**Rhinology**

**Multiple gene expression profiling suggests epithelial dysfunction in polypoid chronic rhinosinusitis**

*Il profilo di espressione genica multipla rivela una disfunzione epiteliale nella rinosinusite cronica polipoide*

A. PISTOCHINI¹,², F. ROSSI¹, S. GALLO¹,², C. PIRRONE¹, A. PRETI³, R. GORNATI¹,⁴, G. BERNARDINI¹,⁴, P. CASTELNUOVO¹,²

¹ Department of Biotechnology and Life Sciences, University of Insubria, Varese, Italy; ² Department of Otorhinolaryngology, University of Insubria and ASST Sette Laghi, Varese, Italy; ³ Department of Otorhinolaryngology, University of Milan and IRCCS Multimedica, Ospedale San Giuseppe, Milan, Italy; ⁴ “The Protein Factory” Research Center, Politecnico of Milano, ICRM-CNR Milan and University of Insubria, Milan, Italy

**SUMMARY**

Chronic rhinosinusitis (CRS) is a heterogeneous inflammatory disorder resulting from a complex gene-environment interaction. Although its aetiology remains elusive, numerous studies reported gene expression alterations of factors apparently implicated in all aspects of the inflammatory response. However, most investigations are limited, unconfirmed analyses of a single gene. Moreover, studies concerning multiple gene expression analyses, usually on inflammatory mediators (e.g. cytokines), show contrasting outcomes in part due to use of heterogeneous samples or methodologies with limited power. In this scenario, our goal was to simultaneously evaluate the expression of a panel of selected genes (*AQP5, MUC5AC, CAV1, LTF, COX2, PGDS, TNFα, TGFβ1, MGB1*) potentially involved in CRS inflammatory mechanisms. While most of the samples collected were excluded from the analysis because of poor quality RNA, we were able to demonstrate statistically significant downregulation of the *AQP5, CAV1, LTF, MGB1* genes in a specific subset of polypoid CRS (patients without typical comorbidities), which might suggest relevant underlying epithelial dysfunction. Further studies are needed to enrich our knowledge on the pathogenesis of CRS. Forthcoming approaches might utilise next-generation RNA sequencing and comprehensive bioinformatics analyses to better characterise the transcriptome profiles of CRS endotypes.

**KEY WORDS:** Chronic rhinosinusitis • Nasal polyps • qPCR • Epithelial damage • Inflammatory cytokines • Tissue remodelling • Immune barrier

**RIASSUNTO**

La rinosinusite cronica (CRS) è un disturbo infiammatorio eterogeneo risultante da una complessa interazione genetico-ambientale. Sebbene l’eziologia rimanga tuttora sfuggente, numerosi studi riportano alterazioni nell’espressione genica di diversi fattori implicati nell’ambito della risposta infiammatoria. Tuttavia, la gran parte di queste sono analisi isolate, non replicate, che prendono in considerazione un singolo gene alla volta. Inoltre, gli studi riguardanti analisi di espressione genica multipla, solitamente su mediatori infiammatori (es. citochine), spesso presentano risultati contrastanti, che in parte possono essere dovuti all’eterogeneità dei campioni o a metodologie analitiche di potenza limitata. In quest’ottica, il nostro obiettivo è stato di verificare simultaneamente l’espressione genica di un pannello di geni (*AQP5, MUC5AC, CAV1, LTF, COX2, PGDS, TNFα, TGFβ1, MGB1*) potenzialmente coinvolti nei meccanismi infiammatori della CRS. Nonostante la gran parte dei campioni sia stata esclusa dall’analisi a causa del deterioramento dell’RNA tissutale, siamo stati in grado di dimostrare una riduzione statisticamente significativa dell’espressione dei geni *AQP5, CAV1, LTF e MGB1*, in uno specifico sottogruppo di pazientes affetti da CRS nella variante con polipi nasali senza le tipiche comorbidità frequentemente associate (asma, allergia, intolleranza all’acido acetil-salicilico). Questi dati sembrano suggerire una disfunzione della barriera epiteliale nella CRS polipoide. Ulteriori studi saranno necessari per incrementare ulteriormente la nostra conoscenza sulla patogenesi della CRS. A tal proposito l’applicazione delle nuove e più potenti tecniche di sequenziamento, come la next-generation RNA sequencing, e la disponibilità di analisi bioinformatiche più complete potranno migliorare la caratterizzazione del transcriptoma negli endotipi della CRS.

**PAROLE CHIAVE:** Rinosinusite cronica • Poliposi nasale • qPCR • Disfunzione epiteliale • Citochine infiammatorie • Rimodellamento tissutale • Barriera immunitaria
Introduction

Chronic rhinosinusitis (CRS) is a heterogeneous disorder characterised by persistent symptomatic inflammation of the nasal and paranasal sinus mucosa. The interest of research in CRS arises from several factors. First, CRS is one of the most commonly reported diseases, estimated as the second most prevalent chronic health condition and affecting 10.9% of the European population. Second, the burden of CRS to society is considerable, with substantial negative impact on several aspects of quality of life and considerable medical costs. Lastly, the current treatment options struggle to be widely effective.

The aetiology of CRS remains unclear. It is becoming widely accepted that CRS arises from genetic, epigenetic, microbial and environmental factors; several conditions, such as asthma, damage of ciliated mechanical barrier, obstruction of nasal drainage, induction of inflammatory cytokines and impairment of the immune system, have been recognised as predisposing or associated factors to CRS. For these reasons, current research has attempted to elucidate the factors leading to persistent sinus inflammation, focusing on innate and acquired immunological mechanisms.

Numerous studies have reported alterations in the gene expression of factors apparently implicated in all aspects of the inflammatory response. However, most investigations are limited, unconfirmed analyses of a single gene. In addition, studies concerning multiple gene expression analyses, usually on inflammatory mediators (e.g. cytokines), demonstrate contrasting outcomes in part due to heterogeneous samples or methodologies with limited power.

Furthermore, even the site of tissue sampling seems to influence the results; indeed, there is evidence of a topographic gene expression in the sinonasal cavities. In particular, nasal polyps in CRS have been shown to exhibit a unique transcriptional pattern, typical of the polypoid tissue itself and not simply related to chronic mucosal inflammation or regional variations. In this scenario, our goal was to simultaneously evaluate, in healthy samples and CRS with nasal polyps (CRSwNP), the expression of a panel of genes involved in: (1) defects in the epithelial barrier and in both innate and adaptive host defence functions (Aquaporin 5-AQP5, Mucin 5AC-MUC5AC, Caveolin 1-CAV1, Lactoferrin-LTF); (2) alterations of eicosanoid pathways (Cyclooxygenase 2-COX2, Prostaglandin D Synthase-PGDS); and (3) induction of inflammatory cytokines and aberrant remodelling processes (Tumour necrosis factor alpha-TNFα, Transforming growth factor beta 1-TGFβ1, Mammaglobin 1-MGB1). Inclusion criteria for CRS patients were tight and painstaking and care was taken in collecting tissue samples in order to minimise the above-mentioned distortions.

Materials and methods

Participants

A retrospective study was carried out on a total of 85 individuals, comprising 52 cases (CRSwNP) and 33 controls (CTL). The local Institutional Review Board approved this study. All participants provided informed consent to use their samples for research purposes. Research was carried out in compliance with the Helsinki Declaration. As the case group, we recruited patients affected by CRSwNP as defined by the European guidelines, aged from 18 to 75 years. All cases presenting with immune-deficiency, autoimmune diseases, genetic disorders, history of sinonasal trauma, tumours, or loco-regional radiotherapy were excluded. Moreover, only CRSwNP patients without asthma, allergy, aspirin sensitivity, or hyper-eosinophilia (> 10%) were considered suitable for enrolment.

As the control group, we recruited subjects undergoing nasal surgery for other reasons (septoplasty, turbinoplasty, skull base reconstruction after trauma, spontaneous cerebrospinal fluid leaks, endoscopic approaches to sellar region), matched to patients for age and sex. We adjusted the control selections for geographic region and ethnicity in order to minimise the environmental differences with the cases. Exclusion criteria consisted of a positive history of asthma, allergy and acute or chronic rhinosinusitis.

Sample collection

Arbitrarily, 20 days before surgery patients suspended local steroid treatment in order to dispose of “pharmacological wash-out” mucosal samples, eliminating the potential effects of steroids on gene expression. At the time of surgery, the absence of acute inflammatory and infectious conditions was verified both in patients and controls. Biopsy specimens of CRSwNP and CTL mucosa were obtained from the anterior ethmoid (uncinate process or bulla ethmoidalis, when available) under general or local anaesthesia and immediately stored at -80°C until further analysis.

RNA extraction and reverse transcription

Total RNA was isolated from 100 mg of tissue using the TRIzol solution (Invitrogen™, Italy) according the standard protocol. The extracted RNA was quantified by
QuantiFluor fluorometer (Promega, Italy) using QuantiFluor® RNA System dye and its quality and integrity was assessed by 1% gel electrophoresis. The first strand cDNA was synthesised using the iScript™ cDNA Synthesis Kit (BioRad, Italy), according to the manufacturer’s instructions and stored at -20°C.

**Quantitative PCR**

For quantitative PCR (qPCR), six genes were chosen as housekeeping genes (glyceraldehyde-3-phosphate dehydrogenase-GAPDH, beta-2 microglobulin-β2M, hypoxanthine phosphoribosyltransferase 1-HPRT1, Actin_beta-Actinβ, TATA box binding protein-Tbp and ribosomal protein L13-RPL13) and the most stable three genes (GAPDH, β2M and Actinβ) were selected according to Palombella et al. 5. Selected target genes are reported in Table I. qPCR was performed using iTaq™ Universal SYBR®Green Supermix (BioRad, Italy). Specific primers were designed using the Beacon Designer Program (BioRad, Italy) within the sequences of the genes shown in Table II. Each reaction was performed according to Rossi et al. 6. Briefly, 7.5 µl of SYBR Green Supermix (2x), 1 µl of forward and reverse primers (6 µM), 5 ng of cDNA and water to a final volume of 15 µl were mixed and run in a CFX 96 Thermocycler (BioRad, Italy). The thermal cycle was as follows: 5 min at 95°C, 10 sec at 95°C and 30 sec at 60°C for 40 cycles. Each experiment was repeated three times.

**Statistical analysis**

Comparative cycle threshold method (ΔCt) was used for qPCR analysis and gene levels expressed as 2−ΔΔCt (ΔΔCt = CtTarget – CtHousekeeping). Data analysis were performed by Student’s t-test. Results were statistically significant with p < 0.05.

**Results**

Despite the large number of samples processed and the maximum care during the sample collection and manipulation, we encountered much difficulty in obtaining good quality RNA from biopsies; among the 85 selected individuals (52 CRSwNP and 33 CTL), only a total of 24 samples, comprising 11 CRSwNP and 13 CTL, were considered suitable for the study, due to extraction of poor-quality RNA.

As can be seen in agarose gel electrophoresis of specimens from both CRSwNP and CTL Groups (Figs. 1, 2), in some samples the RNA appeared to be partially degraded. This occurred particularly in CRSwNP specimens (Fig. 1), showing that the problem was probably due to the extraction of pathologic tissue with poor cellular content.

This problem was clearly less evident in biopsies taken from healthy mucosa from CTL Group (Fig. 2).

In the first group of selected genes, those related to the epithelial barrier and immune functions, APQ5, CAV1 and LTF genes were significantly downregulated in CRSwNP samples compared to CTLs, as reported in Figure 3 (p < 0.05). In the other two groups of genes, statistically significant downregulation was observed for COX2 and MGB1 genes in CRSwNP samples compared to CTLs (p < 0.05), as reported in Figures 4 and 5, respectively.

**Discussion**

It is increasingly acknowledged that CRS is not a single pathological entity, but has a rather broad clinical presentation, histopathology and response to therapy. Several pathophysiological pathways seem to exist, ending in the common point of sinonasal mucosal inflammation. Once chronic inflammation has become apparent, the activation of other pathways inevitably masks the possibility to identify the early cause. Determining which cellular and molecular characteristics of CRS represent the underlying factors that induce inflammation, or more simply the downstream consequences, remains a challenge in the ongoing field of research 7.

In this context, in the attempt to give our contribution
Table I. Target genes selected for qPCR analysis.

| Gene title          | Gene symbol | Family                                                                 | Subcellular localisation                 | Molecular functions                               | Gene ontology biological process                                                                 |
|---------------------|-------------|------------------------------------------------------------------------|-------------------------------------------|---------------------------------------------------|---------------------------------------------------------------------------------------------------|
| Aquaporin 5         | AQP5; PPKB  | MIP/aquaporin (TC 1.A.8)                                               | Apical cell membrane; Multi-pass membrane | Water channel activity; protein binding            | Transport; water transport; excretion; carbon dioxide transport; pancreatic juice secretion       |
| Mucin 5AC           | MUC5AC; LeB; TBM | Mucins                                                             | Secreted                                  | Extracellular matrix structural constituent       | Stimulatory C-type lectin receptor signalling pathway; O-glycan processing; phosphatidylinositol-mediated signalling |
| Caveolin 1          | CAV1; MSTP085; BSLC3; LCCNS; VIP21; CGL3; PPH3 | Caveolin                                                                 | Golgi apparatus membrane; peripheral cell membrane | Receptor binding; patch binding; structural molecule activity; protein binding; cholesterol binding | Negative regulation of transcription from RNA polymerase II promoter; MAPK cascade; inactivation of MAPK activity; angiogenesis; vasculogenesis |
| Lactoferrin         | LTF; GIG12; EC 3.4.21; HEL110; HLF2 | Transferrin                                                          | Secreted; cytoplasmic granule; cytoplasm | Lipopolysaccharide binding; DNA binding; serine-type endopeptidase activity; cysteine-type endopeptidase inhibitor activity; iron ion binding | Ossification; regulation of cytokine production; retina homeostasis; innate immune response in mucosa; immune system process |
| Cyclooxygenase 2    | COX2; PHS II; PGHS-2; EC 1.14.99; GRIPGH5; PGG/HS; HOx-2; PHS-2 | Prostaglandin G/H synthase                                         | Microsome membrane; peripheral membrane; | Peroxidase activity; prostaglandin-endoperoxide synthase activity; protein binding; lipid binding; oxidoreductase activity | Prostaglandin biosynthetic process; angiogenesis; lipid metabolic process; fatty acid metabolic process; fatty acid biosynthetic process |
| Prostaglandin D Synthase | PGDS; EC 5.3.99.2; PGDS2 FDS L-PGDS; LPGDS; PGD2 | Lipocalins                                                             | Rough endoplasmic reticulum; nucleus membrane; Golgi apparatus; cytoplasm, perinuclear region; Secreted. | Prostaglandin-D synthase activity; transporter activity; retinoid binding; fatty acid binding; protein binding | Prostaglandin metabolic and biosynthetic process; lipid metabolic process; fatty acid metabolic and biosynthetic process |
| Tumour necrosis factor alpha | TNFα; TNFSF2; TNFA; APC1; TNLG11F; DIF | Tumour necrosis factor                                               | Cell membrane                             | Protease binding; cytokine activity; tumour necrosis factor receptor binding; protein binding | Protein import into nucleus, translocation; negative regulation of transcription from RNA polymerase II promoter; MAPK cascade; activation of MAPK activity |
| Transforming growth factor beta 1 | TGFβ1; DPD1; LAP; OED | Endogenous ligands                                                   | Secreted; extracellular space; extracellular matrix | Glycoprotein binding; antigen binding; type II transforming growth factor beta receptor binding; cytokine activity; transforming growth factor beta receptor binding | Protein import into nucleus, translocation; negative regulation of transcription from RNA polymerase II promoter; MAPK cascade; vasculogenesis; ureteric bud development |
| Mammaglobin 1       | MGB1; SCGB2A; UGB2 | Secretoglobin                                                         | Secreted; Extracellular matrix            | Protein binding                                   | Biological process                                                                 |

...to better clarify and understand the molecular patterns involved in CRS, we selected healthy and CRSwNP individuals and investigated the expression of several key genes involved in crucial points of CRS pathogenesis. Despite the attention during tissue collection by the surgeon, the expertise of the laboratory technician in manipulating tissue and the care taken in all the steps to preserve the quality and the integrity of biological samples,
Multiple gene expression profiling suggests epithelial dysfunction in polypoid chronic rhinosinusitis

the present work showed some limitations. First, only 24 samples of the 85 collected were deemed suitable for the study due to poor-quality RNA. It is well known that RNA is very susceptible to degradation during sampling, handling and storage. Moreover, previous studies demonstrated a higher concentration of RNases in nasal polyps compared to normal tissue as well as an increased enzyme activity. Second, the selection of patients was flawed by inclusion criteria established exclusively on a clinical basis (patients with polypoid CRS, either without asthma, allergic sensitization, aspirin intolerance or peripheral blood hypereosinophilia) when it is now clear that a clinical phenotypic differentiation does not adequately mirror the underlying immunological profile. As a consequence, our results, though significant, should be interpreted in the light of this deliberate bias.

Disrupted epithelium
The “epithelial barrier hypothesis” of CRS pathogenesis indicates that an intact barrier with tight epithelial junctions is necessary for healthy nasal mucosa. Defects in this protective barrier, including the epithelium itself and its mucous lining, mucociliary clearance, intercellular junctions, ion channels and secreted antimicrobial proteins and enzymes, may cause the passage of pathogenic microbes across the epithelium and subsequent dysregulation of the inflammatory cascade. With the aim of exploring epithelial barrier functionality, we chose to verify the expression of the AQP5, MUC5AC, CAV1, COX2, Cyclooxygenase-2, MUC5AC: Mucin 5AC, PGDS: prostaglandin D, TNFα: tumour necrosis factor alpha, TGFβ1: transforming growth factor beta-1, LTF: Lactoferrin. Fw: forward, Rev: reverse.
CAV1 is a scaffolding membrane protein implicated in vesicular transport, endocytosis and regulation of signal transduction. Moreover, this gene plays a central role in defence against infections. Downregulation of CAV1 has been reported to cause dysregulation of membrane trafficking in the airway epithelium (Fig. 3b).

LTF possess diverse functions, including antibacterial, antifungal and antiviral activities, as well as immune regulatory and anti-inflammatory actions. There is evidence that LTF is significantly decreased particularly in polypoid CRS patients with bacterial biofilms (Fig. 3c).

Taken together our results tend to confirm previous literature reports.

Contrarily, in our analysis, MUC5AC was slightly downregulated in CRSwNP compared to CTL, albeit with no significant difference. MUC5AC is one of the predominant gel-forming mucins in human nasal mucosa and its upregulation, induced by various stimuli such as bacteria and allergens, was found in CRSwNP subjects. Although the increased production of mucus allows for better trapping and clearance, mucin overproduction may lead to airway obstruction and exacerbation of pre-exist-
Multiple gene expression profiling suggests epithelial dysfunction in polypoid chronic rhinosinusitis.

Defects in eicosanoid metabolism
Defects in eicosanoid metabolism, particularly involving leukotriene (LT) and prostaglandin (PG), seem to be associated to CRS, especially in the polyp variant. COX2 has a key role in the synthesis of biologically active prostaglandins that possess different functions. For instance, prostaglandin D2 (PGD2) prolongs the survival of eosinophils and induces the migration of Th2 lymphocytes, whereas prostaglandin E2 (PGE2) stimulates bronchodilation and demonstrates anti-inflammatory effects by reducing inflammation and infection.

Our data showed a single sample with a very high level of MUC5AC compared to the other 10 samples (Fig. 3d).

Fig. 4. Molecular expression of COX2 (a) and PGDS (b) genes in healthy subjects (CTL) and in CRS patients with nasal polyps (CRSwNP) by qPCR. CTL: 13 cases; CRSwNP: 11 cases. (*) indicates data statistically significant (p < 0.05) obtained by Student’s t-test.

Fig. 5. Molecular expression of TNFα (a), TGFβ1 (b) and MGB1 (c) genes in healthy subjects (CTL) and in CRS patients with nasal polyps (CRSwNP) by qPCR. CTL: 13 analysed cases; CRSwNP: 11 analysed cases. (*) indicates statistically significant data (p < 0.05) obtained by Student’s t-test.
the production of LTs. In CRSwNP, increased levels of PGD2 and PGD2 synthase (PGDS) were positively correlated with the eosinophil infiltrate, and imbalance in PGD2/PGE2 metabolism contributes to CRS associated with aspirin intolerance. In line with these findings, in our CRSwNP series, COX2 expression was significantly reduced (Fig. 4a). However, there are controversial reports in the literature on the behaviour of COX2 in CRS. It has been shown that COX2 mRNA displays different kinetics in nasal mucosa and nasal polyps, obliging us to consider that even the sampling site may affect the results. The reduced expression (not significant) of PGDS, apparently in contrast to what previously reported, may be justified by the fact that our population did not include cases of aspirin intolerance, a clinical feature that appears to be strongly associated with eicosanoid metabolism imbalance in favour of the PGD2 cascade (Fig. 4b).

Inflammatory cytokines and remodelling processes
Numerous cytokines have been reported to be expressed at altered levels in CRSwNP tissues. We decided to explore the inflammatory status, remodelling pattern and immune-modulating activities, respectively, through the analysis of expression of the TNFα, TGFβ1 and MGB1 genes (Fig. 5).

Increased levels of TNFα have already been demonstrated in nasal polyp tissues, together with other pro-inflammatory cytokines, and protein levels are elevated in nasal secretion of CRSwNP patients. Although not statistically significant compared to CTL, our CRSwNP samples showed overexpression of TNFα mRNA (Fig. 5a).

In CRS, chronic inflammation results in structural changes referred to as a remodelling process, which tends to balance extracellular matrix production and degradation by several regulation mediators, such as TGFβ1. Previous studies have confirmed its pivotal role in these processes, showing significantly lower expression of TGFβ1 in CRSwNP compared to controls. The downregulation signifying pathway results in oedema formation and lack of collagen production, histologic features that are typical of nasal polyps. TGFβ1 gene expression was also lower in our CRSwNP group, although a significant difference was not reached (Fig. 5b).

Lastly, our results showed a significant downregulation of the MGB1 gene in CRSwNP samples compared to CTLs (Fig. 5c). The role of MGB1 in CRS remain uncertain, but it is known that other proteins of the mammaglobin family are secretory proteins involved in modulation of inflammatory processes, and MGB1 overexpression in nasal polyps has been associated with neoplastic-like growth.

Conclusions
The intent of the present study was to enrich data reported in the literature by analysing, at the same time, the expression of several genes implicated in various inflammatory aspects of CRSwNP. We believe that confirmatory studies are unavoidable in research which strengthen the current knowledge about CRS pathogenesis and allow better definition of endotypes in order to achieve “tailored” treatment. Forthcoming approaches might take into account next-generation RNA sequencing and comprehensive bioinformatics analyses (e.g. hierarchical clustering) to characterise the transcriptome profiles of CRS subgroups. In summary, our attempt at gene expression profiling allowed us to depict a specific subset of CRSwNP (without typical comorbidities), which showed significant down-regulation of APQ5, CAV1, LTF, COX2 and MGB1 genes, data that might suggest relevant epithelial dysfunction in polypoid chronic rhinosinusitis.

Difficulty in mRNA extraction has to be taken into account for future studies, and in this regard the synergy between surgeon and laboratory is of great importance; the surgeon must have full knowledge of what happens during laboratory processing and what is needed. Additional genes could be selected from the literature for similar studies, or the same genes could be retested. In any case, the present results need further study on larger samples.

Acknowledgements
Stefania Gallo is a PhD student of the “Biotechnology, Biosciences and Surgical Technology” course at University of Insubria. Cristina Pirrone is a post-doc funded by Fondazione Cariplo (Rif. 2013-1052). Andrea Preti is a PhD student of “Experimental and Translational Medicine” at University of Insubria.

Conflict of interest statement
None declared.

References
1. Fokkens WJ, Lund VJ, Mullol J, et al. European position paper on rhinosinusitis and nasal polyps 2012. Rhinol Suppl 2012;23:1-298.
2. Tieu DD, Kern RC, Schleimer RP. Alterations in epithelial barrier function and host defense responses in chronic rhinosinusitis. J Allergy Clin Immunol 2009;124:37-42.
3. Stevens WW, Ocampo CJ, Berdnikovs S, et al. Cytokines in chronic rhinosinusitis, role in eosinophilia and aspirin-exacerbated respiratory disease. Am J Respir Crit Care Med 2015;192:682-94.
4. Platt MP, Soler ZM, Kao SY, et al. Topographic gene expression in the sinonasal cavity of patients with chronic sinusitis with polyps. Otolaryngol Head Neck Surg 2011;145:171-3.
Multiple gene expression profiling suggests epithelial dysfunction in polypoid chronic rhinosinusitis.