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Articles

THE DARK SIDE OF BIOFILM

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PERSPECTIVES

Bacterial biofilms are structured multicellular microbial communities embedded in a matrix of self-produced extracellular polymeric substances (EPSs) [1,2]**.**

Anthony van Leeuwenhoek first defined biofilm as an aggregate of bacteria in dental plaque. About 100 years later, Louis Pasteur described aggregates of bacteria that he hypothesized to be the cause of the wine becoming acetic. Since then, for the next century, research has shown no interest in microbial biofilms, long unknown to medical microbiologists[3]. Only recently, the rates of antibiotic resistant Gram-negative bacteria associated with biofilm-forming activity have increased worrisomely, so that biofilms are nowadays considered a serious public-health problem worldwide, capturing the attention of research again[4]. As a matter of fact, although the observation of biofilms can be considered as old as microbiology itself, the mechanisms by which biofilms contribute to the pathogenesis and chronicization of several diseases are still unknown[5]. Moreover, the lack of therapeutic strategies that are truly effective in eradicating them imposes the identification of more effective antimicrobial treatment options[6]. This explains the growing emphasis on biofilms over the past decade, particularly regarding the complex mechanisms involved in biofilm life cycles and their strategies to resist infections[7]. Indeed, biofilms represent more tolerant forms of existence for bacteria than planktonic forms, being protected from several stressors. Their life cycle begins with planktonic microorganisms, which are free-living species that propel themselves to a surface or a tissue, and then produce the EPS matrix, which protects them from a wide variety of stressful conditions. Moreover, microorganisms produce small signaling molecules involved in quorum sensing (QS), which is an intercellular communication mechanism that ensures intercellular communication[8]. The maturation of biofilm ends with the development of a three-dimensional structure that guarantees the efficient transportation of nutrients and signaling molecules. When bacteria leave the biofilm in the planktonic form, they attach to new surfaces and start a new life cycle, and become susceptible to host defenses and antibiotics[9]. On the contrary, pathogenic biofilms are 10- to 1000-fold more drug-resistant than planktonic microorganisms and cause about 80% of all chronic infections worldwide[10,11]. Given the recognized involvement of biofilms in the pathogenesis of difficult to treat acute and chronic infections, due to the elusion of host defenses along with the inherent antibiotic-tolerance, there is a there is a critical need to accurately diagnose, prevent, and treat biofilms[12]. In this context, their early and correct detection, which can be performed by scanning electron microscopy (SEM), transmission electron microscopy (TEM), confocal laser scanning microscopy (CLSM) combined with live/dead staining or fluorescent in situ hybridization (FISH), plays a pivotal role in the diagnostic path of patients suffering from several infectious disease[13]. In the last decade, as regards the field of Rhinology, nasal cytology (NC) has been introduced as a crucial part of the rhino-allergologic

diagnostic path, since it is an easy-to-apply, reproducible and non-invasive diagnostic tool that allows to classify the different sino-nasal pathologies according to the cytotypes of the nasal inflammatory infiltrate and to assess the presence of biofilm identification with good accuracy and lower costs[13]. In particular, in NC samples, which are obtained by Nasal Scraping® from the middle part of the inferior turbinate, air-dried, stained with May-Grunwald-Giemsa (MGG) and then read with optical microscopy, biofilm appears as a cyan-stained "infectious spot" whose polysaccharide nature can be confirmed by periodic acid-Schiff staining[14].

Interestingly, just by observing some nasal cytological samples, we came across a very evocative image (***Fig. 1***) which, in our opinion, symbolizes the dark side of the biofilm. Indeed, it represents a bacterial biofilm present on the surface of the nasal mucosa which, on the cytological sample, recalls the profile of a man with an evil and frightening aspect. This man seems to have a threatening appearance, just like the biofilm, which poses a threat to human health, resisting the common therapies available today and contributing to the recalcitrance of infections. This dark side could also refer to everything that is not yet completely clear about biofilm and which, therefore, represents an important barrier to the identification of new therapeutic strategies. In this sense, just as we have to look for the good in every person, we would also like to find the good in the biofilm. In fact, biofilms are characterized by self-regeneration, sustainability, scalability, and tunability, which are favorable features that make them candidates for diverse applications, including catalysis, electric conduction, bioremediation, and medical therapy[15]. As regards the latter application, we would highlight that the human microbiome consists of different microorganisms and properly the overgrowth of "bad" bacteria, which displaces those microorganisms that constitute a healthy microbiome, leads to microbial dysbiosis, mucosal and chronic diseases[16,17]. In the future, the characteristics of some bacteria considered "good" could be exploited, including for example *Corynebacterium accolens,* which has antimicrobial activity against both planktonic and biofilm forms, paving the way for innovative probiotic therapies and also to the possible use of "good" biofilms[18].

Further studies are therefore required to understand the still unclear mechanisms of biofilm formation and eradication as well as its possible positive applications, to finally bring to light the dark side of biofilm.

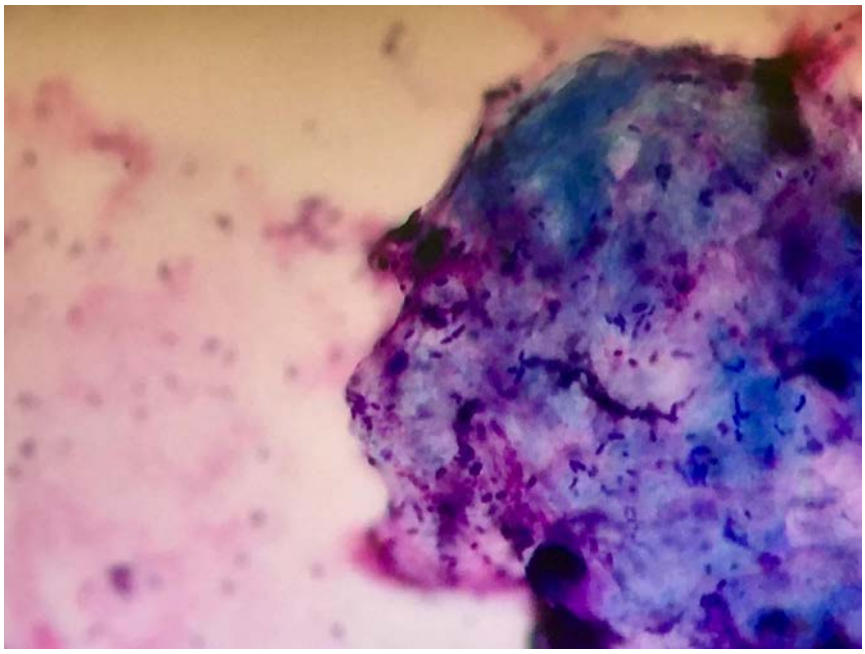

FIGURE LEGEND

Figure 1 - Nasal cytology in a patient with chronic rhinosinusitis. Numerous bacteria incorporated in an exopolysaccharide matrix (biofilm) MGG staining. Magnification 1000X

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Longitudinal studies: an obstacle course. The challenge and the paradox of communication

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Let's assume that a young researcher decides to challenge the great enemy, called "passing time", and to re-contact, after about 10 years, the young patients evaluated by his Professor for rhinological disorders, subjected to Skin-Prick Test (SPT) and nasal cytology. Let's assume that the Professor, with patience and foresight, has not only recorded the clinical data of these 1030 children, but also the landline and mobile phone numbers of their parents, in the hope that one day he could be able to contact them easily for some prestigious study. Let's assume that the young researcher realises that only 578 satisfy the inclusion criteria of the study and thus starts contacting them: this is where he unfortunately comes across the first big obstacle, represented by the telecommunications paradox. As a matter of fact, in the past, each family unit had a unique number in life, indelibly linked to a house and a family. Nowadays, the telephone numbers per household have become at least 2 or 3, and they have a very short life: indeed, only 361 of the re-contacted patients has a telephone number that still exists. The young researcher at least hopes that these patients would pay him due attention. "*I am sure that they will be willing to dedicate a few minutes of their attention! It is still a doctor who is phoning you to talk about the health of your child!*" he thinks naively. But no! Only 213 parents listen to the whole content of the phone call. The list of patients gets shorter and shorter, and the scientific discomfort grows progressively. In addition, the researcher run into another difficult obstacle: the male listener, who certainly does not remember what happened to his son ten years ago, and who suggests contacting his wife. "*She certainly remembers everything, from the period when the child was examined, to the diagnosis, the medicine prescribed, even how much the doctor was paid"*. And so, timidly, the young researcher asks if it is possible to make a follow-up visit: a simple, non-invasive and completely free rhinological examination. Unfortunately, the answers given are not satisfactory: 80

patients does not agree to a follow-up visit, because not interested (33), no longer resident in Apulia (19), or too busy (28); 71 patients fix an appointment immediately; 62 patients initially answer possibly yes, but then only 9 of them get back to the Clinic. In summary, the young researcher visited only 80 (7,8 %) of the 1030 children entered in the database. And no, Covid-19 doesn't matter. We cannot blame negative responses on the fear of going to hospital settings and getting infected. Unfortunately, we have to point out that these investigations were carried out before the start of the pandemic, which would certainly have further reduced the number of positive responses[1].

How, when, and why are patients lost to follow-up is a common question for many doctors and researchers, regardless of their branch. With the advent of technology and the possibility of entering all patient data in computerized databases, studies on the importance of long-term follow-up and on strategies aim at reducing the prevalence of "lost in-follow up" patients are constantly increasing[2,3]. Indeed, beyond the implications for patient care and outcomes, a complete patient follow-up allows to fully understand the risks and benefits of both investigational and established therapies. Therefore, the loss of data inevitably corresponds to a missed opportunity in translating the results of clinical trials into clinical practice[4]. However, according to several studies, percent adherence to follow-up appointments are alarmingly low, also in the field of Oncology[5,6]. Moreover, patients who are teenagers transitioning to adults are considered at higher risk for being lost to follow-up[7]. Hence the importance of additional studies to improve adherence to follow-up and to integrate into the care team figures such as social workers or care coordinators/educators that they can sensitize patients and their parents to the importance of staying in contact with their doctors, adhering to follow-ups.

Meanwhile, the young researcher has also become an expert sociologist, and he just has to search for the remaining 669 on social networks… finger crossed!

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Nasal cytology: a point-of-care testing for Precision Medicine in clinical practice

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Video Abstract

In the last decade, nasal cytology (NC) has become an integral part of the diagnostic-therapeutic pathway of the rhino-allergologic patients[1]. As a matter of fact, NC is a cheap, repeatable and non-invasive diagnostic tool, which allows to evaluate nasal immunophlogosis and to monitor the effectiveness of therapeutic strategies over time[2]. The procedure consists in sample processing, staining, and microscope reading. In particular, samples are obtained from the middle third portion of the inferior turbinate, under anterior rhinoscopy, and immediately smeared on a glass side. After air-drying, samples can be stained with May-Grunwald-Giemsa (MGG) or with MGG Quick Stain®, which is a quick stain kit that has been on the market for a few years[3]. The dyes present in the solutions of the latter kit are the same ones used in the formulation of traditional MGG staining. Nevertheless, the rapidity of the staining process, which lasts 20 seconds, is due to the different degree of dissociation of the active chemical species which make their absorption rapid on the cellular structures. Stained samples are then read ad optical microscopy, with a 1000x objective with oil immersion. A minimum of fifty fields is considered necessary to identify a sufficient number of cells[4]. The proposed video aims to illustrate step-by-step the phases of the diagnostic procedure, paying particular attention to the description of the rapid staining method, and to show the photomicrographic images of the most common conditions detectable by nasal cytology.

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“Juvenile Angiofibroma: Report of an intracranial, PPF-ITF, cavernous sinus extension”

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A 19-year-old male, complaining of a 4-year-long history of respiratory nasal obstruction, frontal headache and nosebleeds was brought to our attention. A CT scan and an MRI with c.e. were performed, showing an expansive formation with contextual vascular elements, entirely occupying the left nasal fossa, paranasal sinuses and extending into the left pterygopalatine and infratemporal fossa. The lesion also caused diffuse bone erosion with intracranial extradural invasion of the anterior frontal region and the left cavernous sinus. Due to the clinical and radiological suspect of Juvenile Angiofibroma (Andrews stage IIIb; UPMC stage IV), no biopsy was performed, and surgical indication was given. The patient, therefore, underwent a diagnostic angiography to identify supplying branches from the external carotid artery, which were contextually embolized, and branches from the internal carotid artery which were preserved, resulting in a partial devascularization of the lesion. 24 hours later, an endoscopic excision was performed and radicality was completely achieved, as shown in the postoperative MRI 72 hours later.

Keywords: JA, JNA, juvenile angiofibroma, endoscopic surgery, endonasal, intracranial, infratemporal fossa, ITF, pterygopalatine fossa, PPF, cavernous sinus, endoscopic resection, vascular malformation.

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Dupilumab treatment in Type 2 CRSwNP: nasal cytology evaluation before and after six months' follow-up and correlation with clinical response to biological therapy.

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Keywords: nasal cytology, chronic rhinosinusitis with nasal polyps, dupilumab, biological therapy, eosinophils

Abstract

Objective. The European Position Paper on Rhinosinusitis and Nasal Polyps 2020 (EPOS2020), the EUFOREA consensus on biologics for CRSwNP with or without asthma (2021) and the ARIA-ITALY multisciplinary consensus on nasal polyposis and biological treatments report guidelines for the use of biological treatment in chronic rhinosinusitis with nasal polyps (CRSwNP), all referring to the need of evidence of Type-2 inflammation to select patients for these therapies. During follow-ups of biological therapies, the same guidelines do not actually consider peripheral blood eosinophilic count nor local count of eosinophils in nasal mucosa as a marker of response to treatment. Nasal cytology is a useful methodology to evaluate inflammation at the level of nasal mucosa and it can assess the presence of eosinophilic flogosis in nasal mucosa. We performed the evaluation of nasal cytological aspects in patients with Type-2 CRSwNP eligible to biological treatment with Dupilumab to monitor during the first 6 months of therapy.

Materials and methods. We selected 20 patients who were candidate to start Dupilumab treatment for CRSwNP (according to EPOS2020 guidelines and Italian Agency of Medicines – AIFA – prescribing information). We performed nasal cytological sampling before the beginning of biological therapy (T0) and then during therapy at 2, 4 and 6 months (T1, T2, T3) after the first administration. We also performed on each patient nasal endoscopy for Nasal Polyp Score (NPS) evaluation, 22-item Sinonasal Outcome Test (SNOT-22) questionnare, and olfactometry with Sniffin' Sticks Identification test.

Results. Data showed a decreasing eosinophilic count in nasal cytology from T0 to T3 and this was associated with the improvement of other standardized parameters for the evaluation of biological therapy's response.

Conclusion. Nasal cytology is a good tool for monitoring nasal mucosa eosinophilic inflammation and it may be used routinely during Dupilumab therapy's follow up, as the peripheral eosinophilic count cannot be used for this purpouse for its known lack of correlation with treatment response.

INTRODUCTION

Prescribing indication for biological therapy in CRSwNP are described in EPOS2020, EUFOREA2019 and, for Italy, in ARIA Italian Guidelines of 2021.(1–3)

In each paper, the first fundamental criteria for prescribing biological therapies is the presence of Type-2 inflammation in considered patients. Type-2 inflammation can be determined using one of the following criteria: tissue eosinophils ≥10/HPF (high power field) or blood eosinophils ≥ 250 cells/ µl or total IgE ≥ 100 kU/L.

Other considered criteria include the impairment of smell with remark of anosmia, the need for systemic corticosteroids or contraindication to systemic steroids, a significantly impaired quality of life with SNOT-22≥ 35 or ≥ 40 (depending on the document considered), the presence of asthma as a comorbidity.(2)

When our study started, only Dupilumab (Dupixent) had been approved for treatment of CRSwNP.

Dupilumab is a fully human monoclonal antibody that inhibits signaling due to interleukin-4 (IL-4) and interleukin-13 (IL-13), which are, together with interleukin-5 (IL-5), substantially the main mediators of type 2 inflammation.(4,5)

IL-4 and IL-13 are mediators with both common and diversified roles: IL-4 is the main factor in the polarization of T-lymphocytes CD4+ naive towards Th2 lymphocytes, it also conditions the switching of B cells towards the production of IgE and activates eosinophil chemotaxis; IL-13 also affects switching to IgE production and activates eosinophil chemotaxis but also determines the hyperplasia of the goblet cells and increases the contractility of the smooth muscle.(6) This direct mechanism of IL-13 on the production of nasal mucus causes changes in the rheological characteristics of the mucus, with alterations that determine some important pathological aspects of CRSwNP. IL-13 also alters ciliated cell differentiation, increases the proportion of secretory cells and decreases ciliary beat frequency in a time- and dose-dependent manner. (7)

In nasal mucosa and other target organs of Type-2 inflammation such as lungs, the effector cell is considered to the eosinophil, with a fundamental role of recruiting and chemotaxis driven by (IL-5).(8,9)

In the already mentioned guidelines, the monitoring of response to biological therapy with Dupilumab involves the use of nasal endoscopy through the use of the Nasal Polyps Score (NPS), a subjective questionnaire as the 22-items Sinonasal Outcome test (SNOT-22), the assessment of smell impairment using a validated test such as the Sniffin' Sticks Smell Test or the UPSIT test (University of Pennsylvania Smell Identification Test). The degree of response to biological therapy is also

assessed on the reduction of need of oral/systemic corticosteroids and on the improvement of comorbidities (e.g. asthma).

In clinical monitoring of patients who are addressed to therapy with Dupilumab, therefore, to date there are no parameters that allow the monitoring of the trend of local eosinophils' level in nasal mucosa, as it also not monitored even at a systemic level. This is due to the fact that transient increases in blood eosinophilia are generally known and peripheral eosinophils count does not meet any correlation with clinical response to biological therapy. (10–12)

To date, local and systemic eosinophilia associated with type 2 inflammation do not represent follow-up parameters, despite its finding being fundamental both from a theoretical and clinical point of view, for eligibility of patients to Dupilumab therapy.

Nasal cytology represents a useful, inexpensive and easy-to-apply diagnostic method to better detail the phenotypic characteristics of mucosal flogosis. In fact, it allows to detect and quantify the cell population within the nasal mucosa at a given time. As it is replicable at any time without any discomfort or adverse effect for patients, it can be performed at different times before and during medical treatments to assess changings in nasal mucosa flogosis associated with the considered therapy.

The technique involves sampling, processing and microscope reading. Sampling requires the collection of cells from the surface of nasal mucosa that is usually done by a sterile disposable curette. Samples should be collected from the middle portion of the inferior turbinate where the ratio ciliate/mucinous cells is usually expected to be of 4:1. This totally painless procedure is performed under anterior rhinoscopy, with an appropriate light source. The sample staining is executed using the common May-Grünwald-Giemsa (MGG). The stained sample is read at optical microscopy with a 1000x objective and with oil detecting the presence of inflammatory elements (eosinophils, mast cells, neutrophils and lymphocytes) in nasal mucosa.(13)

Nasal cytology has also been already used to characterized CRSwNP and its degree of severity before the introduction of biological therapies for CRS with a significant predictive value on risk of relapses and a role in the non-surgical management of the disease.(14)

With these considerations, we conducted the present study to evaluate changings in nasal cytology comparing samples collected before Dupilumab therapy was started and then samples collected at two, four and six months after the beginning of monoclonal therapy.

MATERIALS AND METHODS

We evaluated 20 patients affected by severe CRSwNP who met the necessary criteria for indication to Dupilumab monoclonal antibody therapy and which had never received any biological therapy for CRS or asthma or atopic dermatitis. Patients were evaluated before starting the treatment (T0) with the following assessments:

- Blood sampling for elevated peripheral blood eosinophilic count or elevated total IgE values.
- Nasal endoscopy, for NPS measurement.
- Assessment of the severity of symptoms using SNOT-22;
- Olfactometry using Sniffin' Sticks (Identification test).

Furthermore, medical history was collected for comorbidity of asthma and for the use of systemic and oral corticosteroids in the last year.

We have added the nasal cytological evaluation to all these tests routinely performed in patients with CRSwNP.

Nasal cytology was performed by nasal scraping with the use of a Rhinoprobe from the body of inferior turbinate. We used the May-Grumwald-Giemsa stain. The samples were read by semi-quantitative reading using an optical microscope. (15,16)

The patients were then re-evaluated with nasal cytology and with other listed above tests at 2 months, 4 months and 6 months from the start of the therapy itself (T1, T2, T3).

RESULTS

Patients enrolled were all affected by severe Type-2 CRSwNP (Tab.1). Eight patients were male, and 12 patients were female. Mean age was of 53.8 ± 13.5. Each patients had undergone at least one surgical procedure for nasal polyposis with a mean of 1,65 procedures.

Twelve patients had asthma has comorbidity and three patients and ASA-intolerance. All except one patient had need of oral or systemic corticosteroid during the last 12 months.

SNOT-22 was found higher than 50 in all patients (mean value 64.3) and every patient suffered of anosmia or iposmia with a mean value of Sniffin’ Sticks Identification test of 7.8.

	M/F	Age (yo)	Previous surgery (n)	Asthma	AERD	Need of OCS during last year
1	M	69	1	yes	no	yes
2	F	53	1	yes	no	yes
3	M	49	3	no	no	yes
4	F	54	1	yes	no	yes
5	M	60	1	no	no	yes
6	F	43	1	no	no	yes
7	F	46	2	no	no	yes
8	F	26	1	yes	no	yes
9	M	40	1	yes	yes	yes
10	F	71	1	yes	no	yes
11	M	68	1	no	no	yes
12	M	40	3	yes	no	yes
13	F	47	3	yes	no	yes
14	F	51	1	yes	no	yes
15	M	74	1	no	no	yes
16	F	48	1	yes	no	yes
17	F	74	5	no	no	yes
18	F	70	1	yes	no	yes
19	M	40	2	yes	yes	yes
20	F	52	2	no	yes	no

Tab.1 A brief report of the main characteristics of patients included in the study.

Data from nasal cytology shows at T0 a reduction of the ratio between ciliate/mucinous cells with an increasing of the last type. At T0, nasal mucosa presented a high number of eosinophils in cytological samples (mean value: 15 cells/HPF). (Tab.2)

We compared these results with nasal cytological examination at T3, when ciliated cells appeared to be normally represented in nasal scraping samples, with a decreasing of mucinous cells. Eosinophils were significally reduced at T3 with a mean value of 0.75 cells/HPF, though they did not completely disappear from nasal mucosa. Performing a statical analysis using Mann-Whitney U test we found $p<0.0001$ comparing results from cytological evaluation at T0 and at T3.

T0				T3			
	Ciliated cells	**Goblet cells**	**Eosinophils**		**Ciliated cells**	**Goblet cells**	**Eosinophils**
	++++	+++	++		++++	+	/
	++++	+++	++++		++++	+	+
	++	+++	++++		++++	++	+
	++	+++	++		++	++	++
	++	++	+++		++++	+	/
	++	+++	++++		++++	+	/
	++	+	+++		++++	+	+
	+	+++	++++		+++	+	+
	+++	++++	++++		++++	+	+

+	+++	+	+++	++	+
++	++	++++	+++	++	/
+++	+	++	+++	+	/
++	++	++++	+++	+	/
++++	+++	+++	++++	+	+
+++	+++	+++	++++	+	+
+++	++	+++	+++	++	/
+++	+++	+++	++++	+	/
++	++	+++	+++	+	+
++	+++	+	++++	+	+
++	+++	++	+++	+	/

Tab.2 Results from nasal cytology samples read with a semiquantitive reading.

We report the trend of different parameters (SNOT-22, NPS, Identification Test) and of nasal cytology and it seems that the latter has a correlation with the formers in terms of indicators of therapy response. (Fig.1)

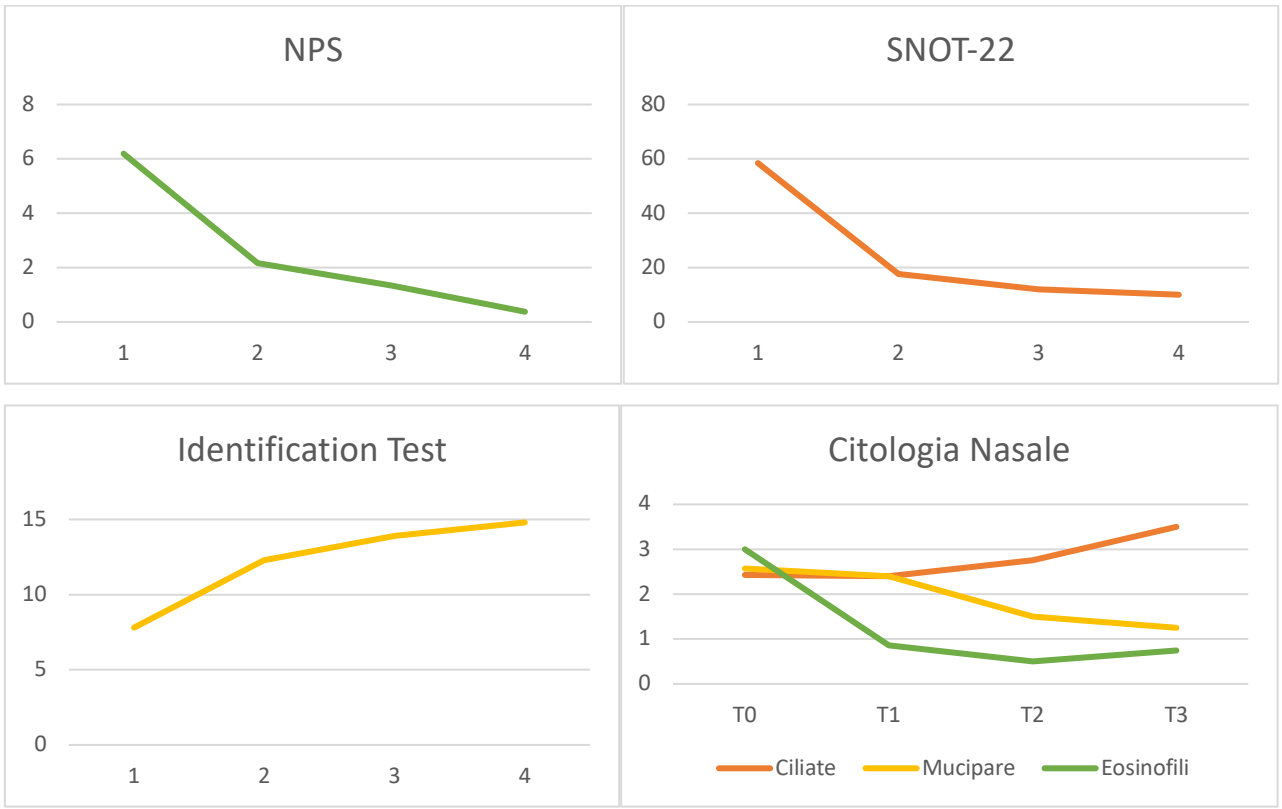

Fig. 1 Trend of standardized parameters of evaluation of response to biological therapy with Dupilumab from T0 to T3 and trend of main cytological aspects considered (ciliated cells, mucinous cells, eosinophils).

DISCUSSION

The use of biological therapy with Dupilumab in CRSwNP, which is directed against IL-4 and IL-13 receptor, has paved the way for the treatment of severe and relapsing forms of this disease, which otherwise cannot be fully controlled with endoscopic sinus surgery, even when correctly performed in order to restore ventilation of paranasal sinuses and allow an improved delivery of drugs for topical use.(2) Traditional medical therapies such as corticosteroids, administered topically, orally o with systemic administration, also resulted in a partial control of severe forms of CRSwNP.

Dupilumab is a targeted therapy that must be addressed to those patients who have the correct indication, which mean a higher probability to properly respond. Current international guidelines, such as EPOS2020 and EUFOREA, put a particular attention, in addition to clinical criteria, on the presence of type 2 inflammation, which represents a specific type of inflammatory response involving specific humoral and cellular mediators: one of the main players is the eosinophil cell. The increasing of peripheral blood eosinophilic count or the presence of eosinophils on nasal biopsy represents a fundamental prescribing information (as an alternative to the detection of elevated total IgE).

The lack of a monitoring system of this important type-2 inflammation "actor" that correlates with the trend of the other parameters used in the follow-up constituted the rationale of our study to investigate the inflammatory status of the nasal mucosa and its improvement induced by Dupilumab therapy.

At baseline, these patients appear to have marked eosinophilic cellularity in nasal mucosa, an aspect that substantially correlates with those indicated by the guidelines and in particular with the increased presence of eosinophils in nasal biopsies.

The finding obtained at the 6-months follow-up is that of a substantial correlation between the decreasing of the eosinophilic count in nasal cytology samples and the improvement of traditional parameters used for clinical evaluation such as NPS, SNOT-22, olfactometry.

Nasal cytology therefore represents a method that is easy to perform and repeatable which could allow the documentation of the improvement over time of the nasal eosinophilic inflammation during therapy with Dupilumab and which could therefore allow the monitoring of a parameter otherwise ignored in the follow-up but of extreme importance from the point of view of the control of type 2 inflammation in these patients. Moreover, nasal cytology had already been studied and classified in the so-called clinical-cytological grading to choose the correct medical therapy for each degree of severity.(14)

On the other hand, the routinary use of this diagnostic method would require a better characterization in cytological terms of type 2 patients especially before starting of biological therapy.(17)

Conclusions.

Nasal cytology is an underused tool in the follow-up of patients with severe Type-2 CRSwNP treated with biological therapies. Our data are intended to be a preliminar study and a further examination in the long term (e.g. twelve months of Dupilumab therapy) may be needed. If data will be confirmed by a larger sample, nasal cytology may be considered an indicator as responsiveness to biological therapy and a way to monitor local eosinophilic count in nasal mucosa, which is not actually being recorded in routinary clinical practice, but it may represent a valuable data.

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