

# Circulatory matrix metalloproteinases as tissue destruction indicators for improving clinical management of pressure ulcers patients

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**Abstract.** Background and objectives: Pressure ulcer remains a major and debilitating health issue. Biomarkers that can evaluate the patient’s status are important tools in monitoring this disease. A less studied family of circulatory biomarkers, matrix-metalloproteinases (MMPs) are gaining interest in research of pressure ulcers. Our study analyses nine circulatory MMPs evaluated in the serum of pressure ulcers patients prior to any treatment. Material and methods: Forty patients with pressure ulcers and 25 healthy subjects as control group matching ages and gender were included in the current study. The control group was chosen on purpose with an increased aged domain in order to mitigate the known healthy age-related serum MMPs increment. The following group of MMPs: MMP-1, 2, 3, 7, 8, 9, 10, 12 and 13 were quantified in the harvested serum using the multi-analyte technology xMAP array. Results: The highest serum concentration was registered for MMP-9, namely 185 times increased in patients compared to the control group, followed closely by serum MMP-1, found increased 54 times. Conclusion: Several serum MMPs could stand out as biomarkers in pressure ulcers patients.

**Key Words:** pressure ulcers, matrix-metalloproteinase, xMAP array, multi-analyte.

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## Introduction

Pressure ulcers are deep injuries to both skin and underlying tissue and are the consequence of prolonged skin pressure. This condition develops mainly on the bony areas such as heels, ankles, hips and tailbone. The main causes are the medical conditions limiting the capability to change positions. These skin lesions develop quickly, are very difficult to treat and, more recently, were associated with post-stroke mortality (Lee et al 2015).

In this light, pressure ulcer remains a major and debilitating health issue. Extended European programs, like Pressure Ulcer Programme Of reSEarch (PURPOSE) are studying patients’ quality of life. Besides several studied parameters, evaluation of healing capacity is a subject of high interest. Biomarkers that can evaluate the patient’s status are important tools in monitoring this disease (Nixon et al 2015) and matrix-metalloproteinases (MMPs) are gaining interest in research of pressure ulcers. MMPs are involved in pathological conditions, such as tissue destruction and tumor metastasis. Traditionally MMPs were considered to cleavage only components of the extracellular matrix, but recently other substrates were discovered (growth factors,

cell receptors), outlining new biological functions (McCawley and Matrisian 2001).

Our study focused on several families of MMPs that are both enzymes in extracellular matrix remodeling but as well as destructive molecules involved in tissue degradation.

MMP-1 (interstitial collagenase), cleaves types I, II, III, VII and X collagens (Desrochers et al 1991) and its production is enhanced by inflammatory cytokines (Nissinen and Kähäri 2014). Wound healing seems to be correlated with circulatory MMP-2 and MMP-9 (Caimi et al 2015). MMP-3 and 10 is produced in chronic wounds by basal keratinocytes (Saarialho-Kere et al 1994). MMP-7 (matrilysin) cleaves collagen type I, III, IV, V, casein, and fibronectin and enhances gelatinases MMP-2 and MMP-9 expression (Quantin et al 1989; Knox et al 1997; Yokoyama et al 2008).

MMP-8 (neutrophil collagenase), a distinct collagenase, cleaves preferentially collagen type I in contrast to fibroblast collagenase which cleaves preferentially the collagen type III (Devarajan et al 1991) and MMP-8 is a feature for inflammatory cells in chronic wounds (Pirila et al 2007).

Table 1. Matrix-metalloproteinases families concomitantly detected using xMAP technology

Analyte	Spectral region	Analyte	Spectral region	Analyte	Spectral region
<b>MMP-1</b>	43	<b>MMP-7</b>	66	<b>MMP-10</b>	62
<b>MMP-2</b>	26	<b>MMP-8</b>	37	<b>MMP-12</b>	53
<b>MMP-3</b>	45	<b>MMP-9</b>	55	<b>MMP-13</b>	47

MMP 9 (type IV collagenase or gelatinase B) is activated by MMP-3 (van den Steen *et al* 2002; Nagase and Woessner 1999; Ramos-DeSimone *et al* 1999) and it is highly expressed in patients with chronic venous ulcers (Bigg *et al* 2007; Serra *et al* 2013). MMP 10 (stromelysin-2) cleaves fibronectin, laminin, elastin, proteoglycan, gelatin and collagen activating MMP-9 proenzyme and MMP-7 (Madlener *et al* 1997; Nakamura *et al* 1998). MMP 12 (macrophage metalloelastase) cleaves collagen type I and III (Belaouaj *et al* 1993; Shapiro *et al* 1993; Shapiro and Senior 1998; Taddese *et al* 2010) and its expression is enhanced in chronic inflammation (Stawski *et al* 2014).

MMP 13 (collagenase-3) cleaves fibrillary type I, II and III collagens being a promoter of fibroblasts survival and proliferation (Toriseva *et al* 2007).

Taking into account the possibly to monitor circulatory MMPs as biomarkers in pressure ulcer patients we have embarked in studying serum MMPs patterns as potential tool for ulcer patients monitoring.

## Material and Method

In the current study patients diagnosed with pressure ulcers and healthy volunteers with matching ages and gender were included. The study was approved by the Ethics Committee of the "Sf. Pantelimon" Emergency Hospital and all the enrolled individuals gave their informed consent regarding the samples usage, data evaluation and presentation.

### Patients group

Forty patients diagnosed with pressure ulcers admitted in "Sf. Pantelimon" Emergency Hospital were enrolled. The age domain was 23 to 90 years (mean± standard deviation 69.56±12.5 years), 60% males. The enrolled patients had hip and/or coccyx pressure ulcers diagnosed in stages II and III. The etiology of pressure ulcers was grade II and III obesity.

### Control group

Healthy 25 normal weight subjects with no ulcer pressure or any associated pathology, no diabetes, no cardiovascular diseases, no neoplasias and no background treatment were enrolled. The age domain was 63 to 85 years (mean± standard deviation 65.33±5 years), 45% males.

### Biological samples

Serum samples were harvested upon admittance in Hospital for patients and upon volunteering for healthy subjects. Blood was taken by venipuncture and harvested on clotting tubes. Serum was immediately aliquoted and kept until investigation at -20°C. Samples were not subjected to any freeze/thaw cycles and were investigated after 7-14 days upon harvest.

### Multi-analyte investigation

Multi-analyte quantification using Bio - Plex® MAGPIX™ System and the standard kit Bio-Plex Pro™ Human MMP

Assays (Bio-Rad Laboratories US) was used. The principle of the test resides in xMAP technology that uses MagPlex polystyrene 6.5 microns beads impregnated with iron and magnetite. These beads are labeled with 2 or 3 fluorochromes and display hence a unique spectral region. Each bead type contains also a unique monoclonal antibody that captures from the biological sample the specific antigen. We have used this technology to quantify concomitantly 9 MMPs from serum samples as presented in Table 1. Standard curves were used for quantifying all MMPs and thus expressing the results in pg mL<sup>-1</sup>.

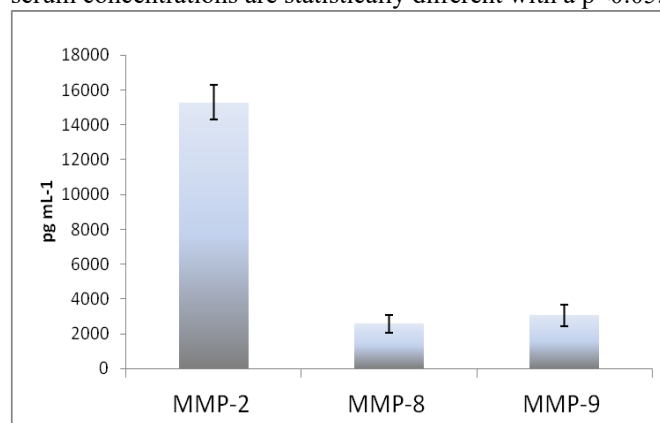
### Statistical analysis

SPSS version 19.0 was used for analysis. Pearson coefficient was used to establish if there is a correlation between each individual age and the corresponding MMPs levels. Independent t-test was used to determine if there is a statistically significant difference between the means of MMPs in control group versus MMPs in patients. Two-tailed t-test with statistical significance 0.05 (5%), 95% confidence level was taken into account. Results were presented as pg mL<sup>-1</sup> and, when indicated, as index = patient value/control value.

## Results

### MMPs normal serum values

In terms of statistics there was no difference between the patients age and control group. Moreover we have intentionally chosen more aged healthy subjects to mitigate the known aged-related increased values of circulatory MMPs. We can pinpoint 3 classes of serum MMPs in terms of their concentrations. The first class comprising MMP-2, 8 and 9 are in the 2000 – 15000 pg mL<sup>-1</sup>, the second one, MMP-1, 3 and 7 in the 100-900 pg mL<sup>-1</sup> and the third one MMP-10, 12 and 13 in the lowest 10-90 pg mL<sup>-1</sup> interval (Figure 1). The levels of MMP-8 and 9 are statistically identical while MMP-2 is statistically increased compared to 8 and 9 (p<0.001). MMP-1, 3 and 7 are statistically different in control serum (p<0.001). MMP-12 and 13 are not statistically different in control serum, while MMP-10 is statistically increased in comparison to MMP-12 and 13. MMP-1 and 10 serum concentrations are statistically different with a p<0.05.



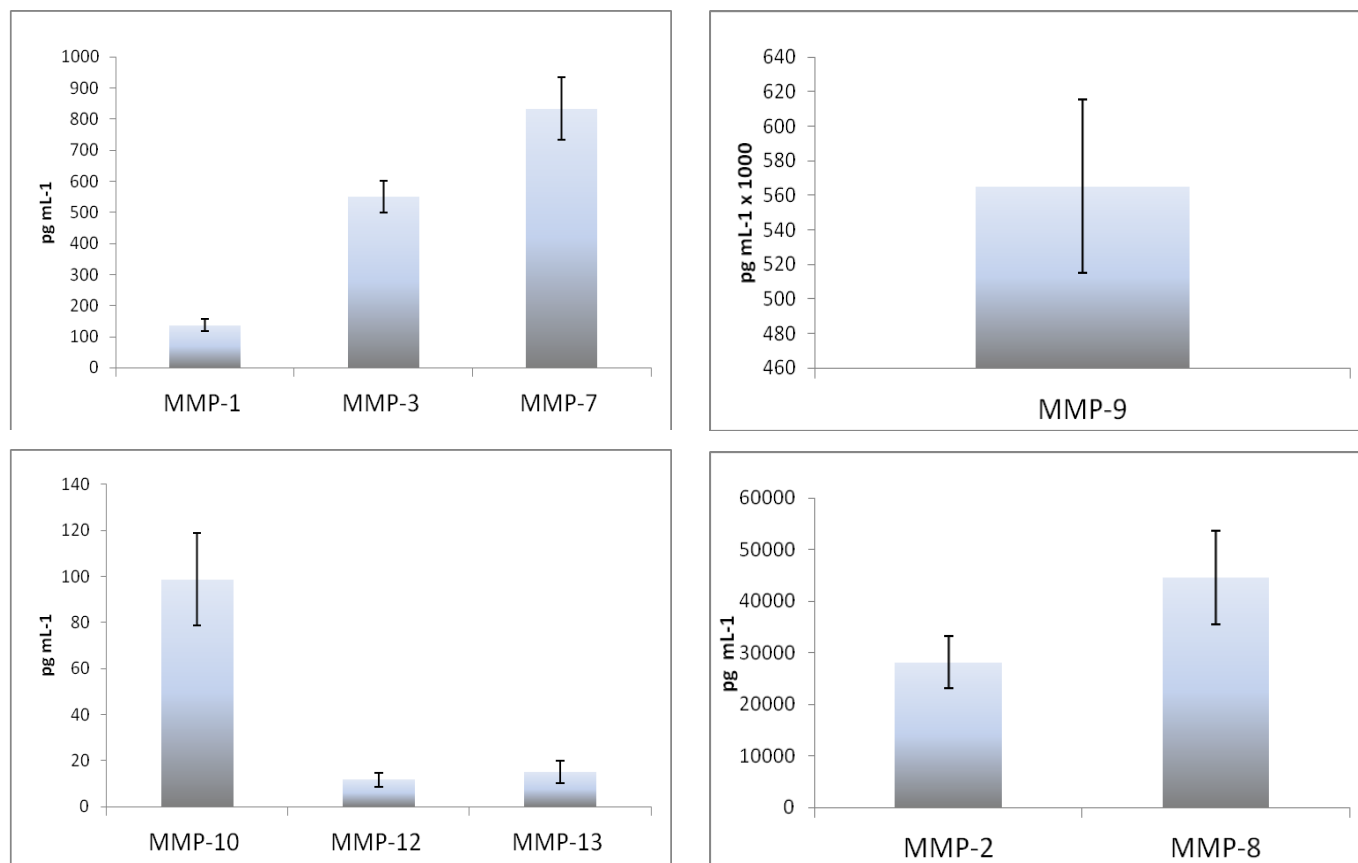


Figure 1. MMPs values detected in control group; A) MMP-2, 8 and 9; B) MMP-1, 3 and 7; C) MMP-10, 12 and 13

**Serum MMPs in patients diagnosed with pressure ulcers**

Grade II and III obese patients were admitted with hip and/or coccyx pressure ulcers. The lesions had diameters ranging from 14 to 64 cm<sup>2</sup> and, according to the standard scale; lesions were staged as II and III (Edsberg *et al* 2014). Pressure ulcers stage II had partial thickness loss of dermis showing an open ulcer, shallow in depth with a red pink wound bed. For stage III, the lesion had full thickness tissue loss, subcutaneous fat clearly visible, no bone, tendon or muscle were visible.

At hospital admittance, patients with extensive pressure ulcers were tested prior to any medication. The overall picture of the serum MMPs are significantly increased for MMP-9, 1, 8, 12, 3, 10 and 13, but no statistical difference compared to control values for MMP-7 and 2. Serum MMP-9 was found 185 times increased in comparison to control values. This MMP had the highest increment for all the investigated MMPs (Table 2). It is worth mentioning that, except for MMP-9, the same concentration ranges categories as in control group were found also in patients. Specifically, MMPs in patient’s serum divide as well in three concentrations categories: high for MMP-2, and 8; medium for MMP-1, 3 and 7, and low for MMP-10, 12 and 13. Besides MMP-9, statistically increased serum values were found for MMP-1 (54 X), MMP-8 (17X), MMP-12 (11X), MMP-3 (10X) and MMP-10 (4.7X) and MMP-13 (3X). There are also two MMPs that are not statistically different in patients compared to controls (MMP-7 and 2) although their concentrations evaluated in patients, are 2X and 1.8X increased respectively.

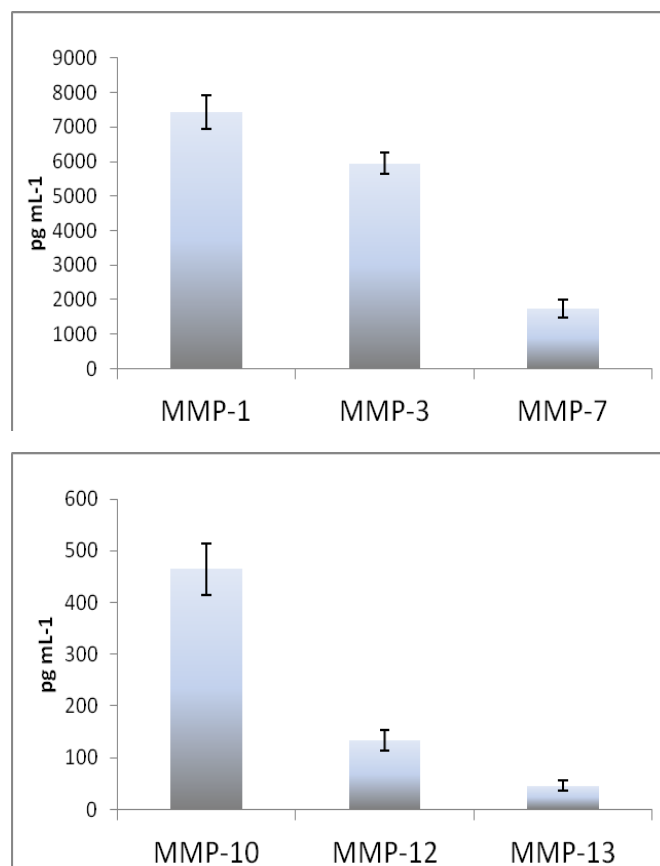


Figure 2. MMPs detected in patients serum; A) MMP-9; B) MMP-2, and 8; C) MMP-1, 3 and 7; D) MMP-10, 12 and 13

Table 2. Matrix-metalloproteinases serum concentrations in pressure ulcer patients compared to controls

Serum values	MMP-1	MMP-2	MMP-3	MMP-7	MMP-8
pg mL-1					
<b>Patients</b>					
<b>MEAN (n=40)</b>	7431.37	28123.4	5949.01	1738.89	44546.53
<b>STDEV</b>	574.8	519.79	1307.03	476.58	12143.7
<b>Controls</b>					
<b>MEAN (n=25)</b>	137.52	15284.86	550.66	833.71	2585.05
<b>STDEV</b>	25.33	1002.01	113.09	265.67	125.98
<b>Index</b>	54.04	1.84	10.8	2.08	17.23

Serum values	MMP-9	MMP-10	MMP-12	MMP-13
pg mL-1				
<b>Patients</b>				
<b>MEAN (n=40)</b>	565205.2	464.32	134.16	45.81
<b>STDEV</b>	11206	77.01	45.28	10.39
<b>Controls</b>				
<b>MEAN (n=25)</b>	3060.58	98.67	11.76	15.12
<b>STDEV</b>	250.01	12.56	3.22	5.09
<b>Index</b>	184.67	4.71	11.41	3.03

Multiplexing technology offered the possibility to concomitantly quantify 9 MMPs in the serum of enrolled patients, thus bypassing any individual ELISA determinations that can be subjected to errors and moreover, are time consuming technique. There are reports stating that there are aged related differences while quantifying serum MMPs (Thraikill et al 2005). This was the reasons of using aged healthy controls. We do not rule out the hypothesis that the MMPs values presented as control or reference values could have different intervals for other age groups. It was shown that MMP-2 is significantly negatively correlated with age while MMP-3 was reported as significantly positively correlated with age (Thraikill et al 2005). In our study we did not find any significant correlation between age and the level of any particular circulatory MMP whether in controls or patients prior to any therapy.

## Discussion

Literature is extremely poor regarding the normal ranges of serum MMPs (Edkins et al 2012). It seems that with normal ageing there are correlations with MMP-3, 10 (Komosinska-Vassev et al 2011) and MMP-2 (Thraikill et al 2005). That is the reason why we had enrolled normal healthy aged individuals to mitigate normal physiological age-related alteration of circulatory MMPs.

Up to our knowledge, there are no reports showing circulatory panels of MMPs in pressure ulcers. There are although several disparate reports showing one or the other of serum MMPs as altered in other pathology like venous leg ulcers. Excessive proteolysis caused by an imbalance between matrix metalloproteinases and their tissue inhibitors is the main mechanism responsible for impaired wound healing in chronic venous leg ulcers. Wound fluids from venous leg ulcers showed significantly increased concentrations of MMP-1 and MMP-3 and decreased

tissue inhibitor TIMP-1 compared with other sources of acute wound fluids, such as inflammatory fluid from skin grafts or post-mastectomy axillary drains. These MMPs are stimulated by inflammatory cytokines, such as interleukin-1 beta and tumor necrosis factor-alpha (Subramaniam et al 2008). Wound healing in venous leg ulcers was associated with an increased collagen III turnover and enhanced MMP-1 activity, compared with both venous leg ulcers resistant to treatment and healthy controls (Meyer et al 2008). Studies on human fibroblasts treated with MMPs show a decrease in synthesis of prostaglandin E2 by IL-1 beta (Nissinen and Kähäri 2014). This finding could be important in treatments regarding tissue repair, since prostaglandin E2 was found to be rapidly induced after injury with beneficial roles in the early inflammatory phase of wound healing (Stratton and Shiwen 2010). Overexpression of the activated collagenase MMP-8 associated with decreased levels of tissue inhibitor TIMP-1 seems to be implicated in the resistance to wound healing in chronic ulcers (Nwomeh et al 1999). Interestingly, plasma and wound fluid of infected chronic venous ulcers showed higher levels of collagenases MMP-1 and MMP-8 associated with increased concentrations of inflammatory cytokines, such as IL-1, IL-6, IL-8, vascular endothelial growth factor and tumor necrosis factor-alpha, compared with those uninfected, where gelatinases MMP-2 and MMP-9 were predominantly expressed (Serra et al 2014). The high MMPs levels found by us could be the main pathway for resistance in wound healing, thus, studies on venous leg ulcers are showing a decreasing pattern of MMPs in the process of healing. For example, MMP-2 and MMP-9, initially found increased in bilateral venous leg ulcers, decline after a good response to alginogel therapy (Grzela et al 2014). Interestingly, MMP-10 is expressed later than MMP-1 in normal healing wounds, and it is induced by keratinocytes migrating to

the dermal matrix. The keratinocytes of inflammatory chronic diabetes and venous ulcers express higher MMP-10 levels compared with other ulcers, such as rheumatoid and pressure ulcers (decubitus ulcers) (Rechardt *et al* 2000). In our study circulatory MMP-10 was found 4 times increased as compared to controls. Moreover, recent identified enzyme substrates such as integrin alpha-6 subunit, cysteine-rich angiogenic inducer 61 and dermokine highlight the complex biological involvement of MMP-10 in cell adhesion, cell invasion, proliferation and differentiation necessary for wound healing but also for cancer progression (Schlage *et al* 2015).

MMP-13 was initially reported in deep fibroblasts of the chronic ulcers, being important in matrix remodeling, in contrast to MMP-1 which is expressed by keratinocytes and dermal cells in the superficial wound, which is critical for re-epithelization (Vaalamo *et al* 1997). As our results show the circulatory levels of MMP-1 are higher than those registered for MMP-13, indicating that our patients were in the phases in which keratinocytes and dermal cells were actively involved in wound healing. Increasing the number of controls and patients can emphasize other aspects such as correlation of the MMPs circulatory levels to ageing and/or gender and/or with the extension and grade of lesions. However our study is one of the first focusing on a large panel of circulatory MMPs, enzymes involved in cutaneous regeneration/degradation and inflammatory processes. Our results indicate that serum MMPs are robust enough markers to indicate an actively on-going pressure ulcer and possible to monitor in the future the regenerative status of the cutaneous lesions.

## Conclusion

An outline can be drawn regarding serum MMPs quantified in pressure ulcer patients prior to any treatment. Therefore, the MMPs portrait is dominated by very high MMP-9 concentrations, followed by a group of MMPs 1, 3, 8, 10 and 12 that are also statistically increased in this pathology, while MMP-2, 7 and 13 are not found statistically different compared to controls.

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