

Immunohistochemical dissimilarity between allergic fungal and nonfungal chronic rhinosinusitis

Ahmed Ragab, M.D., Ph.D.,¹ and Rehab Monir Samaka, M.D.²

ABSTRACT

Backgrounds: Diagnosis of allergic fungal rhinosinusitis (AFRS) is complicated because of the presence of fungi on mucosal surfaces of sinonasal passages. The objectives of this study were to define, using immunohistochemistry, lymphocyte populations associated with noninvasive fungal-related chronic rhinosinusitis (CRS; AFRS and FBs [FB]) relative to CRS with nasal polyposis (CRSwNP) and CRS without nasal polyposis (CRSSNP) as a means of diagnosing different forms of CRS.

Methods: Sinus CT scans, nasal endoscopy scores, and the presence of eosinophilic fungal mucin or FBs were used to prospectively define patient groups with CRS who had failed medical treatment and were undergoing endoscopic sinus surgery. Four patient groups were identified: AFRS, FB, CRSwNP, and CRSSNP. Tissue specimens were studied and graded for histopathological changes. Immunophenotyping of mucosal lymphocytes was performed using anti-CD3, -CD20, -CD4, -CD8, -CD56, and -perforin antibodies.

Results: Nasal polyposis scores were similar between AFRS and CRSwNP. Radiological changes associated with AFRS can also be present in CRSwNP, e.g., heterogeneity in 9/30 (30%), expansion in 25/30 (83%), and bony attenuation of the ethmoid trabeculae in 19/30 (63%). Different grades of basement membrane thickness, edema, and fibrosis were observed. In both types of noninvasive fungal rhinosinusitis, CD3⁺ T lymphocytes were most commonly identified. In cases of AFRS, most T cells were CD8⁺ ($p < 0.001$). In FB cases, CD4⁺ lymphocytes were dominant ($p < 0.001$). In nonfungal CRS cases, CD20⁺ lymphocytes (B lymphocytes) predominated ($p < 0.001$).

Conclusion: Although CT scans and histological examination can assist the diagnosis of rhinosinusitis, tissue immunophenotyping can be used in defining different types of fungal and nonfungal CRS cases.

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Fungal-related diseases of the nose and paranasal sinuses represent a heterogeneous form of chronic rhinosinusitis (CRS). The most commonly accepted classification system divides fungal-related diseases of the paranasal sinuses into invasive and noninvasive forms based on histopathological evidence of host tissues containing fungal elements.^{1–4} Noninvasive fungal-related diseases of the sinonasal passages are an important category of diseases in the temperate zone including the Middle East. Although a sinus fungal ball (FB) is often well defined, substantial confusion surrounds the context of diagnosing allergic fungal rhinosinusitis (AFRS). The most commonly cited criteria used in the diagnosis of AFRS have been previously described⁵ and depend on the presence of nasal polyposis, characteristic findings on CT scan, and the presence of allergic fungal mucin containing fungi on direct microscopic examination without tissue invasion. Specifically, diagnosis is made difficult if fungus can be found throughout over the nasal mucosa in patients presenting with and without sinusitis and further compounded by difficulties associated with the histopathological identification of fungi. Therefore, collection of mucus from involved sinuses using precise techniques and rapid processing procedures is prerequisite when using culture techniques.^{6,7} The use of special fungal stains with variable fungal detection sensitivities are necessary for histopathological detection because some CRS cases presenting with nasal polyposis can be misdiagnosed as AFRS.

On a daily basis, individuals inhale variable numbers of conidia that are successfully cleared by epithelial mucociliary defense mechanisms.⁷ The key determinant of the host interaction with fungal species (and the reason for the diversity of outcomes) is hypothesized to be the nature of the resulting immune response that may clear or contribute to disease progression. In the context of noninvasive fun-

gal sinonasal infections, the nature of the immune response can determine the morphological form, antigenic structure, and physical location of the fungus.⁶ Also, the fungi itself can elicit specific immune responses in the involved mucosa. In healthy, uninfected sinonasal passages the lining is populated with T cells both in the lamina propria and in the surface epithelium.⁸ Previous studies identified approximately twice as many CD3⁺CD8⁺ as CD3⁺CD4⁺ in the surface epithelium and only a slight predominance of CD4⁺ T cells in the lamina propria.⁹ Among the spectrum of CRS diseases, lymphocytes are believed to play an important role in regulating the mucosal immune responses to foreign antigens and, subsequently, play a key role in disease presentation. It is therefore expected that immunophenotyping and characterization of the resident lymphocyte cell populations present in the sinonasal mucosa will reveal different lymphocyte populations associated with different forms of CRS (with and without fungal etiology). This will allow adequate classification, diagnosis, and, subsequently, adequate research layouts and treatment plans for noninvasive fungal infections related to CRS. Also, it will establish a foundation for understanding the etiopathology of the disease.

MATERIALS AND METHODS

Patients scheduled for functional endoscopic sinus surgery after unsuccessful medical treatment for 3 months were enrolled in this prospective study. The criteria for enrollment included the symptoms and signs of rhinosinusitis lasting for at least 3 months with nasal endoscopic findings of CRS confirmed by soft tissue involvement of the paranasal sinuses by CT scan. All of the patients had primary surgery without any medications including corticosteroids 1 month before surgery except for local treatment of asthma. Patients with primary ciliary dyskinesia or cystic fibrosis were excluded. Patients with acute exacerbations were not included. All of the patients had no immunodeficiency disorders and were immunocompetent as shown in their blood pictures. The Ethics Committee of the hospital and Institutional Review Board approved the study.

Each patient had endoscopic assessment with grading of polyps according to the Lund-Kennedy score¹⁰ and a CT scan that was graded in line with the Lund-Mackay score.¹¹ Endoscopic evaluation

From the Departments of ¹Otorhinolaryngology and ²Pathology, Menoufia University Hospital, Shebin El-Kom, Egypt

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Address correspondence and reprint requests to Ahmed Ragab, M.D., Ph.D. (Brussels), Department of Otorhinolaryngology, Head and Neck Surgery, Menoufia University Hospital, Shibin El-Kom, Egypt, or, 73 Saied Street, Tanta, 31111 Egypt

E-mail address: ahmedragab2000@hotmail.com

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allowed for the categorization of CRS patients with and without nasal polyposis.

FB disease was identified clinically in the presence of radiological evidence of sinus opacification with or without radiographic heterogeneity and clay-like materials within the sinus. Histopathological analyses can detect dense conglomerations of hyphae separate from the sinus mucosa associated with nonspecific chronic inflammation without predominance of eosinophils, granulomas, or allergic mucin. Also, no histopathological evidence of fungal invasion of the mucosa could be seen.² AFRS patients met the classic criteria.⁵ They were diagnosed based on the presence of nasal polyposis, characteristic CT scan findings, the presence of allergic fungal mucin (thick tenacious colored mucus at the time of surgery, eosinophilic in histology examination, and containing noninvasive numerous scattered fungal hyphae on direct microscopy), and with allergy to any fungi determined by positive skin-prick test. CRS with nasal polyposis (CRSwNP) patients were diagnosed when nasal polyps were present with or without tenacious mucus, no fungi on direct microscopy, and no allergy to fungi. CRS without nasal polyposis (CRSsNP) patients were diagnosed in the absence of nasal polyps, no fungi on direct microscopy, and no fungal allergy. Using these parameters patients were divided into four groups: AFRS, FB, CRSwNP, or CRSsNP.

Endoscopic sinus surgeries were performed for all patients using the anteroposterior (Messerlinger) technique. The intraoperative criteria for categorization of patients were confirmed and mucus and tissues (of the anterior wall of the bulla ethmoidalis) were collected and analyzed by histopathological and immunohistochemical (IHC) analysis.

Histopathological Assessment

Specimens were fixed in 10% neutral-buffered formalin, sectioned, and stained with hematoxylin and eosin as a means of assessing epithelial ulceration, squamous metaplasia, basement membrane thickness, mucous-secreting glands status, and the degree of edema and fibrosis. The major histopathological changes were clearly categorized.¹² Basement membranes thicknesses were judged as mild, slight and localized thickening; marked, severe and diffuse thickness; and moderate, any abnormality between both. Subepithelial edema and fibrosis were also assessed in a categorical fashion (0 = not present, 1 = focal/mild, 2 = patchy/moderate, and 3 = extensive/marked). Eosinophils were quantified in the foci of the densest cellular infiltrates.¹² Scoring was performed using a wide-angle Olympus CH2 light microscope (field size of 0.274 mm² and field diameter of 0.59 mm²; Olympus Corp., Tokyo, Japan). Counts were recorded in 10 fields per patient sample at 400 $\times$ magnification and reported as absolute numbers. Microscopic assessment was performed three times in three separate settings within 2 months duration by a single board-certified head and neck surgical pathologist. Excellent concordance between the instances of scores was reached (intraobserver reliability, 0.99). Reassessment of the histopathological parameters was performed by two pathologists with interobserver reliability of 0.90. Unintentional bias was prevented by coding tissue samples so that histopathological study was performed without knowledge of the used component characteristics.

IHC Staining Procedures

Five-micrometer-thick sections were deparaffinized and rehydrated in xylene combined with a graded series of alcohol. Antigen retrieval was performed by boiling in 10 mM of citrate buffer (pH 6.0) for 20 minutes, followed by cooling at room temperature. Six slides per patient's tissue specimen were incubated overnight at room temperature with rabbit polyclonal anti-CD3 (pan T-lymphocytes marker; cat. no. 103A-78; Cell Marque Corp., Rocklin, CA), monoclonal anti-CD 20 (pan B lymphocytes marker for all stages of

B-cell development except the first and last; cat. no. 120M-88; Cell Marque Corp.), mouse monoclonal anti-CD4 (pan T-helper lymphocytes marker; product code, RTU-CD4-1F6, clone 1F6, Novocastra; Leica Biosystems Newcastle, Ltd., Newcastle Upon Tyne, U.K.), mouse monoclonal anti-CD8 (pan T suppressor/cytotoxic lymphocytes marker; product code, RTU-CD8-295, clone 1A5), mouse monoclonal anti-CD 56 (natural killer cell marker; cat. No. CMA361; Cell Marque Corp.), and mouse monoclonal anti-perforin (natural killer cell marker; cat. no. MS-1834-S0, 0.1 mL of concentrated mouse monoclonal antibody "dilution is 1:100"; Lab Vision, Fremont, CA). The ultravision detection anti-polyvalent horseradish peroxidase/diaminobenzidine was used for visualize staining (Lab Vision). The reaction was visualized using an appropriate substrate/chromogen (diaminobenzidine) reagent with Mayer hematoxylin as a counterstain. Tonsil tissues were used as a positive control for CD3, CD20, CD4, and CD8 and an NK lymphoma was used as a positive control for CD56 and perforin. Negative control slides were used at each run of immunostaining.

IHC Analysis

All lymphocytes with complete membranous staining were regarded as positively stained, regardless of intensity. Cytoplasmic immunoreactivity was not considered positive. All samples were read in a blind fashion without knowledge of the clinical, radiological, or histopathological findings. The staining for all markers was scored three times. The intraobserver reliability was shown to be 0.99 for CD3, CD20, CD4, and CD8 and 1 for CD56 and perforin. Scoring was performed for all resident lymphocytes in tissue sections and data were expressed as the absolute number of positive immunoreactive lymphocytes among the total number of lymphocytes. We calculated the percent of positive lymphocytes by the following calculation: number of positive lymphocytes $\times$ 100/total number of lymphocytes.

IHC assessment was performed three times in three separate settings within 3 months duration. Excellent concordance between the instances of scores was reached (intraobserver reliability, 0.99). Also, IHC reassessment was performed by two pathologist with interobserver reliability of 0.97. Unintentional bias was prevented by coding tissue samples so that the IHC study was performed without knowledge of the used component characteristics.

Statistical Methods

Statistical analyses were performed using SPSS software, Version 17.1 (SPSS, Inc., Chicago, IL). Mean, medians, SD, and ranges were calculated. For the nonparametric data, χ^2 -test was used in the analysis of differences between the qualitative data, *i.e.*, histopathological data and Kruskal-Wallis test for the quantitative data, *i.e.*, the eosinophil cells numbers. Student's *t*-tests were used in comparison between CRSwNP and CRSsNP immunohistopathological data. As the other quantitative data were skewed, and the two sample sizes were unequal, the most appropriate statistical test was Mann-Whitney *U* test. Therefore, Mann-Whitney *U* tests were used to ascertain differences between each of the two groups concerning eosinophils, CT, and immunohistopathological data. Results yielding a value of $p = 0.05$ are considered on the borderline of statistical significance. If the value of $p < 0.01$, results are considered statistically significant and if it is <0.005 they are considered highly statistically significant.

RESULTS

Four groups were included in the present study: 12 AFRS, 6 FB cases, 30 CRSwNP cases, and 30 cases without nasal polyposis. The demographic data and endoscopic scores are described in Table 1. CT scan evaluation of FBs (Fig. 1 A) revealed unilateral sinus involvement in 6/6 patients (100%) with involvement of the max-

Table 1 Demographic data and nasal polyposis scores in the studied groups

Parameters	FBs <i>n</i> = 6	AFRS <i>n</i> = 12	CRSwNP <i>n</i> = 30	CRSsNP <i>n</i> = 30
Age (mean ± SD)	42.6 ± 5.7	31.3 ± 12.2	32.4 ± 7.6	34.4 ± 10.6
Male/female ratio	1:2	1:1	2:1	1.7:1.3
Allergy	1	12	11	8
History of asthma	0	3	7	2
History of aspirin sensitivity	0	0	1	0
Left side nasal polyposis score (mean ± SD)	0	1.7 ± 1.1	2.3 ± 0.7	0
Right side nasal polyposis scores (mean ± SD)	0	1.5 ± 1.2	2.2 ± 0.9	0

AFRS = allergic fungal rhinosinusitis; CRSsNP = chronic rhinosinusitis without nasal polyposis; CRSwNP = chronic rhinosinusitis with nasal polyposis; FBs = fungal balls.

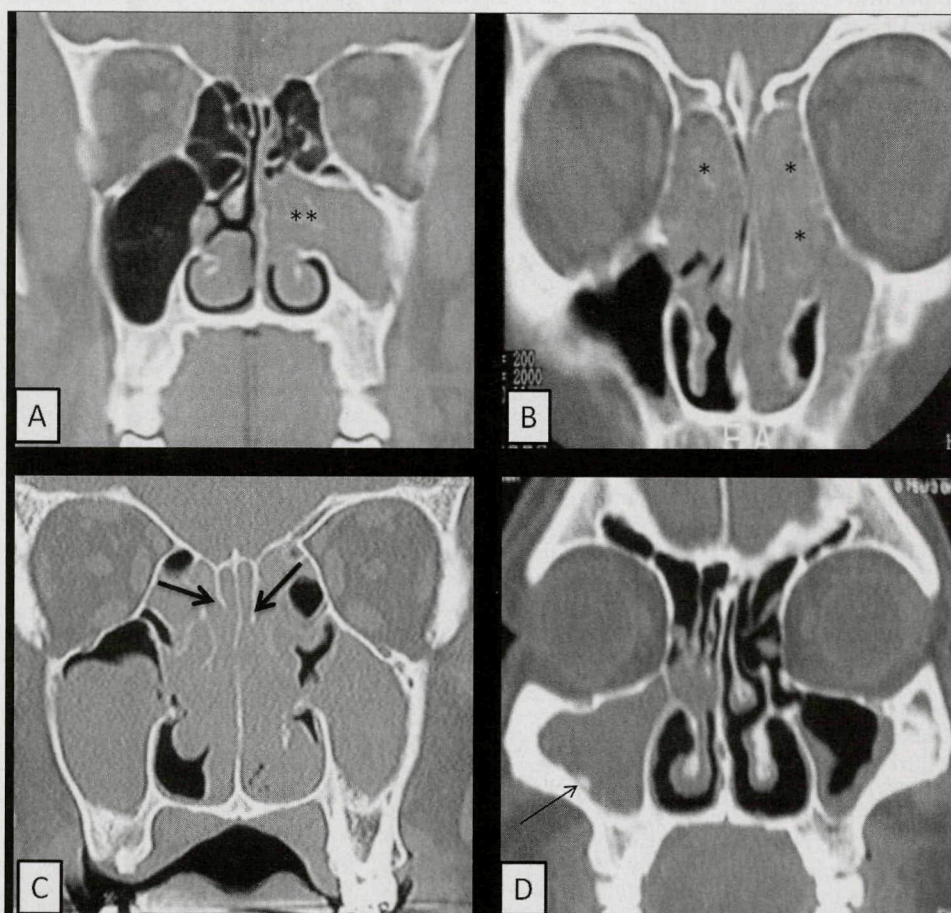


Figure 1. Coronal CT analysis of different chronic rhinosinusitis (CRS) disease forms. (A) Fungal balls (FB) with expansion of the left maxillary sinus toward the middle meatus and nasal cavity with calcification (**). (B) Allergic fungal rhinosinusitis (AFRS) showing expansile heterogeneous soft tissue densities (*) more in the left maxillary and ethmoid with loss of bony ethmoid trabeculae. (C) CRS with nasal polyposis (CRSwNP) with bilateral soft tissue density of the maxillary sinuses and anterior ethmoids, bony attenuation of the ethmoid trabeculae, and truncation of the bony middle turbinate (arrows). (D) CRS without nasal polyposis (CRSsNP) showing complete soft tissue density of the right maxillary sinus with mild calcification in the floor (arrow) and mild soft tissue density of the left maxillary sinus.

illary sinus in 6/6 (100%) cases. Hyperdense areas with flocculent calcification were determined in 3/6 patients (50%). Sinus expansion was observed in 4/6 individuals (66%) and erosions in 3/6 (50%) of the samples analyzed. CT scans of sinuses from AFRS cases (Fig. 1 B) showed heterogeneity in 8/12 (67%), expansion in 10/12 (83%), and erosion in 8/12 (67%). In CRSwNP, heterogeneity was observed in 9/30 (30%), expansion in 25/30 (83%), bony attenuation of the ethmoid trabeculae in 19/30 (63%), and truncation of the bony middle turbinate in 16/30 (53%; Fig. 1 C). CT scores are described in Table 2. The anterior and posterior ethmoid scores were elevated in CRSwNP compared with AFRS ($p < 0.05$) and both scores were higher than values observed in FB or CRSsNP ($p < 0.05$; Fig. 1 D).

Histopathological criteria and grading are described in Table 3 and Fig. 2. Each histopathological category was graded. Fungal hyphae were identified in all fungal cases (12 AFRS and 6 FB) using routine

hematoxylin and eosin and fungal stains. Colonies detected in FB cases consisted of tightly packed hyphal arrays. Larger colonies had pale centers with more-defined morphology present at the periphery. Allergic mucin was grossly thick, glue-like consistency, green-black-brown mucus, and abundant. Histologically, sections of the mucinous material showed a mixture of sloughed epithelial cells, eosinophils, and scattered numerous fungal hyphae. The more definitive branching septate hyphae were more easily detected by fungal stains.

Marked to moderate mucosal edema was more frequently observed in CRSwNP followed by AFRS and FB. Edema was the least in the CRSsNP group. Conversely, 80% of CRSsNP showed moderate to marked fibrosis. Also, a more marked to moderate fibrosis was observed in 75% of AFRS. Mucus-secreting glands proliferations together with moderate to marked degrees of basement membrane thickness were prominent in the nonfungal forms of CRS. Eosinophils

Table 2 CT scan scores of the studied groups

Sinus Involved in CT Scan	FBs <i>n</i> = 6	AFRS <i>n</i> = 12	CRSwNP <i>n</i> = 30	CRSsNP <i>n</i> = 30	<i>p</i> Values*
Left maxillary (mean ± SD)	1.0 ± 1.1	1.2 ± 0.87	1.3 ± 0.5	1.0 ± 0.4	0.029 ⁶
Left OMC (mean ± SD)	0.3 ± 0.8	1.5 ± 0.9	2.0 ± 0.0	1.06 ± 1.0	0.023 ¹ <0.001 ² 0.005 ⁴ 0.000 ⁶
Left anterior ethmoid (mean ± SD)	0.3 ± 0.8	1.3 ± 0.8	1.9 ± 0.3	1.0 ± 0.6	0.039 ¹ <0.001 ³ 0.036 ³ 0.012 ⁴ 0.000 ⁶
Left frontal (mean ± SD)	0.0	0.6 ± 0.9	0.9 ± 0.9	0.1 ± 0.3	0.016 [§] 0.039 ⁵ 0.000 ⁶
Left posterior ethmoid (mean ± SD)	0.16 ± 0.4	0.8 ± 0.9	1.5 ± 0.7	0.1 ± 0.4	0.024 0.001 ² 0.021 ⁴
Left sphenoid (mean ± SD)	0.0	0.6 ± 0.9	0.9 ± 0.8	0.1 ± 0.3	0.017 ² <0.001 ⁶
Total left side (mean ± SD)	1.8 ± 2.7	6.1 ± 4.7	8.5 ± 2.6	3.5 ± 1.7	0.001 ² 0.039 ³ <0.001 ⁶
Right maxillary (mean ± SD)	1.0 ± 1.0	1.3 ± 0.7	1.4 ± 0.5	0.9 ± 0.6	<0.001 ⁶
Right OMC (mean ± SD)	1.0 ± 1.0	1.1 ± 1.0	1.9 ± 0.2	0.7 ± 0.9	0.003 ² 0.003 ⁴ <0.001 ⁶
Right anterior ethmoid (mean ± SD)	1.0 ± 1.0	1.0 ± 0.7	1.8 ± 0.3	0.6 ± 0.6	0.016 [§] <0.001 ⁴ <0.001 ⁶
Right frontal (mean ± SD)	0.3 ± 0.8	0.6 ± 0.8	1.0 ± 0.8	0.1 ± 0.3	<0.001 ⁶ 0.027 ⁵
Right posterior ethmoid (mean ± SD)	0.0	0.9 ± 0.9	1.5 ± 0.7	0.2 ± 0.4	0.000 ¹ <0.001 ² 0.022 ⁴ 0.004 ⁵ <0.001 ⁶
Right sphenoid (mean ± SD)	0.0	0.8 ± 0.9	0.8 ± 0.8	1.0 ± 0.3	0.043 ¹ 0.002 ³ 0.002 ⁵ 0.000 ⁶
Total right side (mean ± SD)	3.3 ± 3.7	6.0 ± 4.5	8.6 ± 2.4	2.7 ± 2.2	0.003 ³ 0.004 ⁵ <0.001 ⁶
Total score (mean ± SD)	5.1 ± 2.5	12.0 ± 4.2	17.0 ± 4.8	6.2 ± 3.6	0.005 ¹ <0.001 ² <0.001 ⁵ <0.001 ⁶

*Mann-Whitney test.

¹Comparison between FB and AFRS.²Comparison between FB and CRSwNP.³Comparison between FB and CRSsNP.⁴Comparison between AFRS and CRSwNP.⁵Comparison between AFRS and CRSsNP.⁶Comparison between CRSwNP and CRSsNP.

AFRS = allergic fungal rhinosinusitis; CRSsNP = chronic rhinosinusitis without nasal polyposis; CRSwNP = chronic rhinosinusitis with nasal polyposis; FBs = fungal balls.

were detected significantly in samples from AFRS and CRSwNP, consequently, in comparison with CRSsNP and FB.

Neither CD56 nor perforin could be detected in any samples (Figs. 3–6). In both types of noninvasive fungal rhinosinusitis, CD3⁺ T cells were the most common cell type. In AFRS, most T cells were (CD8⁺; *p* < 0.001;

Table 4; Fig. 3). In FB cases, CD4⁺ T cells were the dominant T cells subtype (*p* < 0.001; Table 4; Fig. 4). By contrast, CD20⁺ B cells were most common in both types of CRS (CRSwNP and CRSsNP; *p* < 0.001; Figs. 5 and 6; Table 4). In CRSwNP there were no differences in CD20⁺ B cells in patients with (75.82 ± 1.6) or without allergy (75.47 ± 2.7; *p* = 0.05).

Table 3 Histopathological parameter differences between the studied groups

Parameters	FB <i>n</i> = 6 (%)	AFRS <i>n</i> = 12 (%)	CRSwNP <i>n</i> = 30 (%)	CRSsNP <i>n</i> = 30 (%)	χ^2	<i>p</i> Values
Epithelial ulceration					13.66	0.003
Complete	0 (0)	0	6 (20)	14 (46.7)		
Partial	6 (100)	12 (100)	24 (80)	16 (53.3)		
Squamous metaplasia					12.58	0.006
Complete	4 (66.7)	0 (0)	4 (13.3)	8 (26.7)		
Partial	2 (33.3)	12 (100)	26 (86.7)	22 (73.3)		
Basement membrane thickness					27.97	<0.001
Mild	2 (33.3)	4 (33.3)	0 (0)	0 (0)		
Moderate	0 (0)	4 (33.3)	3 (10)	7 (23.3)		
Marked	4 (66.7)	4 (33.3)	27 (90)	23 (76.7)		
Edema					31.49	<0.001
Mild	0 (0)	5 (41.7)	0 (0)	15 (50)		
Moderate	3 (50)	2 (16.7)	10 (33.3)	12 (40)		
Marked	3 (50)	5 (41.7)	20 (66.7)	3 (10)		
Mucous-secreting glands					29.74	<0.001
Normal	4 (66.7)	7 (58.3)	6 (20)	3 (10)		
Proliferated	2 (33.3)	3 (25.0)	24 (80)	27 (90)		
Atrophy	0 (0)	2 (16.7)	0 (0)	0 (0)		
Fibrosis					37.41	<0.001
Mild	3 (50)	3 (25)	20 (66.7)	6 (20)		
Moderate	3 (50)	5 (41.7)	10 (33.3)	24 (80)		
Marked	0 (0)	4 (33.3)	0 (0)	0 (0)		
Eosinophils cells	20.75 ± 11.64	26.67 ± 5.05	23.07 ± 4.34	11.07 ± 3.83	39.28*	<0.001
Mean ± SD						0.001**1 0.001**3 <0.001**4 <0.001**5 <0.001**6

*Kruskal-Wallis test.

**Mann-Whitney test.

¹Comparison between AFRS and FB groups.²Comparison between AFRS and CRSwNP groups.³Comparison between AFRS and CRSsNP groups.⁴Comparison between FB and CRSwNP groups.⁵Comparison between FB and CRSsNP groups.⁶Comparison between CRSwNP and CRSsNP groups.

AFRS = allergic fungal rhinosinusitis; CRSsNP = chronic rhinosinusitis without nasal polyposis; CRSwNP = chronic rhinosinusitis with nasal polyposis; FBs = fungal balls.

DISCUSSION

The clinical and radiological methods used, to date, for the diagnosis of chronic noninvasive fungal-related diseases are not effective in the diagnosis of different forms of disease. Diagnosis of noninvasive fungal diseases of the nose and paranasal sinuses is typically performed using endoscopic detection of nasal polyposis that also present in cases of CRSwNP. Allergic mucin has areas of high protein content and low water concentration that give rise to characteristic "hyperdense" appearance on sinus CT.¹³ Although this is considered as a pathognomonic feature of AFRS, heterogeneity was not only observed <70% of AFRS but also in >30% of CRSwNP patients. Also, in FB, calcification and erosion of the inner wall of the sinus, or both, were found to be the most reliable indicators of the disease.^{14,15} However, significant radiological changes were not always apparent in all of the patients. They may even exist in other forms of CRS without fungal etiology (as documented in the this study), highlighting the difficulties in categorizing different forms of the disease. Ishitoya *et al.* identified increases in the posterior ethmoid sinus scores that were more commonly associated with eosinophilic CRSwNP than with noneosinophilic CRS.¹⁶ In the present study such changes existed in CRSwNP and AFRS.

CRS is a clinical syndrome characterized by chronic inflammation.¹ Such inflammation is multifactorial and results from dysfunction of host-environment interaction. Histological findings can be considered the hallmark for diagnosis of CRS disease process. In clinical settings it became difficult in definite subtyping of CRS categories because pathologists depend on subjective quantification of tissue organization. Both AFRS and CRSwNP^{17,18} are associated with frequent epithelial damage and prominent edematous stromal tissue and coupled with eosinophilic infiltration. In the present study, similar findings were observed. The observed degree of edema and mucosal gland proliferation in AFRS was variable but less than CRSwNP. In addition, the degree of fibrotic elements was more than nasal polyps cases without fungal etiology.

Therefore, focusing on the host interaction, *i.e.*, immunologic response in the tissues of CRS patients, can allow a tool for differentiating between its different subcategories. In this study, we examined lymphocyte infiltrates present in the sinus mucosa to determine if they were altered in AFRS and identified differences between lymphocytes population between CRSwNP, CRSsNP, and other types of noninvasive fungal diseases (*e.g.*, FB). The authors preferred to use a single cell surface IHC technique because it has many more advantages

Figure 2. Histopathology examination in different chronic rhinosinusitis (CRS) disease forms. (A) CRS with nasal polypoid (CRSwNP) presenting with polypoid tissue architecture with marked thickening of basement membrane (arrow) and prominent edema. (B) CRS without nasal polypoid (CRSSNP) presenting with respiratory epithelial covering and partial squamous metaplasia and prominent stromal fibrosis. (C) Allergic fungal rhinosinusitis (AFRS) case presenting with mild edema and mild fibrosis with prominent eosinophilic infiltrates (circles). (D) Fungal ball (FB) case presenting with dense and compact aggregation of fungal hyphae lying in adjacent sinus mucosa without invasion (closer view inset; hematoxylin and eosin, $\times 100$ for panel A, $\times 200$ for panels B and D, and $\times 400$ for panel C).

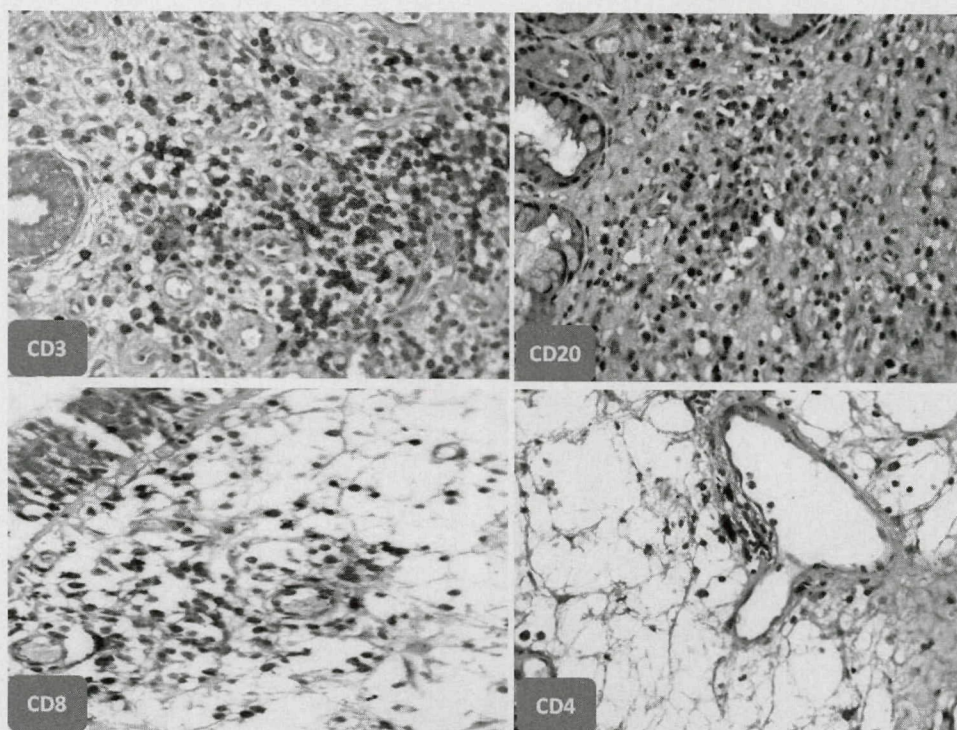
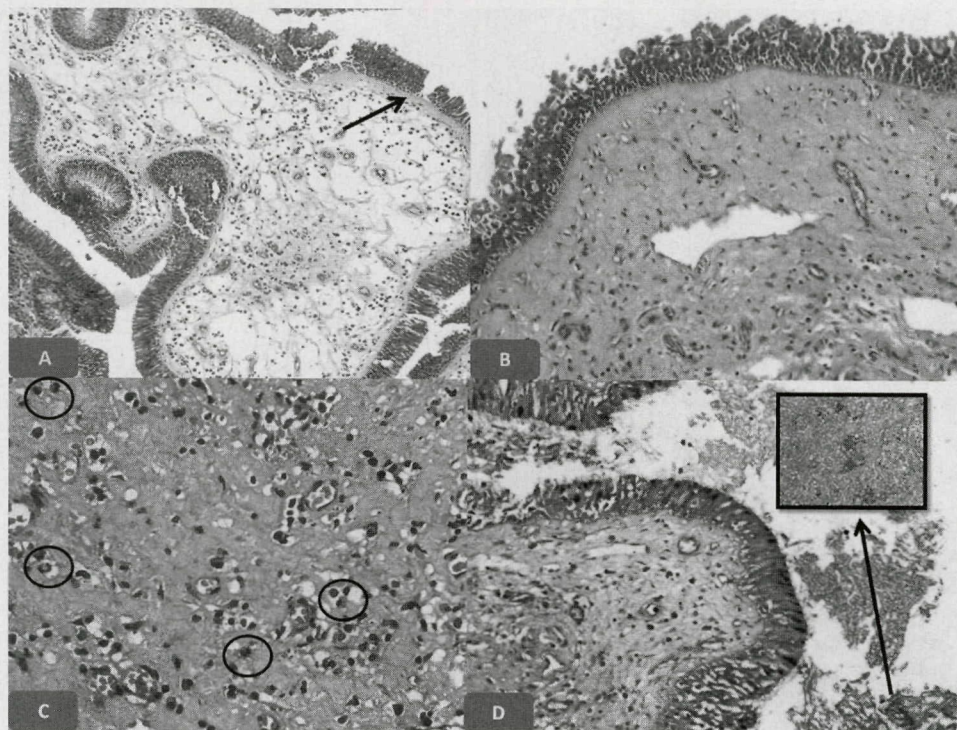


Figure 3. Allergic fungal rhinosinusitis (AFRS) immunophenotyping revealed predominant $CD3^+$ T cells in contrast to $CD20^+$ B-cell populations. Most T cells identified were $CD8^+$ (immunohistochemistry [IHC], $\times 400$ for all).

than flow cytometry. In IHC morphology an antigen label can be visualized simultaneously by light microscopy, which is not the case in flow cytometry, enabling accurate identification of the cells or region of interest. It is considered more suitable in the detection of cells and it is also more beneficial in antigen localization assessment. Another privilege of IHC is the long-term preservation of the biological specimen after processing and the ability to reexamine it at any time.

Fungal interactions with hosts are anticipated mainly in fungal-related diseases of the sinonasal passages, e.g., FBs and AFRS.⁵ In

other forms of CRS disease (CRSwNP and CRSSNP) the view of fungi as a primary antigenic stimulus for the disease is lacking, based on different lymphocyte infiltrates in the present study, immunologic reactions differences,¹⁹ and antifungal treatment response failure.²⁰ The association of fungal diseases with different immune responses can contribute to the understanding of the natural defense mechanisms mounted against mycoses. Depending on an individual's immune responses the fungal/host interaction (combined with the immune status) results in the production of different cytokine profiles

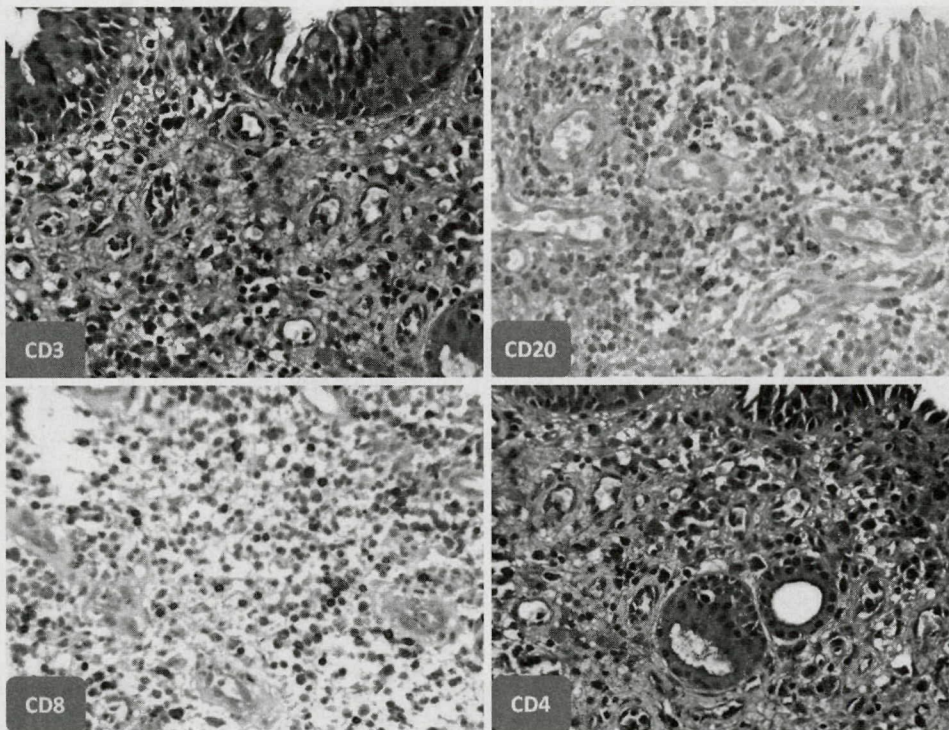


Figure 4. Fungal ball (FB) immunophenotyping showing CD3⁺ T cells as a majority of lymphocytes in comparison with CD20⁺ B cells. The majority of the identified T cells were CD4⁺ (immunohistochemistry [IHC], $\times 400$ for all).

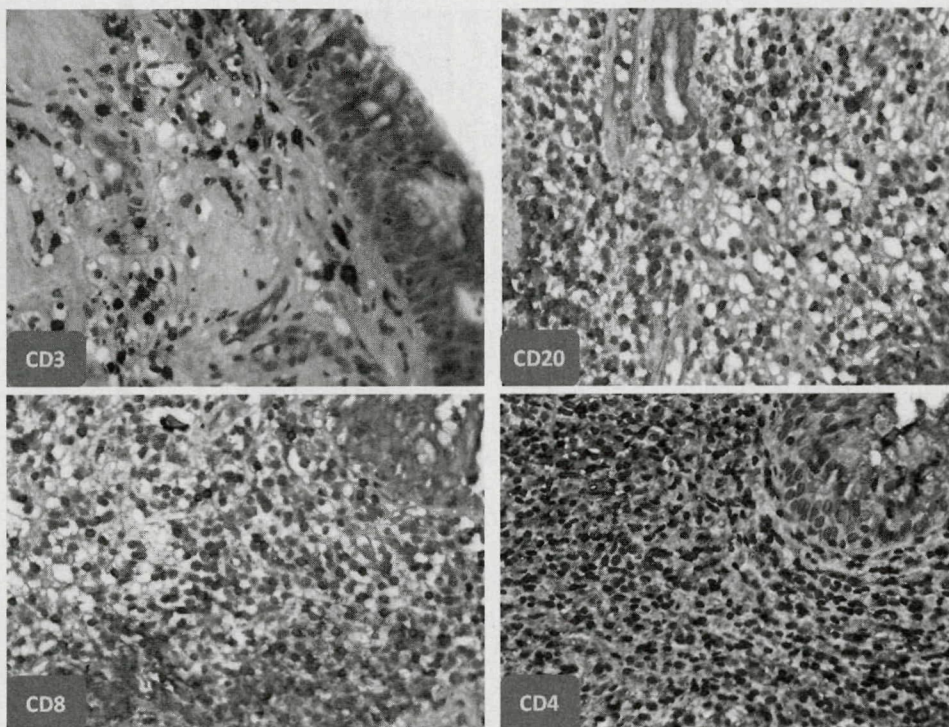


Figure 5. Chronic rhinosinusitis with nasal polyposis (CRSwNP) immunophenotyping showing CD20⁺ B-cell populations as a majority in comparison with CD3⁺ T cells. Most T cells were CD8⁺ with few CD4⁺ cells detected (immunohistochemistry [IHC], $\times 400$ for all).

that can affect fungal clearance and disease presentation.²¹ In the two noninvasive fungal-related sinonasal diseases, the levels of CD3⁺ and CD8⁺ cells were increased in the sinus mucosa of AFRS compared with the elevated number of CD3⁺ and CD4⁺ observed in FB.

In FB, CD4⁺ T cells were the dominant immune cell detected. In this disease germinating large conidia can progress into hyphal forms. A consistent theme arising from studies in a variety of fungal infections is the ability of the immune system to respond according to the germinating stage potential of fungi, *i.e.*, live germinating conidia

elicit inflammatory signals that resting conidia do not.²² Antigen-presenting cells mediated activation of CD4⁺ cells; as evidenced in the present study, results in the secretion of cytokines that attract other cells to the area. The result is an inflammatory response designed to wall off the fungal antigenic material and subsequently protecting against invasive infection.

In cases of AFRS, CD8⁺ cells dominate in the paranasal sinus mucosa, supporting previous work showing that both CD8⁺ cells and eosinophils were more abundant in AFS but not in cases of CRSwNP.²³ The CD8

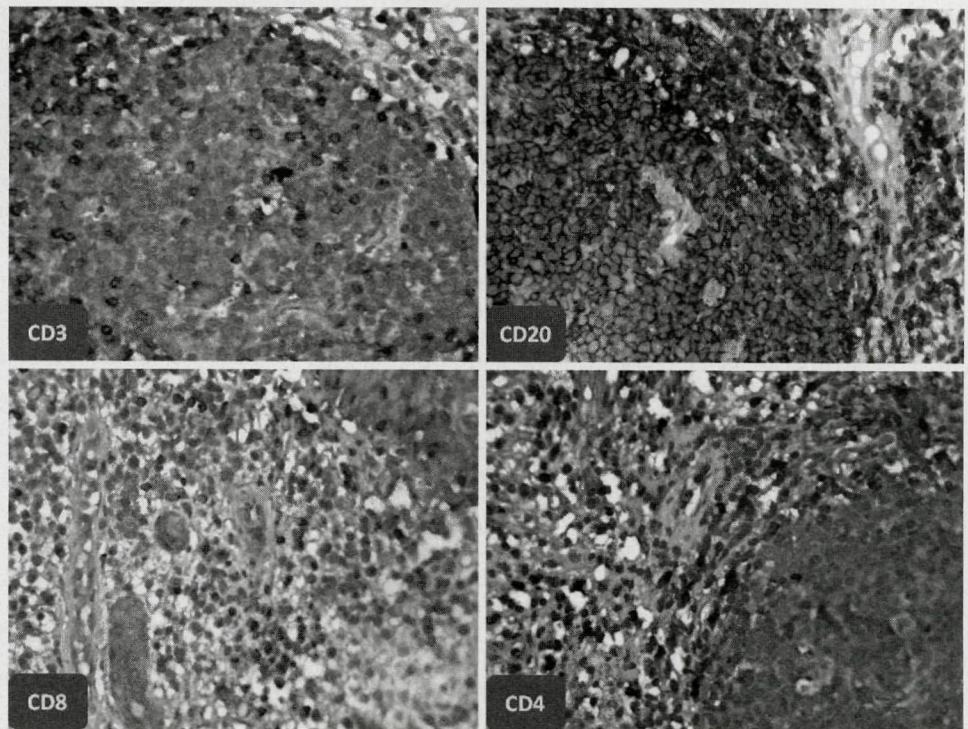


Figure 6. Chronic rhinosinusitis without nasal polypsis (CRSsNP) immunophenotyping showing preponderance of CD20⁺ B cell in comparison with CD3⁺ T cells. Most T cells were CD8⁺ with only a few CD4⁺ cells detected (immunohistochemistry [IHC], $\times 400$ for all).

Table 4 Immunophenotyping variations in the percentages of mucosal lymphocytes in chronic non-invasive fungal related sinonasal diseases and nonfungal CRS

Parameters	FB	AFRS	CRSwNP	CRSsNP	Test of Significance	p Values
CD20					0.42**	0.67 ¹
Mean $\pm$ SD	12.67 $\pm$ 4.08	12.08 $\pm$ 5.52	75.63 $\pm$ 2.34	67.93 $\pm$ 8.33	5.03**	<0.001 ²
Min-Max	5–17	6–20	72–83	60–86	5.02**	<0.001 ³
Median	13.0	10.0	75.0	63.0	3.85**	<0.001 ⁴
					3.83**	<0.001 ⁵
					4.87*	<0.001 ⁶
CD3					0.42**	0.67 ¹
Mean $\pm$ SD	87.33 $\pm$ 4.08	87.92 $\pm$ 5.52	32.07 $\pm$ 8.33	24.37 $\pm$ 2.34	5.02**	<0.001 ²
Min-Max	83–95	80–94	14–40	17–28	5.03**	<0.001 ³
Median	87.0	90.0	37.0	25.0	3.83**	<0.001 ⁴
					3.85**	<0.001 ⁵
					4.87*	<0.001 ⁶
CD4					3.38**	0.001 ¹
Mean $\pm$ SD	73.33 $\pm$ 4.08	18.83 $\pm$ 4.84	7.97 $\pm$ 2.66	5.43 $\pm$ 1.74	5.03**	<0.001 ²
Min-Max	67–78	12–31	2–12	2–8	5.06**	<0.001 ³
Median	74.5	18.5	8.0	5.0	3.88**	<0.001 ⁴
					3.88**	<0.001 ⁵
					4.37*	<0.001 ⁶
CD8					3.38**	0.001 ¹
Mean $\pm$ SD	14.00 $\pm$ 7.32	69.06 $\pm$ 6.72	24.10 $\pm$ 6.34	18.93 $\pm$ 2.41	5.03**	<0.001 ²
Min-Max	8–28	59–78	11–31	14–22	5.05**	<0.001 ³
Median	12.0	71.0	28.0	20.0	2.93**	0.003 ⁴
					2.49**	0.013 ⁵
					4.17*	<0.001 ⁶

*The t-test.

**Mann-Whitney test.

¹Comparison between AFRS and FB groups.

²Comparison between AFRS and CRSwNP groups.

³Comparison between AFRS and CRSsNP groups.

⁴Comparison between FB and CRS wNP groups.

⁵Comparison between FB and CRSsNP groups.

⁶Comparison between CRSwNP and CRSsNP groups.

AFRS = allergic fungal rhinosinusitis; CRSsNP = chronic rhinosinusitis without nasal polypsis; CRSwNP = chronic rhinosinusitis with nasal polypsis; FBs = fungal balls.

coreceptor is predominantly expressed on the surface of cytotoxic and suppressor T cells. Negativity of CD56 and perforin in this study that is specific for cytotoxic/NK, respectively, can direct attention for CD8⁺ cells to be considered as T suppressor cells. CD8⁺ cells have been shown to limit fungal replication,^{24,25} explaining the limited replication observed for fungi in cases of AFRS (unlike FB). Also, it has been shown that repeated fungal vaccinations induced a profile of transcription factors functionally linked to the development of memory CD8⁺ T cells (unlike exposure to bacterial and viral antigens) maintained in the absence of CD4⁺ T cell help or the persistent presence of fungal antigens.^{24,25} This can allow long-term inflammatory reactions observed in AFRS even after different types of treatment were administered. It is also interesting that CD20⁺ cells were not found in high numbers in AFRS—given that many still consider AFRS to be an antibody-driven, IgE-type disease. Eosinophils have been considered a hallmark of AFRS. It has been suggested that these cells are recruited into inflammatory foci where they modulate immune responses to diverse stimuli. In addition, several lines of evidence suggest that eosinophils may also exhibit antigen-presenting properties in certain states.²⁶ These fungal-activated eosinophils are then capable of inducing the proliferation of CD4⁺ and CD8⁺ cells resulting in the production of fungal-specific Th2 immune response.²⁷ The observation that eosinophils are capable of stimulating a primary response to fungi *in vitro*²⁷ suggested that this activity may also occur *in vivo*.

In the present study we observed an increase in CD20⁺ cells in cases of CRSwNP and CRSsNP. In both groups less than one-third of the patients had systemic allergy. Also, no differences were observed in CD20⁺ cells between allergic and nonallergic patients. Therefore, local B-cell class switch recombination and synthesis of IgE and IgA can be considered to mediate activation of airway mast cells and eosinophils, respectively, in response to antigen exposure. In other studies, a large proportion of patients with CRSwNP can present with elevated levels of IgE in tissues without evidence of circulating IgE against the same antigens.^{28,29} A single study had shown an increase in antigen-specific IgE in AFRS as well.³⁰ In the present study CD20 was not increased in this disease category, which may be attributable to CD20 expression of all stages of B-cell development except for the first (early pro-B cells) and the last (plasma cells). Whether specific IgE expression is tightly related to pathogenesis or is merely a by-product of B-cell interactions in local mucosa with colonizing organisms, allergens or irritants remain unresolved.³¹

CONCLUSION

Novel ways of diagnosing fungal and nonfungal inflammation need to be developed. Immunophenotyping was used in this study to categorize different types of fungal and nonfungal chronic inflammation in CRS cases. This approach may lead to better defined diagnoses, therapeutic approaches, and outcome parameters for specific types of inflammation.

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