

## A New Explanation of Inflammation in Rheumatoid Arthritis Patients With Respect to Claudin-5, Matrix Metalloproteinase-9, and Neuroserpin

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### ABSTRACT

**Objectives:** This study aims to investigate the relationship between neuroserpin (NSP) and claudin-5, as well as matrix metalloproteinase-9 (MMP-9), with respect to clinical activity of disease in patients with rheumatoid arthritis.

**Patients and methods:** The study included a total of 75 patients (18 males, 57 females; mean age 48.12±11.23 years; range 20 to 60 years) who were admitted to the rheumatology outpatient facility at the Medical Faculty Hospital, Sakarya University, in October 2014. Patients were divided into four groups based on their Disease Activity Score 28 (DAS28) scores as remission group (n=16, DAS28 <2.6), low disease activity group (n=16, DAS28 between 2.6-3.2), moderate disease activity group (n=28, DAS28 between 3.2-5.1), and high disease activity group (n=15, DAS28 >5.1). Ten healthy subjects (HS) served as controls.

**Results:** Claudin-5, MMP-9, and NSP levels were significantly different in rheumatoid arthritis patients compared to HS (p=0.035, 0.026, and 0.014, respectively). Additionally, there were no differences between claudin-5 levels and disease activity among all RA groups. However, compared to HS, patient groups showed a significant difference (p=0.035) in terms of claudin-5 levels. Serum levels of MMP-9 were significantly different in moderate disease activity group compared to HS (p=0.013). Levels of NSP were significantly different in moderate disease activity and high disease activity groups compared to HS (p=0.008 and 0.031, respectively).

**Conclusion:** Our study demonstrated the differential associations of endothelial function/dysfunction biomarkers and disease activity in rheumatoid arthritis. How and why this impairment occurs is not fully understood and more data regarding NSP, MMP, and claudin expression in plasma are warranted.

**Keywords:** Claudin-5; matrix metalloproteinase-9; neuroserpin; rheumatoid arthritis.

Rheumatoid arthritis (RA) is a major autoimmune disease affecting 1% to 2% of the adult population. RA is characterized by synovial joint inflammation with accompanying destruction of joint.<sup>1,2</sup> Because RA is a chronic inflammatory disease of the joints, plasma levels of inflammatory cytokines, such as interleukin (IL)-6, IL-17, IL-22, and IL-1 $\beta$ , and tumor necrosis factor-alpha, are increased in both RA patients and animal models.<sup>3,4</sup> In addition,

synovial inflammation in RA causes increased cytokine release and abnormally high levels of acute reactive proteins, such as C-reactive protein (CRP).<sup>5</sup>

Endothelial dysfunction (ED) is characterized by a shift of actions by the endothelium towards reduced vasodilation, a proinflammatory and proadhesive state, and prothrombic properties. Therefore, several biological markers, which serve

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as ED indicators, are used to assess the targets of each vascular phenotype.<sup>6</sup> The presence of ED is a characteristic feature of patients with RA.<sup>7</sup> ED markers include endothelin-1, von Willebrand factor, tissue plasminogen activator (tPA), plasminogen activator inhibitor, and adhesion molecules.<sup>6</sup> Poredos<sup>8</sup> showed that the cause of ED in RA may be systemic high-grade inflammation. In addition, asymmetric dimethylarginine, which is an endogenous competitive inhibitor of nitric oxide synthase, has been associated with ED.<sup>9</sup> Tight junctions (TJs) also play a critical role in epithelial barrier function that involve complexes between adjacent epithelial cells, control paracellular passage, and maintain cell polarity.<sup>10,11</sup> TJs exist in several cerebral vascular endothelial cells, such as zonula occludens-1, occluding, and the claudin family.<sup>12</sup> However, endothelial barrier function requires the adhesive activity of both E-cadherin and claudin-5 (Cld-5).<sup>13</sup> Collagen-induced arthritis may cause blood-brain barrier (BBB) hyperpermeability and alter the expression of TJ proteins, including Cld-5. Moreover, the integrity of the BBB is damaged by ED.<sup>14</sup>

Neuroserpin (NSP) is a serine protease inhibitor and member of the serpin family. It is expressed in developing and adult nervous systems, and acts as an inhibitor of protease tPA and a regulator of neuronal growth and plasticity.<sup>15,16</sup> By means of complex formation with tPA, NSP restricts microglial activation; hence, the production of inflammatory mediators decreases, as reflected by reduced serum levels.<sup>17</sup> In other words, NSP displays anti-inflammatory activity.<sup>18</sup> tPA can also activate matrix metalloproteinases (MMPs), specifically MMP-9. MMPs are a family of endopeptidases that primarily catalyze the degradation of the extracellular matrix (ECM), and in turn, increase BBB permeability. During various physiological and pathological events, such as embryogenesis, ovulation, arthritis, and inflammatory conditions, MMP production is stimulated.<sup>19-21</sup> In ischemia-induced brain injury, abnormal upregulation of MMP-9 has been linked to BBB disruption by ECM degradation, basal lamina proteins, and TJs surrounding the BBB. Ischemic injury induces significantly decreased expression of Cld-5, which disrupts BBB integrity and increases MMP-9 expression.<sup>22</sup> MMPs also play critical roles in RA, and the expression of

specific members of the MMP family, including MMP-3, MMP-8, and MMP-9, is significantly related to disease advancement.<sup>23</sup> In this regard, MMP-9 is detected at high levels in active RA patients.<sup>24</sup> However, to the best of our knowledge, there is no combined study that focuses on MMP-9 and Cld-5 in RA patients. Therefore, in this study, we aimed to investigate the relationship between NSP and Cld-5, as well as MMP-9, with respect to clinical activity of disease in patients with RA.

## PATIENTS AND METHODS

The study included a total of 75 patients (18 males, 57 females; mean age  $48.12 \pm 11.23$  years; range 20 to 60 years) who were admitted to the rheumatology outpatient facility at the Medical Faculty Hospital, Sakarya University, in October 2014. RA diagnosis was based on the diagnostic criteria revised in 2010 by the American College of Rheumatology/European League Against Rheumatism.<sup>25</sup> Ten healthy subjects (HS) admitted to our outpatient hospital for a checkup served as controls. Patients who (i) did not meet the criteria for RA; (ii) were treated for another rheumatic disease in the past; (iii) had a history of diabetes mellitus, renal failure, or liver failure; and (iv) were on tumor necrosis factor blockers were excluded. Disease activity was determined by Disease Activity Score 28 (DAS28), which was calculated using results from clinical and laboratory data. DAS28 remission criteria, including levels of CRP, swollen and tender joint counts, and global health assessment, were used to determine whether the disease was in remission. A DAS28 score between 2.6 to 3.2 indicates low disease activity, 3.2 to 5.1 indicates moderate activity, and  $>5.1$  indicates high disease activity.<sup>26</sup>

Patients were then divided into four groups based on their DAS28 scores as remission group (RG) ( $n=16$ , DAS28  $<2.6$ ), low disease activity group (LDAG) ( $n=16$ , DAS28 between 2.6-3.2), moderate disease activity group (MDAG) ( $n=28$ , DAS28 between 3.2-5.1), and high disease activity group (HDAG) ( $n=15$ , DAS28  $>5.1$ ). Patients' demographic data, including age, sex, smoking habit, and clinical data such as duration of symptoms, duration of disease, and delay in diagnosis were recorded.

Rheumatoid factor (RF) was measured by nephelometry (Beckman Coulter IMMAGE® 800, USA); levels greater than 20 U/mL were considered positive. Anti-cyclic citrullinated peptide (anti-CCP) levels were measured using an enzyme linked immunosorbent assay (Abbott Laboratories, Abbott Park, IL, USA); those greater than 0.5 U/mL were considered positive. NSP (Hangzhou Eastbiopharm Co., Ltd, China), MMP-9 (eBioscience, San Diego, California, USA) and Cld-5 (Eastbiopharm, China) were assessed by enzyme linked immunosorbent assay from serum samples.

Ethics approval was obtained from the local Ethics Committee of Sakarya University (approval number: 71522473/050.01.04/86). A written informed consent was obtained from each patient. The study was conducted in accordance with the principles of the Declaration of Helsinki.

### Statistical analysis

Statistical analysis was performed using IBM SPSS software, version 20.0 (IBM Corporation, Armonk, NY, USA). Values are presented as the mean  $\pm$  standard deviation and interquartile range. Differences between groups and HS were determined by one-way analysis of variance. *P* values less than 0.05 were considered statistically significant.

## RESULTS

Age, sex, and smoking habits were similar between RA patients and HS. Average disease duration, duration of symptoms, and delay in diagnosis were  $43.56 \pm 36.95$  months,  $51.01 \pm 38.18$  months, and  $7.77 \pm 5.01$  months, respectively. These results and clinical characteristics of RA patients are

summarized in Table 1. With respect to disease-modifying anti-rheumatic drugs, disease duration and duration of symptoms were determined to be significantly different among RA groups ( $p < 0.001$ ); however, delay in diagnosis did not significantly differ among RA groups (Table 2).

A significant difference was observed between HS and RA patients in terms of erythrocyte sedimentation rate values ( $p < 0.001$ ). Within RA groups, CRP values were significantly different. Moreover, a significant difference was revealed among MDAG individuals and HS ( $p = 0.001$ ). Patients with RA had significantly higher RF values ( $p < 0.001$ ) than HS. Anti-CCP levels of remission group compared to MDAG were different, but not statistically significant ( $p = 0.071$ ). In addition, anti-CCP levels were significantly different ( $p = 0.008$ ) in HS compared to patient groups.

Our results revealed no differences between Cld-5 levels and disease activity. However, when patient groups were compared to HS, a significant difference was detected ( $p = 0.035$ ). Figure 1a shows the relationship between Cld-5 and stage of disease activity. Serum levels of MMP-9 were significantly different in MDAG patients compared to HS ( $p = 0.013$ ). MMP-9 in HDAG patients was only moderately different from HS ( $p = 0.055$ ), whereas all RA groups were significantly different from HS ( $p = 0.026$ ). Figure 1b shows the relationship between MMP-9 and stage of disease activity. When HS were compared to MDAG and HDAG patients, serum levels of NSP were significantly different ( $p = 0.008$  and  $0.031$ , respectively). All RA groups were significantly different from HS in terms of serum levels of NSP ( $p = 0.014$ ). Figure 1c shows the relationship between NSP levels and stage of disease activity.

**Table 1.** Demographic and clinical characteristics of study groups

Characteristics	Rheumatoid arthritis patients		Healthy subjects	
	%	Mean $\pm$ SD	%	Mean $\pm$ SD
Age (years)		48.1 $\pm$ 11.2		46.2 $\pm$ 12.4
Gender (female)	76.0		60.0	
Cigarette smoking	34.7		40.0	
DS (months)		51.0 $\pm$ 38.2		NA
DD (months)		43.6 $\pm$ 37.0		NA
DED (months)		7.8 $\pm$ 5.0		NA
Health Assessment Questionnaire total scores		2.7 $\pm$ 0.1		NA

SD: Standard deviation; DS: Duration of symptoms; DD: Disease duration; DED: Delay in diagnosis.

**Table 2.** Demographic and laboratory parameters among study groups

	HS			RG			LDAG			MDAG			HDAG			Pg*
	n	%	Interquartile range	n	%	Interquartile range	n	%	Interquartile range	n	%	Interquartile range	n	%	Interquartile range	
Patients	10	8.5		16	21.5		16	21.5		28	37.4		15	20		
DS**	NA		33.1±18.6			34.0±23.4			47.7±23.0			47.7±23.0			94.7±54.5	
DD**	NA		28±16.9			26.8±2			39.9±23.1			39.9±23.1			84.9±54.4	
DED**	NA		6.4±4.8			7.1±3.6			7.8±5.3			7.8±5.3			9.9±5.7	
DMARD†	NA		1.1±0.3			2.2±0.5			2.2±0.8			2.2±0.8			3.5±0.5	
ESRF‡	8.4±4.0		21.6±9.5	7		29.3±8.9	11.75		32.0±12.3	14.5		32.0±12.3	22		47.7±18.0	
CRP§	4.7±2.5		6.2±3.7	5		9.7±7.4	10.75		8.9±6.9	9.75		8.9±6.9	11		14.6±6.4	
RFIgM¶	9.9±1.1		33.0±39.0	1.42		64.2±44.6	31.52		72.2±65.3	91.14		72.2±65.3	49.23		37.6±24.4	
Anti-CCP¶	4.0±1.2		114.7±120.3	168.78		201.5±228.3	287.58		332.9±389.8	633		332.9±389.8	159		162.0±176.8	
Cld-5¶	10.4±3.4		11.6±5.5	5.04		14.2±8.0	5.48		16.7±9.4	7.97		16.7±9.4	3.55		11.3±2.26	
MMP-9¶	14.8±1.3		17.8±2.9	8.82		18.2±4.2	3.94		19.2±4.3	7.86		19.2±4.3	6.8		18.9±4.0	
NSP¶	13.2±1.5		15.7±2.7	2.33		16.3±4.1	4.03		17.3±3.8	7.06		17.3±3.8	5.72		17.2±3.1	

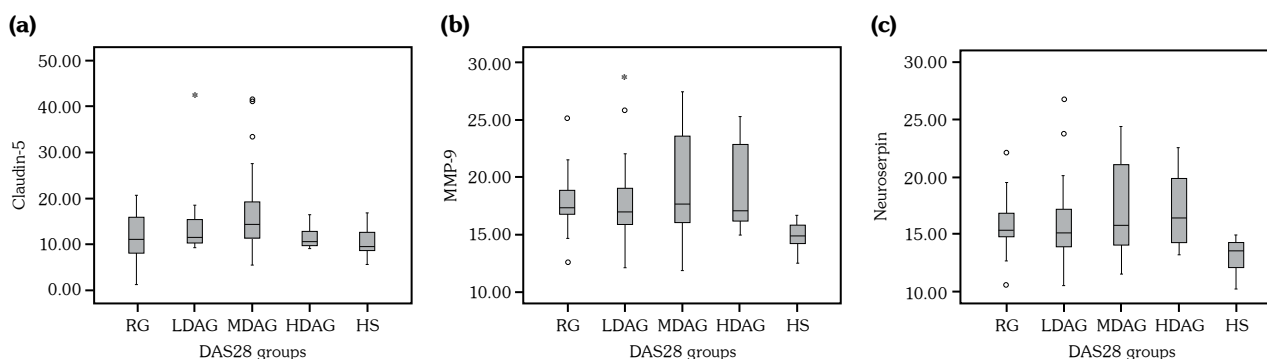
HS: Healthy subjects; RG: Remission activity group; LDAG: Low disease activity group; MDAG: Moderate disease activity group; HDAG: High disease activity group; SD: Standard deviation; DS: Duration of symptoms; DD: Disease duration; DED: Delay in diagnosis; DMARD: Disease-modifying antirheumatic drug; ESR: Erythrocyte sedimentation rate; CRP: C-reactive protein; RFIgM: Rheumatoid factor-immunoglobulin M; Anti-CCP: Anti-cyclic citrullinated peptide; Cld-5: Claudin-5; MMP-9: Matrix metalloproteinases; NSP: Neutrosperin; \* One-way ANOVA (within groups). NA: Not applicable; \*\* Presented data were sorted according to mean±SD (months); † Synthetic DMARDs; ‡ Range in women 10 mm/h; § Range in men 20 mm/h; ¶ Range in men 20 mm/h; †† U/mL; ††† ng/mL.

## DISCUSSION

To the best of our knowledge, this is the first study demonstrating that Cld-5, MMP-9, and NSP levels are significantly different in RA patients relative to HS ( $p=0.035$ ,  $0.026$ , and  $0.014$ , respectively). NSP is a member of the serine protease inhibitor or serpin superfamily of proteins.<sup>15</sup> Thrombolytic proteases, their receptors, and serine protease inhibitors (serpins) play an important role as regulatory counterbalances in homeostasis and vascular inflammation. It has been suggested that NSP inhibits tPA and urokinase plasminogen activator (uPA), in order of descending inhibitory activity.<sup>18</sup> In a previous study, plasma soluble uPA receptor concentrations were evaluated in RA patients. Soluble uPA receptor is believed to be particularly valuable in recognition of inflammatory activity in patients in remission, according to DAS28 scores.<sup>27,28</sup> However, increased levels of uPA and PA inhibitors, such as plasminogen activator inhibitor-1 and -2, are also found in synovial tissue in RA patients.<sup>29</sup> In contrast, Cook et al.<sup>30</sup> indicated that the major effect of uPA in the collagen-induced arthritis model is deleterious, whereas that of tPA is protective. These data and the complexities of PA function highlight that approaches targeting uPA or aimed towards enhancing local tPA activity in joints may be of therapeutic benefit in RA. In our study, NSP levels, as well as CRP and erythrocyte sedimentation values, were elevated in patients with RA compared to HS.

Our findings also suggest that NSP may serve as an inflammatory marker in RA patients. In the literature, there is some evidence of a relationship between serine proteases in clot-forming (thrombotic) and clot-dissolving (thrombolytic) cascades, activated by an inflammatory cytokine storm, which can induce systemic inflammation with a loss of normal serine protease inhibitor (serpin) function.<sup>31</sup> In addition, NSP possesses anti-inflammatory activity in systemic arteries, modifies T helper cell responses, and significantly reduces plaque formation in mouse aortic allografts.<sup>18</sup>

We found an association between anti-CCP/RF immunoglobulin M levels and Cld-5 in MDAG patients based on DAS28 scores. This is supported by several earlier studies suggesting that the presence of anti-CCP antibodies and



**Figure 1.** Relationship between (a) claudin-5, (b) MMP-9 and (c) neuroserpin levels and disease activity. MMP: Matrix metalloproteinases; RG: Remission group; LDAG: Low disease activity group; MDAG: Moderate disease activity group; HDAG: High disease activity group; HS: Healthy subjects; DAS28: Disease Activity Score 28.

RF immunoglobulin M is related to impaired endothelial function independent of cardiovascular risk factors in patients with RA.<sup>32</sup>

Physiologic and pathophysiologic events in tissue alter endothelial cell interactions with soluble and cellular blood components. Recent studies with RA patients have indeed revealed interactions between ED and disease activity; these include soluble human vascular cell adhesion protein 1, von Willebrand factor, pentraxin-3, asymmetric dimethyl-L-arginine, soluble E-selectin, monocyte chemoattractant protein-1, and osteoprotegerin.<sup>33</sup> Many of these substances are affected by inflammatory mediators and cytokines, and as specified earlier, contribute to the mechanisms underlying RA pathogenesis. With respect to therapeutic strategies for RA treatment, especially anti-tumor necrosis factor- $\alpha$  therapies, most are effective at reducing inflammation, as well as improving endothelial function.<sup>34</sup> In this regard, we found higher Cld-5 and NSP levels in MDAG patients compared to HDAG individuals.

After exposure to inflammatory stimuli, endothelial cells in virtually every tissue express plasminogen activator inhibitor-1.<sup>35</sup> In our previous study, we demonstrated increased von Willebrand factor and Cld-5.<sup>36</sup> It is also important to note higher levels of Cld-5, MMP-9, and anti-CCP in MDAG individuals relative to other RA subgroups. There are several potential explanations for these findings. Firstly, this could be due to circulating Cld-5 and MMP, or autoimmunity that has not yet developed in individuals of remission and low disease activity groups. Alternatively, this result may indicate that Cld-5 and/or MMP-9 are in the

circulation in the absence of clinically apparent RA in patients who have demonstrated high disease activity based on their DAS28 scores. Furthermore, our study demonstrated the complex regulation of Cld-5 and intricate balance of protein expression for disease activity in RA patients. Studies with various inducers of inflammatory pain have highlighted similar regulation of TJ proteins. Studies are also needed to evaluate the potential mechanisms of RA-related disease activity responses, including NSP and Cld-5, in brain endothelial cells. Informative animal models will also be important to understand how RA-related disease activity may alter Cld-5 and NSP levels.

Recent studies have investigated the relationship between MMP family members, plasminogen activators and their inhibitors, and RA. MMPs, which are produced primarily in fibroblast-like synoviocytes, are proteases that participate in the irreparable proteolytic degradation and remodeling of the ECM. Activation of inactive pro-MMPs is dependent on other regulatory mechanisms, including the plasminogen-plasmin cascade, which is itself regulated by serine proteinases uPA and tPA. A recent study demonstrated that the level of MMP-9, which is strongly associated with uPA, is increased in the synovial fluid of RA patients.<sup>37</sup> In RA, an imbalance between inhibition and activation of MMPs secreted by endothelial cells can result in excessive degradation of the ECM.<sup>38</sup> Consistent with this report, the level of NSP, a natural inhibitor of tPA, was significantly increased in RA and strongly associated with the level of MMP-9 in RA patients in our study.

We may speculate that NSP may be involved, at least in part, in the pathogenesis of arthritis through activation of MMP-9. This hypothesis is in agreement with previous studies that have described the involvement of serine proteinases and cysteine proteinases in MMP activation and collagen degradation, respectively.<sup>39</sup> However, because tPA can also activate MMPs, we should also consider that an increase in NSP observed between RA patients and HS may involve MMP-9, albeit directly or indirectly (via inflammation-associated mechanisms). We acknowledge that the major limitation of our study was that pro-inflammatory mediators and cytokines, which are abundantly produced in RA and exert numerous effects in endothelial cells, were not assayed.

In conclusion, our study demonstrates differential associations of endothelial function/dysfunction biomarkers and disease activity in RA. Cld-5, MMP-9 and NSP levels are increased in patients with RA compared to healthy controls. However, higher levels of Cld-5, MMP-9, and NSP were also observed in patients with MDAG. Nevertheless, how and why this impairment occurs is not fully understood, and more data regarding NSP, MMP, and claudin expression in plasma are warranted.

#### Declaration of conflicting interests

The authors declared no conflicts of interest with respect to the authorship and/or publication of this article.

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