Original paper

Biochemical markers of inflammatory syndrome in chronic rhinosinusitis

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Abstract
Chronic rhinosinusitis (CRS), defined as a persistent inflammation of the nasal and sinus mucosa, affects approximately 10% of the European adult population. Our study focused on the evaluation of serum chitotriosidase (Cht) and 25-hydroxyvitamin D_3 (25-OH-D3) as potential biomarkers of CRS-associated inflammation. This cross-sectional, randomized, case-control study included 16 CRS patients and 21 non-smoking controls, investigated for nasal septum deviation. No inflammatory conditions were diagnosed in both investigated groups and no anti-inflammatory treatments were administered for at least 3 weeks prior to serum sampling. Serum Cht activity was significantly increased in CRS patients, compared to the controls (Mann-Whitney U test p=0.030), while 25-OH-D3 levels were significantly decreased in patients, versus controls (Mann-Whitney U test p=0.001). No significant Cht or 25-OH-D3 variations were induced by any of the investigated covariates, such as the coexistence of comorbidities, microbial biofilms or previous corticosteroid treatments. Receiver operating characteristic (ROC) analysis indicated a good discriminating capacity between patients and controls, for both Cht and 25-OH-D3, suggesting their possible utility as non-invasive biomarkers in CRS. Patients with polyps exhibited lower 25-OH-D3 levels, while those with microbial biofilms had higher Cht and lower 25-OH-D3 values than their counterparts, suggesting that biofilms may be an aggravating factor in disease pathogenesis.

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Introduction

Chronic rhinosinusitis (CRS) is a frequent pathological condition exhibiting a strong social impact, in terms of reducing the quality of life and increasing the cost of health care. CRS affects approximately 10% of the European adult population and it is defined as a persistent inflammation of the nasal and sinus mucosa, with a length of at least 12 weeks (FOKKENS & al [1]). Based on the potential existence of nasal polyps, CRS is divided into two large subtypes: with nasal polyps (CRSwNP) and without nasal polyps (CRSsNP). The two categories are further differentiated by the type of inflammation and of tissue remodelling (VAN CROMBRUGGEN & al [2]; VAN BRUAENE & al [3]). CRSsNP is characterised by a predominantly neutrophilic inflammatory infiltrate, associated with Th1 lymphocytes (VAN CROMBRUGGEN & al [2]; VAN ZELE & al [4]). Instead, the eosinophilic infiltrate and tissue remodelling based on fibroblast proliferation, with a Th1/Th2 ratio in favour of Th2 cells, are the main features of CRSwNP (VAN CROMBRUGGEN & al [2]; VAN ZELE & al [4]).

The pathogenesis of CRS has been widely studied. However, it is still not fully understood, due to the complexity of host-environment interactions. Microbial elements, such as superantigens, fungal infections or bacterial biofilms, have been described as pathogenic factors (FOKKENS & al [1]; BACHERT & al [5]). Among these, bacterial biofilms are frequently involved, especially in the refractory CRS (FOKKENS & al [1]; HARVEY & al [6]). This three-dimensional structure consists of bacterial cell aggregates incorporated into an auto-secreted exopolysaccharide matrix, attached to either living, or inert surfaces (HARVEY & al [6]). Within the biofilm, bacterial cells have a slower metabolic rate, the exopolysaccharide matrix providing protection against the phagocytic action of macrophages, as well as resistance to antibiotics (SINGH & al [7]; DLUGASZEWSKA & al [8]; GEORGESCU & al [9]).

In the CRS-associated inflammatory process, macrophages are activated by either T cell mediators (such as interferon gamma or RANTES), or by bacterial pathogenicity factors (LEE & LANE [10]).

Chitotriosidase (Cht) is a hydrolytic enzyme secreted by activated macrophages, acting on both chitin and chitin-like substrates. Initially, high levels of circulating Cht have been reported in Gaucher disease and other lysosomal storage disorders, however, recent studies have demonstrated elevated enzymatic activities in other diseases, such as diabetes mellitus, atherosclerosis, pulmonary disorders, parasitic and helminthic infections (MALAGUARNERA [11]; ELMONEM & al [12]; MUSUMECI [13]). As a product of non-specific macrophage activation, Cht may be considered a biomarker of systemic inflammation (MUSUMECI [13]; CIOCAN & al [14]).

Besides its well-known roles on phospho-calcium metabolism, vitamin D3 (VD3), the precursor of the steroid hormone calcitriol, acts as an immunomodulator, with anti-inflammatory and antiproliferative effects. (AKBAR & ZACHAREK [15]; KAMEN & TANGPRICHA [16]). Its dietary precursor, the cholecalciferol, synthesized in the skin following UV exposure, undergoes liver hydroxylation to 25-hydroxy-vitamin D3 (25-OH-D3), which is the storage form of the vitamin and will be released into circulation for peripheral tissue uptake. Its renal conversion to active calcitriol is catalysed by 1-alpha-hydroxylase (GALLIENI & al [17]). VD3 receptors have been found in T-lymphocytes, macrophages and fibroblasts (De LUCA & CANTORNA [18]).

The investigation of CRS-associated inflammatory syndrome could lead to a better understanding of the pathogenesis of this disease and to an improvement in patient care. In this context, we sought to evaluate the inflammatory syndrome, by means of biochemical markers reflecting its extent. Thus, we assessed serum Cht activity and 25-OH-D3 levels in CRS patients and analysed their variations as a function of disease subtype and bacterial biofilm presence.

Materials and Methods

The study was approved by the ethic committee of our University (no.273/16.06.2016) and was conducted within a year, between June 2016 and June 2017, at the Clinical Hospital CF from Cluj-Napoca. All patients and controls involved have agreed to sign the informed consent.

We conducted a cross-sectional, randomized, case-control study. Sixteen patients with CRS were included in the case group. The control group comprised 21 non-smoking subjects investigated for nasal septum deviation, without other known associated pathology. CRS diagnosis was based on disease history, cranial computer tomography (CT) imaging and nasal endoscopy examination. Other inclusion criteria for the case group were the absence of the known inflammatory disease and previously inefficient CRS drug treatment. Exclusion criteria for both patient and control groups were the following: age under 18, immunocompromised patients, administration of vitamin D supplements, antibiotic or corticosteroid treatment until at least 3 weeks prior to hospital admission.

Under endoscopic control, fragments of inflamed sinus mucosa from the middle meatus and the ethmoidal sinus were sampled from each CRS patient and stained with Haematoxylin-Eosin (H-E) and Gram colorations, for confirmation of the bacterial colonisation. Biofilms were detected by Toluidine Blue identification of the bacterial polysaccharide layers, characteristic for the Gram-positive or Gram-negative micro-colonies. The presence of neutrophils, eosinophils, plasma cells and lymphocytes in the cellular infiltrate was revealed by H-E staining.

Immediately after harvest, nasal biopsy tissues were immersed for fixation in 10% neutral-buffered formalin

(NBF), for 48-72 hours. After transverse trimming, samples were dehydrated with an ethylic alcohol gradient (70%, 80%, 95% and 100%), clarified in xylene and embedded in paraffin wax. Multiple sections were cut from each paraffin block at 4 μm thickness and routinely stained with H-E. The stained histological slides were examined with an Olympus BX41 microscope. Images were obtained with an Olympus UC30 digital camera and processed by the Olympus Stream Image Analysis Software (Olympus Corporation, Tokyo, Japan) (HOCHSTIM & al [19]; SRIDHARAN & al [20]).

Serum samples obtained from freshly drawn blood were immediately stored at -20°C. Chitotriosidase activity was measured using a fluorescent artificial substrate, as previously described (HOLLAK & al [21]). One patient and two controls with undetectable chitotriosidase activity, probably because of the homozygosity for a null allele (BOOT & al [22]), were excluded from the analysis of enzymatic activity results.

Serum 25-OH-D3 levels were measured using an enzyme-linked immunosorbent assay (ELISA) according to the instructions of the manufacturer (DiaMetra S.r.l., Italy). For this assay, insufficiency was defined as a level below 30 ng/mL and deficiency as a concentration below 10 ng/mL.

Statistical analyses were performed with the IBM SPSS software version 25.0 (IBM, USA). Using the Shapiro-Wilk test we found that the quantitative variables had no normal distributions and therefore group comparisons were performed with the Mann-Whitney U test. Data were presented as median and interquartile range (IQR). P values less than 0.05 were considered significant.

Results

Patient characteristics

The studied sample comprised 16 CRS patients aged between 18 and 80 years, included in the CRSwNP (68.75%) and CRSsNP (31.25%) subtypes, respectively. 75% of them came from urban areas and 25% declared a rural origin. The control group consisted of 21 non-smoking patients with nasal septum deviation and no past or present diagnosis of an acute or chronic inflammatory condition.

Based on clinical records and interview-based information respectively, 37.50% of CRS patients had been diagnosed with bronchial asthma (BA), and 31.25% declared themselves affected by various allergies. All the patients with known allergies presented BA as well and had been treated with nasal corticosteroids. All the patients with BA belonged to the CRSwNP category.

Bacterial biofilms were detected by H-E (Figure 1) and by Toluidine Blue (Figure 2) staining in 56.25% of CRS patients, without statistically significant differences between CRSwNP and CRSsNP subgroups, respectively.
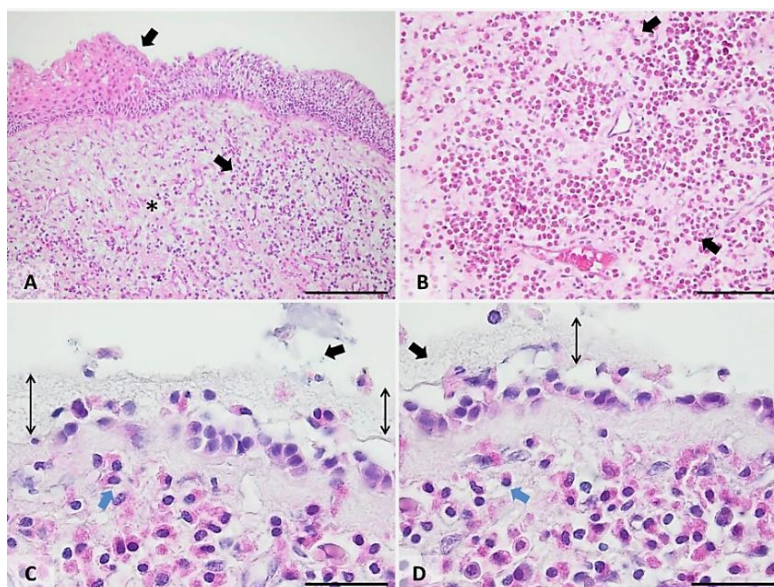

Figure 1. Histopathological aspect of nasal mucosa (Haematoxylin-Eosin staining): presence of biofilms, abundant inflammatory infiltrate with eosinophils, lymphocytes and plasma cells.
A. nasal mucosa with squamous metaplasia (upper arrow), diffuse leucocyte infiltrate (lower arrow) and oedema (asterisk); 4x, scale = 1000 μm;
B. detail of the lamina propria, characterised by massive eosinophilic infiltrate (indicated by arrows); 20x, scale = 200 μm;
C and **D.** superficial nasal mucosa with epithelial desquamation, atrophy and superficial mucoid material, characteristic for the microbial biofilms (reticulate aspect with coccoid bacteria indicated by short arrows, thickness indicated by double arrows); 100x, scale = 40 μm.
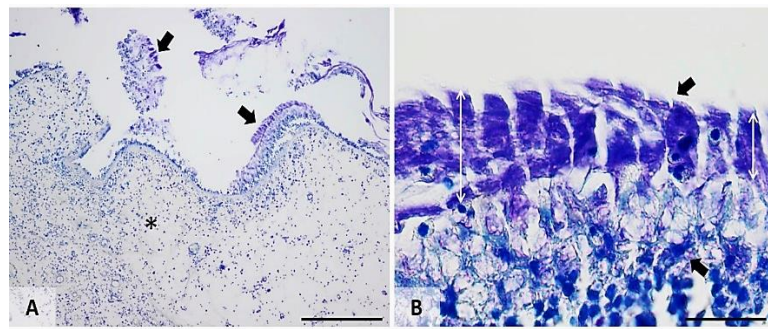
Figure 2. Histopathological aspect of nasal mucosa (Toluidine Blue staining). **A** and **B.** polysaccharide rich material intensely positive with Toluidine Blue, associated with bacterial biofilms; 4x, scale = 1000 µm (image A) and 100x, scale = 40 µm (image B).

Serum chitotriosidase activity and 25-hydroxy-vitamin D3 levels

Serum Cht enzymatic activity and 25-OH-D3 concentrations did not follow a normal distribution: using the Shapiro-Wilk test, we obtained p=0.020 and p<0.001 for Cht activity and for 25-OH-D3 levels, respectively.

For the controls, the enzymatic activities of Cht and the concentrations of 25-OH-D3 were within the reference ranges. However, in CRS patients, the distribution of Cht activity values was significantly different from that of the control group (Mann-Whitney U test p=0.030) (Table 1, Figure 3). Likewise, statistically significant differences in the distribution of 25-OH-D3 values between patients and controls have been found (Mann-Whitney U test p=0.001). These differences are highlighted in Table 1 and illustrated in Figure 3.

Table 1. Results of chitotriosidase activity and 25-hydroxy-vitamin D3 serum level assays

Group	Chitotriosidase activity (nmol/mL/h)		25-hydroxy-vitamin D3 (ng/mL)	
	Median	IQR	Median	IQR
CRS patients	230.00**	150.00-280.00	22.70**	16.70-28.30
Controls	130.00**	100.00-180.00	43.50**	32.20-64.20
Patient origin*	Median	IQR	Median	IQR
Rural	145.00	100.00-310.00	37.10	27.50-73.30
Urban	160.00	130.00-250.00	28.10	23.80-32.20
Nasal polyps*	Median	IQR	Median	IQR
Present	150.00	120.00-300.00	27.35	22.70-32.20
Absent	150.00	130.00-250.00	37.10	27.90-64.20
Bronchial asthma*	Median	IQR	Median	IQR
Present	140.00	120.00-300.00	27.50	23.80-45.60
Absent	160.00	130.00-250.00	32.20	27.90-45.70
Bacterial biofilm*	Median	IQR	Median	IQR
Present	260.00	140.00-300.00	23.45	14.40-29.30
Absent	140.00	120.00-180.00	32.20	27.90-45.60

*Only for the CRS patient group
**Statistically significant differences observed; all other comparisons did not reach statistical significance
CRS: chronic rhinosinusitis; IQR: interquartile range.

Impact of covariates on chitotriosidase and 25-hydroxy-vitamin D3 values

The analysis of serum Cht activities indicated no statistically significant differences between CRSwNP and CRSsNP subgroups (Mann-Whitney U test p=0.694). Cht activity variations displayed no statistically significant differences with respect to the patient origin (Mann-Whitney U test p=0.955) or previous corticosteroid treatment (Mann-Whitney U test p=0.074). Similarly, we checked the potential dependence of Cht values on the association between CRS and BA (Mann-Whitney U test p=0.745) and on the presence of bacterial biofilms (Mann-Whitney U test p=0.070), without revealing statistically significant differences.
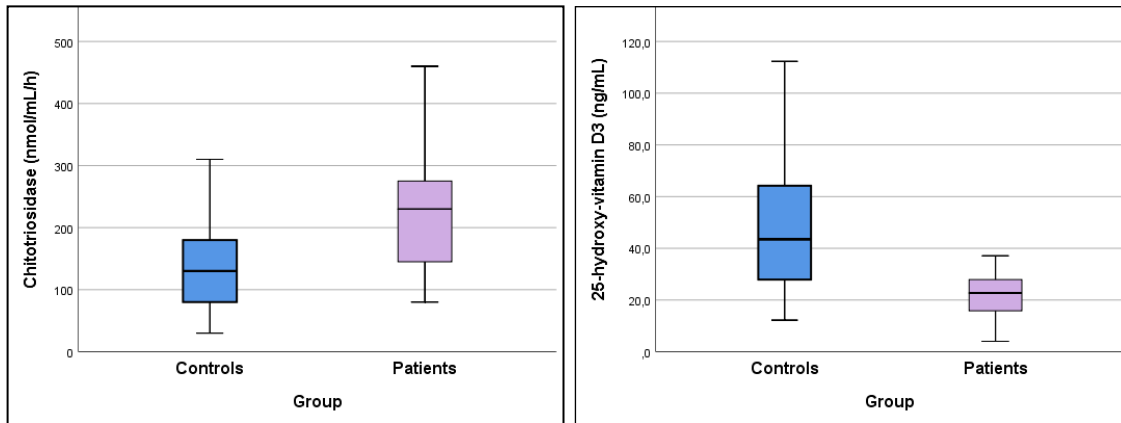
Figure 3. Significant differences observed for the distribution of serum chitotriosidase activities (nmol/mL/h) and 25-hydroxy-vitamin D3 concentrations (ng/mL) in chronic rhinosinusitis patients versus controls.

A similar behaviour was observed for the variation of serum 25-OH-D3 levels. Thus, neither polyposis, nor previous corticosteroid treatment did not induce significant differences in its values (Mann-Whitney U test p=0.236 and p=0.175, respectively. Likewise, the origin of patients had no significant impact on the variation of this parameter (Mann-Whitney U test p=0.358). Moreover, serum 25-OH-D3 values were not significantly influenced by the association between CRS and BA (Mann-Whitney U test p=0.603) or between CRS and bacterial biofilms (Mann-Whitney U test p=0.054).

Although the presence of microbial biofilms did not induce statistically significant differences in Cht activity levels, we recorded higher enzymatic activities in patients with biofilms than in other CRS patients (median and IQR were 260 (140-300) versus 140 (120-80) nmol/mL/h, respectively). We also noticed that 25-OH-D3 concentrations were lower in patients with biofilms, compared to those without this feature (median and IQR were

23.45 (14.40-29.30) versus 32.20 (27.90-45.60) ng/mL, respectively). Similarly, 25-OH-D3 values were lower in patients with polyps, again without reaching statistical significance (Table 1).

Discriminative capacity of chitotriosidase and 25-hydroxy-vitamin D3

As both investigated parameters (Cht and 25-OH-D3) were not influenced by comorbidities, microbial biofilms, previous therapies or the origin of patients, we considered their evaluation as diagnostic tests for CRS and verified their ability to effectively discriminate between patients and controls, using the receiver operating characteristic (ROC) curve (Table 2, Figure 4).

The area under the ROC curve approaches 0.80 for Cht activity and exceeds this value for the concentration of 25-OH-D3, indicating a good discrimination between patients and controls and suggesting their potential utility as biomarkers of systemic inflammation in CRS.
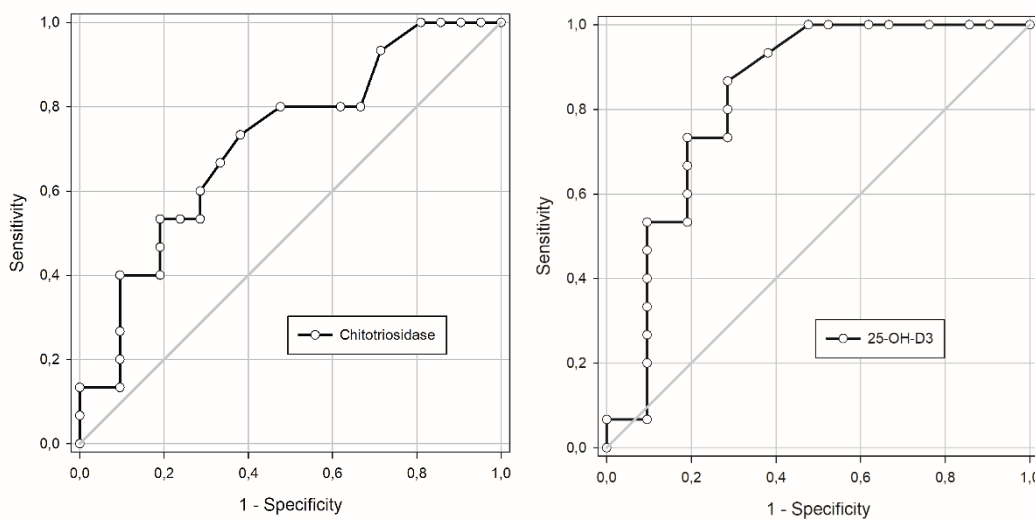

Figure 4. Receiver operating characteristic curve for the evaluation of the discriminative capacity of chitotriosidase (left) and 25-hydroxy-vitamin D3 (right) as biomarkers of chronic rhinosinusitis.

Table 2. Parameters of receiver operating characteristic curves for chitotriosidase
and 25-hydroxy-vitamin D3 as biomarkers in the diagnosis of chronic rhinosinusitis

Parameter	Chitotriosidase activity (nmol/mL/h)	25-hydroxy-vitamin D3 (ng/mL)
ROC curve area [confidence interval]	0.7143 [0.5436 – 0.8850]	0.8286 [0.6912 – 0.9660]
p value	0.0303	0.008

Discussions

Given that CRS affects patients' quality of life to a similar extent as heart failure, angina, chronic obstructive pulmonary disease and back pain (GLIKLICH & METSON [23]) and increases health care costs by the numerous medical appointments, often followed by sick leave days, it is of great interest to evaluate biomarkers that might optimise the monitoring of disease progression and, implicitly, the treatment effectiveness. As CRS is a multifactorial pathology, the inflammatory response is mediated by a large number of biological messengers, acting on different signalling pathways; therefore, one cannot delineate a single mediator, playing a key role in the disease progression and in the pathophysiology (KATO [24]).

In CRS, a persistent inflammatory syndrome was frequently found in association with microbial biofilms (HARVEY & al [6]). Although this association is well documented (HOCHSTIM & al [25]; JUNG & al [26]), the involvement of bacterial biofilms in CRS physiopathology is still being extensively investigated. In this study, we have identified the presence of biofilms in 56.25% of patients with CRS, which is consistent with literature data, showing variable biofilm results (from 28% to over 70% of the investigated patients) (DLUGASZEWSKA & al [8]; HEKIERT & al [27]; WANG & al [28]). Most studies have shown that biofilms may be an aggravating factor of disease progression, being associated with a refractory evolution to the usual therapeutic measures (LEE & LANE [10]; TROMBITAS & al [29]; SINGHAL & al [30]).

In our study, we evaluated serum Cht activity in CRS patients, compared to the control group. The enzymatic activity of Cht was significantly higher in patients than in controls, indicating the activation of tissue macrophages and, consequently, the existence of an important inflammatory syndrome. As none of the covariates considered in this study have had a significant impact on circulating chitotriosidase, we assume that this inflammatory syndrome is most likely an intrinsic element of the pathogenesis and evolution of CRS.

Therefore, we verified the ability of this enzyme to accurately discriminate between patients and controls, in order to evaluate its utility as a CRS biomarker. Based on the results of ROC curve analysis, we believe that circulating Cht may be considered as a non-invasive biomarker of CRS-induced inflammation and it may also be used for patient monitoring and follow-up. To the best of our knowledge, this is the first study that addresses the usefulness of Cht as a biomarker of this disease. Besides its discriminative capacity between CRS patients and controls, the high sensitivity of this enzyme to changes in activated macrophage numbers may indicate its possible use for the monitoring of disease-associated inflammatory syndrome (VAN DUSSEN & al [31]).

Previous attempts to evaluate the chitinolytic activity of Cht in nasal polyps, by gene expression studies and enzymatic activity assays in bioptic fragments from polyps and the inferior turbinate tissue, revealed a significant increase of the enzyme levels in CRSwNP patients, compared with controls (PARK & al [32]; PARK & al [33]). We have also noticed that biofilm-positive patients displayed higher circulating Cht values than other CRS patients, although our results did not reach statistical significance. However, we may speculate that the presence of biofilms could induce a more pronounced inflammation, responsible for the higher Cht values in these patients. This hypothesis could be confirmed by further studies performed on larger numbers of patients, sequentially followed over longer lengths of time.

Another study has shown that the significantly elevated Cht activity in nasal polyps was associated with allergic rhinitis and smoking (HEO & al [34]). In our study, no significant differences have been found between Cht activities in patients with bronchial asthma, compared to other CRS patients, since all our allergic subjects also had bronchial asthma and nasal polyps.

A second line of investigation of the inflammatory response in CRS patients was the evaluation of the status of VD3, reflected by the serum levels of 25-OH-D3. As expected, serum 25-OH-D3 levels were significantly lower in CRS patients than in the control group, this result being consistent with literature data (ABUZEID & al [35]; STOKES & RIMMER [36]). Indeed, previous studies investigated the effect of 25-OH-D3 variation on systemic inflammation, by the evaluation of inflammatory markers and found that lower VD3 levels were correlated with a significant increase in the number of dendritic cells CD209+ and of dendritic cell regulatory products, such as prostaglandin E2 and granulocyte-macrophage colony-stimulating factor (MULLIGAN & al [37]).

No statistically significant differences were noted between CRSwNP and CRSsNP subgroups, although mean 25-OH-D3 values were lower in patients with polyps than among those without polyps and, of course, than in the control group. This result is also in agreement with the literature data, which showed significantly lower values in patients with polyps, compared to those without polyps and to the control group, regardless of age, sex, smoking or atopic status (MULLIGAN & al [37]; MULLIGAN & al [38]; MULLIGAN & al [39]; SCHLOSSER & al [40]). In our study, the lack of a statistical significance of this result is most likely due to the limited number of patients in each investigated subgroup.

Recent studies have pointed out to the relationship between the decreased serum 25-OH-D3 levels and a more severe clinical picture of CRS (MULLIGAN & al [38]; WANG & al [41]). In CRSwNP patients, a decrease of 1-alpha-hydroxylase activity has been observed, a finding that may explain the decreased calcitriol level in the sinonasal epithelium, correlated with excessive fibroblast proliferation in nasal polyps (MULLIGAN & al [38]; CAROLL & al [42]). Since calcitriol is a potent inhibitor of pro-inflammatory cytokine synthesis (such as RANTES and interleukins 6 and 8), its reduced concentration may contribute to the more severe clinical manifestations in CRSwNP patients (CAROLL & al [42]; ROSTKOWSKA-NADOLSKA & al [43]).

Furthermore, the administration of VD3 seems to enhance the effect of corticosteroid therapy in CRS. In cell cultures, topical VD3 analogues (calcitriol and tacalcitriol) have been shown to potentiate the effects of Budesonide application on the suppression of polyp fibroblast proliferation (ROSTKOWSKA-NADOLSKA & al [43]; ROSTKOWSKA-NADOLSKA & al [44]).

Although in our study mean serum 25-OH-D3 levels displayed no statistically significant differences between CRS subgroups with and without microbial biofilms, this metabolite was still lower in patients with biofilms, than among other patients. This result is consistent with the literature data, indicating that the antibacterial effect of VD3 can be explained by its ability to stimulate the synthesis of mediators belonging to the cathelicidin family. In particular, LL-37, an antimicrobial peptide from the human cathelicidin family, has demonstrated a strong inhibitory effect on bacterial biofilm formation (SULTAN & al [45]; PARK & al [46]).

In our patients, circulating 25-OH-D3 levels were not significantly affected by any of the investigated covariates. Therefore, we checked the ability of this metabolite to correctly differentiate between patients and controls, in order to evaluate its usefulness as a biochemical marker of CRS. Based on the results of the ROC curve analysis, we may consider that circulating 25-OH-D3 could be included among the biomarkers of CRS associated inflammation. In this respect, our study is the first to evaluate the possibility of using this VD3 metabolite as a non-invasive biomarker, equally useful for the accurate differentiation between CRS patients and controls and also, in the long term, for the monitoring of disease progression.

The main drawback of this study is related to the limited number of the investigated patients, which may explain the absence of statistically significant correlations between patient subgroups and the investigated parameters. However, these data represent preliminary results that can pave the way for new therapeutic and monitoring opportunities in CRS.

In this respect, an interesting research direction could focus on the dynamic evaluation of the proposed biomarkers, both before and after surgical intervention. Furthermore, the investigation of these parameters among patient subgroups could highlight specific outcomes that may be important for individualised therapy and clinical follow-up.

Although this study is a preliminary one, based on the investigation of a small number of patients, our results indicate that the evaluation of inflammatory biomarkers, such as circulating chitotriosidase and 25-OH-D3 could highlight interesting aspects of CRS pathogenesis and progression. In particular, the chitinolytic activity of Cht deserves further investigation as a potential defence mechanism against the development of bacterial biofilms. In addition, since these biofilms are often associated with refractory CRS, vitamin D adjuvant supplementation may improve the effectiveness of the therapy. Further studies, conducted on larger patient cohorts, monitored over a longer time frame, are nonetheless necessary to confirm these hypotheses.

Conclusion

Both Cht and 25-OH-D3 could be considered as non-invasive biomarkers, whose utility in the follow-up of CRS-associated inflammation requires further investigation. Patients with polyps exhibited lower 25-OH-D3 values, compared to those without polyps, while patients with microbial biofilms had higher mean values of chito-triosidase and a lower mean value of 25-OH-D3 than their counterparts, a finding which may reveal the importance of microbial biofilms as an aggravating factor of the disease pathogenesis.

Conflict of interest

The authors declare no conflict of interest related to the present study.

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