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Rhinology

Calprotectin in nasal secretion: a new biomarker of non-type 2 inflammation in CRSwNP

Calprotectina nel secreto nasale: nuovo biomarker di infiammazione non-tipo 2 nella poliposi nasale

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SUMMARY

Objective. We analysed calprotectin in sinonasal secretions of different chronic rhinosinusitis with nasal polyps (CRSwNP) endotypes to assess its role as a biomarker of non-type 2 inflammation.

Methods. We included primary diffuse CRSwNP patients (n = 41) and three different control groups [non-allergic rhinitis (NAR) (n = 13), non-allergic eosinophilic syndrome (NARES) (n = 10) and healthy subjects (n = 12)]. Calprotectin levels were detected in nasal secretions using a chemiluminescent immunoassay (CLIA).

Results. Calprotectin levels in nasal secretions were significantly higher in all non-type 2 endotypes of CRSwNP compared to healthy controls ($p < 0.05$). In contrast, in type-2 CRSwNP calprotectin was significantly lower compared to controls ($p < 0.05$). A significant correlation between calprotectin levels and neutrophilic count/HPF was found in CRSwNP ($p < 0.01$). Clinically, mean levels of calprotectin and neutrophilia were significantly higher in patients who previously underwent 3 or more endoscopic sinus surgeries ($p < 0.05$).

Conclusions. Calprotectin in nasal secretions may be a biomarker of non-type 2 inflammation. Low levels of calprotectin are indicative of a type-2 immune response in both CRSwNP and non-allergic rhinitis. We observed that calprotectin levels significantly increased based on the number of previous surgeries.

KEY WORDS: sinusitis, nasal polyps, rhinitis, immunophenotyping, precision medicine

RIASSUNTO

Obiettivo. *Abbiamo analizzato i livelli di calprotectina nelle secrezioni nasali di diversi endotipi di CRSwNP, per valutarne il ruolo di marcatore di infiammazione non-tipo 2.*

Metodi. *Abbiamo incluso pazienti con CRSwNP diffusa primaria (n = 41) e tre diversi gruppi di controllo [rinite non allergica (NAR) (n = 13), rinite eosinofila non allergica (NARES) (n = 10) e soggetti sani (n = 12)]. I livelli di calprotectina sono stati dosati mediante test di chemoluminescenza.*

Risultati. *I livelli di calprotectina sono risultati significativamente più alti in tutti gli endotipi non-tipo 2 di CRSwNP, rispetto ai controlli sani ($p < 0,05$). Al contrario, nella CRSwNP tipo 2 essa è risultata inferiore rispetto ai controlli ($p < 0,05$). È stata riscontrata una correlazione significativa tra i livelli di calprotectina e la conta dei neutrofili/HPF ($p < 0.01$). I livelli medi di calprotectina aumentano inoltre nei pazienti in precedenza sottoposti a 3 o più ESS ($p < 0,05$).*

Conclusioni. *La calprotectina nelle secrezioni nasali può essere considerata un biomarcatore di infiammazione non-tipo 2 mentre bassi livelli di calprotectina sono indicativi di immunoflogosi di tipo 2. Abbiamo infine osservato che i livelli di calprotectina aumentano significativamente in relazione al numero di interventi chirurgici pregressi.*

PAROLE CHIAVE: *rinosinusite, polipi nasosinusali, rinite, immunofenotipizzazione, medicina di precisione*

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Introduction

Calprotectin is a member of the S100 protein family, consisting of two sub-units (S100A8/S100A9), produced by neutrophils, monocytes and epithelial cells, which participate in the innate immune response by binding specific receptors (TLR4 and RAGE) [1]. Calprotectin may have several functions in both the internal and the extracellular compartments by promoting anti-microbial and pro-inflammatory responses. Its exact function depends on the local tissue environment in which it is released. Firstly, it may be involved in the recruitment and trans-endothelial migration of neutrophils and monocytes by stimulating epithelial cells to produce IL-8 and TNF-α, an important chemoattractant for these cells. Secondly, it has direct antimicrobial activity against bacteria and fungi, sequestering extracellular divalent metal cations that are essential for bacterial growth [2]. Furthermore, cytoplasmic calprotectin is crucial for cytoskeleton reorganisation and phagocyte migration by conferring resistance to intracellular invading bacteria [3,4]. Finally, the importance of this protein in maintaining epithelium barrier integrity has been demonstrated, which is related to its tissue-protective functions by inhibiting the production of reactive oxygen species (ROS) and activity of matrix metalloproteinases (MMPs) that cleave a vast range of cytokines, chemokines, receptors, proteases, and adhesion molecules to alter their function [5].

There are conflicting reports in the literature regarding whether calprotectin may play protective or pathologic roles in patients with chronic rhinosinusitis (CRS). The expression patterns of calprotectin in CRS patients may significantly differ if measured in nasal secretions, polyp tissue and normal sinonasal mucosa at different subsites. Richer [6] showed that the gene expression of S100A7 and S100A8 in epithelial cells of sinonasal mucosa was significantly decreased in CRS compared with controls, resulting in a decreased proliferative or aberrant regenerative capacity of the respiratory epithelium, particularly in response to injury. On the other hand, Van Crombruggen [7] showed that tissue levels of S100A8/A9 were significantly higher in nasal polyp tissue from CRSwNP patients compared to inferior turbinates of controls. The authors observed a significant correlation between tissue levels of IL-8 and S100A8/A9, demonstrating that IL-8 produced by activated epithelium or resident macrophages was a strong chemoattractant for neutrophils. Finally, Tieu [8] showed that the expression of S100A8/A9 was significantly increased in polyp tissue compared to turbinate and uncinate tissue from chronic rhinosinusitis with nasal polyps (CRSwNP), chronic rhinosinusitis without nasal polyps (CRSsNP) and controls; however, its levels were significantly lower in the nasal lavage fluid of CRSwNP patients compared to healthy controls, reflecting lower epithelial production of these peptides. Furthermore, they observed that calprotectin levels correlated with levels of neutrophils, as assessed by means of quantification of neutrophil elastase. Recently, Sumsion [9] reviewed several manuscripts highlighting the expression patterns of S100 proteins in CRS hypothesising that they may have an important role in mediating immunity in different subtypes of CRS. Unfortunately, studies investigating differential expression of S100A8/9 in endotypes of CRSwNP are lacking.

The objective of this manuscript was to analyse calprotectin (S100A8/S100A9) levels in sino-nasal secretions of different CRSwNP endotypes and investigate the relationship with neutrophilia. We tried to verify the hypothesis that calprotectin may be a biomarker of non-type 2 CRSwNP. Furthermore, we evaluated the expression of nasal calprotectin based on clinical characteristics of CRSwNP.

Materials and methods

Study design and study population

This was a non-profit analytical case-control observational study performed at our institution "A. Gemelli Hospital Foundation IRCCS", Otorhinolaryngology, Rhinology Unit, Catholic University of Sacred Heart-Rome, Italy. Patients were screened and enrolled between October 2019 and January 2021.

Inclusion criteria:

- male or female 18-65 years old;
- primary diffuse CRSwNP according to EPOS 2020 [10];
- significant nasal symptoms (SNOT-22 > 20);
- willingness and ability to provide written informed consent and to comply with all study related procedures.

Exclusion criteria:

- acute exacerbation of CRS (AECRS) as defined in the EPOS 2020 [10];
- local or systemic steroid treatment less than 4 weeks before the inclusion;
- active smokers;
- previous nasal surgery (septoplasty, cosmetic surgery etc.);
- current malignancy;
- previous radiotherapy for head neck cancer.

Based on inclusion and exclusion criteria we enrolled 41 primary diffuse CRSwNP (17/41 females; mean age 42.6; range 18-65). We identified 3 different groups as controls: 13 non-allergic rhinitis (NAR) patients, 10 non-allergic eosinophilic syndrome (NARES) patients, and 12 healthy subjects (no history of rhinitis/CRS, negative allergy tests, no nasal inflammation at nasal cytology, no evidence of na-

Table I. Epidemiological data and clinical features of patients and controls.

	Primary diffuse CRSwNP (n = 41)	NAR (n = 13)	NARES (n = 10)	Healthy controls (n = 12)
Age, years, median (IQR)	42.6	52 (17)	39 (15)	44 (14)
Sex, female, n/total (%)	17/41 (41.5%)	4/13 (30.7%)	6/10 (60%)	6/12 (50%)
Asthma, n/total (%)	14/41 (34.1%)	None	3/10 (30%)	None
ASA intolerance, n/total (%)	9/41(21.9%)	None	2/10 (20%)	None
Atopy, n/total (%)	17/41 (41.5%)	None	None	4/12 (33.3%)
Peripheral blood iper-eosinophilia, n/total (%)	10/41 (24.4%)	None	5/10 (50%)	None
Smoking, yes n/total (%)	16/41 (39%)	None	2/10 (20%)	4/12 (33.3%)
SNOT-22, mean	33.2	12.5	21.9	77.22
LKES, mean	13.3	0	0	0
Mean CT Lund Mackay score	13.3	0	0	None
Eosinophil count/HPF, mean±SD	35.3 ± 5.9	0	52.4 ± 19.4	0
Neutrophil count/HPF, mean±SD	27.9 ± 9.8	3 ± 6.3	0	2.5 ± 4.5
Mean number of surgery±SD	1.6 ±1.0	0.1 ± 0.3	0	0
Calprotectin ng/mL, mean±SD	207.8 ± 97.6 ng/ml	118.6 ± 46.1 ng/ml	62.8 ± 31.2 ng/ml	77.3 ± 31.6 ng/ml

CRSwNP: Chronic Rhinosinusitis with Nasal Polyps; HPF: High Power Field; LKES: Lund Kennedy Endoscopic Score; NAR: non-allergic rhinitis NARES: Non-infectious non-allergic rhinitis with eosinophilia syndrome; Non-EOS: Non-Eosinophilic; SNOT: sinonasal outcome test; SD standard deviation; ASA: acetylsalicylic acid; IQR: interquartile range.

sal polyps at nasal endoscopy). Clinical features of patients and control groups are shown in Table I.

Study protocol

All enrolled patients underwent:

- baseline interview including previous treatments, family history, asthma (based on previous pulmonologists evaluation), hypersensitivity to non-steroidal anti-inflammatory drugs (NSAID) (based on a reported history of adverse reactions or on previous allergological evaluation). Eosinophilia was assumed for blood eosinophil count > 500/microliter. We divided patients into allergic/non-allergic and asthmatic/non-asthmatic according to the ARIA project [11]. Data on recently performed CT scans were collected;
- allergy testing: total immunoglobulinE (IgE), skin prick testing (SPT) for common inhalant allergens (at least 18), specific IgE in serum by Radio-Allergo-Sorbent-Test (RAST) and, if indicated, nasal provocation test to exclude local allergy in patients who were negative by SPT;
- symptom score analysis by the validated Italian version SNOT-22 (Score range: 0-110 [12]);
- nasal endoscopy bilaterally: findings were noted using the Lund-Kennedy Endoscopic Score (LKES) [13]. A combined score (right + left side) of 0-20 was possible;
- nasal cytology: material was obtained by scraping of lower/middle turbinate mucosa bilaterally by rhinoprobe (Farmark snc, Milan). Samples mounted on a slide and stained by the May-Grunwald-Giemsa method. Cell counts were expressed as mean value per high-power-field (HPF) on a mean of at least 10 richest observed fields at high magnification (x 400). Percentage of eosinophils and neutrophils on total cellularity was also provided [14]. Eosinophilic inflammation was assumed if eosinophil count was ≥ 10/HPF (x400) and eosinophils accounted for more than 20% of total cellularity. To date, there is no specific landmark to define type 2 inflammation by nasal cytology; for this reason, in order to be more rigorous, we adopted that used for histological findings (eosinophil count: > 10/HPF) combined with the one used for nasal cytology (eosinophils 20% of total cellularity) suggestive of significant eosinophilia. Neutrophilic inflammation was assumed if the neutrophil count was ≥ 20/HPF (x400) with neutrophil accounting for over 50% of total cellularity. Mixed inflammation if both eosinophilic count ≥ 10/HPF and neutrophilic count ≥ 20/HPF were observed;
- nasal lavage fluid collection was obtained from subjects with the head bent down, as previously described [15-17]. We instilled 5 ml of saline solution (NaCl 0.9%) pre-warmed to 35°C into each nostril and used it to rinse the nostrils several times. All fluid was then collected by asking the subjects to lean forward and blow the nasal contents gently into a funnel connected to a 30 ml universal container. The lavage fluid was filtered to remove any nasal mucus and centri-

fuged immediately at 4000 revolutions per minute (RPM) for 5 minutes. It was then divided in aliquots and frozen at -80°C until the assays were performed.

Eligible patients were grouped, according to nasal disorder, as follows:

- primary diffuse CRSwNP was defined according to EPOS 2020 [10]. All patients underwent CT scan if not performed in the last 6 months and staged by Lund-Mackay score (LMS). Endotypes were established based on the cellular pattern (defined by nasal cytology) and biochemical pathways measuring most relevant cytokines (IL-4, IL-5, IL-17, INF-γ and TNF-α) in nasal secretion by ELISA [10]. Type-2 was defined in case of eosinophilic inflammation at nasal cytology and if IL-4/IL-5 were measured in nasal secretion according to literature data [15,18,19] (Tab. I). In case of neutrophilic inflammation, Type-1 was defined when IFN-gamma/TNF-alpha were detected, whereas Type-3 in case of IL-17 detection; mixed endotypes were also described as shown in Table II. CRSwNP patients were further divided according to the dominant cellular pattern as follows:
- eosinophilic CRSwNP (EOS-CRSwNP) (n = 15): pure Type-2;
- non-eosinophilic (Non-EOS-CRSwNP) (n = 12): pure Type-1 or Type-3;
- mixed CRSwNP (n = 14): mixed Type 2-3, Type 1-2.
- nAR (n = 13): persistent rhinitis, negative allergy tests in vivo/vitro, LKES = 0, LMS = 0; no evidence of eosinophilic inflammation at nasal cytology;
- NARES (n = 10): persistent rhinitis, no evidence of atopy allergy in vivo/vitro, marked nasal hypereosinophilia at nasal cytology.

Biochemical assays

Multiple cytokines were detected in nasal secretions by high-sensitivity ELISA (HS Quantikine Human Cytokine Immunoassay; R&D Systems, Minneapolis, US). Assays were performed in duplicate during a single session and the mean values were calculated. The coefficient of variation (CV) of duplicates was always less than 3%. The sensitivity of cytokines assays were specifically: 1.2 pg/ml for IL-4, 1.08 pg/mL for IL-5, 0.51 pg/mL for IL-17, 5.1 pg/ml for IFN-γ and 6.3 pg/ml for TNF-α.

Calprotectin was determined by DiaSorin-LIAISON assay, an in vitro diagnostic chemiluminescent immunoassay (CLIA) intended for the quantitative measurement of faecal calprotectin. This is a sandwich assay using two monoclonal antibodies for capture and detection of the molecule. The samples are incubated with assay buffer and paramagnetic particles coated with a monoclonal antibody that specifically recognises the heterometric calprotectin (S100A8/A9); following incubation, a wash cycle is performed to remove any unbound material. An isoluminol conjugated monoclonal antibody that recognises calprotectin is then added to the reaction and incubated. The unbound conjugate is removed with a second wash step. Starter reagents are then added, and a flash chemiluminescent reaction is initiated. The light signal is measured using a photomultiplier as relative light units (RLU), which is proportional to the concentration of calprotectin present in the calibrators, controls, or samples. The kit measures the level of calprotectin in faeces in µg/g, then dividing it by 7500, with a value that corresponds to mg/L calprotectin in nasal lavage fluid. In our study, we report the calprotectin value in ng/ml.

Statistical analysis

Statistical analysis was performed using SPSS.25 for Windows (Chicago, Illinois). Continues values such as levels of calprotectin, symptom scores, endoscopic scores, cell count, are expressed as mean ± standard deviation (SD). Comparisons between groups were performed using the Mann-Whitney U-test for non-normally distributed data and t-test for paired samples in normally distributed values; when comparing more than two groups we used 1-way ANOVA and Bonferroni post-hoc analysis. The Chi-square test was used to compare categorical data such as prevalence and incidence. The strength of the correlation between the two parameters was obtained by Spearman's rank correlation test. The results were considered significant for p-values < 0.05.

Results

Calprotectin levels in nasal secretion of different endotypes of CRSwNP

Calprotectin levels based on dominant endotype of CRSwNP are shown in Table II and Figure 1. Mean levels in nasal secretions of type-1 and type-3 CRSwNP were significantly higher compared to healthy controls: 170.37 ± 70.2 *vs* 77.3 ± 31.6 ng/ml ($p < 0.05$) and 434.73 ± 100.2 *vs* 77.3 ± 31.6 ng/ml ($p < 0.05$), respectively. Contrarily, in type-2 CRSwNP calprotectin was significantly lower compared to controls (31.6 ± 15.8 ng/ml *vs* 77.3 ± 31.6 ng/ml; $p < 0.05$). The highest levels were observed in type-3 endotype *vs* type-1 and 2 ($p < 0.05$). Significantly higher levels of calprotectin were also detected in mixed type 2-3 and type 1-2 CRSwNP compared to healthy controls ($p < 0.05$). The inter-group comparisons revealed that mean calprotectin levels in type-1 and type-3 endotype dominant CRSwNP patients were significantly higher compared to type-2: 170.37 ± 70.2 *vs* 31.6 ± 15.8 ($p < 0.05$) and 434.73 ± 100.2 *vs* 31.6 ± 15.8 ($p < 0.05$), respectively. Furthermore, mean calprotectin levels were significantly higher in mixed

Table II. Endotype dominance of patients included with primary diffuse chronic rhinosinusitis with nasal polyps. Values of cytokines are indicated as mean ± SD.

	No. of patients	Eosinophil count/HPF	Neutrophil count/HPF	IL-5 (ng/L)	IL-4 (ng/L)	IL-17 (ng/L)	TNF-α (ng/L)	INF-γ (ng/L)	Calprotectin ng/ml
Type 1	5	3 ± 6.7	37.4 ± 8.0	0.6 ± 0.2	0.9 ± 0.7	0	1.4 ± 0.6	1.7 ± 0.6	170.37 ± 70.2
Type 2	15	36.2 ± 8.2	0.6 ± 1.2	3.0 ± 2.0	2.9 ± 1.3	0.1 ± 0.2	0.1 ± 0.2	0.05 ± 0.2	31.6 ± 15.8
Type 3	6	0	47 ± 6.7	0.9 ± 0.4	1.2 ± 0.9	1.2 ± 0.3	0.4 ± 0.3	0.4 ± 0.3	434.73 ± 100.2
Type 2-3	5	16.7 ± 5.1	41.3 ± 10.6	3.3 ± 2.8	1.6 ± 0.3	2 ± 0.8	0.5 ± 0.9	0.5 ± 0.9	270.86 ± 89.2
Type 1-2	10	27 ± 3.5	39 ± 6.9	3.7 ± 1.8	1.9 ± 0.9	0	0.4 ± 0.7	0.4 ± 0.7	268.42 ± 87.2

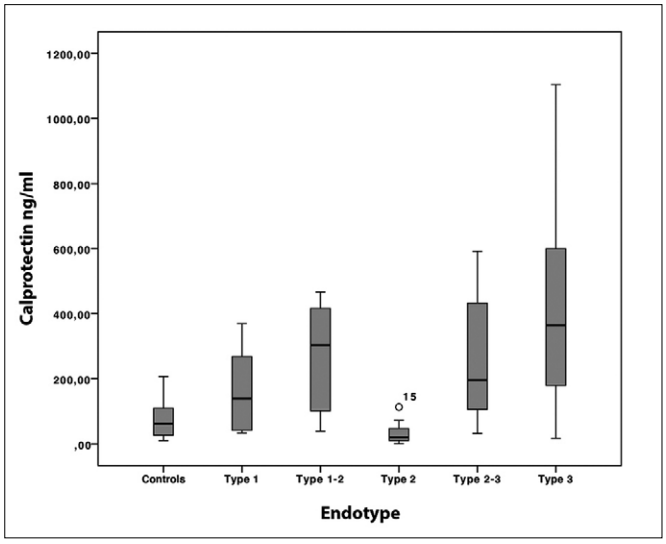

Figure 1. Box plots showing mean levels of calprotectin (ng/ml) based on CRS endotyping. The box plot shows the median and interquartile range and the error bars show the 5th and 95th percentiles. Statistical significance is given as $p < 0.05$.

type 2-3 and type 1-2 compared to type-2: 270.86 ± 89.2 *vs* 31.6 ± 15.8 ($p < 0.05$) and 268.42 ± 87 *vs* 31.6 ± 15.8 ($p < 0.05$), respectively.

Mean concentrations of calprotectin in nasal lavage of different subtypes of CRSwNP based on the cellular dominant pattern is reported in Table III. The mean concentration of calprotectin in non-EOS-CRSwNP and Mixed-CRSwNP was significantly higher compared to healthy subjects: 324.5 ± 159.7 ng/ml *vs* 77.3 ± 31.6 ng/ml ($p < 0.05$) and 269.6 ± 94.5 ng/ml *vs* 77.3 ± 31.6 ng/ml ($p < 0.05$), respectively. Mean levels of calprotectin in EOS-CRSwNP were significantly lower compared to healthy controls (31.6 ± 15.8 ng/ml *vs* 77.3 ± 31.6 ng/ml; $p < 0.05$).

At the inter-group comparison, we observed higher mean levels of calprotectin in non-EOS-CRSwNP and in mixed-CRSwNP compared to EOS-CRSwNP ($p < 0.05$). A difference was observed between non-EOS-CRSwNP and mixed-CRSwNP ($p > 0.05$).

By Spearman's rank test, we observed a significant correlation between calprotectin levels and neutrophilic count/HPF in all patients (rs: 0.692; $p < 0.01$) (Fig. 2) and in all CRSwNP patients (rs: 0.882; $p < 0.01$) (Fig. 3). A significant correlation was found between calprotectin levels and eosinophil count/HPF (rs: 0.127; $p > 0.05$).

Calprotectin levels in nasal secretion based on different clinical characteristics of CRSwNP

The mean calprotectin in nasal lavage of CRSwNP patients was significantly higher compared to healthy subjects (200.2 ± 71.3 ng/ml *vs* 77.3 ± 31.6 ng/ml; $p < 0.05$). Analysing concomitant factors, calprotectin levels were higher in non-atopic versus atopic (237.1 ± 109.3 ng/ml *vs* 153.9 ± 98.2 ng/ml; $p > 0.05$) and in non-asthmatic versus asthmatic (213.4 ± 98.9 ng/ml *vs* 186.2 ± 101.1 ng/ml; $p > 0.05$) even though the difference was not statistically significant. No significant correlation was found between calprotectin levels and SNOT-22 ($p > 0.05$), LMS ($p > 0.05$) and LKES ($p > 0.05$) by Spearman's rank test.

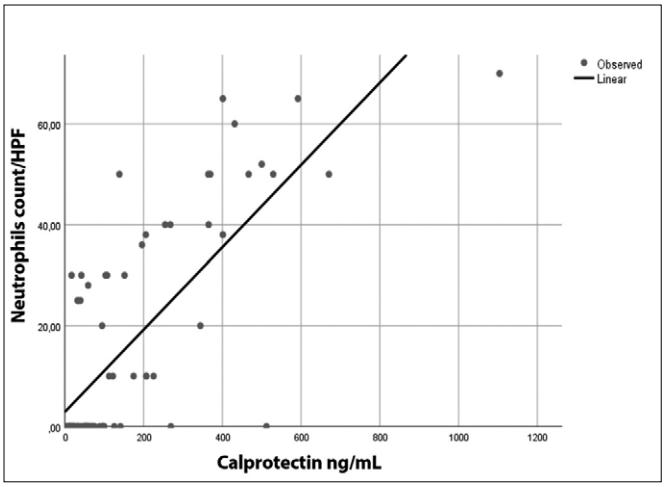

Figure 2. Scatter plot showing intra-individual correlations between calprotectin and neutrophil count/HPF in all patients (NAR; NARES and CRSwNP) excluding healthy subjects (rs = correlation coefficient assessed by Spearman's rank correlation test. Statistical significance is given as $p < 0.05$).

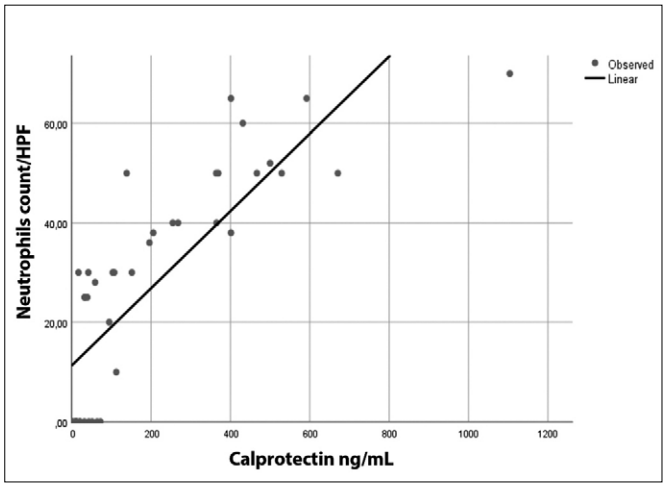

Figure 3. Scatter plot showing intraindividual correlations between calprotectin and neutrophil count/HPF in CRSwNP patients (rs = correlation coefficient assessed by Spearman's rank correlation test). Statistical significance is given as $p < 0.05$).

Finally, we observed that levels of calprotectin were significantly different based on the number of previously performed endoscopic sinus surgeries (ESS) (Tab. IV). Calprotectin progressively increased with the number of surgeries, as shown in Figure 4. The inter-group comparison revealed that mean levels of calprotectin and neutrophilia were significantly higher in patients who previously underwent 3 or more ESS compared to the other groups ($p < 0.05$). Furthermore, we performed a subgroup analysis in Type-2 patients observing that levels of calprotectin were significantly higher in patients who underwent 3 or more surgeries compared with those who underwent fewer than 3 surgeries (280.4 ± 109.3 ng/ml *vs* 92.5 ± 41.2 ng/ml; $p < 0.05$). In conclusion, the levels of calprotectin significantly increased in both Type-2 patients with multiple surgeries and in non-Type 2, but the difference was not statistically significant (280.4 ± 109.3 ng/ml ng/ml vs 222.9 ± 92.1 ng/ml; $p > 0.05$).

Nasal lavage calprotectin in CRSwNP vs non-allergic-rhinitis

In order to compare CRSwNP patients to upper respiratory diseases with overlapping pathophysiology, but without nasal polyp development, we evaluated calprotectin levels in non-allergic-rhinitis patients with eosinophilic inflammation (NARES) or without it (NAR). The average concentration of calprotectin in non-EOS CRSwNP was significantly higher compared to NAR (324.5 ± 159.7 ng/ml *vs* 118.6 ± 46.1 ng/ml; $p < 0.05$) and NARES (324.5 ± 159.7 ng/ml *vs* 62.8 ± 31.2 ng/ml; $p < 0.05$). The difference between EOS-CRSwNP and NAR was significant (31.6 ± 15.8 ng/ml *vs* 118.6 ± 46.1 ng/ml; $p < 0.05$), whereas the difference between EOS-CRSwNP and NARES was not (31.6 ± 15.8 ng/ml *vs* 62.8 ± 31.2 ng/ml; $p > 0.05$). Box plots summarising the levels of calprotectin in all groups are reported in Figure 5.

Table III. Clinical features of patients based on cellular dominant patter of inflammation associated to CRSwNP.

	Non-EOS CRSwNP (Type 1; Type 3) (n = 12)	Eosinophilic CRSwNP (Type 2) (n = 15)	Mixed CRSwNP (Type 1-2; Type 2-3) (n = 14)	Healthy controls (n = 12)
Age, years, median (IQR)	44 (17)	38 (12)	46 (14)	44 (14)
Sex, female, n/total (%)	2/12 (16.6%)	11/15 (73.3%)	4/14 (28.5%)	6/12 (50%)
Asthma, n/total (%)	2/12 (16.6%)	5/15 (33.3%)	7/14 (50%)	None
ASA intolerance, n/total (%)	1/12 (8.3%)	3/15 (20%)	5/14 (35.7%)	None
Atopy, n/total (%)	3/12 (25%)	5/15 (33.3%)	9/14 (64.3%)	4/12 (33.3%)
Peripheral blood hyper-eosinophilia, n/total (%)	None	6/15 (40%)	4/14 (28.5%)	None
Smoking, yes n/total (%)	5/12 (41.6%)	5/15 (33.3%)	6/14 (42.8%)	4/12 (33.3%)
SNOT-22, mean	29.12	39.77	40.05	77.22
LKES, mean	11.1	13.3	14.9	0
Mean CT Lund Mackay score	11.1	13.3	14.9	None
Eosinophil count/HPF, mean ± SD	0	36 ± 5.0	34 ± 7.3	0
Neutrophil count/HPF, mean ± SD	43 ± 12.8	0.6 ± 2.5	40 ± 14.9	2.5 ± 4.5
Mean number of surgery ± SD	1 ± 1.0	1.4 ± 0.7	2.2 ± 1.0	0
Calprotectin ng/mL, mean ± SD	324.5 ± 159.7 ng/ml	31.6 ± 15.8 ng/ml	269.6 ± 94.5 ng/ml	77.3 ± 31.6 ng/ml

CRSwNP: Chronic Rhinosinusitis with Nasal Polyps; HPF: High Power Field; LKES: Lund Kennedy Endoscopic Score; NAR: non-allergic rhinitis NARES: Non-infectious non-allergic rhinitis with eosinophilia syndrome; Non-EOS: Non-Eosinophilic; SNOT: sinonasal outcome test; SD standard deviation; ASA: acetylsalicylic acid; IQR: interquartile range.

Table IV. Mean levels of calprotectin (ng/mL) based on number of previous surgeries.

Number of previous surgeries	Number of patients	Neutrophils (N)/HPF	Mean calprotectin ng/ml
0	36	29.4	110.5
1	22	22.5	141.5
2	10	23.5	184.4
3	5	43.3	341.7
4	4	42.5	363.2

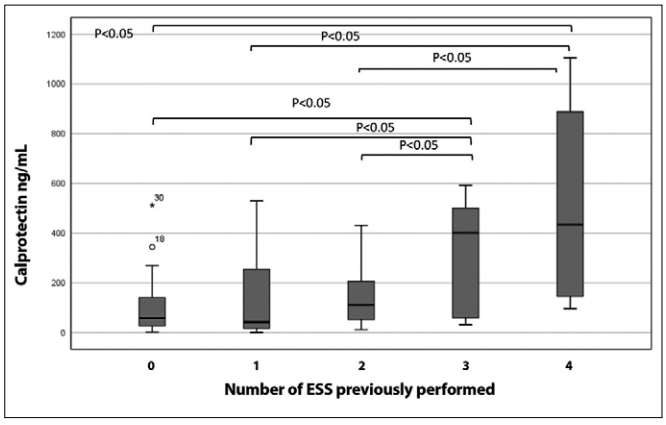

Figure 4. Box plot showing mean levels of calprotectin (ng/ml) of CRSwNP patients based on the number of previous endoscopic sinus surgery performed. The box plot shows the median and interquartile range and the error bars show the 5th and 95th percentiles.

Discussion

CRSwNP is a complex and heterogeneous disorder with distinct pathophysiologic mechanisms. With the advent of more individualised treatments, the efforts of researchers have been focused on identifying the molecular pathway(s)/inflammatory endotypes that have been activated in an individual patient rather than analysis of the unknown and aetiologic factors that may cause CRSwNP [10].

Over the last years, significant progress has been made in characterising the inflammatory endotypes in CRSwNP. Nowadays, it is clear that chronic inflammatory response in CRS may utilise Type-1, 2 or 3 pathways of the immune response. The selective or combined expression of Type-1, 2, or 3 immune responses which gives rise to significant disease heterogeneity [20]. Type-2 inflammation associated with CRSwNP has been widely investigated and seems to respond better to biologic therapy compared to non-Type 2 driven CRSwNP (Type-1 and Type-3) [21]. Nevertheless, it is still unclear which biomarkers are most accurate at predicting specific endotypes and their impact on clinical disease. In particular, biomarkers of Type-1 or Type-3 inflammation are especially lacking because they have received less attention, and much information has not been confirmed at the protein level [22,23].

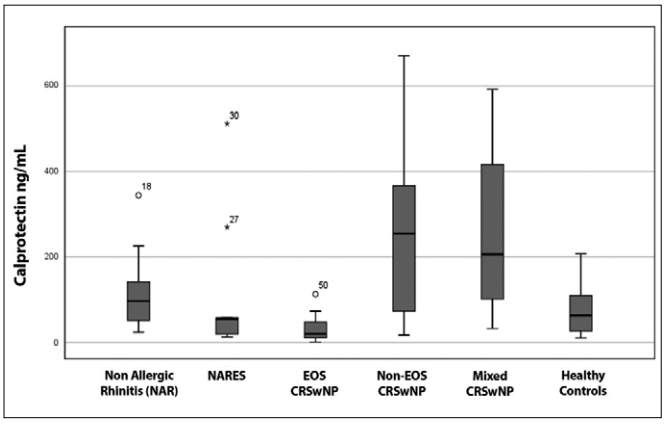

Figure 5. Box plots showing mean levels of calprotectin (ng/ml) in nasal secretions (NAR: non-allergic rhinitis; NARES: non-allergic eosinophilic syndrome; CRSwNP chronic rhinosinusitis with nasal polyps). The box plot shows the median and interquartile range and the error bars show the 5th and 95th percentiles. Statistical significance is given as $p < 0.05$.

To our knowledge, this is the first study analysing calprotectin in nasal secretions of different CRSwNP endotypes. According to our previous experience [15-17], we believe that nasal secretion may be representative of the different inflammatory cytokine patterns associated with CRSwNP. We firstly demonstrated that levels of calprotectin were significantly higher compared to controls in all endotypes associated with significant neutrophilia (Type-1, Type-3, mixed Type 1-2 and Type 2-3). The highest levels were observed in type-3 consistent with literature data showing a more pronounced neutrophilia in this endotype [10]. Accordingly, we observed a significant correlation between calprotectin levels and neutrophil count in all patients (rs:0.692; $p < 0.01$) and a stronger one considering only CRSwNP (rs:0.882; $p < 0.01$). Interestingly, calprotectin levels observed in Type-2 CRSwNP were significantly lower than in healthy subjects.

The analyses based on cellular dominant pattern of inflammation associated with CRSwNP further support our hypothesis that calprotectin may be considered as a biomarker of non-Type 2 inflammation. In fact, we observed that the levels of calprotectin in nasal secretions of non-EOS CRSwNP (Type 1-3) were significantly higher compared to EOS-CRSwNP (Type-2). In addition, we demonstrated that mixed CRSwNP (type 1-2 and 2-3) had significantly higher levels of calprotectin compared to the control group and to EOS-CRSwNP; these patients showed the same magnitude of calprotectin levels compared to non-EOS CRSwNP, probably due to a comparable mean neutrophil count/HPF

in the two groups. Accordingly, in the past, some authors [8] demonstrated an increased level of calprotectin in nasal polyp tissue which reflects neutrophil recruitment as a compensatory mechanism. Our data support the hypothesis that levels of calprotectin in nasal secretions reflect the magnitude of neutrophil recruitment. As demonstration of this, mean calprotectin levels in NARES patients were comparable to those in EOS-CRSwNP and significantly lower compared to non-EOS CRSwNP. NARES patients have overlapping pathophysiology with EOS-CRSwNP, and neither are typically associated with neutrophilia.

The analyses of calprotectin based on the clinical characteristics of CRSwNP revealed higher mean calprotectin levels in atopic and asthmatic CRSwNP compared to non-atopic and non-asthmatic, even though the differences were not statistically significant. The relationship between atopy and calprotectin is still matter of discussion. Interestingly, Kato et al. [24] observed in vitro on cultured scraped epithelial cells that the intracellular calprotectin concentration in unstimulated cells from EOS-CRS patients was significantly lower than those from non EOS-CRS patients. Nevertheless, they observed that allergen stimulation significantly induced calprotectin production in epithelial cells from EOS-CRS patients. Our data seem to be in contrast with those results, although a direct comparison cannot be performed because we measured calprotectin levels in nasal secretions in vivo. Furthermore, literature data [6-9] suggest that expression patterns of calprotectin in CRS patients may significantly differ if measured in nasal secretions, polyp tissue, or normal sinonasal mucosa. For this reason, we believe that future studies are required to further define a correlation between atopy and calprotectin secretion.

We finally observed that a significant increase of calprotectin level in nasal secretions was correlated with a high number of previous ESS. Patients who underwent multiple ESS (> 3) showed more pronounced neutrophilia and a corresponding significantly higher level of calprotectin in both Type 2 and non-Type 2. Inter-group comparison revealed that the difference between multiple operated (> 3) type 2 and non-type 2 patients was not statistically different. We previously hypothesised that calprotectin levels in these cases are related to the increased neutrophilia as a consequence of progressive epithelial barrier breakdown that is generally observed in patients who underwent multiple surgeries [25,26].

Among the limitations of this study there is the absence of confirmation of the endotype by histological analysis. However, this limitation has been exceeded by combining nasal cytological features and cytokine dosage in nasal secretion. Another limitation of the study is the exclusion of CRSsNP patients. Future investigations should confirm if our conclusions may also be applied to CRSsNP; in fact, recent evidence [10] suggests that they may be associated with Type-2 or non-Type 2 inflammation as well as CRSwNP.

In conclusion, our data suggest that the role of nasal secreted calprotectin in CRS seems to be promising as biomarker of non-Type 2 inflammation; our data suggest that increased levels of calprotectin in nasal secretions may be the expression of a pure non-type 2 mechanism or of the neutrophilic component in a mixed pattern of inflammation. To our knowledge, this is the first study analysing calprotectin levels in nasal secretions based on different CRSwNP endotypes. The conflicting data in the literature regarding calprotectin in nasal secretions may be explained by the heterogeneity of the disease and the different proportion of non-type 2 endotypes included in different series. For this reason, we suggest that the levels of calprotectin should always be defined based on endotypes of the disease and on the proportion of neutrophilic infiltration in mixed patterns.

The role of biomarkers in the identification of different endotypes of CRSwNP seems to be crucial to improve the results of targeted therapy and the efforts of researchers should focus on this goal [23]. The most prevalent endotype of CRSwNP in Italy shows a type-2 inflammatory response [15,18,19] and exciting and promising new treatments with biologics, primarily developed as treatments for asthma, have shown good results for type-2 CRSwNP [19]. Our data support the hypothesis that calprotectin may be a diagnostic biomarker of non-type 2 inflammation (type-1 and 3 inflammation); in fact, low levels of calprotectin in nasal lavage seem to be more indicative of a type-2 immune response. Bearing in mind that biologics may offer greater efficacy in type-2 inflammatory disease, future studies should be performed to determine if high levels of calprotectin in nasal secretions may be predictive of poor response to type-2 biologics in CRSwNP, whereas low levels might be predictive of good response.

Conflict of interest statement

The authors declare no conflict of interest.

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Author contributions

EDC and JG: conception and design of the study, acquisition of the data, analysis and interpretation of the data; drafted the article and revised it for important intellectual content; gave final approval of the version to be submitted; agree to be accountable for all aspects of the work. SB, MEO, LT, SS, GM, ET, RFM, DF, FC and AU: acquisition of the data, analysis and interpretation of the data; drafted

the article and revised it for important intellectual content; gave final approval of the version to be submitted; agree to be accountable for all aspects of the work.

Ethical consideration

This study was approved by the Institutional Ethics Committee ("A. Gemelli Hospital Foundation IRCCS", Otorhinolaryngology, Rhinology Unit, Catholic University of Sacred Heart-Rome, Italy) (protocol n. 11177/20; ID number: 3036).

The research was conducted ethically, with all study procedures being performed in accordance with the requirements of the World Medical Association's Declaration of Helsinki.

All subjects signed a written informed consent form.

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