Evaluation of MMP-12 expression in chronic rhinosinusitis with nasal polyposis*

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Abstract

Background: The purpose of this study was to evaluate the expression of MMP-12 in patients with chronic rhinosinusitis with polyps (CRSwNP).

Methodology: Tissue samples from 37 patients with CRSwNP undergoing functional endoscopic sinus surgery and healthy mucosa specimens from 12 healthy controls were obtained intraoperatively. The mRNA and protein expression levels of MMP-12 were quantified by real-time polymerase chain reaction and Western blotting, respectively.

Results: mRNA levels of MMP-12 were significantly elevated in the CRSwNP tissue samples compared to those in control ones. The protein levels of MMP-12 showed a trend of increasing but with no statistical significance.

Conclusions: Elevation of MMP-12 in patients with CRSwNP suggests its potential implication in the pathogenesis of the disease. The difference in the expression profile observed between mRNA and protein levels could be due to post-translational gene expression regulation. Our findings provide evidence that MMP-12 along with other MMPs may serve as a biomarker and therapeutic target in the management of the disease.

Key words: matrix metalloproteinases, nasal mucosa, nasal polyps, sinusitis

Introduction

Chronic rhinosinusitis (CRS) constitutes an heterogeneous disease of multifactorial etiology characterized by inflammation of the nasal and paranasal sinuses mucosa, accompanied by tissue remodeling in both mucosa and bone, resulting in osteitis, mucosal hypertrophy, fibrosis and basement membrane thickening, through squamous metaplasia, collagen deposition, hyperplasia of mucous glands and goblet cells, altered epithelial cell differentiation and gland morphology, as well as edema and extracellular matrix deposition [(1,2)].

The definition of CRS according to the European Position Paper on Rhinosinusitis and Nasal Polyps (EPOS2020) includes sinonasal inflammation with a duration of at least 12 weeks, presenting with two or more symptoms, one of which should be either nasal blockage/congestion/obstruction or nasal discharge (anterior/posterior nasal drip) associated with facial pain/pressure or reduction/loss of smell. Additional findings may comprise endoscopic signs of nasal polyps, and/or mucopurulent discharge primarily from middle meatus, and/or edema/mucosal obstruction in middle meatus. Computed Tomography (CT) changes include mucosal changes within the ostiomeatal complex and or sinuses [(3)]. Its prevalence is estimated to be as high as 10.9% in Europe [(4)] and 16% in the United States of America (USA) [(5)].

CRS with nasal polyps (CRSwNP) is a subset phenotype of CRS, affecting 1–4% of the general population in the USA [(6)]. It is characterized by a predominantly Th2 inflammatory response with increased eosinophils, mast cells, and basophils and elevated

type 2 cytokines [7]. Furthermore, a high prevalence (20-60%) of asthma has been reported in CRSwNP [8].
Coexistence of upper and lower airway disease has been identified and thus justifies the rationale of a 'united airway' with common pathophysiological processes [9]. In fact, as stated above, sinonasal inflammation has been associated with asthma of allergic or non-allergic etiology as well as chronic obstructive pulmonary disease (COPD), while the presence of CRSwNP in patients with bronchiectasis also supports the connection between upper and lower airway disease [10,11].

Matrix metalloproteinases (MMPs) are a group of proteins belonging to the zinc- and calcium-dependent endoproteases family, which serve as key molecules in extracellular matrix (ECM) as well as non-matrix components turnover [12]. Due to their established contribution in the airway remodeling, MMPs has become the focus of interest in the field of research for CRS over the past decade [13]. Namely, the expression of MMP-1, 2, 3, 7, 8, 9 and their tissue inhibitors (TIMP-1 and 2) have been studied in CRS patients [14-17].
MMP-12 is a potent elastase, produced mainly by macrophages, with a known involvement in diseases such as atherosclerosis, COPD and liver fibrosis [18-19]. In the present study we investigated the expression of MMP-12 in patients with CRSwNP in comparison to subjects with normal nasal mucosa, aiming to elucidate its role in the tissue remodeling observed in CRSwNP. To our knowledge, this is the first study to evaluate MMP-12 expression in patients with CRSwNP.

Materials and methods

Study population and inclusion criteria

The study population consisted of a total of 49 subjects (37 CRSwNP patients, aged between 18 and 72 years, average 46.3 years and 12 controls, average 45.2 years). All subjects were enrolled in the time period between September 2017 and January 2021.
Inclusion criteria for enrollment in the study group were patients over the age of 18 with CRSwNP scheduled to undergo functional endoscopic sinus surgery (FESS). Patients with CRSwNP met the diagnostic criteria set by the guidelines of the latest EPOS2020, while the diagnosis was confirmed by standard preoperative computed tomography (CT) and endoscopy accordingly [3].
Patients suffering from CRS without nasal polyps, allergic fungal rhinosinusitis, antrochoanal polyps, neoplastic disease and extranasal complications of CRSwNP were excluded from this study. In 2 cases, an inverse papilloma, identified intraoperatively, was then confirmed by pathology. These patients were also excluded. Participants were selected randomly solely based on consecutive appointments for scheduled surgery.
The control group included 12 participants who underwent septoplasty for nasal septum deviation without any other nasal pathology. Controls were also selected based on the date of presentation for surgery.

Table 1. Demographic and clinical characteristics of the patient and control group.

	Patients		Controls	
Age	n	%	n	%
Average (years)	46.3 (31.1 - 61.4)		45.2 (29.2 - 61.1)	
<30	5	13.5		
31-40	9	24.3		
41-50	8	21.6		
51-60	7	18.9		
>61	8	21.6		
Gender				
Male	30	81.1	8	66.7
Female	7	18.9	4	33.3
Asthma				
Yes	14	37.8	2	16.7
No	23	62.2	10	83.3
Smoking status				
Yes	14	37.8	5	41.7
No	22	59.5	7	58.3
Discontinued	1	2.7	-	-

The study protocol was approved by the Ethics Committee of the University Hospital of Patras (number 403/01.08.2017). Written informed consent was obtained from all participants prior to their induction in the study regarding processing personal data and allowing tissue excision and its use for the purposes of the study.
All study procedures and research conducted were in accordance with the principles of the Declaration of Helsinki.

Biological samples acquisition

Tissue samples of nasal polyps were received during FESS from 37 patients (30 male and 7 female) with CRSwNP between 18 and 72 years of age. Part of the excised tissue was sent for pathology, while the remainder was stored at -80°-C until they were processed.
In the control group, healthy mucosa tissue samples were obtained from the inferior turbinate after separation from the underlying bone.

Real time Polymerase chain reaction (qRT-PCR)

RNA was extracted using the NucleoSpin RNA/protein kit (Macherey-Nagel) according to the manufacturer's instructions. The specimens were cryopreserved at -80°C and then lysated by adding 700μl Lysis solution (RP1) and 3.5μl of β-mercaptoethanol.

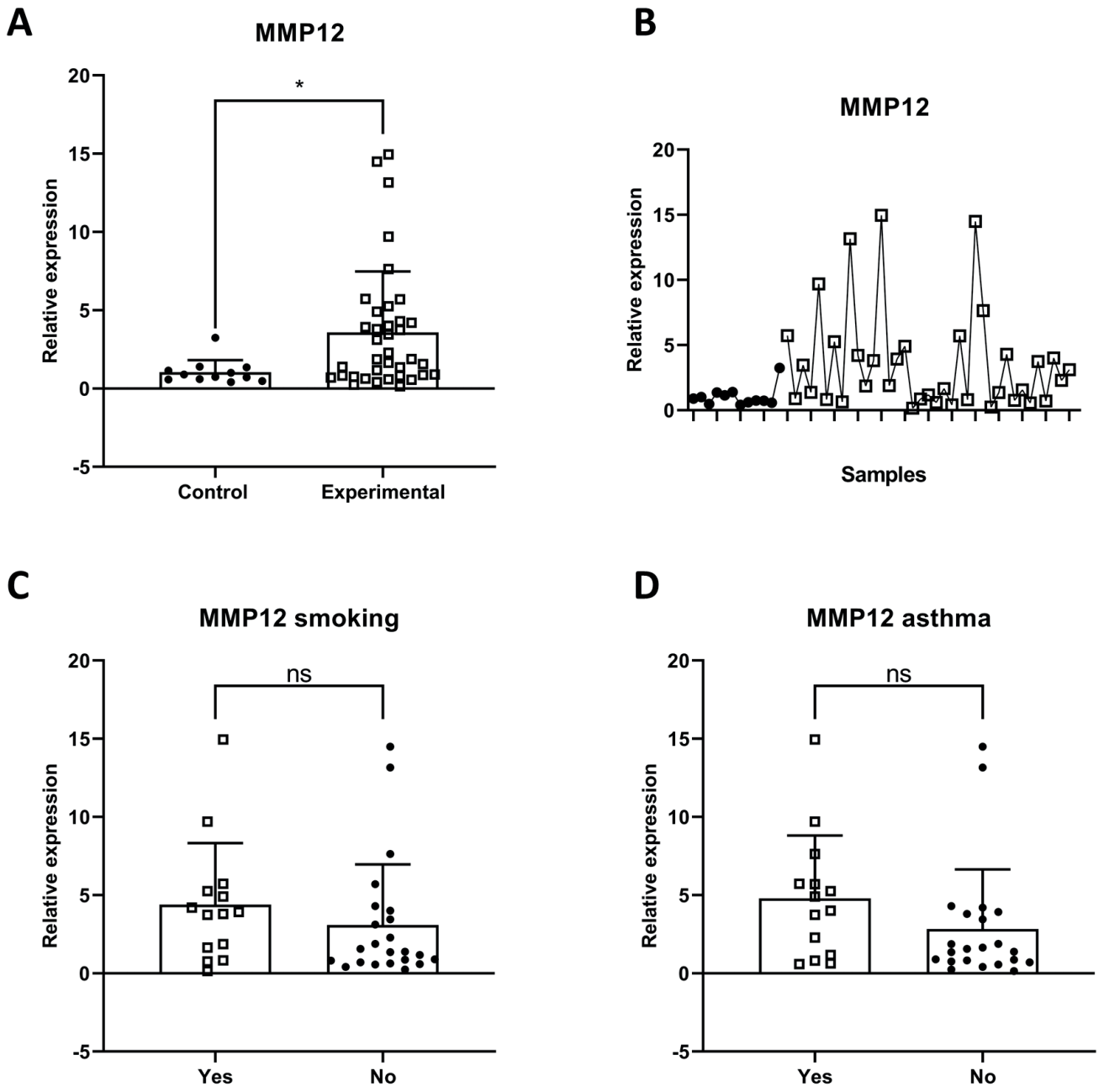

Figure 1. mRNA levels of MMP-12 in CRSwNP. A-B) Relative expression of MMP-12 in controls and patients. MMP-12 expression was significantly increased in the patient group. C) Comparison of relative expression of MMP-12 between smoking and non-smoking. CRSwNP patients showed no significant difference between the two groups. D) Asthma was not associated with a significant increase in MMP-12 expression in CRSwNP patients. CRSwNP: Chronic rhinosinusitis with nasal polyps.

Following homogenization, the homogenization product was added in the column provided for disposal of particles not homogenized by centrifugation at 11,000 g for 1 minute. 350µl of ethanol 70% were added to the repeatedly eluted suspension until a clear suspension was obtained to extract total RNA. Tissue elute was further centrifuged at 11,000 g for 1 min and was stored at 4°C until RNA extraction was complete. 350µl MDB were added and the elute was recentrifuged at 11,000g for 1 minute. Total RNA was measured with an Multiskan Sky High Microplate Spectrophotometer (ThermoFischer). Measurements were performed at 230nm and 280 nm to ensure RNA purity. The estimated ideal absorbance ratio at 260 nm (DNA) versus 280 nm (proteins) was 1.8. The quality of RNA in all specimens was also assessed with a 1.5% agarose gel in the presence of formaldehyde which allows visualization of the 28SrRNA and 18SrRNA bands as markers of RNA integrity. A total of 5 µg mRNA of total RNA per sample was reverse transcribed to complementary DNA (cDNA) using the reverse transcriptase enzyme

Table 2. Quantification of total RNA for each sample after photometry at 260nm.

Sample	Concentration	260/230	260/280	Sample	Concentration	260/230	260/280
NP1	391 μg/mL	2	2.11	NP26	952 μg/mL	2.24	2.14
NP2	54.8 μg/mL	1.94	2.09	NP27	86.2v	1.93	2.11
NP3	149 μg/mL	1.3	2.12	NP28	674 μg/mL	2.22	2.13
NP4	68.1 μg/mL	1.76	2.02	NP29	43.5 μg/mL	1.93	2.08
NP5	42.2 μg/mL	1.57	2.1	NP30	91 μg/mL	1.78	2.14
NP6	153 μg/mL	2.01	2.13	NP31	1440 μg/mL	2.26	2.13
NP7	303 μg/mL	1.67	2.12	NP32	214 μg/mL	2.12	2.14
NP8	115 μg/mL	2.07	2.11	NP33	140 μg/mL	2.12	2.14
NP9	1280 μg/mL	2.22	2.14	NP34	38.2 μg/mL	1.98	2.09
NP10	518 μg/mL	2.21	2.11	NP35	59 μg/mL	1.69	2.11
NP11	587 μg/mL	2.19	2.13	NP36	116 μg/mL	1.58	2.12
NP12	117 μg/mL	2.15	2.1	NP37	329 μg/mL	2.05	2.14
NP13	28.3 μg/mL	1.52	2.14	C1	302 μg/mL	2.23	2.15
NP14	495 μg/mL	2.26	2.12	C2	997 μg/mL	2.27	2.15
NP15	168 μg/mL	1.4	2.13	C3	223 μg/mL	2.11	2.14
NP16	198 μg/mL	2.2	2.14	C4	376 μg/mL	2.2	2.12
NP17	60.8 μg/mL	1.75	2.11	C5	286 μg/mL	2.05	2.14
NP18	29.9 μg/mL	0.873	2.13	C6	454 μg/mL	2.24	2.12
NP19	77.4 μg/mL	1.97	2.11	C7	937 μg/mL	2.21	2.15
NP20	209 μg/mL	1.96	2.11	C8	131 μg/mL	1.72	2.13
NP21	138 μg/mL	1.54	2.13	C9	410 μg/mL	1.93	2.12
NP22	749 μg/mL	2.25	2.13	C10	140 μg/mL	2.16	2.12
NP23	42.9 μg/mL	1.92	2.05	C11	108 μg/mL	0.737	2.11
NP24	258 μg/mL	2.01	2.12	C12	70.4 μg/mL	2.17	2.11
NP25	39.8 μg/mL	1.78	2.09				

NP: nasal polyp, C: control

Superscrip tII (Invitrogen). cDNA was synthesized using random nucleotide hexamers as primers to ensure DNA synthesis from the whole mRNA. qPCR reactions were performed using KAPA SYBR FAST qPCR Kit (Kapa Biosystems) using 50ng cDNA as template. Reactions were set up in 96-well plates and performed on an MX3000P qPCR system (Agilent). The Ct values were analyzed using the $2^{-\Delta\Delta CT}$ method after normalization against ACTB levels [20]. The normalization procedure was performed using one of the control samples, which was analyzed in every 96-well plate to ensure that all samples were equally compared to the same control. All reactions were performed in triplicates and the primers sequences are the following: ACTB_F: AGCGAGCATCCCCCAAAGTT, ACTB_R: GGGCACGAAGGCTCATCATT, MMP12_F: CCCATGCTTTTGGACCTGGA, MMP12_R: TGCCACGTATGTCATCAGCA.

Western blotting

Protein lysates were extracted using the NucleoSpin RNA/protein kit (Macherey-Nagel) according to the manufacturer's instructions. 30-50μg of total protein extracts were separated by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to Immobilon-P PVDF membranes (Millipore, MA, USA). Blocking in 5% (w/v) non-fat dry milk in TBS/0.05% Tween 20 was followed by incubation with primary antibodies overnight at 4°C and with goat anti-rabbit secondary HRP-conjugated antibody for 1h at room temperature. The band intensity measurements of each experiment were analyzed using the Image Lab software (Bio-Rad, version 6.1). The protein levels were normalized against β-actin levels in each separate set of experiments. The normalization procedure was performed using one of the control samples, which was separated in every SDS-PAGE to ensure that all samples were equally compared to the same control. The antibodies used are the following: anti-β-actin (#4967, Cell Signaling), anti-MMP12 (CSB-PA12379A0Rb, Cusabio).

Statistical analysis

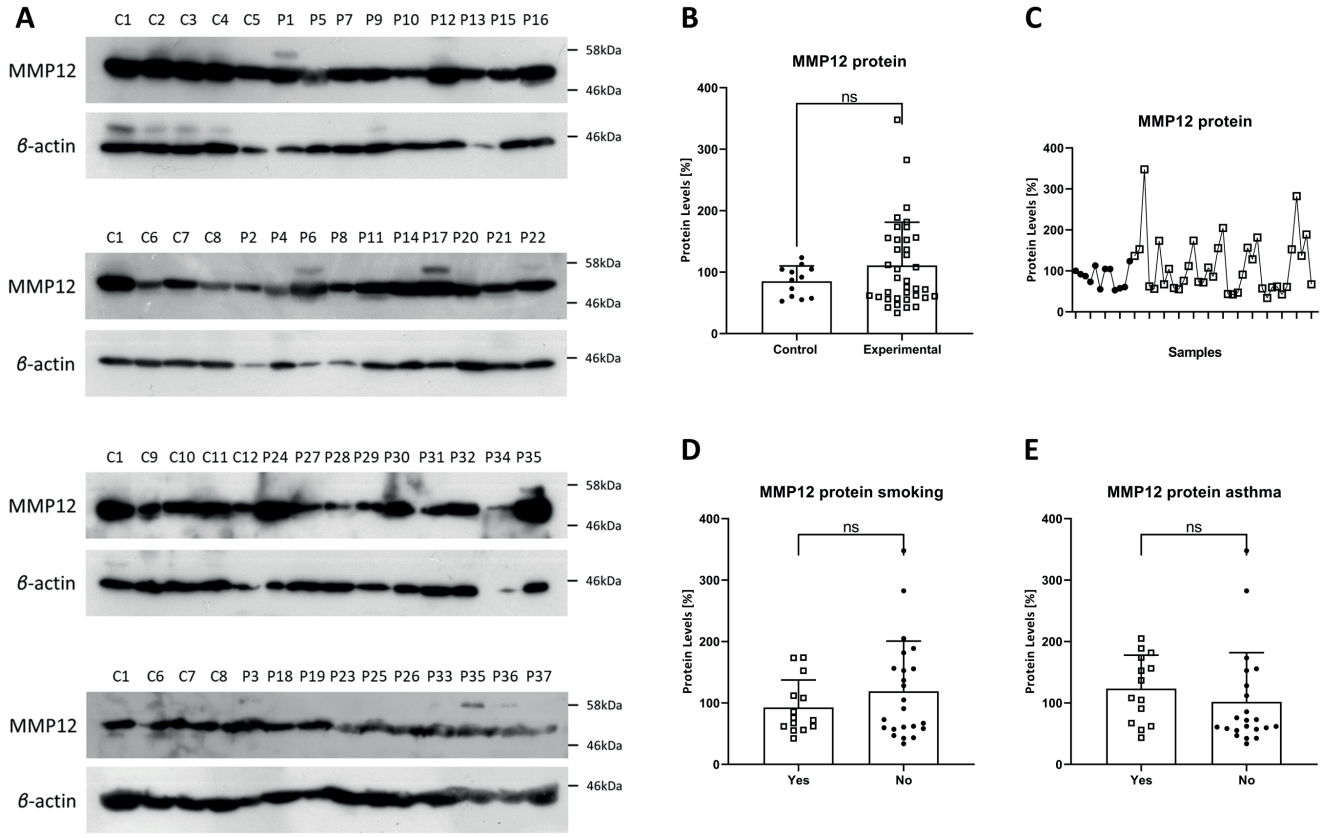

Figure 2. Protein levels of MMP-12 in CRSwNP. A. Western blots of MMP-12 in controls (C1-C12) and patients (P1-P37). B-C. Relative % protein levels of MMP-12 in controls and patients. D. Comparison of relative expression of MMP-12 between smoking and non-smoking CRSwNP patients showed no significant difference between the two groups. E. Asthma was not associated with a significant increase in MMP-12 expression in CRSwNP patients. CRSwNP: Chronic rhinosinusitis with nasal polyps.

Statistical analysis was performed using the GraphPadPrism 8 software package. More specifically, the unpaired parametric Student's t-test was applied and measurement data for all samples were expressed as mean ± standard deviation (SD). A P-value <0.05 was considered statistically significant.

Results

Patient characteristics

Clinical and demographic data of the patient and control group are summarized in Table 1. The two study groups did not differ significantly with respect to age and gender (P<0.05). In the CRSwNP group 59.5% of the patients were smokers and 37.8% had a medical history of asthma, while in the control group 58.3% of the participants were smokers and 16.7% were previously diagnosed with asthma (Table1).

mRNA levels of MMP-12 in CRSwNP

Expression profiles of MMP-12 in CRSwNP

In nasal polyps tissue samples an increased expression of the MMP-12 gene was observed (Figure 1A and B). More precisely, in the study group (n=37) MMP-12 increase was estimated 3.59-fold higher in comparison to the control group (n=12). This increase was statistically significant (p<0.05) with a standard deviation of ± 3.89. Smoking was not associated with a statistically significant increase in MMP-12 expression in patients with CRSwNP (Figure 1C). The relative MMP-12 expression was estimated to be 4.39 (± 3.93) in smokers as opposed to 3.09 (± 3.87) in non-smokers. In addition, no significant increase in MMP-12 expression was noticed in asthmatic patients compared to patients without asthma. The relative MMP-12 expression in asthma patients was 4.79 (± 4.01), while in non-asthmatic patients 2.84 (± 3.80) (Figure 1D).

Protein expression of MMP-12 in CRSwNP

Western blot analysis showed a trend to increased expression of the protein levels of MMP-12, but with no statistical significance (Figure 2A, B and C). More precisely, in the study group (n=37) MMP-12 expression was calculated at 111.04% (± 70.19) in comparison to 85.31% (± 24.73) in the control group (n=12). Smoking was not associated with a statistically significant increase in MMP-12 expression in patients with CRSwNP (Figure 2D). The relative MMP-12 expression was estimated to be

92.76% (± 44.61) in smokers as opposed to 118.94% (± 81.76) in non-smokers. In addition, no significant increase in MMP-12 expression was noticed in asthmatic patients compared to patients without asthma. The relative MMP-12 expression in asthma patients was 123.40% (± 54.40), while in non-asthmatic patients 101.80% (± 80.07) (Figure 2E).

Discussion

MMP-12 is a 54kDa proenzyme, proteolytically converted into a 45kDa and 22kDa active form, the mature protease [21]. It is mainly produced by macrophages and determines a crucial role in macrophage migration through ECM as well as degradation of elastin and other ECM components. The gene responsible for MMP-12 expression is located in chromosome 11 as part of a cluster of MMP genes.

MMP-12 serves as a proinflammatory mediator via different interactions. For instance, MMP-12 released by tissue macrophages acts by decreasing neutrophils and monocytes chemotaxis and increased neutrophils apoptosis, while it increases M2 macrophage and Th2 lymphocyte differentiation, as well as IL-13 activity [22]. Upregulation of MMP-12 after exposure to inflammatory cytokines such as IL-1β and TNF-α leads to activation of other MMPs like MMP-2, -9, -13 and -14 associated with ECM degradation as well as migration of inflammatory cells such as monocytes and macrophages. As a result, increased elastolytic activity and tissue remodeling is observed following MMP-12 increase [23]. Increased MMP-12 also results in activation of fibroblasts and TGF-β1 activity and in endothelial cells it is implicated in increased migration and decreased activation. In macrophages MMP-12 leads to increase of active TNF-α and activation and alterations in epithelial cells. Moreover, MMP12 has anti-inflammatory properties through promoting cleavage of complement components and phagocytosis [24]. Therefore, altered MMP-12 expression may result in disequilibrium in inflammatory processes and ECM breakdown.

In spite of the fact that the role of MMPs has been studied extensively in CRS over the last few years, there are no data available in literature regarding the role of MMP-12 in the disease.

In the present study we aimed to evaluate for the first time the expression of MMP-12 in patients with CRSwNP. Our findings demonstrated that MMP-12 expression is enhanced in polyps tissue samples from patients with CRSwNP in terms of mRNA and provide indirect evidence of the contribution of MMP-12 in the pathogenesis of CRSwNP. The protein levels of MMP12 showed a slight upregulation but with no statical significance. This observation does not coincide with the mRNA levels of MMP12 gene, where we report a statistically significant upregulation. This could be partially explained by the small number of experimental samples. However, this observation could also be due to the possible involvement of post-transcriptional gene expression regulation. This is mainly mediated by several classes of non-coding RNAs (ncRNAs), especially microRNAs(miRNAs). miRNAs regulate gene expression either by binding on specific regions of the target mRNAs and therefore blocking the ribosome from scanning the mRNA, or by inducing cleavage of the mRNA. Several miRNAs have been reported to possibly regulate the expression levels of MMP12, including miR-452 [25] and miR-647 [26], while other unidentified miRNAs could also be involved [27]. Moreover, novel classes of ncRNAs that can regulate gene expression are tRNA-derived fragments (tRFs) [28] and long ncRNAs [29], whose role in the pathogenesis of CRSwNP has not been reported so far.

MMP-12 has been found to be involved in acute and chronic pulmonary inflammatory diseases characterized by intense airway remodeling and fibrosis, such as asthma and COPD [30]. MMP-12 upregulation has been originally demonstrated in an experimental smoke-induced emphysema mice model [31], as well as in bronchial biopsies and bronchoalveolar lavage samples [32] and sputum from COPD and asthma patients [33,34]. This increase has been positively correlated with relevant inflammation and tissue injury, and negatively with functional tests. Moreover, an association of MMP-12 expression levels with the severity of these underlying diseases has been observed [34,35]. In fact, genetic polymorphisms of the MMP-12 gene can lead to abnormal expression of MMP12, and may be involved in the development of COPD [36,37].

The induction of elastin degradation, a vital protein for small airway elasticity also explains the role of MMP-12 in asthma [38]. A study evaluating 6 different endotype groups of patients with severe asthma demonstrated a correlation between the severity of the disease and the levels of MMP-12 in patients' sputum [39]. A study focusing on gene polymorphisms found a correlation between MMP-12 gene and sensitivity to asthma in a Japanese population [40]. Another study including asthmatic children and young adults observed a correlation between MMP-12 gene expression and the severity of the disease [41]. In the same study pharmacologic inhibition of MMP-12 in allergen sensitized sheep resulted in reduced responses to allergic stimuli. The abovementioned studies highlight that MMP-12 determines an important role in inflammatory processes and tissue remodeling in the respiratory system and asthma in particular. MMP-12 has consequently been used as a therapeutic target for lung disease. Recent studies using MMP-12 inhibitors have demonstrated a reduction in both the inflammatory process and airspace enlargement in lung tissue [42,43].

Taking into consideration the established role of MMP-12 in

asthma, a comparison of MMP-12 expression between asthmatic and non-asthmatic CRSwNP patients was conducted. In our study, patients with asthma did not exhibit significantly increased MMP-12 expression compared to non-asthmatic CRSwNP patients. These finding may suggest that MMP-12 may be stably increased in CRSwNP, irrespectively of the underlying asthma, supporting the hypothesis of a united airway with common pathophysiological pathways for both diseases.

Moreover, MMP-12 has been correlated with the inflammatory process caused by cigarette smoking [31,43]. Therefore, we also tried to assess the effect of the smoking status of CRSwNP patients on MMP-12. No significant difference in MMP-12 mRNA levels and protein expression emerged between smoking and non-smoking CRS patients. Comparably, Demedts et al. found MMP-12 to be increased in sputum of both active smokers with COPD, as well as COPD patients after cessation of smoking and thus attributed this upregulation of MMP-12 not to cigarette smoking but to the course of the disease itself [32]. On the other hand, in another study, sputum MMP-12, enzyme activity and expression in smokers with asthma was significantly elevated in comparison to healthy non-smokers. However, MMP-12 did not differ significantly between smokers with asthma and healthy smokers [34].

Our study had certain limitations. For instance, it involved tissue samples from CRSwNP patients undergoing FESS. Therefore, due to disease progression, our samples are not capable of reflecting the early pathophysiological stages of the development of nasal polyposis. In addition, the small size of the evaluated subgroups of smokers and asthma patients in the study population may preclude drawing safe conclusions concerning the association of these parameters with MMP-12 expression.

Conclusion

According to our results, MMP-12 appears to be increased in CRSwNP at the mRNA level, indicating its possible contribution in the pathophysiological changes seen in the disease, as seen with other MMPs. Larger future studies may help establish the connection between our findings and the physiological mechanisms of the disease, determine possible therapeutic targets and contribute to an overall better understanding the pathogenesis of CRS.

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Authorship contribution

SL: Literature collection and review of literature, data collection, draft preparation and final review of the manuscript. GD: Data collection, review of the manuscript GCK: Data processing and analysis, review of the manuscript. KG: Data processing, review of the manuscript. FT: Literature collection and review of the manuscript. CS: Design of the research, supervision of data analysis, review of the manuscript. VD: Design, implementation and supervision of the research and final review of the manuscript.

Conflict of interest

The authors have no conflict of interest to declare.

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None.

Abbreviations

CRS: chronic rhinosinusitis; CRSwNP: chronic rhinosinusitis with nasal polyps; COPD: chronic obstructive pulmonary disease; MMP: matrix metalloproteinase; TIMP: tissue inhibitors of matrix metalloproteinases; ECM: extracellular matrix; FESS: functional endoscopic sinus surgery; qRT-PCR: quantitative real-time polymerase chain reaction; SDS-PAGE: sodium dodecyl sulphate–polyacrylamide gel electrophoresis; IL-13: interleukin 13; IL-1β: interleukin 1 beta; TNF-α: tumor necrosis factor alpha; TGF-β1: transcription growth factor beta-1; ncRNA: non-coding RNA; miRNA: microRNA; tRFs: tRNA-derived fragments.

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