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***Gene expression evaluation in patients affected by
inflammatory diseases of paranasal sinuses***

***Valutazione dell'espressione genica in pazienti affetti
da patologia infiammatoria dei seni paranasali***

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1 INTRODUCTION

1.1 General concepts about chronic rhinosinusitis

Chronic rhinosinusitis is defined as a persistent symptomatic inflammation of the nasal and paranasal sinus mucosa, resulting from the interaction of multiple host and environmental factors.

It is one of the most commonly reported diseases, being estimated as the second most prevalent chronic health condition, affecting 12.5% of the United States (US) population [Hamilos DL, 2011], and with an overall prevalence of 10.9% in Europe (ranging from 6.9 to 27.1% in different countries) [Hastan D et al., 2011].

The burden of CRS to society is considerable and related to loss of productivity, office visits and medical expenses. Costs of medical and surgical care for CRS are estimated at about 8.6 billion dollars yearly in the US [Bhattacharyya N, 2011].

CRS has been shown to have a considerable negative impact on several aspects of quality of life [Birch DS et al., 2001] and has a greater impact on social functioning than chronic heart failure, angina or back pain [Gliklich RE et al., 1995; Suh JD et al., 2010].

CRS is clinically characterized by two or more symptoms, one of which should be either nasal blockage/obstruction/congestion or nasal discharge (anterior/posterior nasal drip), and facial pain/pressure or reduction/loss of smell. Duration of symptoms has to be longer than 12 weeks, without complete resolution and with the possibility of periodical exacerbations [Meltzer EO et al., 2004]. Nasal endoscopy and sinuses computed tomography are important for objective confirmation of the diagnosis, because of the false-positive and false-negative rates arising from subjective criteria alone [Bhattacharyya N et al., 2010; Tomassen P et al., 2011].

The widespread adoption of the term “rhinosinusitis” in preference to “sinusitis” indirectly supports the perspective that foreign material brought in through the

airway, or perhaps from the nasopharynx, acts on the nasal mucosa first, with secondary effects direct and indirect on the sinus mucosa [Van Crombruggen K et al., 2010]. In a very small percentage of cases, such as dental and iatrogenic sinusitis, this pathway is reversed with processes in the sinus cavity leading to secondary inflammation. CRS may also, in rare cases, develop secondary to inflammatory processes intrinsic to the mucosa in the presumed absence of exogenous stimuli (e.g. Wegener's granulomatosis, Churg-Strauss syndrome, Sarcoidosis). Lastly, CRS may occur in association with distinct host genetic factors (e.g. Cystic fibrosis) or systemic immunodeficiency [Fokkens WJ et al., 2012, European Position Paper on Nasal Polyposis 2012, EPOS 2012].

In the overwhelming majority of CRS cases, however, the aetiology and pathogenesis remain unclear. Idiopathic CRS has been typically divided into 2 distinct phenotypes based on endoscopic findings, CRS with nasal polyps (CRSwNP) and CRS without nasal polyps (CRSsNP). CRSsNP is more tightly linked to mechanical obstruction of the ostio-meatal complex, while CRSwNP is generally attributed to a different inflammatory pattern of mucosal response [Leung RM et al., 2011], though these broad phenotypes do not provide full insight into the potential underlying pathophysiologic mechanisms of CRS and significant overlap between the two forms can exist.

CRS is a complex inflammatory disease with several variants resulting mainly from dysfunctional host-environment interactions [Kern RC et al., 2008]. Different attempts to sub-classify CRS have been based mainly on clinical and histopathological features [Han JK, 2013; Stammberger H, 1999]. However, last trends support a deeper concept, that is, CRS consists of multiple biological subtypes, or "endotypes", which are defined by distinct cellular and molecular mechanisms that might be identified by corresponding biomarkers and might differ in therapeutic responses [Akdis CA et al., 2013]. The characterisation of the heterogeneity of the underlying inflammatory process should then define the

treatment plan. In other words, a specific kind of medication should be used for a specific kind of sinus inflammation.

Medical therapy remains the cornerstone of CRS management and relies on combinations of antibiotics and oral or topical corticosteroids. The most recent medical treatment evidences and recommendations are reported in the last EPOS publication [Fokkens WJ et al., 2012] (Table 1; Table 2).

Table 1 - Treatment evidence and recommendations for adults with CRSsNP

Therapy	Level	Grade	Relevance
steroid - topical	Ia	A	yes
nasal saline irrigation	Ia	A	yes
bacterial lysate (OM-85 BV)	Ib	A	unclear
oral antibiotic therapy short term < 4 weeks	II	B	during exacerbations
oral antibiotic therapy long term ≥ 12 weeks	Ib	C%	yes, especially if IgE is not elevated
steroid - oral	IV	C	unclear
mucolytics	III	C	no
proton pump inhibitors	III	D	no
decongestant oral / topical	no data on single use	D	no
allergen avoidance in allergic patients	IV	D	yes
oral antihistamine added in allergic patients	no data	D	no
herbal en probiotics	no data	D	no
immunotherapy	no data	D	no
probiotics	Ib(-)§	A(-)°	no
antimycotics - topical	Ib(-)§	A(-)°	no
antimycotics- systemic	no data	A(-)°	no
antibiotics - topical	Ib(-)§	A(-)°	no

[Adapted from Fokkens WJ et al., 2012]

Some of these studies also included patients with CRSwNP

Acute exacerbations of CRS should be treated like acute rhinosinusitis

§ Ib(-): Ib study with a negative outcome

° A(-): grade A recommendation not to use

% Level of evidence for macrolides in all patients with CRSsNP is Ib and strength of recommendation C, because two double-blind placebo controlled studies are contradictory; indication exist for better efficacy in CRSsNP patients with normal IgE (recommendation A). No RCTs exist for other antibiotics

Table 2 - Treatment evidence and recommendations for adults with CRSwNP

Therapy	Level	Grade	Relevance
topical steroids	Ia	A	yes
oral steroids	Ia	A	yes
oral antibiotic therapy short term < 4 weeks	Ib and Ib(-) [§]	C [#]	yes, small effect
oral antibiotic therapy long term ≥ 12 weeks	III	C	yes, especially if IgE is not elevated, small effect
capsaicin	II	C	no
proton pump inhibitors	II	C	no
aspirin desensitisation	II	C	unclear
furosemide	III	D	no
immunosuppressants	IV	D	no
nasal saline irrigation	Ib, no data in single use	D	yes for symptomatic relief
topical antibiotics	no data	D	no
anti IL-5	no data	D	unclear
phytotherapy	no data	D	no
decongestant topical / oral	no data in single use	D	no
mucolytics	no data	D	no
oral antihistamine in allergic patients	no data	D	no
antimycotics - topical	Ia(-) [%]	A(-) [°]	no
antimycotics - systemic	Ib(-) [§]	A(-) [°]	no
anti leukotrienes	Ib(-) [§]	A(-) [°]	no
anti-IgE	Ib(-) [§]	A(-) [°]	no

[Adapted from Fokkens WJ et al., 2012]

Some of these studies also included patients with CRSsNP

[#] Short term antibiotics show one positive and one negative study, therefore recommendation C

[§] Ib(-): Ib study with a negative outcome

[%] Ia(-): Ia level of evidence that treatment is not effective

[°] A(-): grade A recommendation not to use

While a combination of these therapies is often effective in relieving symptoms, at least temporarily, they are rarely curative. In individuals failing to respond to medical therapy, surgical management is indicated and required, in the form of functional endoscopic sinus surgery (FESS), to remove diseased tissue and clear obstructed sinus drainage passages. FESS restores sinus health with complete or moderate relief of symptoms in about 90% of patients with recurrent or medically unresponsive CRS [Senior BA et al., 1998]. However, approximately in 20% of operated patients FESS fails [Hopkins C, Slack R. et al., 2009].

Those patients who complain persisting signs and symptoms of CRS, despite technically adequate endoscopic sinus surgery and well-tailored, specific, medical treatment, are considered to have a refractory/recalcitrant or difficult-to-treat CRS [Desrosiers M, 2004]. Many reports point out a role for more radical or extended surgeries in this group of patients, with the aim of reducing the high inflammatory load [Bassiouni A et al., 2012], combined with medical therapies, yet more determined by the individual experience of the single ENT centre/physician than by standardized clinical trial [Fokkens WJ, 2010]. A preoperative assessment giving a prognostic index of relapse based on clinical and cytological features has been proposed [Gelardi M, 2009]. Novel treatment strategies are about to spread [Desrosiers MY et al., 2008] but their efficacy is still unpredictable and needs to be proven on a large scale. The characterization of precise endotypes will be of great help in tailoring these novel treatments.

1.2 Aetiology and pathogenesis of chronic rhinosinusitis

1.2.1 Predisposing factors

The current diagnosis of CRS requires cardinal nasal symptoms and one objective sign of nasal inflammation. These rudimentary diagnostic criteria result in a

heterogeneous group of “CRS” patients, making it difficult to investigate etiologies and outcomes [Orlandi RR et al., 2016].

Several conditions have been recognized as predisposing or associated factors to CRS. All these conditions predispose CRS by different mechanisms, such as alterations of the epithelial respiratory structure and remodelling, deposition of extracellular matrix (ECM) proteins, damage to ciliated mechanical barrier, induction of inflammatory cytokines, impairment of the immunity system and obstruction of the nasal drainage and accumulation of secretions in the nose. Some of the more examined associations are listed below.

1.2.1.1 Ciliary impairment

The inability of the cilia to transport the viscous mucus causes obstruction of the sinus ostia, secondary ciliary malfunction and consequently CRS with recurrent polyposis and infections, mainly sustained by *Pseudomonas aeruginosa* [Fokkens WJ et al., 2012].

1.2.1.2 Allergy

It has been postulated that swelling of the nasal mucosa in allergic rhinitis at the site of the sinus ostia may compromise ventilation and even obstruct sinus ostia, leading to mucus retention and infection [Fokkens W et al. 2007].

1.2.1.3 Asthma

Bronchial asthma is considered a comorbid condition of CRS, as strongly suggested by a recent large-scale European survey, reporting an association of CRS, in the absence of nasal allergies, with late-onset asthma [Jarvis D et al., 2012].

1.2.1.4 Aspirin sensitivity

The presence of aspirin or other non-steroidal anti-inflammatory drugs (NSAIDs) hypersensitivity in a patient with CRSwNP is associated with a particularly persistent and treatment-resistant form of the disease, coexisting usually with severe asthma and referred to as the “Samter’s triad” [Samter M et al., 1968].

Moreover, several arachidonic acid metabolism abnormalities have been linked to AERD patients, mainly in terms of cyclooxygenase (COX) inhibition [Kowalski ML et al., 2000] and increased production of leukotrienes [Pérez-Novo CA et al., 2005; Owens JM et al., 2006; Adamjee J et al., 2006].

1.2.1.5 Immunocompromised state

Primary and acquired immunodeficiencies, determining dysfunction of the immune system, may manifest with CRS [Fokkens WJ et al., 2012].

Studies investigating other dubious predisposing factors, including pregnancy and the endocrine state, local host factors (e.g. anatomic variations), environmental factors (e.g. cigarette smoke and lifestyle-related), iatrogenic factors (surgery-related), *Helicobacter pylori* and laryngopharyngeal reflux, are often contrasting and unable to verify a clear causal correlation with CRS [Fokkens WJ et al., 2012].

1.2.2 Pathogenetic hypotheses

Historically, idiopathic CRS was attributed to either the end stage of an incompletely treated case of acute rhinosinusitis (CRSsNP) or severe atopy (CRSwNP). The limitations of these assessments were clear to many but relatively few hypotheses have been proposed as alternatives.

The first attempt to address aetiology and pathogenesis in broad terms was the “*fungus hypothesis*” which attributed all CRS to an excessive host response to *Alternaria* fungi [Ponikau JU et al., 1999] (Figure 1-1). This theory proposes that patients with CRS mount an eosinophilic response to fungi. [Braun H et al., 2003; Ponikau JU et al., 2007].

Defects in the eicosanoid pathway, most closely associated with aspirin intolerance [Kowalski et al., 2007], have also been proposed as a potential cause of CRSwNP in general. Specifically, increased synthesis of pro-inflammatory leukotrienes, down-regulation of COX-2 and reduced levels of anti-inflammatory prostaglandins (PGE₂) have been proposed as a mechanism not just for aspirin-sensitive nasal polyps but also aspirin-tolerant CRSwNP [Pérez-Novo CA et al., 2005; Van Crombruggen K et al., 2011; Roca-Ferrer J et al., 2011]. While some theoretical evidence supports this line of thought in CRSwNP, enthusiasm is muted by the limited clinical efficacy of leukotriene pathway inhibitors [Fokkens WJ et al., 2012].

1.2.3 The role of microorganisms in chronic rhinosinusitis

Bacteria have an established role in the aetiology of acute rhinosinusitis and it has long been speculated that incompletely treated bacterial acute rhinosinusitis leads to the development of CRS. While bacteria may trigger acute infectious exacerbations, the role of bacteria in the initial establishment of CRS remains unclear. Studies of the bacteriology of the sinonasal cavities in patients with CRS has yielded highly variable results, although many have found evidence for greater prevalence of certain bacteria, such as *Haemophilus influenzae*, *Moraxella catarrhalis*, *Pseudomonas aeruginosa*, *Streptococcus* species and *Staphylococcus aureus*, particularly in patients with acute exacerbations of CRS [Benninger MS et al., 2003].

Different theories were pointed out to explain the role of bacteria in the persistent inflammation of the sinuses in CRS, their ability to escape the host innate and adaptive defences and their increased resistance to antibiotics:

- establishment of bacterial biofilms on the mucosal layer
- intracellular residence of bacteria

- qualitative and quantitative modifications in the sinusal microbiome

Biofilms have been suggested as a potential entity that can cause CRS [Foreman A et al., 2011]. It can be speculated that a defect in the immune barrier might facilitate formation of biofilms (q.v. section 1.4 for details). The mechanism of biofilm formation and worsening of CRS remain unclear but biofilms on the sinus mucosa have been linked to those mediating periodontal disease [Ohlrich EJ et al., 2009]. Once established, biofilm induce significant changes in the mucociliary layer, as demonstrated by the marked destruction of the epithelium with complete absence of cilia in biofilm-associated CRS [Galli J et al., 2008; You H et al., 2011]. The resulting mucociliary impairment is then likely to promote further bacterial adherence and prevent bacterial clearance by the host's immune system. Interestingly, when bacterial biofilms are observed in areas of epithelial damage, there is a consistent elevation of T-lymphocyte and macrophage numbers, indicating a local inflammatory response [Wood AJ et al., 2011].

The “*staphylococcal superantigen (SAg) hypothesis*” proposed that exotoxins foster nasal polyposis via effect on multiple cell types. *Staphylococcus aureus*, perhaps protected by biofilms or sequestered within epithelial cells, secrete SAg toxins that result in a generalized stimulation of T-cells, cytokines release and local polyclonal IgE response, all of which stimulate eosinophil recruitment and the clinical and histopathological changes associated with CRSwNP [Bachert C et al., 2001; Seiberling KA et al., 2005].

Although the fungal and super antigen hypotheses are often presented as opposing or competing viewpoints, they essentially agree on one salient feature: both imply that unnamed host factors determine disease susceptibility to common environmental elements.

The concept of a dysfunctional host-environment interaction actually forms the basis of another line of current research into CRS aetiology and pathogenesis. The host sinonasal epithelium serves as the site of interface with inhaled irritants, commensal organisms and pathogens. Mucociliary clearance (MCC), physical exclusion and the innate and acquired immune responses are used to separate host from environment. Broadly speaking, when components of these defenses fail, chronic mucosal inflammation ensues and the CRS syndrome is the symptomatic result. This line of thought gives rise to the “*immune barrier hypothesis*” of CRS, wherein host defects are the key to aetiology and pathogenesis [Kern RC et al., 2008]. The shifting emphasis away from environmental and microbial agents toward identifying host susceptibility is well established in other chronic inflammatory diseases involving epithelial surfaces such as atopic dermatitis, psoriasis, asthma and inflammatory bowel disease (IBD) [O'Regan GM et al., 2008; Groschwitz KR et al., 2009; Swindle EJ et al., 2009]. This theory proposes that defects in the co-ordinated mechanical barrier and/or the innate immune response of the sinonasal epithelium manifests as CRS. These defects theoretically lead to increased microbial colonization with a panoply of microbial agents, accentuated barrier damage and a compensatory adaptive immune response [Tieu DD et al., 2009].

1.3 Focus on the sinus mucosa

1.3.1 Mechanical and immunologic barrier

Structurally, the nasal mucosa consists of an epithelial layer of ciliated, pseudostratified, columnar cells joined by tight junctions, interspersed with goblet cells. Beneath the epithelium reside lymphocytes, plasma cells, macrophages, dendritic cells (DC), vascular arcades and glands. Ciliary motility and the structural

integrity of the epithelium serve as mechanical factors limiting antigenic stimulation.

Under optimal conditions, the upper airways and sinuses readily clear these materials and destroy or eliminate them without involvement of the adaptive immune system. When this process fails, microorganisms may flourish on the mucosa and acute or chronic inflammation can result. If the barrier function of the epithelial layer fails, otherwise innocuous materials may gain access to cells of the immune response in and below the lamina propria, further stimulating the inflammatory process [Schleimer RP et al., 2009]. Regardless the inciting antigen, allergens, fungi and bacteria often have proteolytic activity, which may diminish epithelial integrity, while viruses often have the capacity to lyse epithelial cells; all of these agents expose the underlying tissue to foreign stimulation. Despite these exposures, epithelial integrity is usually maintained and, when injury does occur, repair processes restore the mechanical barrier.

Thus, mechanical barriers, effective MCC and optimal healing limit the degree of antigenic stimulation of immune cells residing in the mucosa. Despite this impressive barrier function, animate and inanimate matter will stimulate the mucosal immune system, which must distinguish between commensal organisms and potential invading pathogens without excessive tissue damage.

1.3.1.1 Defects in the mechanical barrier

A number of genes implicated in chronic inflammatory mucosal disorders other than rhinosinusitis (asthma, psoriasis, atopic dermatitis, IBD) have been tested also on epithelial cells from CRS patients. These studies uncovered a marked decrease in the expression of mRNA for S100A7, S100A8, S100A9 and SPINK5 [Richer SL et al., 2008]. S100 family proteins are antimicrobial peptide acting as nonchemokine chemoattractants of inflammatory cells, regulated by T-cell

cytokine IL-22, and appear to have a role in epithelial growth dynamics and repair. SPINK5 is a secreted antiprotease necessary for barrier function of the skin. It protects gap junctions from the attack of proteases derived from host sources or microbes and allergens. The effect of the subsequent loss of epithelial integrity is an increase in epithelial cell death with exposure of TRL ligands to PAMPs and an accentuated inflammatory reaction [Holgate ST, 2007].

1.3.2 Regulation of immune responses of the epithelium

1.3.2.1 Innate Immune response

The innate immune system refers to inborn resistance that is present before the first exposure to a pathogen. Innate responses are initiated by membrane-bound and cytoplasmic pattern recognition receptors (PRRs) that recognize pathogen-associated molecular patterns (PAMPs) [Janeway CA Jr et al., 2002], which are conserved molecular patterns common among significant numbers of pathogens. Recognition of PAMPs by PRRs serves as a “danger” signal to the host immune system [Akira S et al., 2006]. PRRs also identify cellular damage through detection of debris from necrotic cells and the combined recognition of danger and damage signals sets in motion a response consisting of endogenous antimicrobial, antiviral and antiprotease products designed to aid pathogen clearance and preserve the epithelial barrier [Meylan E et al., 2006]. In addition to the release of innate protective agents, PRRs activation triggers the release of chemokines and cytokines mediating the activation of antigen-presenting cells (APCs) and the attraction of innate cellular defenses such as neutrophils and ultimately determines the nature of the acquired immune response [Iwasaki A et al., 2004].

The two best-characterized classes of PRRs are the toll-like receptor (TLR) family and the NOD-like receptor (NLR) family [Akira S et al., 2006; Meylan E et al., 2006]. TLRs are transmembrane receptors expressed on multiple cell types

including respiratory epithelial cells [Lane AP, Truong-Tran QA, Myers et al., 2006]. On activation of TLRs, epithelial cells may initiate production of defensive molecules specific to a particular pathogen (e.g. TLR2 plays a prominent role in responses to Gram-positive bacteria [Akira S et al., 2006]) and transmit the danger signal to alert the appropriate elements of the adaptive immune system [Matzinger P, 2002]. The NLR family includes NOD1 and 2, which are important in the recognition of bacterial cell wall products including staphylococci [Fournier B et al., 2005].

1.3.2.1.1 Defects in the innate immune barrier

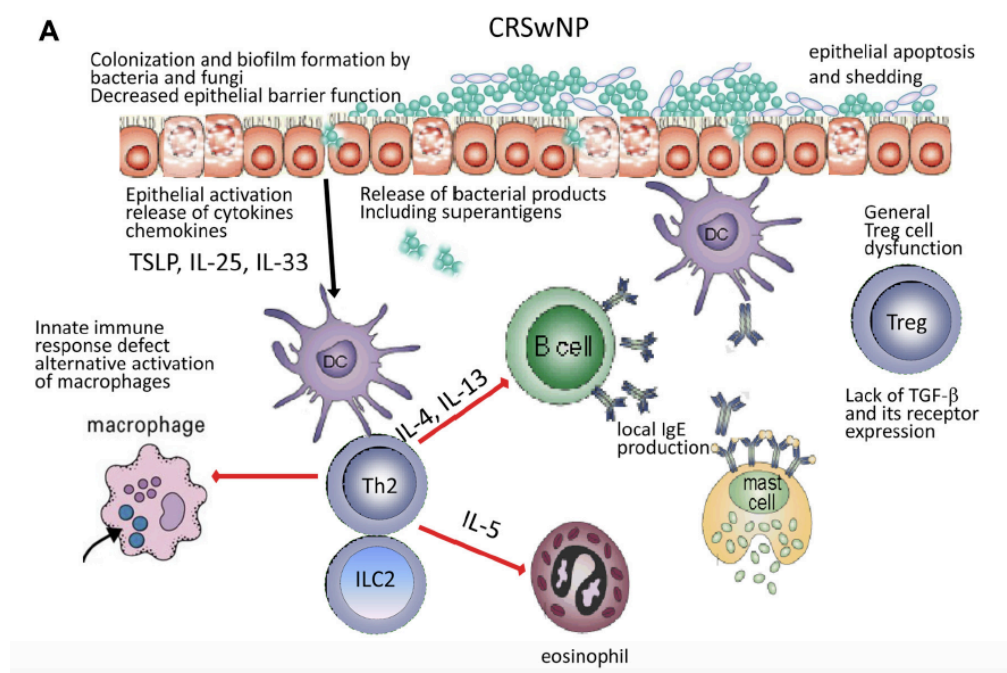
A diminished IL-6/STAT3 response may lead to minimal Th17 response, increased IgE and *Staphylococcus aureus* colonization [Peters AT et al., 2009], as if there was a local equivalent of the hyper-IgE syndrome (Job's syndrome) [Holland SM et al., 2007].

1.3.2.2 Acquired Immune response

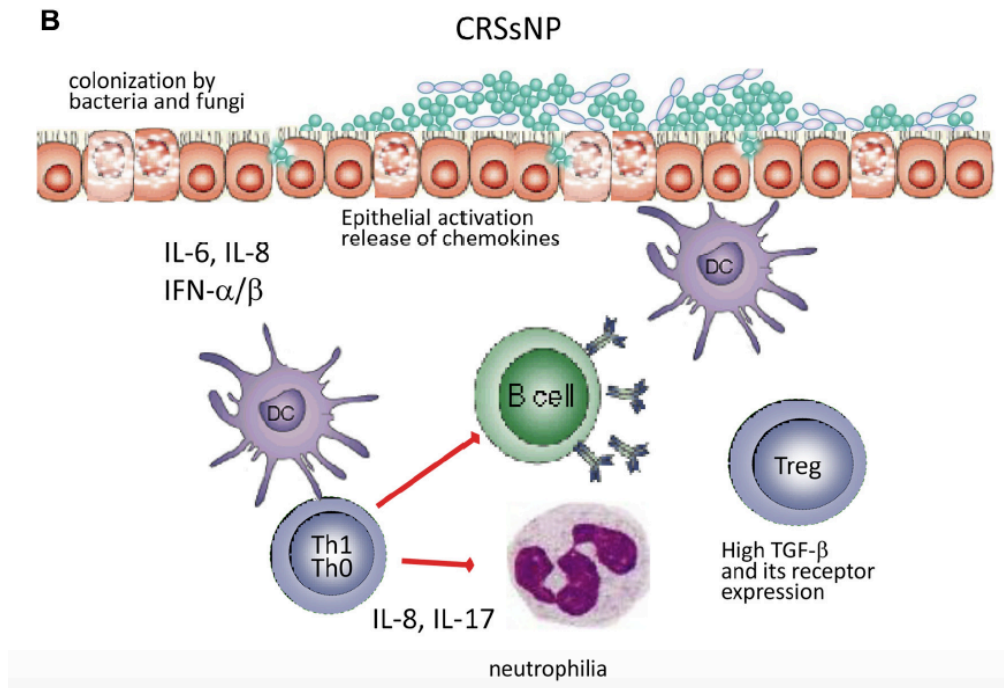
The transition to an acquired immune response is the result of a sufficiently strong PAMP stimulus. Tissue DCs are particularly important in generation of the acquired immune response, acting as APCs. After stimulation by PRRs through PAMP recognition, DCs become activated, cease phagocytic activity and acquire chemokine receptors that lead them to migrate to lymph nodes where they present antigen to Th cells. IL-6 has been proposed to be a key cytokine mediating the transition between the innate and acquired immune responses, helping to shut down many components of the innate response and promoting the acquired response [Jones SA, 2005].

The subsequent Th responses have classically been divided into Th1 and Th2 based on cytokine profiles. Th1 responses (IL-12 and IFN- γ) facilitate defense against intracellular pathogens. Th2 responses (IL-4, IL-5 and IL-13) are of primary importance in parasitic immunity and are associated with allergy and asthma. The type, duration and intensity of the PAMP stimulus shape the cytokine milieu and are believed to be critical in determining the Th profile.

Figure 1 – Pathogenetic mechanisms of CRS (Reprinted with permission 3997040216197 from [Bachert C et al., 2016])



A, CRSwNP. In a TH2-type microenvironment with general lack of regulatory T (Treg) cell function, the epithelial cell activation by microbes induces thymic stromal lymphopoietin (TSLP), IL-25, and IL-33 production. This leads to Th2 and ILC2 response. IL-5 induces eosinophilia, and IL-4 and IL-13 induce local IgE production. An alternatively activated macrophage subset contributes to the inflammation. Activated epithelial cells die, with apoptosis resulting in a compromised epithelial barrier.



B, CRSsNP. Instead of a TH2-skewed T-cell response, a TH1, TH17, or a mixed TH0 response predominates, neutrophilia is often associated, and expression of TGF- β and its receptors is increased. IL-6, IL-8, IL-17, and type 1 interferons play important roles. CRS, Chronic rhinosinusitis; CRSsNP, chronic rhinosinusitis without nasal polyps; CRSwNP, chronic rhinosinusitis with nasal polyps; DC, dendritic cell.

Additional Th subsets besides Th1 and Th2 have recently been recognized, including Th17 and Treg cells [Tato CM et al., 2006]. Th17 responses are thought to play a role in defense against extracellular bacteria and Treg cells mediate immunosuppression and immune tolerance. Several cytokines, including IL-6, TGF- β 1 and IL-23, appear to be key factors in fostering a Th17 response. TGF- β 1 also promotes Treg differentiation, except in the presence of high IL-6, in which case this response is suppressed. Th1 and Th2 responses reciprocally inhibit one another and both suppress Th17 responses [Tato CM et al., 2006]. Treg cells appear to suppress Th1, Th2 and Th17 responses, acting to limit excessive immune responses [Romagnani S, 2006]. Treg responses are inactivated in situ by

strong PRR stimulation, most prominently TLR2 [Liu H et al., 2006]. These permit active protective responses to be mediated at the sites of a strong PAMP stimulation while suppressing excessive or inappropriate immune responses.

In summary, the mechanical and innate immune barriers across the nasal mucosa serve to appropriately repel the constant load of exogenous stimulation and limit activation of the acquired immune response. Genetic and/or acquired defects in this complex process may at least theoretically lead to the development of chronic inflammation seen in CRS [Ramanathan M Jr et al., 2007; Schleimer RP et al., 2007].

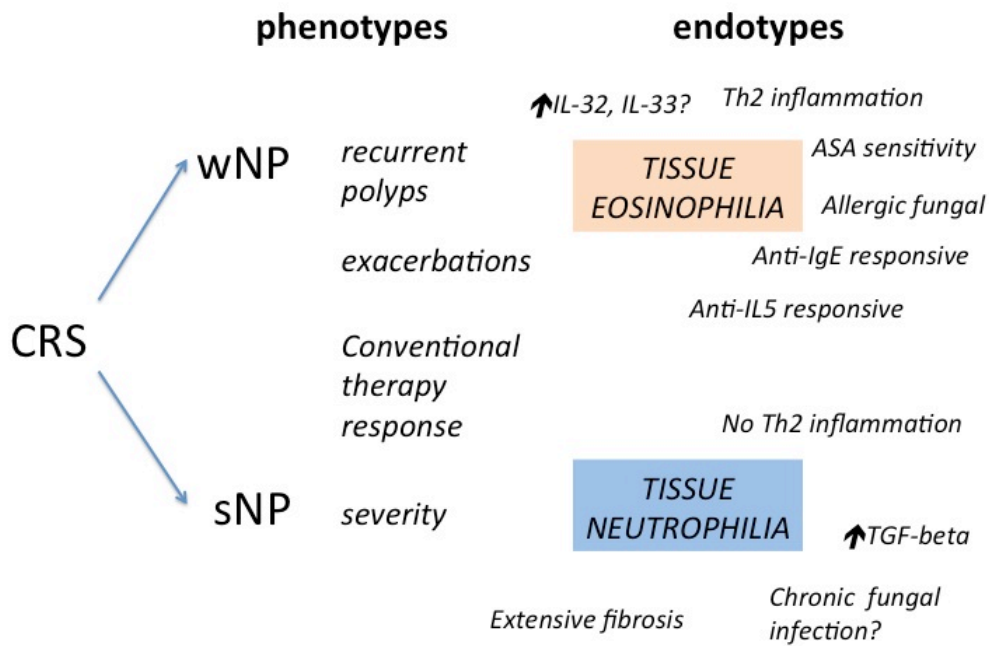
1.3.2.2.1 Defects in the transition from innate to acquired immune response

An aberrant communication and signaling between the innate and acquired responses may represent another potential mechanism for CRS development. As previously mentioned IL-6 has been proposed as key cytokine mediating this transition. Its main action is to free helper and effector T-cells from the suppressive effects of IL-10 secreted by Treg. The described association of elevated IL-6 and IL-6 receptor in CRSwNP (possibly mediated by TLR2 or PAR stimulation) might be sufficient to inhibit local innate immune responses and dampen local adaptive immunosuppression mediated by Treg cells, suggesting that derangement of this signaling pathway may be significant for polyp formation [Peters AT et al., 2010].

Lastly, there are studies also suggesting a dysfunction of the BAFF (B-cell activating factor of the TNF family) regulatory pathways in CRSwNP, together with elevated IgA. BAFF is a secreted epithelial factor instrumental in fostering local immunoglobulin responses, in particular B-cell proliferation and class switch recombination. The hypothesis is that BAFF, through B-cell proliferation, class switch recombination and production of IgA, indirectly influence mediator release

from eosinophils and subsequent mucosal oedema characteristic of NPs [Kato A et al., 2008] (Figure 2).

Figure 2 - Key phenotypes and proposed endotypes of CRS and their possible associations (Adapted from [Akdis CA et al., 2016])



ASA, Aspirin; CRS, chronic rhinosinusitis.

1.3.3 Histomorphological features and tissue remodelling

Remodelling is a critical aspect of wound repair in all organs and it is a dynamic process resulting in both ECM production and degradation. This may lead to a normal reconstruction process with production of normal tissue or may result in pathological reconstruction with formation of pathological tissue [Bousquet J et al., 1992].

Remodelling in the lower airway disease has been extensively studied. It includes changes in airway epithelium, lamina propria and submucosa, resulting in airway wall thickening. The main histologic features of remodelling are macrophage and lymphocyte infiltration, fibroblast proliferation, angiogenesis, increased connective tissue formation (fibrosis) and tissue destruction [Bai TR, 2010].

There is evidence that remodelling is also present in chronic sinus disease and distinct remodelling features differentiate different subgroups of CRS.

As already mentioned, CRS is divided in CRSsNP and CRSwNP on the basis of different inflammatory and “remodelling patterns” [Huvenne W et al., 2009].

Several factors have been implicated in remodelling. TGF- β is a pleiotropic and multifunctional growth factor with immunomodulatory and fibrogenic activity, known in 3 different isoforms (TGF- β 1, β 2 and β 3), which can bind to 3 membrane receptors (TGF- β RI, RII and RIII). It is considered a counter regulatory cytokine to resolve inflammation and to initiate the repair process. Indeed, it acts as chemoattractant and proliferation factor for fibroblasts, inducing synthesis of EMC proteins [Van Bruaene N et al., 2008].

Furthermore, TGF- β regulates the function of immune cells. It is a strong suppressor of T-cell activation and of antibody secretion by B-cells and acts both as an effector and an inducer of Treg function.

TGF- β 1 protein expression was found increased together with TGF- β RI expression, indicating an enhanced TGF- β signalling in CRSsNP. In contrast, in CRSwNP a low TGF- β 1 protein concentration and a decreased expression of TGF- β RII indicate a low level of TGF- β signalling. These findings were reflected by the remodelling patterns observed, characterized by a lack of collagen in CRSwNP and excessive collagen production with thickening of the collagen fibres in the ECM in CRSsNP [Van Bruaene N et al., 2009].

Another possible mechanism implicated in pathologic tissue remodelling is the imbalance between matrix metalloproteinases (MMPs), a family of zinc-dependant and calcium-dependant endopeptidases, and the tissue inhibitors of MMP (TIMPs) [Van Bruaene N et al., 2011]. In CRSsNP, elevated levels of MMP-9 and TIMP-1 together with high levels of TGF- β 1 are found. TGF- β 1 induces the release of TIMP-1, inhibiting the proteolytic activity of MMP-9. In CRSwNP, only MMP-9, but not TIMP-1, is up-regulated, due to the relative lack of TGF- β 1.

Lastly, even fibrinolytic components may have a role in tissue remodelling in CRS, inducing ECM degradation and breakdown. Plasminogen activators (in particular urokinase plasminogen activator [uPA]) play an important role in the fibrinolytic system, as these proteins convert the proenzyme plasminogen into the active enzyme plasmin. Plasmin degrades fibrin and converts inactive promatrix MMPs into active MMPs. This activity is counteracted by plasminogen activator inhibitor-1 (PAI-1), which is activated by TGF- β 1. The PAI-1/uPA ratio of CRSwNP was significantly lower when compared with CRSsNP or controls, suggesting that the activity of uPA may be dominant in CRSwNP compared with the other groups. In CRSsNP, TIMP-1 up-regulation together with MMP-9, high level of TGF- β 1 and low activity of uPA were observed, so that fibrosis is considered to proceed in the ECM [Eloy P et al., 2011] (Table 3). In contrast, in CRSwNP, TIMP-1 is not up-regulated, and high level of MMP-7 and MMP-9, high activity of u-PA, and low level of TGF- β 1 are observed, so that fibrinolysis is considered to proceed in the ECM [Sejima T et al., 2011].

Table 3 - Inflammatory pathways, cytokine profiles, biomarkers, and tissue remodeling in CRS (Reprinted with permission 3997110379476 from [Eloy P et al., 2011])

	CRS without polyps	CRS with polyps	CRS in Chinese patients
T-cell profile	Th1	Th2	Th17
Inflammatory cells	Prominent neutrophils (low percentage of eosinophils, mast cells), T cells	Prominent eosinophils, B cells, T cells	Neutrophils (MPOs), T cells
Cluster of differentiation	CD3, CD25, CD68	CD3, CD25, CD138, CD68	CD4, CD8
Cytokines and chemokines	IFN- γ ↑, TGF- β 1↑, IL-1↑, IL-3↑, IL-6↑, IL-8↑; TNF- α , IL-5 not increased	IL-4↑, IL-5↑, IL-13↑, ECP↑; overproduction of IL-8, RANTES, eotaxin (from epithelial cells)	IL-17, IL-6, IFN- γ , IL-4, IL-5, IL-10, TGF- β
Immunoglobulins	IgE↑ in cases of allergic rhinitis; IgA unknown	Local production of polyclonal IgE (<i>Staphylococcus aureus</i> , enterotoxin); IgA, IgG, IgM elevated	–
Growth factors	GM-CSF increased	GM-CSF (epithelial cells), VEGF	–
Adhesion molecules	VCAM-1 and IL-5 not increased	Upregulated ICAM-1 and VCAM-1, E-selectin, P-selectin	–
Transcription factors	FoxP3 upregulated; Tbet and GATA-3 are similar to controls	Tbet and GATA-3 upregulated; FoxP3 downregulated.	Tbet, GATA-3
Matrix remodeling proteins	Collagen, MMP-9 counterbalanced by natural inhibitor TIMP-1, fibrosis	MMP-1, MMP-2, MMP-9, and MMP-7 upregulated; epithelial shedding pseudocyst formation containing albumin	–
Subsequent tissue remodeling	Basement membrane thickening, goblet cell hyperplasia, limited subepithelial edema, prominent fibrosis and mononuclear cells	Epithelial damage, epithelial shedding, pseudocyst formation containing albumin thickened basement membrane, reduced number of blood vessels and glands, no neuronal structures	–
Genes	Polymorphisms within IL receptors, α 1-antitrypsin	Polymorphisms within COX1/COX2 pathways, leukotriene pathways, and receptors related to AA metabolites	–
Comorbidities	Recurrent URTIs; allergic rhinitis can be associated	Asthma, AERDs	–

AA arachidonic acid, AERD aspirin-exacerbated respiratory disease, COX cyclooxygenase, CRS chronic rhinosinusitis, ECP eosinophil cationic protein, FoxP3 forkhead box P3, GATA-3 GATA-binding protein 3, GM-CSF granulocyte-macrophage colony-stimulating factor, ICAM intercellular adhesion molecule, IFN interferon, IL interleukin, MMP matrix metalloproteinase, MPO myeloperoxidase, RANTES regulated on activation, normal T-cell expressed and secreted, Tbet T-box transcription factor, TGF transforming growth factor, Th T-helper type cell, TIMP tissue inhibitor of metalloproteinases, TNF tumor necrosis factor, URTI upper respiratory tract infection, VCAM vascular cell adhesion molecule, VEGF vascular endothelial cell growth factor.

1.4 Genetics of chronic rhinosinusitis: state of the art

Classic evidence for a genetic component of a disease is the heritability of the condition. Although formal heritability studies are not available for CRS, a genetic basis has long been suspected [Bossé Y et al., 2009]. One early report documented

cases of concordant monozygotic twins who had CRSwNP despite distinct environmental exposure [Drake-Lee A, 1992]. Similarly, familial aggregation of a disease points to a genetic basis, and indeed, reports of families with an unusually high prevalence of CRS are available [Cohen NA et al., 2006; Delagrang A et al., 2008]. Additionally, patients with CRS are more likely to report a positive family history than those without CRS [Greisner WA 3rd et al., 1996]. Supportive evidence for a genetic basis also includes the fact that several syndromes associated with known genetic defects have CRS as a clinical component. This includes primary ciliary dyskinesia [Noone PG et al., 2004], as well as monogenic diseases, such as cystic fibrosis [Armengot M et al., 1997; Sobol SE et al., 2002]. Lastly, the inflammatory features of CRS have similarities to those seen in patients with allergic rhinitis and asthma, two complex diseases with well-established genetic components [Dávila I et al., 2009; Ober C et al., 2011] and strong clinical associations with CRS [Fokkens WJ et al., 2012]. Indeed, studies have demonstrated common molecular and cellular aspects of these disorders, leading to the unified airway concept, which postulates that the common clinical manifestations of CRS, allergic rhinitis and asthma suggest a shared cause [Krouse JH et al., 2007].

1.4.1 Limitations of existing literature on genetics of chronic rhinosinusitis

No large genetic studies of CRS have been performed to date.

The aetiology of CRS is likely multifactorial and genes relevant to CRS are likely to have small effect sizes, similar to other complex multifactorial diseases [Cho JH et al., 2011]

The majority of studies, available in the international literature, testing whether genetic variation is associated with CRS, have used a *candidate gene approach*.

This compares the allele frequencies of SNPs in genes suspected a priori to be involved in CRS among patients with CRS and those without CRS (control subjects). However, there are several limitations to this strategy: (1) the difficulty to generate novel information on disease mechanisms because candidates are selected based on what is already known (or suspected) about disease biology, (2) the difficulty of achieving adequate power, (3) the potential for confounding by ancestry when subject groups are heterogeneous and (4) the caveat that an association between CRS and a polymorphism does not necessarily indicate causality. Subjects groups have to be very carefully selected.

Genome-wide approaches overcome the first limitation because of their ability to interrogate the entire genome in an unbiased, hypothesis-independent fashion. However, to date, such studies are rare in patients with CRS. There are several reasons for the paucity of genome-wide association studies (GWASs), including the need for a large number of well-phenotyped subjects, the expense of high-density genotyping and the lack of research consortia in this field. By the way, GWASs have proved to be fruitful for identifying novel pathways and providing new insights into disease biology.

1.4.2 Current evidences from candidate gene approaches

A recent extensive review extracted the most promising findings from candidate gene studies present in the current literature regarding the genetics of CRS [Hsu J et al., 2013].

Epidemiologic and genetic reports support a relationship between CFTR and CRS independent of cystic fibrosis. The high prevalence of CRS among patients with CF [Ramsey B et al., 1992] suggests that CFTR might contribute to the pathogenesis of CRS and this relationship might extend beyond patients with clinical features of CF. Indeed, a landmark case-control study demonstrated that

patients with CRS were more likely to be CFTR carriers compared with control subjects (7% vs 2%), although CFTR mutations were present in only a minority of the whole group of CRS patients (5%) [Wang X et al., 2000].

A relationship between CRS and HLA genes variation (both class I and class II) is intriguing but still unclear. HLA plays a critical role in antigen presentation and represents an important link between innate and adaptive immunity [Chaplin DD, 2010]. HLA genes variation has been strongly associated with several inflammatory diseases, such as insulin-dependant diabetes mellitus and ankylosing spondylitis [Cho JH et al., 2011]. Several groups have examined variation in HLA in patients with CRS (mainly CRSwNP) and identified alleles associated with the disease, the most robust of which is represented by the association between CRS and HLA-DRB1*04 allele (OR, 2.2; 95% CI 1.2-4.2; $p = 0.009$) [Ramírez-Anguiano J et al., 2006], but only few attempts have been made to replicate them. Nevertheless HLA loci remain attractive candidate genes because of the significant immune dysregulation that is characteristic of CRS [Lane AP, Truong-Tran QA, Schleimer RP, 2006].

A growing body of evidence suggests that aberrant immunity and aberrant remodelling of sinonasal epithelial tissue might underlie the pathogenesis of CRS [Tan BK et al., 2010].

A few studies have investigated whether genetic variation in the arachidonic acid pathway is associated with CRS, with controversial results [Pérez-Novo CA et al., 2005; Gosepath J et al., 2005].

1.4.3 Insight from genome-wide approaches: pooling-based GWAS

Two pGWASs of patients with CRS have been published. Unlike traditional GWASs, this approach relies on pooled DNA from multiple subjects for analysis, which reduces the genotyping costs.

The first study identified novel associations between CRS and variation in genes involved in the basement membrane formation: laminin $\alpha 2$ (LAMA2), laminin $\beta 1$ (LAMB1) and the acyloxyacyl gene (AOAH) [Bossé Y et al., 2009]. The AOAH gene is particularly interesting because it contributes to host defence against bacterial lipopolysaccharide and has also been linked to asthma. Moreover, the association between AOAH (rs4504543) and CRSsNP has been replicated in a candidate gene study of Chinese patients (OR, 0.30; $p = 8.11 \times 10^{-11}$) with a statistical significance even after multiple statistical testing [Zhang Y et al., 2012]. The second pGWAS found an association between CRS and variation in the epithelial tumor protein p73 gene (TP73) [Tournas A et al., 2010], which is supported by separate studies that reported abnormal expression of the p73 protein in the sinonasal tissue of patients with CRSwNP [Li CW et al., 2011].

1.4.4 Gene-environment interactions: is there a role for epigenetics?

The variation of clinical phenotype indicates that even in CF, the most straightforward case of genetic CRS, multiple factors in an individual patient strongly determine disease expression [Hull J et al., 1998]. Alterations in expression of genes other than CFTR, mediated via genetic variation or environmental effects, apparently combine to affect disease phenotype.

The effects of changing environment on prevalence of CRS have not been directly studied but it is certainly reasonable to hypothesize that many of the same environmental factors that influence the prevalence of atopy also influence the prevalence of CRS [Liu AH et al., 2003].

In brief, CRS phenotype most likely results from the combined effect of genetic variation and acquired epigenetic effects across critical pathways that control the immunobiology of the nasal mucosa. Epigenetic changes create de facto genetic changes by altering gene expression without directly altering the DNA sequence.

1.5 Gene-expression studies

The gene-expression is the process through which the information codified in a gene are used to synthesize a functional gene product, usually a protein. This process is applied by all known life, from viruses to multicellular eukaryotes.

Measuring gene-expression is an useful tool in many life sciences since the ability to quantify the level at which a particular gene is expressed in a cell, a tissue or an organism can provide lot of helpful information, for instance determining an individual's susceptibility to a cancer by the identification of an oncogene expression.

Gene-expression studies are based on the identification of a "target" gene thought to be involved in the pathological mechanism of a disease through the analysis of the transcriptome, i.e. the set of all messenger RNA molecules in one cell or a population of cells. Even if it is known that the expression of a lot of genes is performed by pre-transcriptional mechanisms and, by consequence, high concentration of mRNA are not always correlated with high concentration of the corresponding protein, it remains a mainstay of the gene-expression measurement.

The levels of mRNA can be quantitatively measured by northern blotting: a sample of RNA, separated on an agarose gel, is hybridized to a RNA probe marked with a radioactive reagent and then detected by an autoradiograph. The main disadvantages of this technique are related to the relatively large quantities of RNA needed and the not complete accuracy in the quantification of the RNA, since it is based on the measuring of the band strength in an image of a gel. On the other hand the main advantage of this technique lays on the possibility to discriminate alternately spliced transcripts.

Another approach for the quantification of mRNA is the reverse transcription followed by the quantitative PCR (RT - qPCR). The RT PCR generates a DNA template from the mRNA. The single-stranded template is called cDNA and is

then amplified in the quantitative step. During this second procedure the fluorescence emitted by a labelled hybridization probe of the target gene change as the DNA amplification process progress. With a standard curve “ad hoc” made qPCR produce an absolute measurement of the number of copies of the original mRNA (usually in units of copies per nanolitre of homogenized tissue). Generally the expression of the target gene is normalized to the expression of an housekeeping gene that should be equal represented in the sample analysed.

This technique is very sensitive (even the detection of a single mRNA copy is theoretically possible) and may be utilized for numerous genes simultaneously (expecially in low-density array), but can be expensive in relation to the kind of used probe.

As alternative The Real-Time PCR can be performed using the iTaq™ Universal SYBR® Green Supermix (BioRad, Italy), which we used in our study.

The SYBR Green is an aromatic organic compound (molecular formula C₃₂H₃₇N₄S) part of the asymmetric cyanine group, molecules with fluorofor activity and is used in Molecular Biology as colorant of Nucleic Acid.

The SYBR Green is an intercalating agent (molecule, usually planar, capable to fit transversely in the DNA filaments, through a mechanism discovered by Leonard Lerman and called intercalation) and binds preferentially a double-stranded DNA (dsDNA). Complex DNA-colorant absorbs blue light at a wavelength $\lambda_{\max} = 488$ nm and emits green light $\lambda_{\max} = 522$ nm.

Thanks to their hydrophobic properties, intercalating agents are able to fit between two adjacent nitrogen bases along the strands of the double helix. When the cell begins his replicative cycle and the point where the intercalating agent inserted is reached from DNA polymerase, this does not distinguish it from the adjacent nitrogen bases and proceeds by inserting a nucleotide which makes pair with the intercalating agent.

The reading of the fluorescence is made at the end of each amplification cycle of the PCR. The use of intercalating fluorescent molecules is an effective and relatively economic method, however this system is not able to discriminate between the specific amplification products and other nonspecific products such as primer dimers. It's therefore essential to draw the primer with low-risk of dimerization.

Other methods used to quantify simultaneously many different mRNA is the hybridization microarray, in which a single array or "chip" may contain probes to identify transcript levels for all the genome of the organism.

Studies on gene expression related to nasal polyposis are present in literature but still limited.

1.6 Replication studies

A replication study involves repeating an original study using the same methods but with different subjects and experimenters. The researchers will apply the existing theory to new situations in order to determine generalizability to different subjects, age groups, races, locations, cultures or any such variables.

The main determinants of a replication study include:

- To assure that results are reliable and valid
- To determine the role of extraneous variables
- To apply the previous results to new situations
- To inspire new research combining previous findings from related studies

When we perform the deliberate repetition of previous research procedures in our clinical setting we can be able to strengthen the evidence of previous research finding, and correct limitations, and thus overall results may be in favor of the

results of previous study or we may find completely different results.

A replication study of an original study is possible and should be carried out, when:

- The original research question is important and can contribute to the body of information supporting the discipline
- The existing literature and policies relating to the topic are supporting the topic for its relevance
- The replication study if carried out carries the potential to empirically support the results of the original study, either by clarifying issues raised by the original study or extending its generalizability.
- The team of researchers has all expertise in the subject area and also has the access to adequate information related to original study to be able to design and execute a replication.
- Any extension or modifications of the original study can be based on current knowledge in the same field.
- Lastly, the replication of the same rigor as was in original study is possible [Explorable, 2009].

2 MATERIALS AND METHODS

Our study was carried out in the DBSV biology laboratories of the Insubria University Varese, in collaboration with the Otorhinolaryngoiatric Clinic of the Ospedale di Circolo and Fondazione Macchi, and the aim was to verify the expression of certain genes in patients suffering from chronic rhinosinusitis.

We decided to replicate some of the most significant studies leaded on this field, concerning gene expression evaluation in patients affected by inflammatory diseases of paranasal sinuses, trusting in the importance of replication studies.

2.1 Selected genes

We decided what genes have to be investigated analysing the studies published in the international literature over the past 10 years concerning gene expression in CRS and, given the significant results, we chose the following nine genes as possible target genes involved in the physio-pathogenesis of chronic rhinosinusitis:

- 1) *COX 2*
- 2) *Acquaporin*
- 3) *Caveolin*
- 4) *Mammaglobin*
- 5) *MUC5AC*
- 6) *PGDS*
- 7) *TNF alpha*
- 8) *TGF beta*
- 9) *Lactoferrin*

1- COX 2

It is believed that one of the possible pathogenic mechanisms involved in nasal polyposis is due to an impairment in prostanoid metabolism. Prostanoids are produced from arachidonic acid when this is released from the plasma membrane by means of phospholipases and is metabolised by cyclooxygenation (COX) and specific isomerases. Prostanoid production therefore depends upon the activation of the two COX isoenzymes associated with the cells. While COX 1 is expressed in a constitutive manner in many cells, the COX 2 isoenzyme is only minimally expressed, though it can be induced as a response to various forms of stimulation [Mullol J et al., 2002], such as cytokines or growth factors. COX 2 resulted to be over-expressed in inflammatory diseases [Pujols L et al., 2004].

2- Aquaporin:

Aquaporins are a family of small hydrophobic membrane proteins that appear to play a role in the homeostasis of mucosal barrier function by regulating tissue water transport. In chronic rhinosinusitis, and particularly when this is associated with nasal polyposis, there is probably a fault in the nasal epithelial barrier and reports of a decrease in AQP5 in subjects suffering from CRS demonstrate that this has a fundamental role in the relative homeostasis [Frauenfelder C et al., 2014; Shikani AH et al., 2013].

3- Caveolin

Caveolin-1 is a 22kDa scaffolding protein with a central role in defence against infections and in tumour suppression by reducing the production of Cyclin D1 and endothelial nitric oxide synthase. Its role in the pathogenesis of rhinosinusitis inflammation was considered after a reduction in the gene expression in this protein was discovered in patients affected with CRS, especially in the form associated with polyposis [Lin H et al., 2014].

4- Mammaglobin

Mammaglobulin A is a 10kDa glycoprotein that probably belongs to the family of proteins in which rat estramustin binding protein and human uteroglobin are to be found. As yet, the role of mammaglobin is unknown, though the other proteins in this family are secretory ones that can both bind steroids and modulate inflammatory processes [Forsgren B et al., 1979; Miele L et al., 1994].

The mRNA of this protein has been investigated in the oncological field, principally in primary breast tumours since it is over-expressed in 50% of the cell lines in breast cancer in humans and in 23% of primary breast tumours [Fleming T et al., 2000].

The role of mammaglobin in nasal polyposis was postulated in the literature after the discovery that its expression in the mucosa of healthy subjects differed from that in patients with chronic polypoid rhinosinusitis [Chusakul S et al., 2008].

5- MUC5AC

MUC5AC is one of the most profusely produced mucins in the respiratory tract, and in the sinus areas in particular. Its production is controlled by numerous mediators such as interleukin 1beta, 6, 13, 17, and by TNF-alpha [Ding GQ et al., 2007; Kim YJ et al., 2014].

Over-production of this mucin has been considered related to the hypoxic conditions believed to be a predominant pathophysiological factor in chronic rhinosinusitis. Over-production of mucous results to be one of the prevalent characteristics of inflammatory rhinosinusitis.

6- PGDS – prostaglandin D synthase

Prostaglandin D is a derivative of arachidonic acid and is one of the major cyclooxygenase products. It is generated by PGDS. Together with prostaglandin E, it is responsible for mediating airway inflammation through multiple biological

activities that range from vasodilatation to bronchoconstriction [Okano M et al., 2006].

Certain authors have suggested that PGDS might contribute to the development of hyper-eosinophilia, which is known to be one of the factors considered the greatest risk for an outcome of chronic rhinosinusitis that is difficult to treat.

7- Tumour necrosis factor alpha (TNF-alpha)

TNF α is a cytokine implicated in systemic inflammation and plays a central role in reaction during the acute stage of the inflammation. It is produced primarily by macrophages, though it can also be produced by many other cell types such as CD4⁺ and NK lymphocytes, neutrophils, mast cells and eosinophils [Swardfager W et al., 2010; Park SJ et al., 2013]. Along with other cytokines, mainly IL-6, IL-12A and IL-13, it is over-expressed in patients with chronic rhinosinusitis.

8- TGFb

Transforming Growth Factor Beta-1 is a dimer that is intracellularly synthesised and extracellularly stored in latent form. Two proteins bind to TGFb1 and control its activation: LAP (latency associated peptide) and TGFb binding protein. TGFb1 belongs to the cytokine family and exists in 3 isoforms (1, 2 and 3). Generally, when TGFb is referred to, isoform 1 is intended, since this was the first to be discovered in this family.

The role of TGFb 1 is to control proliferation, differentiation and other functions in the majority of cells.

Some studies have demonstrated how down-regulation of TGFb expression activation might be associated with chronic inflammatory diseases in the respiratory tract, RSC being one of these [Go K et al., 2010].

9- Lactoferrin

Lactoferrin is an approximately 80kDa protein binding to iron, first discovered in breast secretion, and is synthesised by the majority of the tissues in mammals. [Zhang Y et al., 2014].

Lactoferrin has various biological properties (anti-cancerogenic, anti-bacteric, anti-viral, anti-fungal, anti-inflammatory and immunomodulation). One of its characteristics that has been the focus of discussion in the literature for several years is that it appears to inhibit the growth of fibroblasts in polypoid mucosa [Psaltis AJ et al., 2007; Psaltis AJ et al., 2008; Acioglu E et al., 2012].

2.2 Patients selection

We decided to compare the gene expression in healthy subjects (not affected by CRS; Control Group; C Group) with that of patients with CRS with nasal polyposis not associated with plasmatic eosinophilia and other immunologic diseases (CRSwNP; Polyps Group; P Group).

Hyper-eosinophilia is a well-known risk factor for the type of CRS that is poorly responsive to treatment and is often one of the implications leading to more serious forms of the disease.

All the patients both from Group C and P, underwent accurate history collection, allergologic and pneumologic evaluation and blood tests.

Patients referring or presenting allergies, ASA intolerance, asthma, immunologic diseases, plasmatic hypereosinophilia or presence of infection were excluded from the study.

Diagnosis (Group P) or exclusion (Group C) of CRSwNP was also obviously supported by endoscopic examination of the nasal fossae and CT scan without mean of contrast of the head.

If we consider a phenotypical / immunological Classification like the one proposed by Stammberger H [Stammberger H, 1999], this study has not taken into account classes I and II (responsive to surgery alone and apparently not linked to an immunological substrate), and class IV / V of nasal polyposis (that are, the types of nasal polyposis that are associated with Hypereosinophilia and systemic diseases, such as Kartagener's syndrome or Cystic Fibrosis); the only class we considered was class III.

2.3 Specimens collection

In our study on gene expression we obtained biopsy specimens of hyperplastic/polypoid mucosa (Group P) and of healthy mucosa (Group C) taken from the anterior ethmoid of the subjects enrolled in the study and submitted to general anesthesia for FESS or local anesthesia for polypectomy (Group P) or submitted to general anesthesia for septoturbinoplasty (Group C).

When the biopsies were performed, the patients had to be free from acute inflammatory and infectious conditions.

The patients were also invited to suspend all local corticosteroid treatment (the standard maintenance treatment given to NP and ECRSWNP patients) at least 20 days prior to the biopsy.

Adequate informed consent was obtained (Figure 3).

Figure 3 – Informed consent given to patient before biopsies procedures (Italian and English versions)



Ospedale
di Circolo
Fondazione
Macchi

Reparto – Day Surgery – Day Hospital - Ambulatori
Clinica Otorinolaringoiatrica
Direttore: Prof. Paolo Castelnuovo

Dipartimento di Biotecnologie e Scienze della Vita
dell'Università degli Studi dell'Insubria di Varese
Direttore: Prof. Giovanni Bernardini



Facoltà di Medicina
e Chirurgia

**FOGLIO INFORMATIVO E MODULO DI CONSENSO INFORMATO PER IL
SOGGETTO PARTECIPANTE A STUDIO OSSERVAZIONALE/SPERIMENTALE**

Università degli Studi dell'Insubria

***“Valutazione dell'espressione genica in pazienti affetti da patologia infiammatoria e/o
neoplastica del distretto nasosinusale e/o del basicranio”***

Coordinatore Scientifico: Dott. Andrea Pistochini

Co-Sperimentatori: Dott.ssa Stefania Gallo, Dott. Andrea Preti, Dott.ssa Cristina Esposito, Dott.ssa Federica Rossi

Tutors: Prof. Paolo Castelnuovo, Prof. Giovanni Bernardini

Gentile Signore/a,

la nostra Università intende condurre uno studio medico-scientifico, che si svolgerà con la collaborazione tra la U.O. di Otorinolaringoiatria e il Dipartimento di Biotecnologie e Scienze della Vita dell'Università degli Studi dell'Insubria di Varese.

Affinché si possa effettuare questa ricerca, abbiamo bisogno della collaborazione di pazienti che, come Lei, sono candidati ad interventi di chirurgia funzionale nasosinusale e/o del basicranio per patologia infiammatoria e/o neoplastica oppure di pazienti “sani” candidati a procedure per le quali sia utilizzata una tecnica endoscopica transnasale (es. interventi di settoplastica, approcci sellari transetmoido-sfenoidali, ...).

Prima che Lei decida in merito ad un'eventuale partecipazione, è importante che comprenda lo scopo per cui la ricerca viene effettuata. La preghiamo di leggere con attenzione questo documento e, se desidera, di discuterne con i suoi familiari e con il suo medico curante, prendendo tutto il tempo che Le necessita ed invitandoLa a chiederci chiarimenti qualora le informazioni fornite non fossero chiare o avesse bisogno di ulteriori precisazioni.

PERCHÉ VIENE EFFETTUATO QUESTO STUDIO?

Questo studio è volto alla caratterizzazione dei meccanismi biologici che entrano in gioco durante la genesi e l'evoluzione della patologia flogistica (es. poliposi) e neoplastica dei seni paranasali e della base cranica.

Pertanto, lo studio di queste patologie mediante l'analisi dell'espressione di specifici geni candidati, mediante analisi dell'mRNA (il “messaggero” che trasporta le informazioni genetiche), fornirà dati preziosi e fortemente significativi riguardo il ruolo svolto da specifici meccanismi cellulari durante la genesi e la progressione di tali patologie.

COME AVVERrà IL PRELIEVO DEI CAMPIONI?

Nei casi prescelti e autorizzati verrà prelevata una porzione millimetrica della mucosa naso/sinusale patologica o di neoplasia.

Ad alcuni pazienti verrà prelevata una porzione millimetrica della mucosa sana da utilizzare come campioni controllo.

I campioni di tessuto verranno conservati a -80°C fino al loro processamento che avverrà presso il Dipartimento di Biotecnologie e Scienze della Vita dell'Università degli Studi dell'Insubria di Varese.

Da essi si estrarrà l'RNA, utilizzato per indagare se l'espressione di geni specifici vari tra i campioni sani e quelli patologici.

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**Facoltà di Medicina
e Chirurgia**

I dati ottenuti dal Dipartimento di Biotecnologie e Scienze della Vita dell'Università degli Studi dell'Insubria di Varese verranno quindi incrociati con i dati clinici a disposizione dell'U.O. di Otorinolaringoiatria dell'Ospedale di Circolo Fondazione Macchi di Varese, allo scopo di ricavare informazioni utili a determinare nuovi potenziali bersagli molecolari per lo sviluppo futuro di farmaci di nuova generazione.

L'adesione allo Studio è completamente volontaria.

Nel caso in cui Lei decidesse di prendere parte allo studio, Le sarà chiesto di firmare il modulo per il consenso informato.

Qualora avesse dei ripensamenti è libero/a di ritirarsi; i dati raccolti verranno cancellati e il materiale prelevato verrà dismesso.

RISERVATEZZA DEI DATI PERSONALI

Il medico che La seguirà nello studio La identificherà con un codice: i dati che La riguardano, raccolti nel corso dello studio, ad eccezione del Suo nominativo, saranno registrati, elaborati e conservati unitamente a tale codice identificativo, presso l'U.O. di Otorinolaringoiatria dell'Ospedale di Circolo Fondazione Macchi di Varese di questo ospedale. Soltanto il medico e i soggetti autorizzati potranno collegare questo codice al Suo nominativo.

La informiamo che i Suoi dati personali verranno raccolti ed archiviati elettronicamente e saranno utilizzati esclusivamente per scopi di ricerca scientifica, in accordo alle responsabilità previste dalle norme della buona pratica clinica (D.L. 211/2003); i Suoi dati personali, in particolare quelli sulla salute, saranno trattati soltanto nella misura in cui sono indispensabili in relazione all'obiettivo dello studio. Lei ha il diritto di conoscere quali informazioni saranno memorizzate e di aggiornare o modificare dati erronei.

L'accesso a tali dati sarà protetto dallo Sperimentatore (il medico che La segue durante lo Studio). Autorità regolatorie (Ministero della Sanità, AIFA, EMA), personale medico, addetti al monitoraggio e alla verifica delle corrette procedure (Comitato Etico Indipendente) potranno ispezionare l'archivio senza che vi sia la possibilità di risalire alla Sua identità personale.

I risultati dello Studio a cui parteciperà potranno essere oggetto di pubblicazione ma la Sua identità rimarrà sempre segreta, nel pieno rispetto della Normativa vigente sulla tutela della persona (D.L. 675/1996) e rispetto al trattamento dei dati personali (D.L. 196/2003).

Il consenso al trattamento dei dati personali è indispensabile per il Suo arruolamento nello Studio Sperimentale, altrimenti non potrà parteciparvi.



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DICHIARAZIONE DI CONSENSO

alla partecipazione allo studio osservazionale/sperimentale *“Valutazione dell'espressione genica in pazienti affetti da patologia infiammatoria e/o neoformativa del distretto nasosinusale e/o del basicranio”* e al trattamento dei dati personali

Io sottoscritto.....

Nato a..... il.....

DICHIARO

Di avere ricevuto dal Dottor..... esaurienti spiegazioni in merito alla richiesta della Mia partecipazione allo Studio sopra descritto. Copia della relativa scheda informativa mi è stata consegnata.

Di aver potuto discutere tali spiegazioni, di aver potuto porre domande e di avere ricevuto risposte soddisfacenti in merito.

Di aver avuto la possibilità di discutere in merito ai particolari dello Studio anche con altre persone di mia fiducia.

Di essere consapevole che la Mia partecipazione alla Ricerca sia volontaria e che ho la facoltà di ritirarmi in qualsiasi momento, senza che tale fatto pregiudichi le cure mediche di cui potrei necessitare.

Per tali motivi ACCONSENTO/NON ACCONSENTO quindi di partecipare alla Ricerca, avendo perfettamente compreso tutte le informazioni sopra riportate.

TRATTAMENTO DEI DATI PERSONALI

Sono stato/a informato/a del Mio diritto di avere libero accesso alla documentazione relativa alla Ricerca. Sono inoltre consapevole che, secondo il rispetto della normativa vigente, i Miei dati personali saranno utilizzati esclusivamente per scopi di ricerca scientifica.

Per tali motivi AUTORIZZO/NON AUTORIZZO l'utilizzo e la divulgazione, in forma anonima, per sole finalità scientifiche ed amministrative e nell'osservanza delle vigenti norme sulla tutela della riservatezza, dei risultati dello Studio, compresi i dati clinici che mi riguardano, nel pieno rispetto della normativa vigente in Italia sulla tutela della persona (D.L. 675/1996) e dei dati personali (D.L.196/2003).

Data .../.../.....

Firma del Paziente Timbro e Firma del Medico

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Nel caso in cui il/la paziente non possa leggere e/o firmare

Io sottoscritto _____

Testimonio che il Dottor _____

ha chiaramente spiegato al/la Signor/a _____

le caratteristiche dello Studio, secondo quanto riportato nella scheda informativa allegata, e che lo/a stesso/a, avendo avuto la possibilità di fare tutte le domande che ha ritenuto necessarie, ha accettato liberamente di partecipare allo Studio.

Data .../.../.....

Firma del Testimone Indipendente

Timbro e Firma del Medico



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**INFORMATIONAL SHEET AND INFORMED CONSENT FORM FOR THE SUBJECT
PARTICIPANT TO AN OBSERVATIONAL / EXPERIMENTAL STUDY**

University of Insubria, Varese

***“Gene expression evaluation in patients affected by inflammatory diseases of paranasal
sinuses or skull base”***

Scientific Coordinator: Andrea Pistochini
Co-Experimenters: Stefania Gallo, Andrea Preti, Cristina Esposito, Federica Rossi
Tutors: Prof. Paolo Castelnuovo, Prof. Giovanni Bernardini

Dear Sir / Madam,

our University plans to conduct a medical-scientific study, which will take place with the cooperation between the U.O. of Otolaryngology and the Department of Biotechnology and Life Sciences at the University of Insubria in Varese.

So that we can conduct this research, we need the cooperation of patients who, like you, are candidates for functional sinus surgery and / or skull base surgery for inflammatory and / or neoplastic disease or "healthy" patients candidate to procedures for which a transnasal endoscopic technique is used (eg. septoplasty, turbinoplasty, transetmoido-sphenoidal sellar approaches, ...).

Before you decide on a possible participation, it is important that you understand the purpose for which the research is performed. Please read this document and, if desired, discuss it with your family and with your doctor, taking all the time that's needed, and inviting you to ask for clarification if the information provided were not clear or needed more clarifications.

WHY IS THIS STUDY CARRIED OUT?

This study is aimed at the characterization of the biological mechanisms that come into play during the genesis and evolution of the inflammatory (eg. Polyposis) and neoplastic disease of paranasal sinuses and skull base.

Therefore, the study of these diseases through the analysis of the expression of specific candidate genes, using mRNA analysis (the "messenger" that carries genetic information), will provide valuable and highly significant data regarding the role played by specific cellular mechanisms during the genesis and progression of these diseases.

HOW THE SAMPLE COLLECTION WILL BE PERFORMED?

In selected cases and if authorized a millimetric portion of the nose / sinus mucosa or of a pathological lesion will be taken, using surgical instrumentation during nasal endoscopy.

In some patients a millimetric portion of the healthy mucosa will be taken, to be used as control samples.

The tissue samples are stored at -80 ° C until their processing that will take place at the Department of Biotechnology and Life Sciences of the University of Insubria in Varese.

From these samples RNA will be extracted and used to investigate whether the expression of several specific genes varies between healthy and pathologic samples.

The data obtained from the Department of Biotechnology and Life Sciences, University of Insubria, Varese will then be crossed with the clinical data available dell'U.O. otolaryngology Macchi Foundation Hospital of

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Circolo di Varese, in order to extract useful information in determining potential new molecular targets for future development of new generation drugs.

Adherence to the Study is completely voluntary.

In case you decide to join the study, you will be asked to sign the form for informed consent.

If you had second thoughts feel free to retire; the collected data will be erased and the collected material will be decommissioned.

CONFIDENTIALITY OF PERSONAL DATA

The doctor who will follow you in the study will identify you with a code: the data concerning you, collected during the study, with the exception of your name will be recorded, processed and stored together with the identification code, at the U.O. of Otolaryngology Hospital of Circolo Fondazione Macchi di Varese of this hospital. Only the doctor and authorized subjects related to the study may link this code to your name.

We inform you that your personal data will be collected and stored electronically and will be used exclusively for scientific research purposes in accordance with the responsibilities provided for by the rules of good clinical practice (Law Decree 211/2003); your personal data, particularly those on health, will be processed only to the extent that they are essential to the objective of the study. You have the right to know what information will be stored, and to update or modify erroneous data.

Access to these data will be protected by the investigator (the doctor who follows you during the Study), regulatory authorities (Ministry of Health, AIFA, EMA), medical staff, employees monitoring and verification of the correct procedures (Independent Ethics Committee) will be able to inspect the archive without the opportunity to go back to your personal identity.

The results of the study may be subject to publication but your identity will always remain secret, in full compliance with current legislation on the protection of people (Law Decree 675/1996) and in the processing of personal data (Law Decree 196/2003).

The consent to the processing of personal data is essential to your enrollment in the Experimental Study, you could not otherwise participate.

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DECLARATION OF CONSENT

to participate to the observational / experimental study

“Gene expression evaluation in patients affected by inflammatory diseases of paranasal sinuses or skull base” and to the processing of personal data

I, the undersigned

Born in..... at.....

DECLARE

I have received from Dr.
detailed explanations on the request of my participation in the study described above. Copy of the
information sheet was given to me.

To have been able to discuss these explanations, that I could ask questions and have received satisfactory
answers about.

To have had the opportunity to discuss about the details of the study also with other people whom I trust.

To be aware that my participation in the research is voluntary and that I have the right to withdraw at any
time, without this fact affecting the medical care I might require.

For these reasons I AGREE / I DO NOT AGREE then to participate in the research, having perfectly
understood all the above information.

PROCESSING OF PERSONAL DATA

I was informed of my right to have free access to the records relating to the search.
I am also aware that, according to the compliance with current legislation, my personal data will be used
exclusively for scientific research purposes.

For those reasons I AUTHORIZE / NOT AUTHORIZE the use and disclosure, anonymously, for only
scientific and administrative purposes and in accordance with current regulations on the protection of
confidential data, the results of the study, including clinical data concerning me, in full compliance with
current legislation in Italy on the protection of the person (DL 675/1996) and personal data (DL196 /
2003).

Date .../.../.....

Signature of the Patient Stamp and Signature of the Doctor

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In the case in which the patient can not read and / or sign

I, the undersigned _____

I testify that Dr. _____

clearly explained to Mr/Mrs _____

the characteristics of the Study, as reported in the attached information sheet, and that, having had the opportunity to ask any questions that he considered necessary, he/she has freely agreed to participate in the study.

Date .../.../.....

Signature of Independent Witness

Stamp and Signature of the Doctor

The biopsies took place in operating rooms of the Day Hospital, Day Surgery and Ordinary Otorhinolaryngoiatric Clinic of the Circolo and Fondazione Macchi Hospital.

The biopsies for the study required at least 100 mg of tissue.

At the end of the study, RNA has been isolated from the samples of 85 patients.

Only the RNA of 24 (28.2%) patient was considered adequate (13 on 35 Group C, 37,1%; 11 on 50 Group P, 22%)

DEMOGRAPHY

10 Female - 14 Male

Median Age 49,2

13 patients C group - 11 patients P Group

Median Age C group 48,77 - Median Age P Group 49,31

In the interval before starting the investigation, the extracted material was preserved either under dry ice and/or in a freezer at -80°C.

All the material was handled with sterile instrumentation.

The specimens were processed in the DBSV biology laboratories of the Insubria University Varese.

The laboratory process we used, leading to analyse the mRNA expression in the biopsy tissue, is the common procedure reported also in literature in all comparable studies and is illustrated below. The author was involved in all the following steps of the process, supported by experienced Biologist.

2.4 RNA extraction

The total RNA was isolated starting from approximately 100 mg of tissue, as already specified. Each sample was homogenised in 100 µl of H₂O+DEPC (diethylpyrocarbonate for eliminating the RNases), using an UltraTurrax homogeniser. Then 1 ml of TRIzol reagent (SIGMA Invitrogen, Italy) was added to approximately 100 mg of the homogenised material obtained. After 5 minutes incubation at room temperature, 0.3 ml of chloroform were added for every ml of the initial TRIzol; the samples were agitated by hand for 15 seconds, incubated for 3 minutes and then centrifuged at 12000 x g for 15 minutes at +4°C. This produced two phases: an upper aqueous phase containing the total RNA and a lower organic phase containing DNA, proteins and fragments of membrane.

The upper aqueous phase was retrieved from every sample and the RNA was precipitated by adding 0.5 ml of cold isopropanol for every ml of initial TRIzol and agitated manually for a few seconds.

The samples were left under ice for 10 minutes then they were centrifuged at 12000 x g at +4°C for 10 minutes, thus obtaining pellets of RNA. After eliminating the supernatants, the pellets were rinsed with 1 ml of 75% ethanol in H₂O+DEPC for every ml of TRIzol and centrifuged at 7500 x g for 5 minutes at +4°C. Each pellet was then air-dried and re-suspended in RNase-free water (treated with 0.1% DEPC).

2.5 RNA quantification

The extracted RNA was quantified by QuantiFluor fluorometer (Promega, Italy) using QuantiFluor® RNA System dye, according to the instructions of the manufacturer. Briefly, at 1 µl of RNA, diluted with 99 µl of TE buffer 1x, were added 100 µl of working solution (QuantiFluor® RNA Dye diluted 1:200 with TE

buffer 1x). The prepared samples were incubated at room temperature for 5 minutes, protected from light, and then the fluorescence was detected.

The quality and integrity of the extracted RNA was assessed by gel electrophoresis (RNA migration on 1% agarose gel in TAE buffer 1x containing ethidium bromide 250 ng/ml, intercalant of the nucleic acids, which permits visualisation with a UV transilluminator).

For each sample, 1 µl of RNA was used, adding 2 µl of 6X Loading Buffer and H₂O+DEPC to reach a final volume of 12 µl.

Then RNA was stored at -80 °C until reverse transcription.

2.6 Reverse Transcription

Reverse transcription was performed using the iScript™ cDNA Synthesis Kit (BioRad, Italy), starting from 1 µg of RNA, added with H₂O+DEPC to a final volume of 15 µl. After adding 4 µl of iScript reaction mix and 1 µl of iScript Reverse Transcriptase the samples were incubated at 25°C for 5 minutes, 42°C for 30 minutes, 50°C for 15 minutes and, lastly, at 85°C for 5 minutes in order to inactivate the reaction. The samples were then placed under ice and briefly centrifuged. In this manner, the complementary DNA (cDNA) was obtained for each sample and was then preserved at -20°C.

2.7 Qualitative PCR

The cDNA of a control sample was amplified by qualitative PCR using the primers designed for Real Time PCR, to assess its performance.

The reagent mix without template was used as the negative control.

A reagent mix composed as follows:

- 5 μ l of Buffer Green 5X (Promega)
 - 0.5 μ l of 10mM dNTPs
 - 0.5 μ l of forward primer 50 μ M
 - 0.5 μ l of reverse primer 50 μ M
 - 0.15 μ l of Go Tag (Promega)
 - milliQ H₂O to reach a final volume of 25 μ l
- was added to 1 μ l of each cDNA.

Amplification of the templates was carried out in a thermal cycler (Therma Cyclor, Applied Biosystems) set as follows:

- 94°C for 3 mins: first denaturation of the DNA.

The successive 25 cycles:

- 94°C for 30 secs: second denaturation.
- 60°C for 30 secs: annealing temperature, the primers bind to the complementary areas on the DNA.
- 72°C for 30 secs: lengthening of the filaments.

Lastly, 72°C for 4 mins to complete the lengthening process.

2.8 Quantitative PCR – Real Time PCR

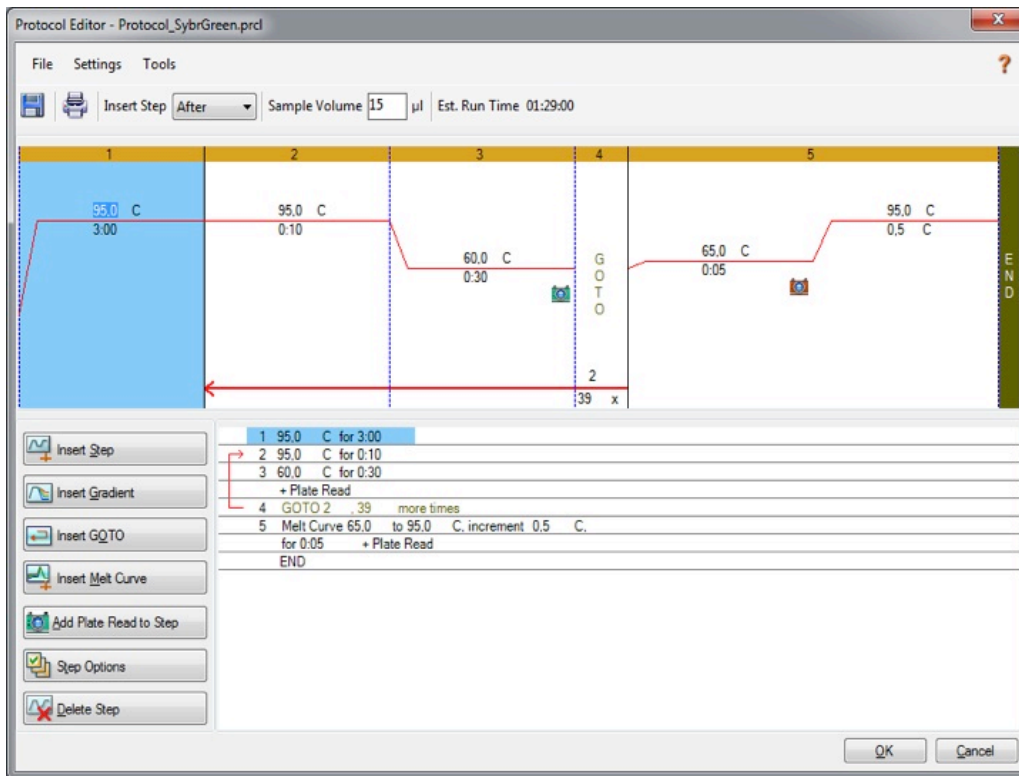
The Real-Time PCR was performed using the iTaq™ Universal SYBR® Green Supermix (BioRad, Italy). Specific primers were designed by Beacon Designer Program (BioRad, Italy) within the sequences of the genes showed in Table 4.

Table 4 – Sequences used for the selected genes

Gene Name	Sequence 5'-3'	Melting Temp (°C)	bp	Sequence Accession Number	
TGFβ1	<i>FW Primer</i>	CTGCCAGAGTGGTTATC	65.9	120	NM_000660.5
	<i>Rev Primer</i>	GTGTTATCCCTGCTGTCA	65.4		
Lactoferrin	<i>FW Primer</i>	CTAATCTCTGTGCTCTGTGTATTG	63.0	88	M93150.1
	<i>Rev Primer</i>	CCAGTGTAGCCGTAGTATCTC	63.2		
TNFα	<i>FWPrimer</i>	ATGGCGTGGAGCTGAGAG	65.3	78	HQ201306.2
	<i>Rev Primer</i>	TGAAGAGGACCTGGGAGTAGAT	65.8		
Mammaglobin A	<i>FW Primer</i>	GAAGTTGCTGATGGTCCTC	62.0	106	NM_002411.3
	<i>Rev Primer</i>	TTGTGGATTGATTGTCTTGGA	61.7		U33147.1
AQP5	<i>FW Primer</i>	GCTCAACAACAACAACG	62.1	90	NM_001651.3
	<i>Rev Primer</i>	TCAGTGGAGGCGAAGATG	62.9		
Cox-2	<i>FW Primer</i>	GTCTGGTGCCTGGTCTGA	65.3	115	M90100.1
	<i>Rev Primer</i>	GTCTGGAACAAGTCTCATCA	64.5		
MUC5AC	<i>FW Primer</i>	CATAACTTGTGGTCTGGAATA	63.9	107	L46721.1
	<i>Rev Primer</i>	CCGAGATTGTGCTGGTTGTA	64.2		
Caveolin 1	<i>FW Primer</i>	TGAGCGAGAAGCAAGTGTAC	64.2	80	BT007143.1
	<i>Rev Primer</i>	GTCATCGTTGAGGTGTTTAGGG	65.1		
PGDS	<i>FW Primer</i>	TGTAACCTGGGCAGACTTCTACT	65.3	123	NM_014485.2
	<i>Rev Primer</i>	GCAGGAATGGCTTGGACTT	64.6		

Each reaction tube was set up as follows: 7.5 µl of Sybr Green Supermix (2x), 1 µl of forward and reverse primer (6 µM), 1 µl of cDNA (diluted 1:5) and water to a final volume of 15 µl were mixed and run in the CFX 96 Thermocycler (BioRad, Italy). Thermal cycle was set as here reported: 5 minutes at 95 °C; 10 seconds at 95 °C and 30 seconds at 60 °C for 40 cycles (Figure 4).

Figure 4 - Thermal Cycles 1) Denaturation step (2-3-4) PCR (40 cycles) 5) Thermal Protocol for Melting Curves



In a first stage we tested the primers of the following three genes to be used in the role of housekeeping (Table 5):

GAPDH (Human glyceraldehyde-3-phosphate dehydrogenase):

GAPDH is an enzyme of approximately 37 kDa and is principally involved in the catalysis process in the 6th passage of anaerobic glycolysis [Tarze A et al., 2007]. It is an enzyme that is to be found in various tissues and is therefore a good candidate for the role of housekeeping gene.

Act_b (Homo sapiens actin beta): actin is a 43kDa globular protein and constitutes an abundant proportion (5-10%) of all eukaryote cells.

beta2-microglobulin (Homo sapiens beta-2 microglobulin):

beta2microglobulin is a component of the major histocompatibility complex of class 1 (MHC class 1) which is present in all the nucleated cells (except in the erythrocytes) [Güssow D et al., 1987].

Table 5 – Sequences used for housekeeping selected genes

Gene Name	Sequence 5'-3'	Melting Temperature (°C)	bp	Sequence Accession Number
GAPDH	<i>FW Primer</i>	ATCATCAGCAATGCCTCCT	60.9	M17851.1
	<i>Rev Primer</i>	GAGTCCTTCCACGATACCAA	60.5	
Act_b	<i>FW Primer</i>	ATGGGTCAGAAGGATTCC	59.8	NM_001101.3
	<i>Rev Primer</i>	CTCGATGGGGTACTTCAG	60.3	
Beta2-MG	<i>FW Primer</i>	CTATCCAGCGTACTCCAA	88	AF072097.1
	<i>Rev Primer</i>	GAAACCCAGACACATAGC		

Melting Curves, which give an indication about the purity of reaction's product (Figure 5, 6, 7) and reveal the possible presence of primer dimers, gave the following results for the three housekeeping genes.

Figure 5 - Melting curve for GAPDH.

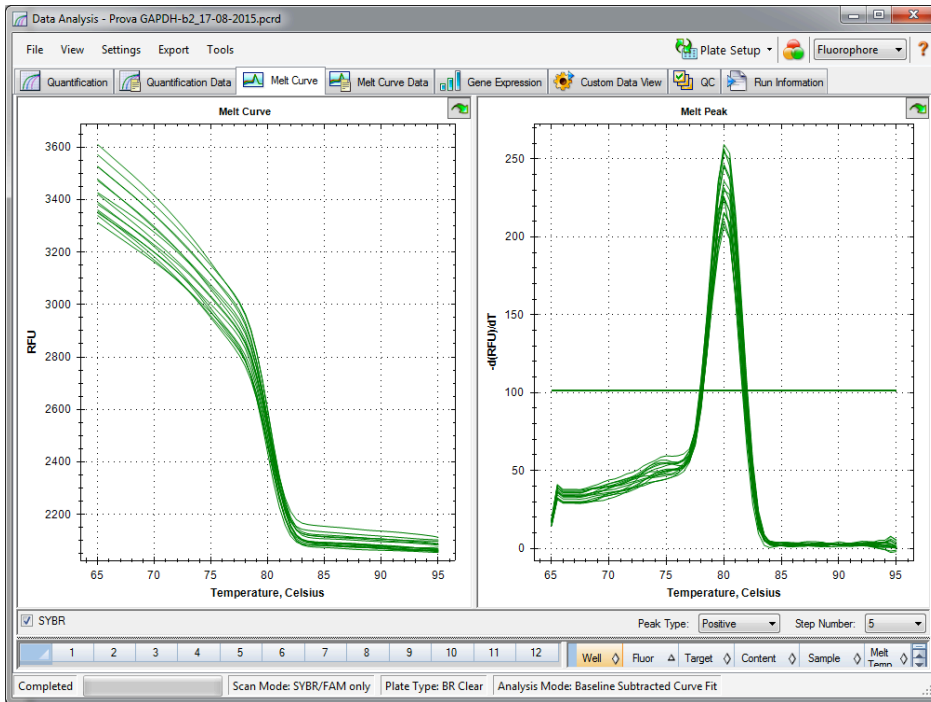


Figure 6 - Melting curve for BETA-2 microglobulin.

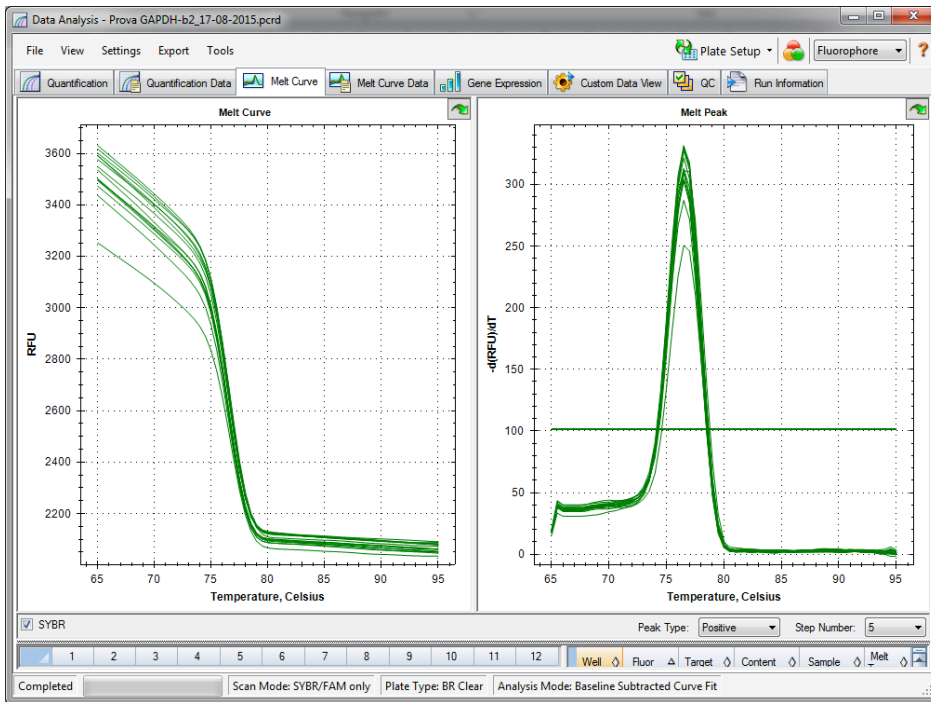
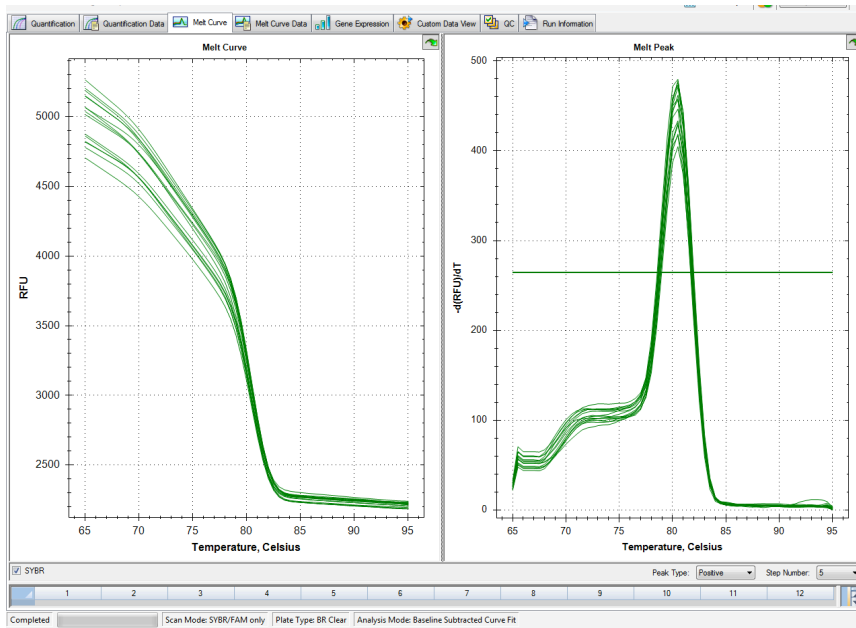
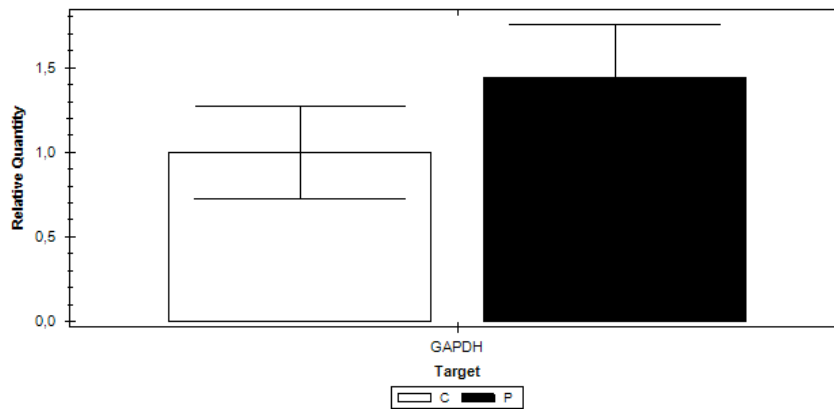


Figure 7 - Melting curve for BETA ACTIN.



After evaluating the expression of the three candidate genes, GAPDH was chosen as housekeeping gene, because of his variability, between the C and the P group, lower than the other two analyzed genes (Figure 8).

Figure 8 - Evaluation of the difference of expression of GAPDH between C Group and P Group , in choosing the housekeeping gene



2.9 Statistical analysis

The Ct values were recorded and the relative gene expression, expressed as $2^{-\Delta\Delta Ct}$ ($\Delta Ct = Ct_{\text{Target}} - Ct_{\text{GAPDH}}$ and $\Delta\Delta Ct = \Delta Ct_{\text{Polyp samples}} - \Delta Ct_{\text{Control samples}}$), was taken as dependent variables. Data analysis was performed by *t di Student* analysis ($p < 0.05$) in order to determine which mRNAs, measured in polyp tissues, were significantly different compared to those evaluated in the control tissues.

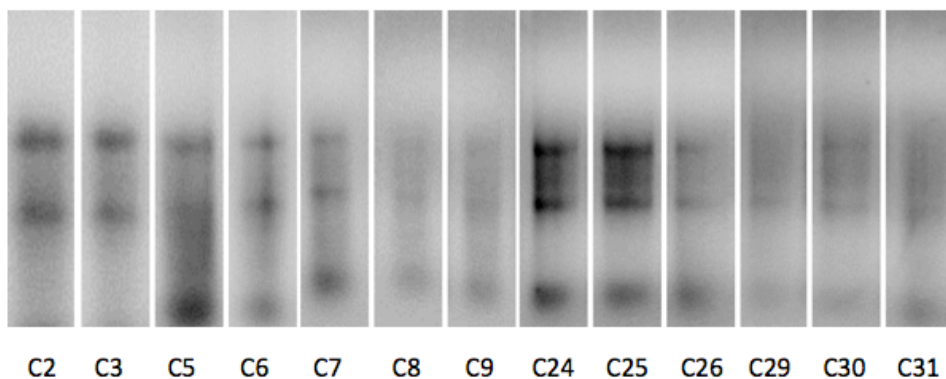
3 RESULTS

Despite we processed 85 samples, only 24 (28.2%) resulted to have a good quality RNA, in which intact 18S and 28S bands were observed.

3.1 RNA extraction – quality

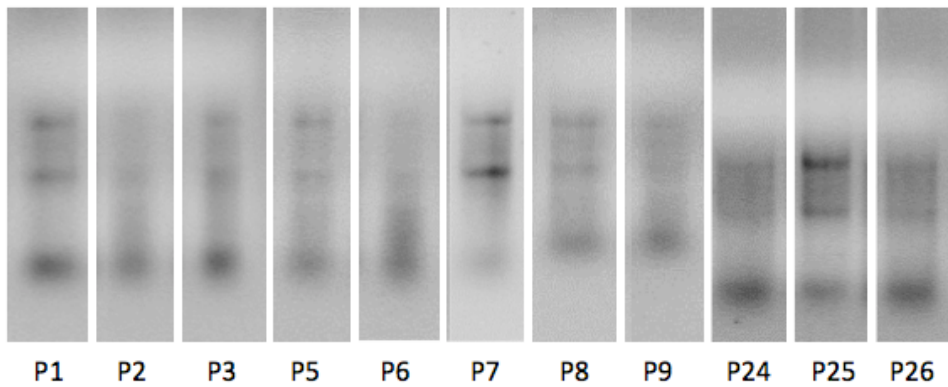
As can be seen in agarose gel electrophoresis of the two Groups (Figures 9, 10), in certain samples the RNA results to be partially degraded. This occurred particularly in the polypoid tissue specimens (Figure 9), showing that the problem was probably due to the extraction of non-sufficiently moist tissue or tissue with poor cell content.

Figure 9 -1% Agarose gel electrophoresis of C GROUP RNA



This problem was clearly less evident in the biopsies taken from healthy mucosa from Group C (Figure 10) (37,1% vs 22% of adequate biopsies).

Figure 10 - 1% Agarose gel electrophoresis of P GROUP RNA

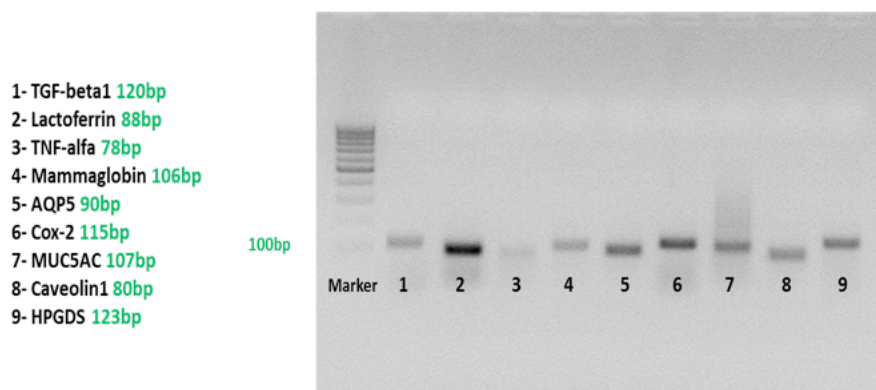


This problem does not seem to have been recurrently reported before in the literature and could well be the topic of further investigation as only rarely cited [i.e. Nicolis E et al., 1999].

3.2 Qualitative PCR

The primers designed for Real Time PCR (Beacon Designer 7.91) were tested by qualitative PCR to assess functioning (Figure 11).

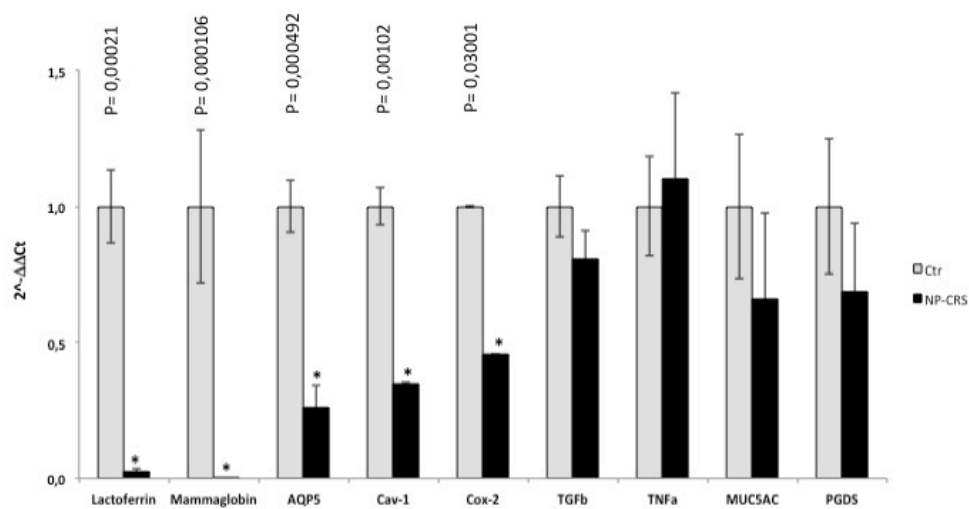
Figure 11 – 1% Agarose gel electrophoresis: amplification test with primers designed for Real Time PCR.



3.3 Quantitative PCR – Real Time PCR

Detected GAPDH as housekeeping gene, we proceeded to evaluate the expression of the target genes in Group 1 compared to control Group (Figure 12).

Figure 12 - Evaluation of the difference of expression of the selected genes between C Group and P Group



As reported in figure 12, in P Group samples five out of nine analyzed genes showed a statistically significant ($p < 0.05$) change in expression compared to controls.

They all showed a decrease in their expression in NP-CRS group, whether they are involved in innate host defense, or in various metabolic pathways, ionic balance or

transport systems; in particular, lactoferrin and mammaglobin expression decreased in a very highly significant way respect to the control.

The other genes tested showed no significant variations between the two groups.

4 DISCUSSION

The pathogenetic hypotheses, mentioned in the introduction chapter, for chronic rhinosinusitis are less in conflict than might appear. The interplay between exogenous agents and host defects conceptually links the theories together, although their single validity remains in flux, and under study.

Host factors that determine susceptibility to CRS depend, in part, on genetic variation across key pathways governing the immunobiology of the nasal mucosa. Even in case of cystic fibrosis, the prototype of genetic-CRS, a wide variation of sinus disease expression is nevertheless observed and multiple genes are involved in an individual patient determining clinical phenotype.

Attempts to identify additional genetic causes have been undertaken, drawn on multiple studies on other chronic inflammatory disorders, and this is a work in progress. These studies suggest not only the involvement of multiple genetic loci but also the importance of environmentally determined epigenetic changes.

CRS is definitely an heterogeneous disease that can be clinically phenotyped according to diverse variables: duration, presence of nasal polyps, recurrence of disease, severity, conventional therapy response, nature of triggering events, etc.

Although the CRSwNP phenotype can be readily determined using endoscopy, it is impacted by surgical state and medical therapies and has limited correlation to symptom severity. Additionally, there is likely a spectrum of disease from CRSsNP with mucosal edema to development of true nasal polyposis that is not accounted for using stiff phenotypic classifications.

However extensive scientific evidence is accumulating evidences that justifies a differentiation of sinus disease not only by phenotype (i.e. defined by an observable characteristic) but also by recognition of more detailed endotypes (i.e. defined by differences in pathogenetic mechanisms that can be discerned by the presence of particular biomarker patterns). Indeed CRS was classically divided into

CRSsNP, intended as a Th1 disorder, and CRSwNP, possibly defined as a Th2 disorder, but however it has been demonstrated that this paradigm does not apply worldwide [Wang X et al., 2016].

This is why there is a call for further endotyping, based on other patterns, including for example TGF-beta expression, Th2 bias and SAg-specific IgE, but current data are until now insufficient to propose a full endotypic characterization of CRS patients.

There has been significant progress toward understanding the aetiology and pathogenesis of CRS. CRS is still described as multifactorial and there is no clearly delineated single molecular pathway that explains the journey from injury to tissue change. There is however an emerging consensus that the persistent inflammation that defines CRS results from a dysfunctional host-environment interaction involving various exogenous agents and changes in the sinonasal mucosa. In concert with the definition of CRS as an inflammatory disorder, there has been movement away from pathogen-driven hypotheses. This overall concept is in agreement with the current understanding of the pathogenesis of chronic mucosal inflammatory disorders in general, which describe a balance of interactions between the host, commensal flora, potential pathogens and exogenous stresses. More original and replication studies are surely needed on this field to corroborate these last achievements.

During last years of biomolecular research mRNA expression of certain specific products have been considered an early marker of the cellular modifications and have also been used as prognostic indicator for many pathologies [i.e. Geomela et al., 2013]; so we wanted and tried to give our contribution to the characterization of this disease using the gene expression analysis technique, replicating some of the most significant recent studies to strengthen, or not, the evidences of previous research findings.

4.1 Difficulties in RNA extraction

Probably because of the high RNase content of the mucosa of the airways, we found major problems in getting good quality RNA from biopsies, often obtaining samples with degraded RNA even if it collected and processed with the maximum care. Moreover the procedure is quite simple and managed by expert hands both in our operating rooms and in our laboratories, where other comparable procedure on different tissues usually leads to excellent results.

For this reason, despite the 85 samples processed, the gene expression analysis has been conducted using 13 samples of healthy mucosa (control – C group, total 35 patients) and 11 samples of hyperplastic/polypoid mucosa (CRSwNP patients – P group, total 50 patients), chosen according to the integrity of the RNA in which intact 18S and 28S bands were observed.

Regarding this point is also to notice that, taking separately the initial number of C group samples (35) and the one of P group samples (50), the proportion of degraded RNA is greater in group P, since only 11/50 specimens were suitable for analysis (22%) while 13/35 in C group (about 37%).

We thought that this low proportion between the specimens taken and those suitable for the genetic study could be due to the fact that RNA degrades very quickly because of the ribonucleases that are present practically everywhere in the mucosa of the airways [Nicolis E et al., 1999]; if the tissue extracted is not immediately placed under dry ice, the specimen will quickly become unsuitable for RNA analysis. All samples that require longer handling time, such as when mucosa is separated from a fragment of bone attached to it, risk deterioration. It is implicit that preservation at -80°C requires dry ice or liquid nitrogen being available inside the operating room where the biopsies are performed. If these materials are not routinely available in an operating room, the time lapsing between sampling and

preservation, even if it was less than few minutes, might have been longer than necessary.

However in our specimen collection we tried to have maximum care of these aspects reducing the time of biopsy till few seconds, so we also suppose that polyp tissue is more easily degraded yet because the lower cell density in swollen/hyperplastic tissue.

During the first part of the study, due to the difficulty of extraction on the nasal polyps, we considered as an alternative to collect tissue from nasal septum or floor of the nasal fossa (usually not involved by polypoid/hyperplastic degeneration, but we decided not to proceed to avoid the risk to have biopsy from a wrong, not affected, mucosa. So at now we don't have any realistic solution to improve the score of validity of the samples.

We have not experience with solution to stabilize RNA, such as RNAlater, because we used dry ice storage that is considered to be an "ideal" method to preserve and store the RNA samples, available in our case.

4.2 Statistically significant results

Lactoferrin:

has become a topic of increasing interest in international literature, due to its numerous protective anti-bacterial, anti-viral, anti-fungal, anti-tumoural and anti-inflammatory activities [Zhang Y et al., 2014]. In effect, this latter activity of the protein has aroused particular interest in scientific research fields; its implication in sinonasal inflammatory disease is a relatively new aspect and opens the way to potential new therapeutic perspectives. Some studies highlight the capacity of this protein to inhibit fibroblast growth in polyps [Psaltis AJ et al., 2007] and its reduction in the tissues of patients with nasal polyposis (our P Group) [Acioğlu E et al., 2012]. Our results seem to strengthen these findings.

It is known that expression of antimicrobial peptides, such as lactoferrin and lysozyme, are part of the innate immune system that appeared dysregulated in biofilm-associated CRS [Psaltis AJ et al., 2007; Psaltis AJ et al., 2008; Wang X et al., 2014], in which the host's innate bacteria-clearing mechanisms is impaired, facilitating surface attachment and providing more adherence targets in order to reach the bacterial quorum required for biofilm formation [Tan L et al., 2010]. This is another point that could justify the downregulation of Lactoferrin in our CRSwNP patients (P group).

It is interesting to notice how lactoferrin levels in tissues seemed to be not influenced by CCS therapy [Acioglu E et al., 2012]. In our opinion, this aspect actually confirms that there is a possibility of associating topic CCS therapy, which is the current gold standard for maintenance treatment of nasal polyps, with one based on lactoferrin, and there are also in literature suggestions to dose mucosal levels of lactoferrin protein in CRS patients with the intent to provide grounds for its possible use in the treatment of nasal polyposis [Psaltis AJ et al., 2007].

Caveolin 1:

Damaging of host defense in innate immunity, such as the mechanism of epithelial barrier linked to the effectiveness of junction proteins [Hallstranda TS et al., 2014] may be involved in CRS pathogenesis [Hamilos DL, 2013] and is also well known that primary and acquired immunodeficiencies, determining dysfunction of the immune system, may manifest with CRS [Fokkens WJ et al., 2012].

Epithelial barrier damage may be due in part to the downregulation of junction proteins [Coyne CB et al., 2002] through inflammatory mediator release, directly via allergen proteolytic activity, or through dysregulation of membrane trafficking by downregulation of caveolin-1. This pathogenical mechanism was seen in the airway epithelium from asthmatic patients, which share with CRS patients many features.

Moreover Caveolin1 plays a central role in defence against infections and also in tumour suppression by reducing the production of Cyclin D1 and endothelial nitric oxide synthase. Saying that the significant downregulation of its gene in our P Group sounds plausible, and it is in agreement with what literature reports [Lin H et al., 2014; Hackett TL, 2013].

Aquaporine:

Aquaporine acts as a key tight junction protein in the maintenance of mucosal water homeostasis regulating cellular water transport and cell volume. Altered expression of AQPs has been revealed in several types of tumours upon their specific tissue localization [Jung HJ et al 2011].

Hypothesizing that AQP5 plays a possible role in the pathophysiology of mucosal edema, polyp formation and the production of thick secretion that is typical in CRSwNP, the down regulation of the gene codifying for AQP5, also reported by other Authors, suggest that the treatment of patients with polyps may necessitate strategies that target the epithelium, and possibly modulate AQP5, as other junction proteins, i.e. cavelolin-1 as expressed above [Frauenfelder C et al., 2014; Shikani AH et al. 2013]

Mammaglobin:

As already explained in the materials and methods chapter, the function of mammaglobin is unknown. Since it was found out that mammaglobin is overexpressed in breast cancer [Fleming T et al 2000], it began to be investigated in the oncological field. Its role in nasal polyps started to be postulated when it was discovered that its expression differs in CRSwNP patients compared to the healthy ones [Chusakul S et al 2008]. His role remain uncertain, but it is now known that other protein of Mammaglobin family are secretory proteins involved

in the modulation of inflammatory process and can bind steroids [Forsgren B et al., 2008; Miele L et al., 1994].

As far as its connection with nasal polyposis is quite concerned, certain studies have reported results that differ from one to another [Chusakul S et al 2008; Fritz SB et al., 2003]. A study demonstrated that mammaglobin is overexpressed in nasal polyps, on the grounds of these results, the authors have postulated that nasal polyps might show a neoplastic-like growth behaviour [Fritz SB et al., 2003]. The results we obtained in our series of samples suggest that mammaglobin A is significantly hypo expressed in pathological tissue according to what is reported in literature by other authors [Chusakul S et al 2008]. According to these same authors, the role that mammaglobin plays in polyposis might lie in its capacity to modulate inflammatory response and to bind to CCS. Only further studies will discover whether the expression of this protein is actually related to nasal polyposis and if it is one of the factors involved in the pathophysiology of this disease.

Cox-2:

In our study a significant decrease of the expression in CRSwNP group (P group). This result is agreement with the results of other Authors [Miłoński J et al., 2015]. As these Authors we believe that this result is due to a dysregulation of the inflammatory response involving the prostanoid metabolism considered to be a possible pathogenic mechanism involved in polyps formation.

By the way is not clear why Cox-2, normally induced in inflamed tissues, in NP results to be downregulated. Pujols L et al stated that COX isozymes appear to function coordinately in inflammatory processes and the lack of one COX isozyme can be compensated by the other: the variability of COX-1 mRNA in nasal polyps may represent the intention of COX-1 to compensate for the incapacity of COX-2 to respond to a stressful situation [Pujols L et al., 2004].

Of course this statement seems to be plausible also for our results, even if, in our own work we didn't evaluate Cox-1 gene expression.

4.3 Not statistically significant results

MUC5AC, TGFbeta and hPGDS were examined (Table 6) and showed a downregulation of their expression in the NP-CRS respect to the controls, but the differences are not statistically significant ($p > 0.05$). For MUC5AC and TGFbeta the result was however in accord with literature, not for hPGDS .

TNF upregulation was not statistically significant, but was in accord with literature.

Table 6 – Table of result's significance (statistically significant in our results are marked with *) related to literature

	Literature's	Ours
MUC5AC	↓	↓
Lactoferrin	↓	↓ *
TGFβ	↓	↓
AQP5	↓	↓ *
TNFα	↑	↑
Cox-2	↓	↓ *
Mammaglobin	↑ ↓	↓ *
Caveolin	↓	↓ *
hPGDS	↑	↓

5 CONCLUSIONS

Below the clinical observation level of CRS, there likely are several consistent pathophysiological mechanisms, different from each other and important in the presentation of the underlying inflammation: so we could say that there are “inflammatory endotypes” of CRS [Tomassen P et al., 2016]. These endotypes are “subtypes of disease defined functionally and pathologically by a molecular mechanism”. Each endotype might be identified by specific biomarkers, allowing reading the type of mucosal inflammation in nasal biopsies, nasal secretions, or better in the serum.

Biomarker originate from the encoding of specific genes.

Analysis of gene expression is the first signal of biologic modifications and in the last years it's often reported in association with proteomics.

In our study we analysed the comparison between the expression of selected genes in C Group (control Group, healthy patients) and in P Group (patient affected by chronic rhinosinusitis with nasal polyps without hypereosinophilia and/or other comorbidities). These candidate genes were selected from literature because of their demonstrated/theoretical hyper/hypo-expression at level of pathological polypoid tissue.

From the perspective of researcher, replication studies do not carry the same weight as an original piece of work, this mainly because Journals aren't eager to publish such works, not qualified as new researches. But from the perspective of contributing to scientific research, replication studies are really very important for the continued progress of science. Without validation, future researchers won't know whether to build on the findings of an original work.

Specifically on this field, concerning CRS, we think that a lot has been done in the last years in terms of trying to validate new theories, but few replication studies have been done to validate the original studies.

In our study most of the analysis we performed on the selected genes confirmed the results published in literature.

In this study for the first time in literature, in the same population many genes involved in the pathogenesis of the disease were analysed at the same time (in the past these genes were analysed one by one) and we obtained statistically significant result in 5 genes out of 9 analysed, and 4 of these corroborate literature results. These 5 genes are the ones towards which future researches could address further efforts.

Difficulty in mRNA extraction has to be taken into account for future studies, the technical problems may be addressed to the quality of the polypoid tissue, poor in cells and difficult to be treated. In this scenario the synergy between surgeon and laboratory is of great importance and the surgeon must have full knowledge of what happens during laboratory processing and what is needed.

In this kind of studies also collaboration between different specialists is of utmost importance (i.e. Pneumologist, Allergologist, Immunologist, ...) both for patients endotyping and in elaborating new treatments, in the perspective of a multidisciplinary treatment.

The next steps of our study may consist on the analysis of a Group of patients affected also by immunologic associated diseases, Recalcitrant Group (when CRSWNP is resistant to treatment, even if considered adequate, it is termed 'recalcitrant'), R Group (such as Hypereosinophilia, Asthma,...) comparing them to Groups C and P for the selected genes. Also new genes could be selected from literature for replication studies or original genes could be tested.

Lactoferrin, which gave us the best results in statistical terms, could be dosed also in blood of selected patient to correlate this information to our results and, in future, attempts of clinical application of our results could be made. Generally all the significant results given by this kind of studies can lead to further new experimental therapies.

Our results, enriching literature, will surely help us in the comprehension of the pathogenetic mechanism of CRS and in identifying its specific endotypes. Using well-defined endotypes in clinical studies might in fact allow to identify patient groups that best benefit from existing as well as new treatments [Bachert C et al., 2016] and this could result in personalized treatments and in a substantial improvement in future patient care.

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6 ABBREVIATION

AERD: aspirin exacerbated respiratory disease
AOAH: acyloxyacyl gene
APC: antigen-presenting cell
ASA: acetylsalicylic acid
BAFF: B-cell activating factor of the TNF family
CF: cystic fibrosis
CFTR: cystic fibrosis transmembrane conductance regulator
COX: cyclooxygenase
CRS: chronic rhinosinusitis
CRSsNP: chronic rhinosinusitis without nasal polyps
CRSwNP: chronic rhinosinusitis with nasal polyps
DC: dendritic cell
DNA: deoxyribonucleic acid
ECM: extracellular matrix
FESS: functional endoscopic sinus surgery
GWAS: genome-wide association study
HLA: human leukocyte antigen
IBD: inflammatory bowel disease
IFN- γ : interferon gamma
Ig: immunoglobulin
IL: interleukin
LAMA2: laminin alfa 2
LAMB1: laminin beta 1
MCC: mucociliary clearance
MET: met proto-oncogene
MMP: metalloproteinase
mRNA: messenger ribonucleic acid
NLR: NOD-like receptor
NOD: nucleotide-binding oligomerization domain
NP: nasal polyp
NSAID: non-steroidal anti-inflammatory drug
OR: odd ratio
PAI: plasminogen activator inhibitor
PAMP: pathogen-associated molecular pattern
PAR: protease activated receptor

PCR: polymerase chain reaction
PGE: prostaglandin
pGWAS: pooling-based genome-wide association study
PRR: pattern recognition receptor
qPCR: quantitative PCR
RT PCR: reverse transcription PCR
S100: calcium binding protein
SAg: superantigen
SERPINA: alfa 1 antitripsina
SNP: single nucleotide polymorphism
SPINK5: serine protease inhibitor Kazal-type 5
STAT3: signal transducer and activator of transcription 3
TGF- β R: transforming growth factor beta receptor
TGF- β : transforming growth factor beta
Th: T helper lymphocyte
Th1: T helper 1 lymphocyte
Th17: T helper 17 lymphocyte
Th2: T helper 2 lymphocyte
TIMP: tissue inhibitor of metalloproteinase
TLR: toll-like receptor
TNF: tumor necrosis factor
Treg: rugulatory T cell
uPA: urokinase plasminogen activator

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