and glycoproteins [35]. Changes of ECM composition, because of degradation of its components, can result in its altered architecture. The most significant enzymes in ECM remodelling are matrix metalloproteinases e.g. MMP-2 and -9 degrade denatured collagen, MMPs also catalyse the hydrolysis of elastin, laminin, and fibronectin. The new ECM forms a scar with disturbance of the alignment of collagen fibres and excessive dermal fibrosis, which can be a cause of significant morbidity [35]. In abnormal hypertrophic scars and keloids, decreased levels of MMP-1, MMP-2, MMP-9 and increased levels of TIMP-1 were observed [35]. Moreover in hypertrophic scars Abergel et al. showed high collagen production of collagens, and also high ratio of collagen I-III [36]. In our study, we did not observe statistically significant differences between the levels of circulating MMP-2, laminin-5 and collagen type IV in burned children, probably due to small sample size. We studied only 10 patients with severe burns, who after the hospitalization, were treated in the outpatient service and in whom formation of hypertrophic scars was observed, vs. 8 children with minor burns, and 13 patients with moderate burns, whose wounds healed without leaving visible scars. In our group of our burned patients, there was no correlation between matrix metalloproteinase-2, collagen type IV or laminin-5 concentrations, and thermal injury severity, although we observed the tendency, that median concentrations of those markers were higher in patients with severe burns ($p > 0.017$). Probably they are not direct markers of tissue damage, but they are rather involved in wound healing processes. Also Matsuyama et al. found elevated levels of MMP-2 in patients with Takayasu arteritis, but no correlation between serum MMP-2 and disease activity score [30]. More observations are needed to evaluate if targeting components of the ECM during burn wound healing, could influence the formation of hypertrophic and keloid scars.

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It is worth to note, that matrix metalloproteinases, including MMP-2, play also an important role in the progression of cancer, by stimulation of cancer cell growth, invasion, metastasis and angiogenesis [6,36–38]. According to many authors, MMPs synthesis and activity are increased in almost all types of human cancers and correlate with cancer severity, its increased invasiveness, ability of cancer cells to metastasize, and with shorter survival of patients [36–38].

In our study we used novel technique — SPRI MMP-2 biosensor, and SPRI biosensors for laminin-5 and collagen type IV. SPRI biosensor is based on the sensitive and specific reaction between the analyte and its receptor (inhibitor or antibody) [6,15–17]. In the literature data concerning MMP-2, collagen type IV and laminin-5 concentration in human blood plasma were obtained using ELISA enzyme immunoassays [39–42]. However ELISA is the most popularly used method for the determination of biologically active species, but it is an indirect method.

SPRI biosensors for MMP-2, laminin-5 and collagen IV, as compared with ELISA are a more rapid, valuable and reproducible tool for the quantitative determination of this biomolecules in biological samples. SPRI method does not require labelling or addition of a second reagent and it can quantitatively measure molecules concentration in real time. In SPRI measurements very low quantities of reagents and samples are used which do not require special procedure of preparation. The biosensors co-operating with SPR imaging measurement are suitable tool for MMP-2, laminin-5 and collagen IV determination in blood plasma or serum and are able to compete with ELISA method. In perspective, commercial biosensors may be produced, and when miniaturized, could be used at the bed side.

We believe, that our study can help in the understanding of molecular mechanisms of burn wound healing, which is important to develop therapies to effectively treat patients after thermal injuries.

5. Conclusions

Current work is the first follow-up study regarding MMP-2 in burns. MMP-2, laminin-5 and collagen type IV levels were elevated early after burn injury in the plasma of studied patients, and were highest 12–16 h after the injury. MMP-2, laminin-5 and collagen type IV levels were not proportional to the severity of the burn. We believe in the possibility that the gradual decrease of MMP-2, collagen type IV and laminin-5 concentrations could be connected with the process of healing, but to prove it, more investigation is needed in this area. The SPR imaging biosensor is a good diagnostic tool for determination of MMP-2, laminin-5 and collagen type IV in plasma of patients with burns.

Conflicts of interest

The authors have declared that they have no conflict of interest.

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