UNIVERSITÀ DI FOGGIA

Doctoral Program

"INNOVATIVE SCIENCES AND TECHNOLOGIES FOR HEALTH"
XXXVII Cycle

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The Role of MUC1 in Chronic Rhinosinusitis with Nasal Polyps (CRSwNP): the Correlation with Disease Severity

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Academic Year 2023/2024

INDEX

CHAPTER 1

ANATOMY AND PHYSIOLOGY OF THE NOSE AND PARANASAL SINUSES

1.1 Nasal pyramid

The external nasal pyramid, composed of both osseous and cartilaginous structures, serves as a robust anatomical framework that protects the internal components of the nasal cavities. The external contour of the nose resembles a three-sided pyramid, with its base positioned inferiorly and its apex anchored to the frontal region. At the level of the frontonasal suture, a slight depression known as the nasion or radix nasi is present. Superior to this structure, at the level of the frontal sinus, lies a flattened bony region called the glabella, which is situated between two bony arches descending from the supraorbital region. The base of the pyramid houses the two nares, which are medially separated by a membranous septal structure known as the columella.

The nasal bones compose a major part of the nasal pyramid, form the upper third of the nose and make their junction at the level of the bridge of the nose. They are attached laterally to the maxilla by a strong bony attachment. Along with those maxillary structures, the nasal bones comprise the bony inlet to the face called the piriform aperture. In the middle of the inferior border of this aperture, there is the anterior nasal spine, which is a prominence that serves as landmark in septal surgery.

The middle third of the pyramid is made up of the upper lateral cartilages, which represent bilateral triangular extensions of the septal cartilage. These cartilages articulate with the undersurface of the nasal bones and are partially overlapped by them.

The lower third of the nasal pyramid is supported by the lower lateral or alar cartilages that are horseshoe-shaped structures composed of medial and lateral crus and provide the framework of the naris. The weak medial crus are incorporated with the contralateral one from the other side in the substance of the columella. The

lateral crura are delicate cartilages comprising the lateral aspect of the nostrils. The caudal margins of the upper lateral cartilages usually overlie the upper margins of the lower lateral cartilages, and by elevating the tip of the nose, this submucosal elevation can be seen within the nose. The junction is called the *limen nasi*. The internal nasal valve, the narrowest segment of the nasal airway, is delineated by the limen nasi, the head of the inferior turbinate, and the nasal septum. Small sesamoid cartilages are interposed between the upper and lower lateral cartilages, connected via fibrous tissue. This region is critical in regulating total nasal airflow resistance, and anatomical variations or inflammatory conditions can significantly impact nasal ventilation (**Fig. 1**).

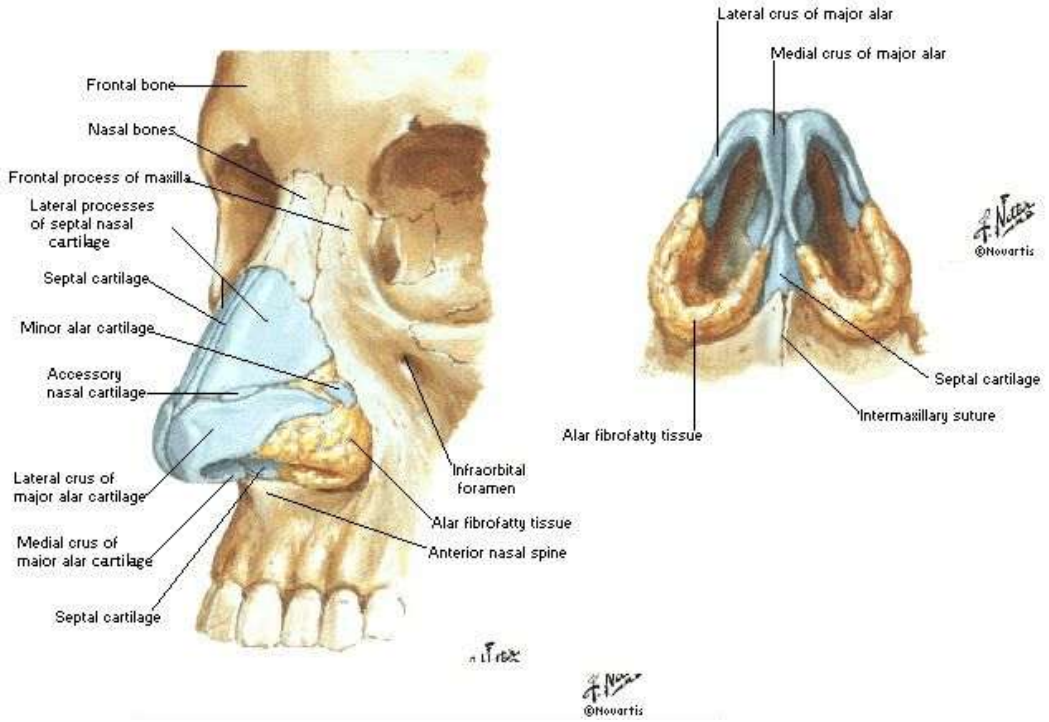

Fig. 1 The bony-cartilaginous structure of the external nose

The external nose, composed of both bony and cartilaginous structures, is enveloped by musculature and skin. The procerus muscle, a continuation of the musculus frontalis, functions to shorten the nasal length upon contraction. Additionally, the alar fibers of the nasalis muscle and the levator labii superioris alaeque nasi muscle contribute to nasal shortening while simultaneously facilitating nostril dilation. These muscles play a continuous role in modulating nasal airflow dynamics. Conversely, the transverse fibers of the nasalis muscle exert a

constrictive effect on the nares, reducing their diameter. The depressor septi muscle acts to lower the nasal tip, further decreasing the caliber of the nasal apertures.

1.2 Nasal septum

The nasal septum is a midline anatomical structure that bisects the nasal cavity into two symmetrical halves. It is composed of both cartilaginous and osseous components, including the quadrangular cartilage, the vomer, the perpendicular plate of the ethmoid bone, and the bony crests of the maxillary and palatine bones. Anteriorly, the quadrangular cartilage is anchored to the nasal spine of the maxilla via fibrous connective tissue. Posteriorly, it articulates with the vomer and the perpendicular plate of the ethmoid bone.

The septal cartilage maintains continuity with the upper lateral cartilages, forming a structural attachment to the nasal bones, which provide essential support to the septum. As a key component of the internal nasal valve, any deviation or malposition of the nasal septum can lead to functional nasal obstruction. The septal cartilage plays a crucial role in the development of the midface and typically reaches its adult dimensions by the age of two years. The posterior border of the nasal septum remains unattached, forming the posterior nasal apertures, also known as the choanae. Inferiorly, the nasal septum rests upon the crista nasalis of the bony palate. (***Fig.2***).

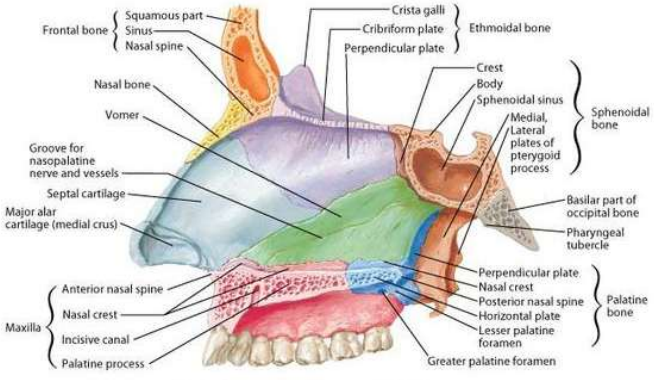

Fig.2 Bones and cartilagesof the nasal septum

1.3 Nasal Cavity

The nasal cavity is a roughly cylindrical, midline airway passage that extends from the nostrils anteriorly to the choana posteriorly. The choanae are formed by the horizontal plate of the palatine bone inferiorly, the vomer medially, the vaginal process of the sphenoid bone superiorly, and the medial pterygoid plate laterally. The floor of the nasal cavity consists in the palatal processes of the maxilla and in the horizontal processes of the palate bones. The alar or lower lateral cartilages, nasal bones, nasal processes of the frontal bones, and bodies of the ethmoid and sphenoid bones form the roof of the nose. The cribriform plate makes up the greatest part of the roof of the nasal lumen[1].

The lateral wall is formed by the inner surfaces of the maxillae and lacrimal bones and supports the three turbinates: the inferior, middle, and superior turbinate. A supreme turbinate can be observed in some patients. The middle and superior turbinates are, embryologically, extensions of the ethmoid bones. The inferior turbinate instead is a separate bone attached by its superior border to the nasal wall. The inferior turbinate forms an important part of the internal nasal valve. Any anatomic or physiologic disorders of this turbinate or the surrounding structures significantly can influence the nasal resistance[2]. The meatuses are three spaces located beneath each turbinate.

The superior meatus drains the sphenoid and posterior ethmoid sinuses.

The middle meatus drains the frontal, anterior ethmoid, and maxillary sinuses. These sinuses open by way of the hiatus semilunaris and into the infundibulum, a deep crescentic groove on lateral wall of this meatus. The groove is hidden by the anterior half of the overhanging middle turbinate. The inferior medial wall of the infundibulum forms a shelf-like ledge known as the uncinate process and above the ledge a hemispheric prominence termed the ethmoid bulla. This area under the middle turbinate into which the maxillary sinus, anterior ethmoidal air cells, and frontal sinus drain also is referred to as the ostiomeatal complex. This complex is an important structure in the intrinsic physiology of the paranasal sinuses. Blockage can occur because of crust formation or inflammation of the respiratory mucosa surrounding the ostium, leading to mucosal swelling, accumulation of secretions,

and superinfections. The ostiomeatal complex is also an important landmark for surgeons performing sinus surgery.

The inferior meatus contains the orifice of the nasolacrimal duct, located on the lateral surface, 1 cm beyond the head of the inferior turbinate (***Fig. 3***).

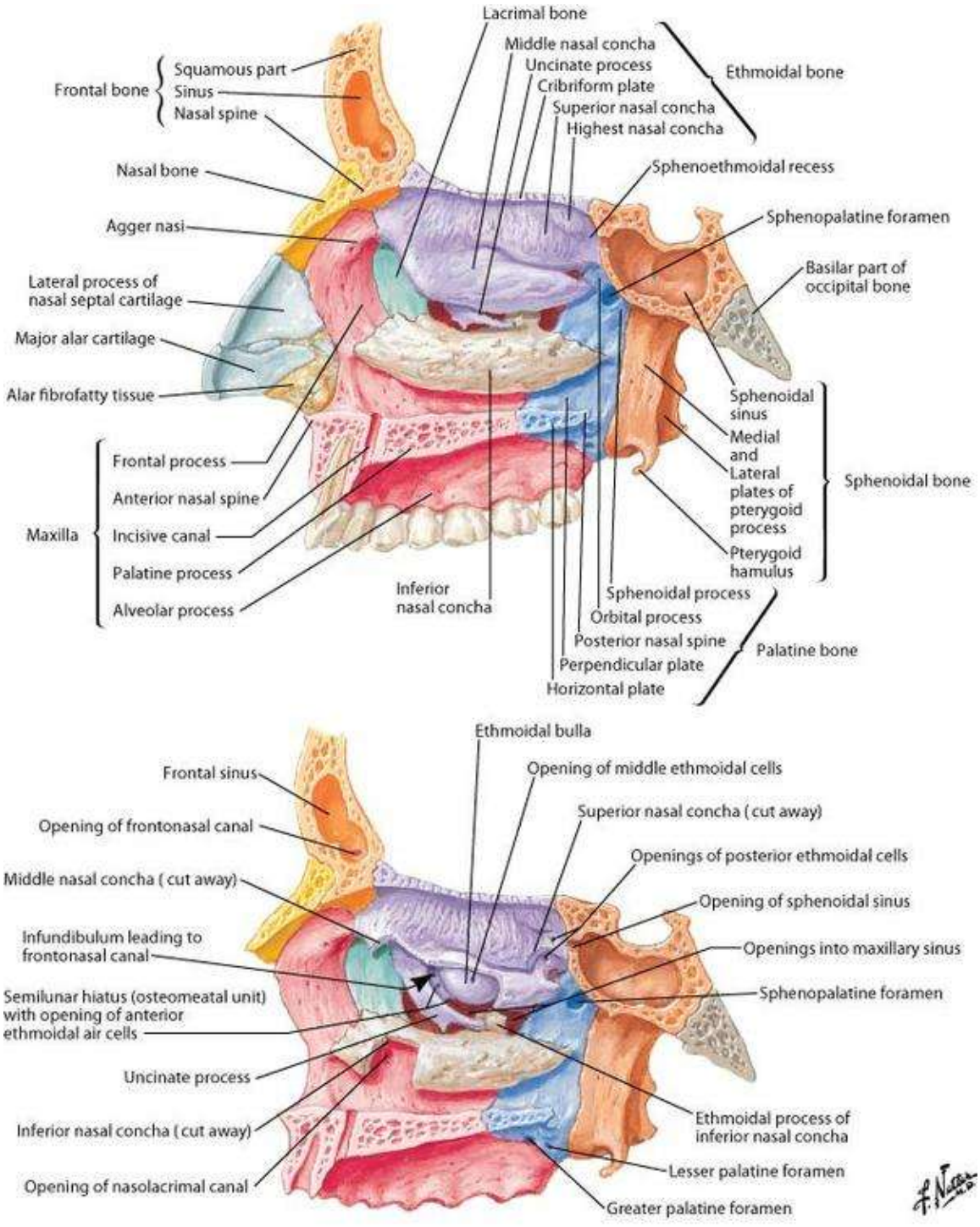

Fig. 3 Anatomy of the lateral wall

1.4 Paranasal sinuses

The nasal cavities are flanked on each side by the maxillary sinuses and roofed by the frontal, ethmoid, and sphenoid sinuses in an anterior to posterior fashion. All sinuses are lined with pseudostratified columnar epithelium. The maxillary sinuses are the largest ones, located under the eyes in the maxillary bones. The frontal sinuses are located superiorly to the eyes, within the frontal bone- The ethmoid sinuses are formed from several air cells within the ethmoid bone, between the nose and eyes. The sphenoid sinuses are located within the body of the sphenoid bone.

1.4.1 Maxillary Sinus

The maxillary sinus is a pneumatic cavity within the maxillary bone, exhibiting a pyramidal morphology. Its base faces the nasal cavity, while it is enclosed by anterior, posterior, superior walls, and a laterally directed blunt apex extending into the zygomatic process of the maxilla. In adults, its average volume is approximately 15 mL, making it the largest of the paranasal sinuses.

The anterior wall corresponds to the facial surface of the maxilla and contains three key anatomical landmarks: the canine fossa, the infraorbital foramen, and the infraorbital groove. The infraorbital foramen is located 5 to 8 mm inferior to the midpoint of the infraorbital margin. The posterior wall aligns with the maxillary tuberosity, which forms the anterior boundary of the pterygopalatine fossa. This wall is in close proximity to the neurovascular structures of the pterygopalatine fossa, including the pterygopalatine ganglion and several branches of the maxillary artery, vein, and nerve.

The superior wall forms the floor of the orbit, through which the infraorbital artery (a branch of the maxillary artery) and the infraorbital nerve (a branch of the maxillary division of the trigeminal nerve) traverse, entering the infraorbital groove.

The sinus floor is intimately related to the apices of the posterior maxillary teeth, separated only by a thin layer of compact bone. The mean distance between the dental apices and the sinus floor is approximately 1.97 mm, with molar roots exhibiting closer proximity compared to premolars.

Maxillary sinuses often contain septa, which are thin cortical bone partitions arising from the sinus floor and are best visualized on cone-beam CT scans. Primary septa develop during sinus formation, while secondary septa appear as a consequence of tooth loss. The maxillary sinus is lined by ciliated pseudostratified columnar epithelium, specialized for mucus production and clearance. This epithelium lacks a periosteal layer, and its cilia play a crucial role in draining mucus toward the ostium, with a higher density of cilia located near the ostium to facilitate efficient drainage.

1.4.2 Ethmoid sinus

The ethmoid sinus is situated in the anterior base of the skull and consists of a complex bony labyrinth of thin-walled cells. At birth, a few cells are already present, and they are, along with the maxillary sinus, the only sinuses that are large enough to be of clinical importance. In adulthood, about 6 to 10 ethmoid cells are present with a total volume of 2 to 3 mL. The lateral wall of the labyrinth constitutes the orbital plate, or lamina papyracea. The roof is formed by the frontal bone anteriorly and by the face of the sphenoid and the orbital process of the palatine bone posteriorly. The cells are divided into anterior and posterior ethmoid cells according to their location inside the ethmoidal complex and of the attachment of the middle turbinate. The anterior ethmoid cell drains into the infundibulum of the middle meatus and the posterior ethmoid cell into the superior meatus.

The complex ethmoidal labyrinth can be reduced into a series of lamellae based on embryologic precursors. These lamellae are obliquely oriented and lie parallel to each other. The first lamella is represented by the uncinate process. The second lamella corresponds to the ethmoid bulla. The third lamella is also known as the basal or ground lamella of the middle turbinate, which serves as the division of the anterior and posterior ethmoids. The anterior part inserts vertically into the crista ethmoidalis. The middle portion and the posterior third attaches into the lamina papyracea, respectively in an obliques and in a horizontal fashion. The fourth lamella is the superior turbinate. The agger nasi cell is the most anterior of the anterior ethmoid cells. It is found anterior and superior to the middle turbinate

attachment to the lateral wall. The posterior wall of the agger nasi cell forms the anterior wall of the frontal recess.

Some anatomic variations can induce disorders of ventilation or drainage of the paranasal sinuses. Occasionally, the middle turbinate can incorporate an ethmoid cell, called a concha bullosa. This pneumatization of the middle turbinate can obstruct the sinus drainage, but usually does not cause symptoms. Another anatomic variation of localization of ethmoid cells is the agger nasi cells. These cells are localized anterior to the anterosuperior attachment of the middle turbinate and lie in close proximity to the frontal recess. They can interfere with the frontal ventilation and drainage. Haller cells are ethmoid cells, which can obstruct the maxillary sinus drainage. Posteriorly, ethmoid cells can extend laterally or superiorly toward the nearby sphenoid sinus and the optic nerve, called Odoni cells. There are many variations of pneumatization of the ethmoid cells. The frontal and sphenoid sinuses frequently present asymmetrically.

1.4.3 Frontal sinus

Frontal sinuses are the most variable sinuses and are almost as individual as fingerprint. The typical volume of frontal sinuses is 4 to 7 mL. Up to 5% of the population present agenesis of one or both frontal sinuses. The posterior wall of the frontal sinus corresponds to the anterior wall of the anterior cranial fossa. The floor of this sinus cavity forms the upper part of the orbits. The sinus opens into the anterior part of the middle meatus by way of the frontonasal duct.

1.4.4. Sphenoid Sinus

The sphenoid sinus is located centrally and posteriorly within the body of the sphenoid bone, and it is posteriorly and superiorly bounded by the sella turcica. The sphenoid sinus, present only in humans and primates, can be identified from the age of two. It keeps developing throughout life but matures in size at around 12 to 14 years of age The typical adult size is 0.5 to 8 mL. Several important structures have a close relation to the sphenoid sinus, including the internal carotid artery and the optic nerve.

The carotid artery is located adjacent to the lateral wall of the sinus, and in 25% of patients, it is dehiscent in this area. The optic nerve leaves an anteroposterior recess on the roof of the sphenoid, and the overlying bone can be dehiscent in around 4% of individuals. The sphenoid sinus is also adjacent to the cavernous sinus and hypophysial gland. The patterns of the sphenoidal sinus pneumatization are classified into conchal, pre-sellar, and sellar, depending on the extension of pneumatization concerning the sella turcica. The sphenoid ostium drains into the sphenoethmoidal recess located within the superior meatus[3,4] (***Fig. 4***)

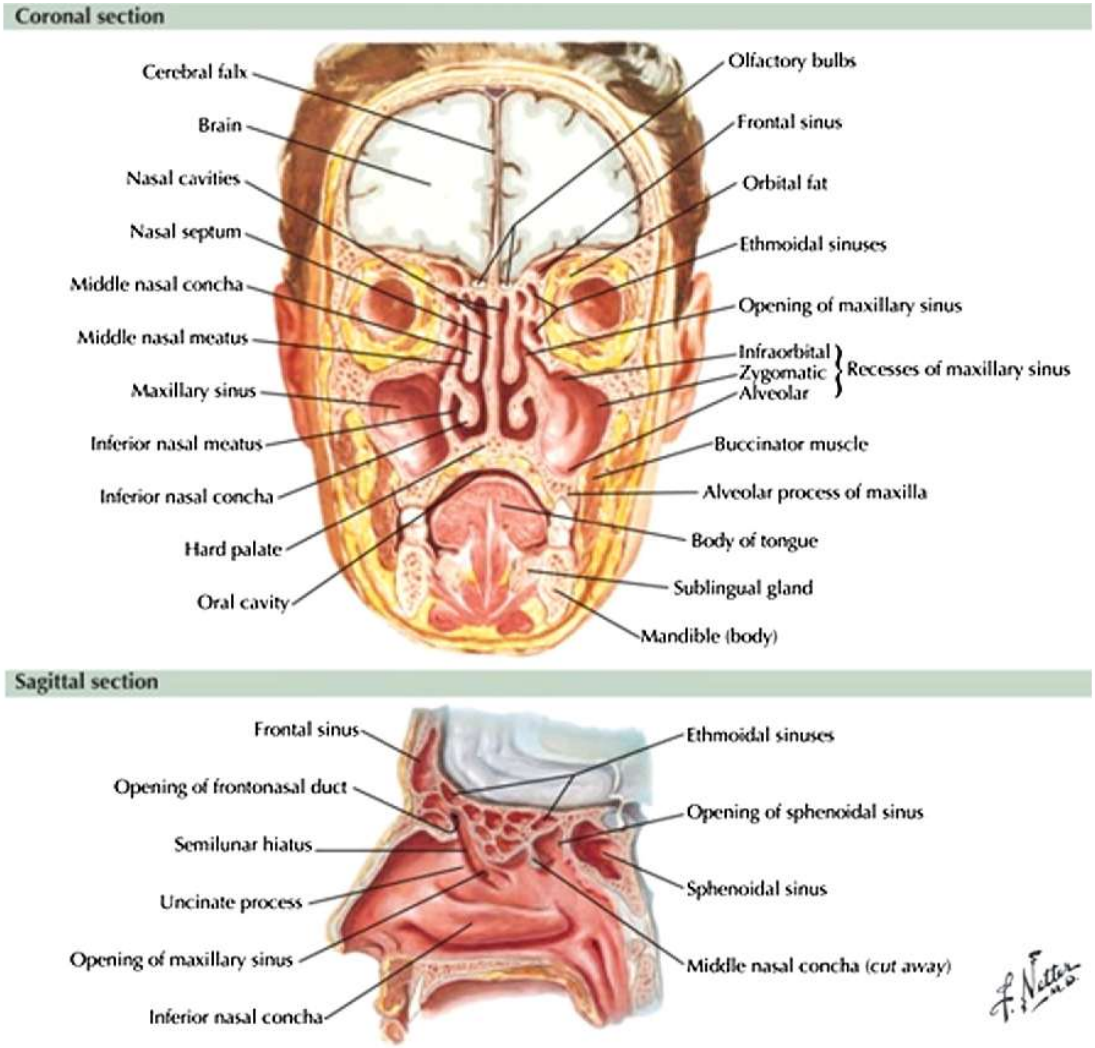

Fig. 4 Anatomy of the paranasal sinuses: coronal and sagittal view.

1.5 Histology of the nasal and paranasal mucosa

The anterior part of the nasal cavity, corresponding to the nasal vestibule, is lined with a squamous epithelium with vibrissae or coarse hairs, sebaceous glands, and sweat glands.

Inside the nasal cavity, three different types of epithelia can be observed. The anterior third of the nasal cavity and lines the anterior ends of the middle and inferior turbinates are covered with squamous and transitional epithelium. This epithelium contains cuboidal cells with microvilli. The posterior two thirds of the nasal cavity are lined by a pseudostratified columnar epithelium, called respiratory epithelium. This epithelium is composed of four major types of cells: ciliated (columnar) cells, non-ciliated (columnar) cells, goblet cells, and basal cells. This epithelium protects the upper and lower airways with the mucociliary clearance activity. Goblet cells produce an acidic mucin with a certain viscoelasticity, essential for good clearance. Ciliated cells are covered by cilia originating from basal bodies that also serve to anchor them to the cell. These cilia beat with a frequency of 1000 strokes per minute, and this beat consists of a rapid forward beat (effective stroke) and a slow return beat (recovery beat). The ratio of the columnar to goblet cells is about 4-5:1. Both of these epithelia lie on a basement membrane and a lamina propria[5,6].

The third epithelium is the olfactory epithelium, which covers the superior turbinate and adjacent septum. It is a pseudostratified epithelium containing olfactory cells (bipolar neurons acting like peripheral receptors and first-order ganglia), basal cells, and Bowman's glands (small serous tubulo-alveolar glands). Basal cells are small polygonal stem cells that give the olfactory epithelium the unique property for neural tissue regeneration after viral damage. The olfactory system is connected with the limbic system, reticular formation of the brain stem for odor-alerting response, hippocampus, thalamus, hypothalamus, and frontal lobe[7].

1.6.6 Vascularization and innervation

The mucosa of the nasal cavity is nourished by branches of the external and internal carotid arteries. The branches of the external carotid artery include the sphenopalatine artery (internal maxillary artery), the greater palatine artery (internal

maxillary artery), and the septal branch of the superior labial artery (facial artery). The sphenopalatine artery terminates into multiple branches including the posterior septal branch. The posterior septal branch crosses the face of the sphenoid sinus and supplies the posterior septum. The internal carotid artery system provides arterial supply to the nasal cavity via the anterior and posterior ethmoid arteries. These collectively supply the roof the of nasal cavity and the superior and dorsal portions of the septum[8].

The maxillary sinus is irrigated by branches of the maxillary artery: the infraorbital artery, the posterior superior alveolar artery, and the posterior lateral nasal artery. The infraorbital artery travels through the infraorbital groove and canal and then through the infraorbital foramen. The posterior superior alveolar artery runs along the sinus' medial wall. The posterior lateral nasal artery can also be found within the medial wall of the maxillary sinus. The frontal sinus vasculature consists of the supraorbital and supratrochlear arteries and ophthalmic and supraorbital veins. The sphenopalatine artery supplies the sphenoid sinus, and venous drainage is via the maxillary vein. The ethmoid sinuses are supplied by the anterior and posterior ethmoid arteries, respectively. These arteries are branches of the ophthalmic artery, a branch of the internal carotid artery. Ethmoid sinus venous drainage is done by the maxillary and ethmoid veins.

The innervation of the nasal cavities is sensory, through the olfactory nerve, and somato-visceral, through the trigeminal nerve and the sympathetic and parasympathetic systems. The center of the parasympathetic innervation is represented by the sphenopalatine ganglion, located in the pterygo-palatine fossa, which, through the sphenopalatine and vidian nerves, performs a secretory and vasodilatory action. Sympathetic fibers with vasoconstrictive action are part of the sphenopalatine nerve and reach the nasal mucosa without interrupting the sphenopalatine ganglion.

The innervation of the maxillary sinus is provided by branches of the maxillary nerve: middle, anterior, and posterior superior alveolar nerves, and infraorbital nerve. The sphenoid sinus' innervation is provided by the sphenopalatine nerve, which comprises parasympathetic fibers and CN V2. The frontal sinus is innervated

by the supraorbital and supratrochlear nerves (CNV1). The anterior and posterior ethmoid nerves provide innervation to ethmoid sinuses.

1.7 Physiology of the nose and paranasal sinuses

The nose and the paranasal sinuses perform the following functions: respiratory; detergent; defensive; air conditioning inspired; phonatory; reflexogenic; olfactory. In healthy individuals, inspired air is filtered, warmed, and humidified by the nasal mucosa before reaching the lung alveoli, assuring the protection for the lower airways. Moreover, part of the inspired air reaches the olfactory cleft to allow the recognition of the different olfactory substances. The nasal mucosa has also a specific role in the primary defense system against allergens, microorganisms, and other irritating products. This defense is sustained by mucociliary transport, inflammation, and humoral and cellular immune responses. The nasal cavity plays also has a role in resonance during phonation.[9]

The function of the paranasal sinuses is debated. However, they are believed to be implicated in several roles: decreasing the relative weight of the skull, increasing the resonance of the voice, providing a buffer against facial trauma, insulating sensitive structures from rapid temperature fluctuations in the nose, and humidifying and heating inspired air. Furthermore, they also play a role in immunological defense.

CHAPTER 2

CHRONIC RHINOSINUSITIS

Chronic rhinosinusitis (CRS), affecting approximately 5 to 12% of the general population, is a chronic inflammatory disease of the nasal mucosa, characterized by the presence of cardinal symptoms, such as nasal obstruction, rhinorrhea, facial pain and hyposmia or anosmia lasting longer than 12 weeks. According to EPOS 2020 Guidelines, the diagnosis of CRS also requires the presence of both endoscopic and radiological signs, including the presence of nasal polyps, mucopurulent discharge from the middle meatus and/or mucosal edema[10]. Given the significant burden on society, due to the impaired quality of life (QoL), losses in productivity and high direct and indirect health care costs, the understanding of pathophysiological processes underlying CRS and, therefore, of effective therapeutic strategies is of utmost importance[11]. Depending on the presence or the absence of nasal polyps, CRS has traditionally been classified in two major phenotypes: CRS with nasal polyps (CRSwNP) and CRS without nasal polyps (CRSsNP). Among CRS patients, 31% of them suffer from CRSwNP, which tends to be a clinically more severe disease, compared with CRSsNP. CRSwNP results from the dysfunctional interplay between host immunity, defective epithelial barrier, and environmental factors[12].

2.1 The emerging clinical relevance of CRS pathophysiology

Until about 20 years ago, the etiology and pathogenesis of CRS did not attract research attention, by virtue of decades-old presumptions that CRSsNP was the result of an incompletely treated acute bacterial infection and that CRSwNP had a relationship to local or systemic allergy[13]. According to these presumptions, in rough overview, CRSsNP patients were commonly treated with antibiotics and CRSwNP patients were treated with corticosteroids, while surgery was considered the option for failures.

Only recently, the advent of biological agents has led research to investigate the pathogenetic mechanisms underlying CRS and, in particular, CRSwNP. Nowadays, CRS is emerging to be a multifactorial disease resulting from the dysfunctional

interaction between the host immune system and environmental factors. However, the extent to which external factors contribute to the development of this pathology is not yet fully understood. Initially, fungi were indicated as the main pathogens responsible for the development of CRS. Than, *Staphylococcus aureus* was proposed as a rival pathogen, involved in biofilm formation and resistant infections[14,15]. Later, the general hypothesis of microbial dysbiosis replaced the previous ones that tried to identify a single pathogen responsible for the CRS[16]. However, antimicrobial therapies directed against fungi, bacteria such as *S. aureus* and the microbiome in general have not been effective. For this reason, the attention of research has focused on correcting any immune dysfunction in CRS patients. As a matter of fact, both the nose and the sinuses are not sterile. In healthy subjects, the nasal mucosa acts as a barrier that modulates the interaction with the immune system, promotes tolerance and symbiosis and prevents or limits inflammation. In CRS patients, the epithelial barrier damage leads to the penetration of exogenous agents, resulting in an inflammatory response that fails to resolve and induces tissue remodeling[17]. EPOS2020 Guidelines define three types of inflammatory patterns: type 1 immune responses targeting viruses; type 2 responses targeting parasites and type 3 targeting extracellular bacteria and fungi. In Western Countries, Type 2 inflammation, characterized by IL-4, IL-5, and IL-13 and by the activation of eosinophils, typically underlies CRSwNP. Type 2 endotype has been shown to be more prone to relapse compared with Type 1 and Type 3 endotypes, since it is associated with greater remodeling of sino-nasal tissues, including polyp formation, globet cell hyperplasia and epithelial barrier abnormalities[10].

2.2 Type 1, Type 2 and Type 3 patterns of inflammation

There is growing evidence that the clinical classification of CRS into 2 phenotypes, CRSwNP and CRSsNP, cannot provide full insight into the underlying pathophysiology in CRS, and that heterogeneous groups of disorders with distinct pathogenic mechanisms could be included in the two latter phenotypes. This heterogeneity of CRS influences the efficacy of medical and surgical treatments and contributes to the inadequate disease control experienced when using “one-size-fits-all” treatment approaches based on traditional phenotype-based

classification[18]. The increasing understanding of the pathophysiological mechanisms underlying CRS has led to a new classification of CRS, based on the different endotypes[19]. In Western countries, CRSwNP is well known to be characterized by Type 2 inflammation with pronounced eosinophilia and the presence of high levels of Type 2 cytokines, such as IL-4, IL-5 and IL-13[20]. In Type 2 CRSwNP, nasal polyps tissues are characterized by dysregulated epithelium, elevated Th2 cells, type 2 innate lymphoid cells (ILC2s), B cells, M2 macrophages, dendritic cells, mast cells, eosinophils, and basophils. The sino-nasal epithelium is the principal source of thymic stromal lymphopoietin (TSLP) which is essential for this patter on inflammation and activates ILC2s and effector Th2 cells. Other epithelial-derived innate Type 2 inducers, such as IL-25 and IL-33, have been reported to be elevated in nasal polyps. Type 2 inflammation leads to nasal polyps formation by promoting fibrin deposition, retention of plasma proteins and edema. On the other hand, non-Type 2 CRS is highly heterogeneous and can be subdivided based on the presence of inflammatory cytokines, including the Type 1 endotype with IFN-γ signaling and Type 3 endotype with IL-17 signaling[21]. In the two latter immune responses, epithelial cells secrete osteopontin in response to environmental triggers, which subsequently stimulates dendritic cells to induce Th1 and Th17 cells differentiation. In conjunction with Tc1 and Tc17 cells, Th1 and Th17 cells orchestrate neutrophilic inflammation by generating IFN-γ, IL-17A and IL-22. IFN-γ induces apoptosis of nasal epithelial cells, disrupts tight junction protein and stimulates neutrophil oxidative outburst, phagocytosis and chemotaxis. IL-17A upregulates IL-36γ in epithelial cells, which further amplifies neutrophilic inflammation. By synergistically combining with IL-17 and TNF-γ, IL-22 stimulates the production of cytokines and chemokines, as well as the recruitment of neutrophils. Neutrophils in turn secrete Transforming Growth Factor β2 (TGF-β2), which may contribute to fibrosis in CRSsNP and non-eosinophilic CRSwNP. Although CRSsNP has been traditionally considered to exhibit a Type 1-based immune response, recent studies have shown that there is geographical variation, and that the main endotype of CRSsNP is type 2 predominant[19,22].

2.3 Comorbidities

According to the "unified airway diseases theory", CRS and asthma often occur simultaneously as comorbidities, complicating their overall management[23]. Indeed, the prevalence of asthma is around 25% in patients with CRS, compared to 5% in the general population. Among patients with CRSwNP, up to 30% of them suffer from asthma[24].

CRS is also associated with Chronic Obstructive Pulmonary Disease (COPD), hypogammaglobulinemia, and Gastro-Esophageal Reflux Disease (GERD). Moreover, smoking, air-pollution and occupational exposure are negatively correlated with CRS symptoms[25,26].

The prevalence of allergy in CRS may vary according to the different phenotypes and a subgroup of patients with CRSwNP suffer from intolerance to nonsteroidal anti-inflammatory drugs (NSAID) and develop NSAID-exacerbated respiratory disease (NERD)[27].

Patients suffering from CRSwNP with comorbid NERD, asthma and allergy, together known as Widal's Triad, are more severe and difficult-to-treat[28].

2.4 Diagnostic tools for CRS

The diagnosis of CRS requires the presence of two or more symptoms, one of which should be either nasal obstruction or nasal discharge, eventually associated with facial pain/pressure and olfactory disorders together with endoscopic signs of nasal polyps and/or mucopurulent discharge primarily from middle meatus and/or oedema or mucosal obstruction primarily in middle meatus. Moreover, the diagnosis requires Computer Tomography (CT) changes, such as mucosal changes within the ostiomeatal complex and/or sinuses. Therefore, the evaluation of a CRS patient requires a complete medical history evaluation and clinical examination. The clinical history should be supported with an atopic evaluation, performed using skin-prick test, blood eosinophil count and serum total IgE[29]. An essential part of the rhinological examination is also represented by nasal endoscopy, which has been shown to have diagnostic accuracy comparable to CT[30]. The most commonly used scoring system for assessing nasal polyps size is represented by the Meltzer endoscopic polyp score (Total Nasal Polyp Score, TNPS) which assesses a score

between 0 and 4 to each nasal cavity as follows: 0= no polyps; 1= small polyps in the middle meatus/edema; 2= blocked middle meatus; 3= polyps extending beyond the middle meatus without complete obstruction; 4= massive nasal polyposis[31] (***Fig. 5***)

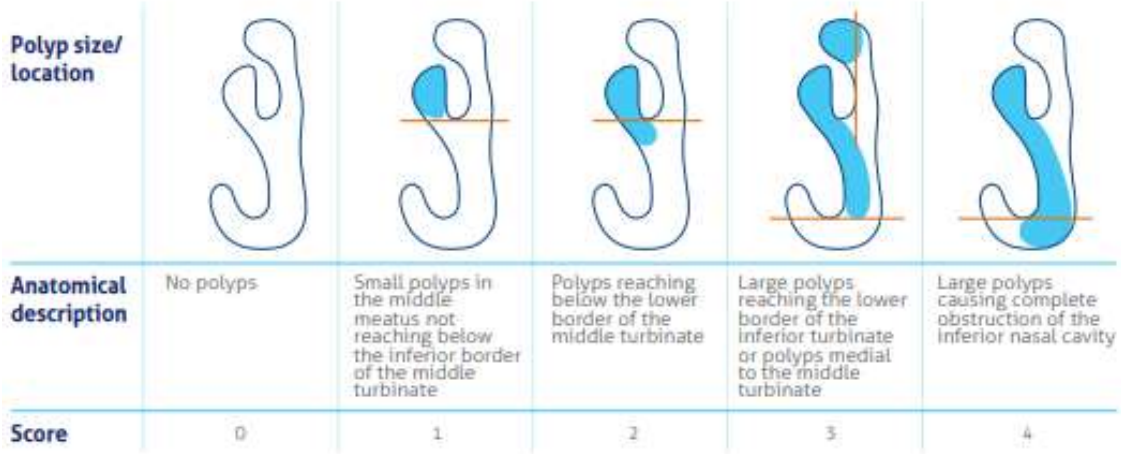

Fig. 5 Graphical representation of Meltzer endoscopic polyp score system.

As regards the different imaging modalities in diagnosis, among CT, cone beam CT and magnetic resonance imaging (MRI), CT scan represents the gold standard in the radiologic evaluation of rhinological diseases, notably CRS[32]. However, CT is not recommended in acute rhinosinusitis, unless the condition persists despite treatment or in case of complications[33]. The Lund-Mackay score is a widely used method for radiologic staging of CRS, that involves scoring 6 bilateral areas of sinus opacification (frontal sinus; anterior ethmoidal cells; posterior ethmoidal cells; maxillary sinus; sphenoid sinus; ostiomeatal complex) from 0 (no abnormality) to 2 (complete opacification), for a possible range of scores between 0 and 24[34] (***Fig. 6***).

Sinus	Right sinus	Left sinus
Frontal	0–2	0–2
Anterior ethmoids	0–2	0–2
Posterior ethmoids	0–2	0–2
Maxillary	0–2	0–2
Sphenoid	0–2	0–2
Ostiomeatal complex	0 or 2	0 or 2

For the sinuses: 0 = no inflammation; 1 = partial inflammation; 2 = 100% inflammation.
For the ostiomeatal complex: 0 = not occluded; 2 = occluded.
Maximum total score: 24.

Fig. 6 Lund-Mackay score.

Nasal obstruction can be objectively evaluated with peak nasal inspiratory flow (PNIF), (active anterior) rhinomanometry (AAR), and acoustic rhinometry (AR). The olfactory impairment instead can be assesses with the North American University of Pennsylvania Smell Identification Test (UPSIT), its short version (SIT, B-SIT) and the European Sniffin' Sticks[35].

The inflammatory cells infiltrating nasal mucosa can be assessed with nasal cytology.

The impairment of patients' QoL can be measured with the Sino-Nasal Outcomes (SNOT)-22 and the 12-item Patient Reported Outcomes in Chronic Rhinosinusitis (CRS-PRO)[36]. SNOT-22 items are scored on a scale of 0 ("no problem") to 5 ("problem as bad as it can be") and are categorized into the following 5 validated domains for CRSwNP: nasal (8 items), ear/facial (4 items), sleep (4 items), function (3 items), and emotion (3 items).14 The total score ranges from 0 to 110, and the domain scores are presented as the average item score per domain (0–5)[37](***Fig. 7***).

1. Considering how severe the problem is when you experience it and how often it happens, please rate each item below on how "bad" it is by circling the number that corresponds with how you feel using this scale: →	No Problem	Very Mild Problem	Mild or slight Problem	Moderate Problem	Severe Problem	Problem as bad as it can be		5 Most Important Items
1. Need to blow nose	**0**	**1**	**2**	**3**	**4**	**5**		○
2. Nasal Blockage	**0**	**1**	**2**	**3**	**4**	**5**		○
3. Sneezing	**0**	**1**	**2**	**3**	**4**	**5**		○
4. Runny nose	**0**	**1**	**2**	**3**	**4**	**5**		○
5. Cough	**0**	**1**	**2**	**3**	**4**	**5**		○
6. Post-nasal discharge	**0**	**1**	**2**	**3**	**4**	**5**		○
7. Thick nasal discharge	**0**	**1**	**2**	**3**	**4**	**5**		○
8. Ear fullness	**0**	**1**	**2**	**3**	**4**	**5**		○
9. Dizziness	**0**	**1**	**2**	**3**	**4**	**5**		○
10. Ear pain	**0**	**1**	**2**	**3**	**4**	**5**		○
11. Facial pain/pressure	**0**	**1**	**2**	**3**	**4**	**5**		○
12. Decreased Sense of Smell/Taste	**0**	**1**	**2**	**3**	**4**	**5**		○
13. Difficulty falling asleep	**0**	**1**	**2**	**3**	**4**	**5**		○
14. Wake up at night	**0**	**1**	**2**	**3**	**4**	**5**		○
15. Lack of a good night's sleep	**0**	**1**	**2**	**3**	**4**	**5**		○
16. Wake up tired	**0**	**1**	**2**	**3**	**4**	**5**		○
17. Fatigue	**0**	**1**	**2**	**3**	**4**	**5**		○
18. Reduced productivity	**0**	**1**	**2**	**3**	**4**	**5**		○
19. Reduced concentration	**0**	**1**	**2**	**3**	**4**	**5**		○
20. Frustrated/restless/irritable	**0**	**1**	**2**	**3**	**4**	**5**		○
21. Sad	**0**	**1**	**2**	**3**	**4**	**5**		○
22. Embarrassed	**0**	**1**	**2**	**3**	**4**	**5**		○

2. Please mark the most important items affecting your health (maximum of 5 items)_____ ↑

Fig. 7 SNOT-22 questionnaire

While SNOT-22 is the highest quality and most widely used CRS-specific instrument, CRS-PRO is a newly developed questionnaire which investigates 12 items concerning rhinological, psychological and sleep-related symptoms and which is proving to be valid in establishing both patient's QoL and the effectiveness of medical treatments over time[38] (***Fig. 8***).

CRS-PRO - Please answer each of the following questions about how your chronic rhinosinusitis affects you. In the past 7 days…					
Physical Symptoms	Not at all	A little bit	Somewhat	Quite a bit	Very Much
1) I had difficulty breathing through my nose	0	1	2	3	4
2) I felt pressure in my face	0	1	2	3	4
3) My face hurt	0	1	2	3	4
4) I had to blow my nose	0	1	2	3	4
5) I have been coughing	0	1	2	3	4
6) I had mucus in my throat	0	1	2	3	4
7) I had mucus in my nose	0	1	2	3	4
Sensory Impairment	Not at all	A little bit	Somewhat	Quite a bit	Very Much
8) I had problems with my sense of smell	0	1	2	3	4
Psychosocial Effects	Not at all	A little bit	Somewhat	Quite a bit	Very Much
9) My symptoms kept me awake at night.	0	1	2	3	4
10) I felt fatigued	0	1	2	3	4
11) I worried that my condition will get worse.	0	1	2	3	4
12) I was frustrated by my condition.	0	1	2	3	4

Fig. 8 CRS-PRO questionnaire.

2.5 Nasal polyps biopsies

To exclude differential diagnoses, nasal polyps can be biopsied, especially in the case of unilateral ones. From a histopathological point of view, nasal polyps were initially classified into four different histological patterns: edematous, fibroinflammatory, with pronounced hyperplasia of seromucous glands and with atypical stroma. The most common type was represented by edematous so-called "allergic" polyps, characterized by edema, goblet cell hyperplasia of the epithelium, thickening of the basement membrane, and of numerous leukocytes, predominantly eosinophils. The second histological type was the fibroinflammatory polyp, characterized by chronic inflammation and metaplastic changes of the overlying epithelium. Rare variants were represented by polyps with respectively pronounced hyperplasia of seromucous glands and with atypical stroma[39]. Over time, however, this classification was abandoned.

Today, Pathology Outlines, one of the most authoritative and updated anatomopathological databases, defines nasal polyps as benign, nonneoplastic inflammatory outgrowth of sino-nasal mucosa characterized by edematous stroma

infiltrated by mixed inflammatory cells. In particular, the edematous, fibrotic or loosely myxoid stroma is covered by respiratory epithelium, which is infiltrated by mixed inflammatory cells, including lymphocytes, plasma cells, eosinophils, neutrophils and mast cells. The surface epithelium can show ulceration or squamous metaplasia or rarely osseous metaplasia. Stroma cells can be bizarre, large, and pleomorphic. Submucosal glands are decreased or absent. Concurrent fungal infection may be seen[40].

Nevertheless, precisely because they are benign neoformations, over the years nasal polyps have not attracted the attention of pathologists and thus pathological evaluations have not been adapted to the current knowledge about the etiopathogenetic inflammatory mechanisms underlying the disease. This is the reason why histological reports of nasal polyps often end up being mere "copy and paste", mainly useful for excluding differential diagnoses but not for characterizing polyps.

2.6 New biomarkers of immunophlogosis

Since in Western countries CRSwNP is typically characterized by Type 2 (T2) inflammation with upregulated production of T2 cytokines (IL-4, IL-5 and IL-13) and pronounced tissue eosinophilia, several biomarkers of eosinophilic inflammation have been recently identified, including Galectin-10 (Gal-10). This glycan binding protein, also known as the Charcot-Leyden crystal protein (CLCP), is a major constituent of the cytoplasm of eosinophils and characteristically forms bi-pyramidal hexagonal crystals during cytolytic extracellular trap cell death (ETosis)[41]. Several studies have demonstrated elevated CLC protein levels in eosinophilic inflammatory diseases such as asthma, allergic rhinitis (AR), non-allergic rhinitis with eosinophilia syndrome (NARES), atopic dermatitis, amdparasitic infections [42,43]. Since CLC protein concentrations are positively correlated with percentages of eosinophils in both polyp tissue and peripheral blood, a great amount of CLC indicates a conspicuous eosinophilic infiltration and therefore an increased risk of recurrence after medical and/or surgical treatment[44]. The concentration of the CLC protein can be estimated in nasal secretions using commercial ELISA kits or through histological investigations on polyps

samples[45,46]. Moreover, a recent study conducted with immunofluorescence and confocal microscopy, has shown a strong correlation between Galectin-10 and the severity of CRSwNP, according to Clinical-Cytological Grading (CCG) scores. This study has also shown that Gal-10 extensively colocalize with CD15+ eosinophils and Tryptase+ mast-cells mainly within the *lamina propria*, while limited Gal-10 deposits can be detected around mucosal epithelial cells. Moreover, both Gal-10-positive eosinophils and mast-cells increased as CCG increases. Intriguingly, the analysis of Gal-10-positive deposits within the mucosal epithelium has revealed that they express a specific marker of eosinophils' derived vesicles, CD63, thus suggesting their origin from infiltrating eosinophils. The greater frequency of eosinophil-derived CD63+ Gal-10 deposits could explain the greater cytopathic effect observed within the *epithelium*, with increasing CCG[47].

The reliability and accuracy of Galectin-10 levels and CCG highlights an important problem: nowadays, the SNOT-22 test is the most widely used questionnaire for assessing the severity of CRSwNP, whereas the TNPS score is used to define the severity of nasal polyposis from an endoscopic point of view. In fact, according to EPOS2020 Guidelines, a SNOT-22 score ≥ 40, is one of the criteria needed for the administration of the biological drug[10]. However, even though the SNOT-22 questionnaire is a validated and widely used survey in the CRS, it represents a subjective diagnostic tool, less accurate than CCG[48].

Therefore, the SNOT-22 cannot objectively express the severity of CRSwNP. On the contrary, the CCG proved to be a true index of the severity of nasal polyposis as well as the risk of recurrence.

CHAPTER 3

NASAL CYTOLOGY

Nasal cytology (NC) is a non-invasive, cheap and easy-to-apply diagnostic tool, which, in the last decade, has become an integral part of the diagnostic process of sino-nasal disorders[49]. NC has a crucial role in rhinopaties follow-up, since thanks to its reproducibility, it allows to monitor the effectiveness of therapeutic strategies over time. This has significant therapeutic and prognostic implications, since the understanding of the cellular characteristics of diseases allows, in the era of Precision Medicine (PM), not only to guarantee the patient tailored treatments, but also to establish patients' prognosis, avoiding false expectations of recovery and identifying *a priori* the most difficult to treat and more prone to relapse forms[50].

3.1 Nasal cytology procedure

NC technique consists in sampling, processing and microscope reading. Cytological samples are obtained under anterior rhinoscopy by Nasal Scraping® (EP Medica, Italy) from the middle part of the inferior turbinate, where the optimal ratio of goblet cells to ciliated cells is 1:4, and immediately smeared on a glass side. After air-drying, samples are stained with May-Grunwald-Giemsa (MGG) and then read at optical microscopy, with a 1000x objective with oil immersion. A minimum of fifty fields is considered necessary to identify a sufficient number of cells (***Fig. 9***). According to the predominant type of inflammatory cell, four cytologic phenotypes are identified: neutrophilic, eosinophilic, mast-cell and mixed cellularity (eosinophil and mast-cells)[51]. Since NC is a cheap and painless procedure, it can be performed on the same patient periodically to monitor the progress of the inflammatory infiltrate.

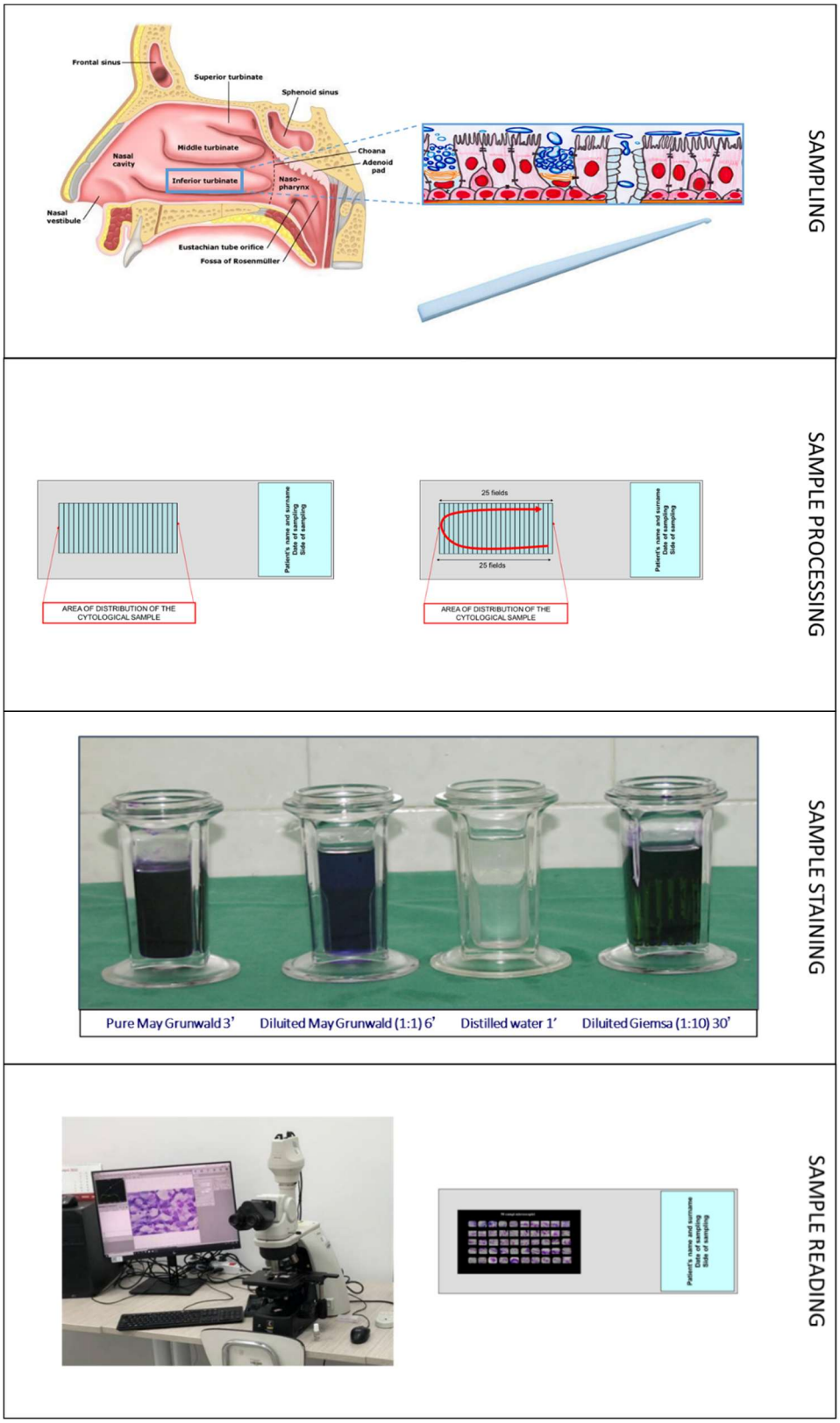

Fig. 9 This figure summarized the main steps of nasal cytology procedure: sampling, processing, staining and reading.

3.2 Epithelial barrier damage

Although the nasal mucosa has traditionally been considered a mere physical barrier between the host and its environment, it is becoming increasingly clear that it is metabolically active, playing a crucial role in maintaining the immunological barrier, producing various inhibitory substances and secretory IgA[52]. The integrity of the nasal mucosal barrier as well as any defects can be directly detected through histological samples. On the other hand, NC allows to indirectly detect such alterations. In particular, the Hyperchromatic Supranuclear Stria (SNS) is considered a specific cytological marker for the anatomic and functional integrity of ciliated cells and for the mucosal barrier integrity. Indeed, ciliated cells exhibit distress phenomena including the disappearance of the hyperchromatic SN in case of diseases affecting nasal mucosa epithelium. Therefore, the absence of the SNS is considered a useful prognostic sign of nasal disorders[53].

Among the mechanisms responsible for epithelial toxicity, the eosinophilic inflammatory infiltrate has been shown to play a noteworthy role, since eosinophils secrete several cationic proteins, including major basic protein (MBP), which induces a marked decrease in the number of desmosomes and an exfoliation of epithelial cells[54]. This explains why, in CRSwNP, the eosinophilic inflammatory infiltrate perpetuates the barrier damage, allowing the penetration of exogenous agents and the generation of a vicious circle that induces chronic inflammation.

3.3 The role of NC in CRSwNP patient's work-up

Over the past 20 years, advances in the field of technology and scientific research have dramatically changed the clinical approach to diagnosis and treatment of CRSwNP, paying greater attention to inflammatory infiltrate and to the cytokine inflammatory patterns that establish CRS. Therefore, several diagnostic procedure have been introduced to refine the diagnostic accuracy, including fibreoptic endoscopy, immunohistochemistry, biomarkers of inflammation and NC[46,55]. Among these procedures, NC has been widely recognized to non-invasively define the predominant inflammatory cells and, thus, the endotype of CRSwNP. Indeed, thanks to nasal cytology, a CCG has been proposed to assesse the severity of CRSwNP as a function of NC findings and comorbidities. In particular, each

comorbidity corresponds to a certain score: ASA sensitivity corresponds to 1, asthma to 2, allergy to 3, ASA sensitivity combined to asthma to 3. Likewise, a score is assigned to each inflammatory cell pattern and, in particular, neutrophilic infiltrate corresponds to 1, mast cell to 2, eosinophilic to 3, mixed eosinophilic-mast cell to 4. The final CCG score corresponds to the sum of the scores attributed to the endotype, represented by the predominant cellular infiltrate, and to the phenotype, represented by the various comorbidities.

The severity of CRSwNP is strictly associated with the refractoriness of the disease. In fact, the CCG corresponds to the Prognostic Index of Relapse (PIR), which is precisely the prognostic expression of CCG and in particular, as the score increases, from 1 to 10, the probability of relapse and non-responsiveness to medical and surgical treatments increases(***Fig. 10***).

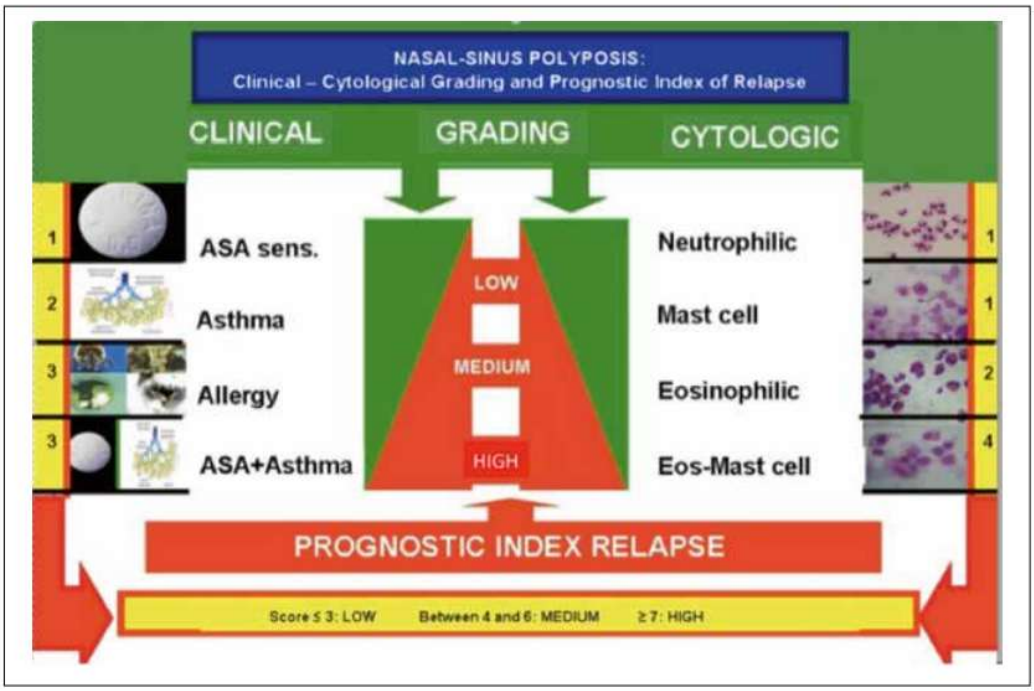

Fig. 10 Clinical-Cytological Grading and related Prognostic index of relapse. This scheme summarizes how to calculate the total CCG score, attributing a specific score to each comorbidity and to each inflammatory pattern.

Therefore, a patient with multiple comorbidities and a predominantly eosinophilic or mixed eosinophilic-mast cell infiltrate is more prone to relapse. As a matter of fact, the degree of eosinophilic infiltration is widely recognized as one of the main

indicators of severity and predictors of relapse and several biomarkers of eosinophilic inflammation have been identified[20]. NC has shown that not only eosinophils but also mast cells are involved in contributing to the severity of the disease, orchestrating the inflammation underlying CRSwNP and producing a series of type 2 inflammatory cytokines[56]. This cytologic point of view is of utmost importance, since in the era of Precision Medicine, several biological agents have been approved or are under evaluation for the treatment of CRSwNP[57,58]. These agents exhibit a broad mechanism of action on type 2 inflammation, acting on several targets. Hence the importance of clearly defining the CRSwNP endotype with diagnostic tools available today, first of all NC, and choosing a suitable treatment.

In this context, NC represents a useful tool for the follow-up of CRSwNP since it allows to monitor the changes of the nasal inflammatory infiltrate over time and to evaluate the effectiveness of the chosen treatment.

CHAPTER 3

BACKGROUND

In recent years, the study of mucins has gained significant attention due to their critical role in the pathophysiology of chronic respiratory diseases, including asthma, Chronic Obstructive Pulmonary Disease (COPD) and CRSwNP. Mucins, primarily composed of glycoproteins, are key structural components of mucus, which serves as the first line of defense against pathogens and harmful environmental agents in the airway. In CRSwNP, the overproduction and altered composition of mucins are associated with the chronic inflammation and tissue remodeling characteristic of the disease. Specifically, mucins such as MUC1, MUC5AC, and MUC5B are thought to play important roles in the regulation of the immune response and in the maintenance of airway epithelial integrity[59].

The understanding of mucin expression in CRSwNP, particularly in relation to disease severity, is still evolving. While MUC1 has been traditionally described as an anti-inflammatory molecule in various airway diseases, emerging evidence suggests that its role might be more complex. Some studies have shown the involvement of MUC1 in promoting inflammation, especially in the context of eosinophilic inflammation, which is prevalent in Type 2 CRSwNP[45]. Furthermore, MUC1 has been implicated in regulating the survival of eosinophils, a key feature in the inflammatory response in CRSwNP. Given its possible dual role, the relationship between MUC1 expression and disease severity in CRSwNP remains unclear, necessitating further investigation.

Based on this background, the current study aimed to investigate the expression of MUC1 in nasal polyps, its correlation with disease severity as assessed by Clinical Cytological Grading (CCG), and its interaction with other inflammatory cells, such as eosinophils and mast cells

CHAPTER 4

MATERIALS AND METHODS

Eighteen consecutive patients, including 13 males (72,22%), who underwent endoscopic sinus surgery (EES) for chronic rhinosinusitis with nasal polyposis (CRSwNP) at the Department of Otolaryngology of the University Hospital of Foggia were enrolled in the study. Diagnosis of CRSwNP was based on EPOS2020 criteria for symptoms and endoscopic and sinus CT findings.

The patients' ages ranged from 30 to 74 years, with a median age of 54.5 years. Before undergoing EES, Before surgery, each patient underwent a thorough clinical and anamnestic evaluation, as well as nasal cytology and nasal endoscopy. During surgery, under general anesthesia, endoscopically guided biopsy samples from nasal polyps were collected using Weil-Blakesley forces.

Specific exclusion criteria were presence of acute or chronic upper respiratory infections, previous or current specific immunotherapy, and use of nasal or oral corticosteroids, nasal or oral vasoconstrictors, antileukotrienes, antihistamines and monoclonal antibodies during the previous 6 weeks.

All participants provided written informed consent. The study was approved by the Area 1 Ethics Committee of the University Hospital of Foggia (44/CE/2022 dated April 4, 2022, and DCS n. 131 dated April 19, 2022).

Medical History

Each patient's clinical history was carefully assessed, with a specific focus on atopy, asthma, asthma, aspirin and other nonsteroidal anti-inflammatory drugs sensitivity (NSAIDs), as well as the number of previous polypectomy procedures.

Moreover, in order to assess, patients' health-related quality of life (HRQoL), they were asked to fill the Sino-Nasal Outcome Test (SNOT-22), a disease-specific questionnaire designed to assess five distinct domains including: rhinologic symptoms, extra-nasal rhinologic symptoms, ear / facial symptoms, psychological dysfunction, and sleep dysfunction[60].

Nasal Endoscopy

Preoperative nasal endoscopy was carried out by a 3.4 mm diameter flexible fiberscope (Vision-Sciences® ENT-2000) to confirm the diagnosis of CRSwNP. The size of sinonasal polyps was assessed using Meltzer endoscopic polyp scores (Total Nasal Polyp Score, TNPS). Based on this classification, each nasal cavity is scored from 0 to 4, with 0 indicating no visible nasal polyps and 4 indicating complete obstruction of the nasal cavity by nasal polyps. Combined left and right scores give a total possible score range of 0–8, with higher scores indicating larger nasal polyps and greater disease severity[61].

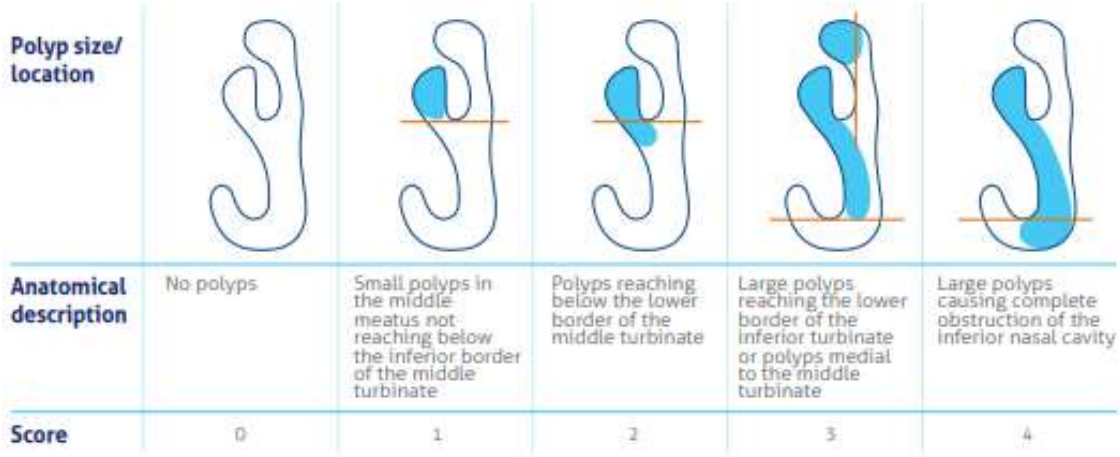

Fig. 5 Graphical representation of Meltzer endoscopic polyp score system.

Nasal Cytology

Cytological samples were preoperatively obtained using Nasal Scraping® (EP Medica, Italy). Under anterior rhinoscopy, samples were collected from the middle part of the inferior turbinate following validated criteria[49]. Immediately after collection, samples were smeared onto glass slides, air-dried, and stained with May-Grunwald-Giemsa (MGG). Stained samples were read at optical microscopy using a 1000x oil immersion objective. A minimum of fifty fields was analyzed to ensure an adequate number of cells[62]. The predominant inflammatory cell type in each sample was considered, allowing classification into four cytologic phenotypes: neutrophilic, eosinophilic, mast-cell, and mixed cellularity (eosinophils and mast cells) [63].

Clinical-Cytological Grading

The CCG is a score developed to determine the severity of CRSwNP based on nasal inflammatory infiltrate, defined by nasal cytology, and comorbidities, including asthma, allergy, and ASA sensitivity. Each comorbidity and cellular infiltrate pattern (such as eosinophils, neutrophils, and mast cells) is assigned a corresponding score. The total score categorizes three severity grades: low (1–3), moderate (4–6), and severe (>7)[64]. The CCG score corresponds to a Prognostic Index of Recurrence (PIR), which helps manage patients' expectations and consequently their adherence to personalized medical treatments and follow-up ambulatory visits

The recruited patients were stratified based on CCG and were subdivided in three groups according to their CCG score: group A: low grade, n=6; group B: moderate, n=6; and group C: high, n=6.

Indirect immunofluorescence and confocal laser scanning microscopy

Histological samples from 18 patients enrolled in the present study were processed at the Clinical Pathology Unit and Center of Molecular Medicine of our University. Serial sections 4 μm-thick were cut from formalin-fixed paraffin-embedded tissues, deparaffinized using xylene, rehydrated through a graded ethanol solution, rinsed for 5 minutes in distilled water and mounted on poly-L-lysine-coated glass slides.

To evaluate the expression of MUC-1, CD15, Tryptase and their eventual co-localization a double-label immunofluorescence was performed. The following primary antibodies were employed: rabbit polyclonal IgG anti-MUC-1 (clone EPR11197, Sigma Aldrich, Darmstadt Germania), mouse monoclonal IgM anti-CD15 (clone MMA; Ventana Medical System, Arizona USA); mouse monoclonal IgG_1 anti-Tryptase (clone G3; Ventana Medical System, Arizona USA). After padeparaffinization, tissue sections were permeabilized with 0.2% Triton x-100, blocked with 0.05% fetal bovine serum (FBS) and 2% bovine serum albumin (BSA) and then incubated overnight at 4°C with a mixture of primary antibodies diluted in the blocking solution.

Immune complexes were detected using the appropriate secondary antibodies: Alexa-Fluor 488 goat anti-rabbit IgG, 546 goat anti-mouse IgM, and 546 goat anti-mouse IgG1, as appropriate (all from Alexa, Thermo Fisher, Waltham, MA). After

three five-minute washes in PBS, the sections and negative controls were incubated at room temperature for one hour with the secondary antibodies diluted 1:250 in PBS.

For nuclear staining, the sections were washed in PBS pH 7.4 (3x5') and incubated with TO-PRO (1:3000 dilution in PBS, pH 7.4; Invitrogen-Molecular Probe, Thermo Fisher, Waltham, MA). Lastly, slides were mounted in Gel Mount (Sigma) and sealed.

Specific fluorescence was evaluated using a Leica TCS SP5 confocal microscope (Leica, Wetzlar, Germany) equipped with argon-krypton (488 nm), green-neon (543 nm), and helium-neon (633 nm) lasers. Fluorescence quantification was performed as described in previous studies.

Moreover, inflammatory cells expressing MUC-1, CD-15 and Tryptase were counted by confocal microscopy. In detail, ten high-power fields (HPF) for each sample were recorded and inflammatory cells were counted by two independent expert pathologists.

Statistical analysis

Statistical analysis was conducted using SPSS Statistics version 25.0 (SPSS Inc, Evanston, IL). Normality of data distribution was assessed using the Kolmogorov-Smirnov test. For comparisons between different groups, Student's t-test was used for normally distributed data, while the Mann-Whitney U test was applied for non-parametric data. One-way analysis of variance (ANOVA) was performed to compare means across more than two groups. A p value of ≤ 0.05 was considered statistically significant.

CHAPTER 5

RESULTS

The study population was subdivided into three groups based on the CCG of CRSwNP: low (n=6), medium (n=6), and high (n=6). The demographic characteristics, comorbidities, nasal cytology, CCG, as well as SNOT-22 and TNPS scores of each patient are summarized in **Table 1**.

Patient	Sex	Age, years	Surgical procedures underwent, N	Allergy	Asthma	ASA sensitivity	Predominant cytotype	CCG	SNOT-22	TNPS
1	M	66	1	No	No	No	Eos	Low (2)	24	5
2	M	59	1	No	No	No	Eos	Low (2)	8	6
3	M	31	1	No	No	No	Eos	Low (2)	20	5
4	M	48	1	No	No	No	Eos	Low (2)	17	5
5	M	63	1	No	No	No	Eos	Low (2)	22	6
6	M	71	1	No	No	No	Eos	Low (2)	22	7
7	M	55	1	Yes	No	No	Eos	Medium (5)	17	5
8	F	34	1	Yes	No	No	Eos	Medium (5)	27	6
9	M	63	2	Yes	No	No	Eos	Medium (5)	13	6
10	M	60	1	Yes	No	No	Eos	Medium (5)	8	7
11	M	40	1	Yes	No	No	Eos	Medium (5)	11	5
12	F	32	1	Yes	No	No	Eos	Medium (5)	27	5
13	M	54	1	Yes	Yes	No	Eos	High (7)	30	6
14	F	48	1	Yes	Yes	Yes	Eos-Mast cell	High (10)	23	7
15	F	30	2	Yes	Yes	Yes	Eos-Mast cell	High (10)	27	8
16	F	74	2	Yes	Yes	No	Eos-Mast cell	High (9)	16	5
17	M	33	2	Yes	Yes	No	Eos-Mast cell	High (9)	24	6
18	M	72	2	Yes	Yes	No	Eos-Mast cell	High (9)	42	8

Confocal microscopy analysis showed MUC-1 expression in tissue samples from the three study groups. MUC-1 was detectable in all examined tissues from CRSwNP patients. In the Low panels, characterized by a low CCG, MUC-1 expression was limited. In the Medium panels, a significant increase in MUC-1 expression was observed. Finally, in the High panels, MUC-1 expression was high, showing strong fluorescence and uniform distribution, with an elevated number of MUC-1 highly positive cells (***Figure 1A-I***). This observation was strengthened by quantification of specific fluorescence ($p<0.001$; ***Figure 1J***).

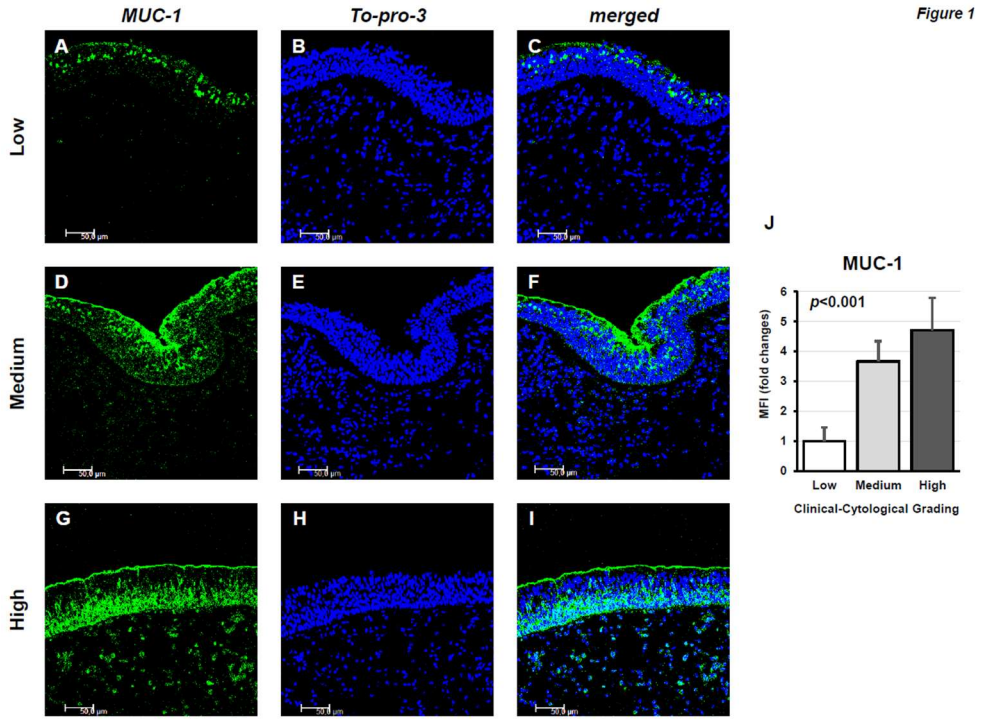

Figure 1. MUC-1 expression in nasal mucosa from patients with different CCG CRSwNP. A-I, immunofluorescence showing expression of MUC-1 (green) by nasal mucosa from patients with low (A-C), medium (D-F) and high (G-I) CCG CRSwNP. To-pro-3 counterstains nuclei (blue). Results are representative of 6 patients for each group. Magnification 400x. Bar = 50 μm. J, quantification of specific MUC-1 mean fluorescence intensity (MFI) among groups showing its increasing expression as low, medium and high CCG tissues were examined (ANOVA, $p<0.001$).

The expression of MUC-1 by both resident and inflammatory cells was then investigated. Interestingly, MUC-1 significantly colocalized with CD15+ eosinophils (***Figure 2***) and but not with Tryptase+ mast-cells (***Figure 3***). In particular, in low GCC patients, a limited expression of both MUC-1 and CD15 was shown. On the contrary, the expression of MUC-1 and CD15 significantly increased as medium and high CCG tissues were examined, respectively. Moreover, if the inflammatory CD-15+ cells expressing MUC-1 were evaluated, their number increased as low, medium and high CCG grading tissues were examined. A specific quantization of double-positive MUC-1+ CD15+ eosinophils confirmed their rising number depending on the CCG grading ($p<0.001$), suggesting a potential

synergistic interaction between the two markers, related to the progression and severity of the disease (***Figure 2A-L***).

Evaluating the expression of MUC-1 and Tryptase, it was observed that in low CCG samples, the expression of both markers was limited or absent, with no significant co-localization between the two markers. In medium CCG samples, the expression of both markers increased, with limited co-localization. However a statistically significant correlation between the expression of MUC-1 and Tryptase was found only in high CCG samples, although without colocalization, a suggesting distinct but potentially related processes (***Figure 3A-L***).

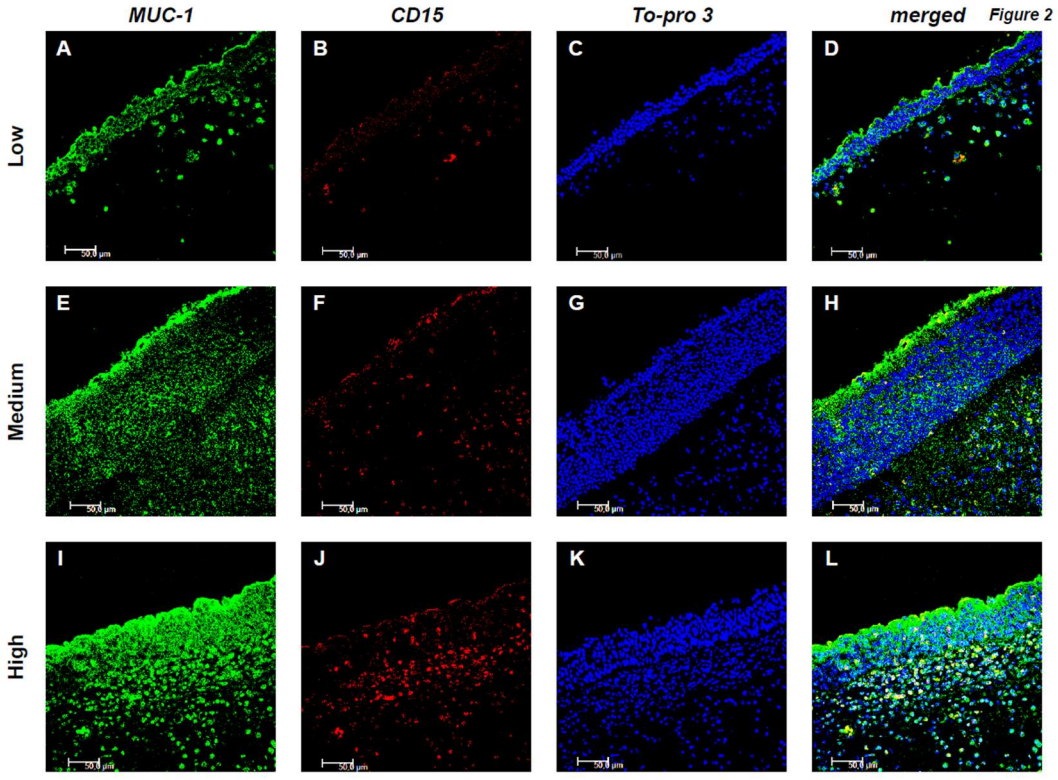

Figure 2. Colocalization of MUC-1 and CD15+ eosinophils. A-L, double-label immunofluorescence showing expression of MUC-1 (green) and CD15 (red) by nasal mucosa from patients with low (A-D), medium (E-H) and high (I-L) CCG CRSwNP. To-pro-3 counterstains nuclei (blue). Merged images (yellow) demonstrate significantly increased expression of MUC-1 by eosinophils with increasing CCG. Results are representative of 6 patients for each group. Magnification 400x. Bar = 50 μm.

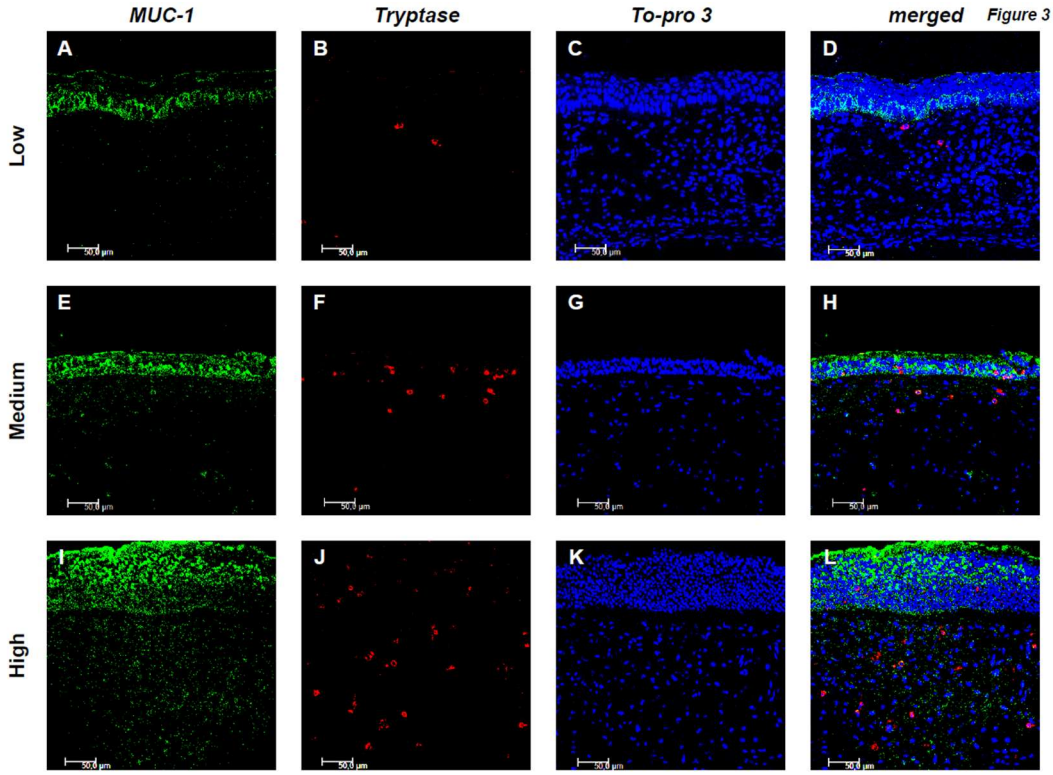

Figure 3. Colocalization of MUC-1 and Tryptase+ mast cells. A-L, double-label immunofluorescence showing expression of MUC-1 (green) and Tryptase (red) by nasal mucosa from patients with low (A-D), medium (E-H) and high (I-L) CCG CRSwNP. To-pro-3 counterstains nuclei (blue). Merged images (yellow) demonstrate significantly increased expression of MUC-1 by mast-cells with increasing CCG. Results are representative of 6 patients for each group. Magnification 400x. Bar = 50 µm.

Aspecific quantization of both CD15+ eosinophils and Tryptase+ mast-cells confirmed their rising number depending on the CCG ($p<0.001$ for eosinophils and mast-cells, respectively; Figure ***4A-B***).

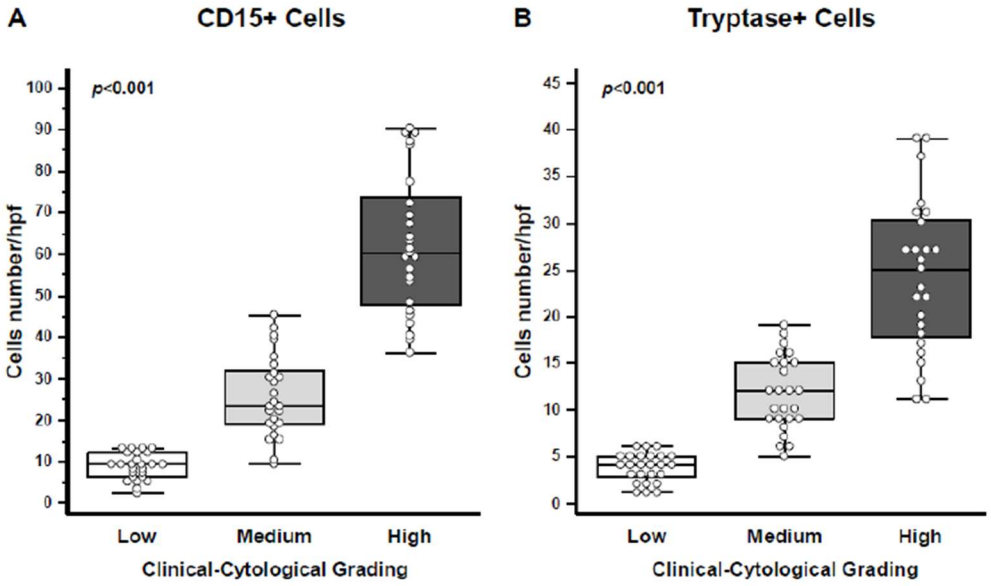

Figure 4. Quantification of eosinophils and mast-cells. A, quantification of CD15+ eosinophils showing increased number of cells per HPF cells with increasing CCG (ANOVA $p<0.001$). B, quantification of Tryptase+ mast-cells showing increased number of cells per HPF with increasing CCG (ANOVA $p<0.001$). Data in the graphs are expressed as median and 25th and 75th percentiles in boxes and 5th and 95th percentiles as whiskers, while circles represent individual data.

CHAPTER 6

DISCUSSION

Airway mucin overproduction is nowadays considered the hallmark risk factor of several chronic respiratory diseases, including asthma, chronic obstructive pulmonary disease (COPD) and cystic fibrosis[65]. According to the unified airway theory, which suggests that the nose and lungs share common pathogenic mechanisms and structural changes, mucins play a critical role in the pathophysiological changes in CRS as well[66].

Since no effective targeted treatment for mucin overproduction in the airways has been developed to date, there is growing interest among researchers in developing personalized and effective treatments aimed at regulating mucins expression, which are the main components of mucus.

Mucus is the first line defense barrier of airway epithelial against several pathogens and environmental agents. In healthy individuals, mucociliary clearance (MCC) is part of the innate defense mechanism and consists of two equally important components: mucus production and mucus transport. When MCC is compromised, airways become vulnerable to a vicious cycle of infection and obstruction. This phenomenon is particularly evident in patients with CRS who experience relentless cycles of infection and inflammation, resulting in ciliary loss and hyper viscous mucus blanket, which generate dysfunctional mucociliary coupling[67].

The sources of mucus secretion are goblet cells present in an outer epithelial layer and submucosal glands. The human respiratory mucus is mostly composed of water (95%). The solid phase is composed of mucins, non-mucin proteins, enzymes, salts, lipids, and cellular debris, which allows the mucus to be classified as a gel with properties of both a soft, elastic solid and a viscous fluid[68].

The hydration status of the mucus is measured as the wet-to-dry content of mucus or as the mucin concentration, which are the major component of mucus. The secreted mucin polymers interweave to form mesh-like gels with mesh sizes that are concentration dependent. Mucins are distributed in two different layers: mucus

layer and periciliary layer. The periciliary layer is a dense gel composed of transmembrane or membrane-bound glycopolymers, including the MUC1, MUC 3, MUC4, MUC12, MUC16 and MUC17 mucins, which are tethered to epithelial-cell surfaces and cilia. The mucus layer is composed of secreted/gel forming mucins, such as MUC2, MU5, MUC6, MUC19. When there is a balance of non-aqueous components, the evaporation of water can lead to crystallization of the electrolytes, and the fern formation occurs[69]. The fern pattern varies depending on the patient's health status and can be evaluated according to Rolando's score: healthy patients have a type I (uniform fern pattern with closely branching arborization) or type II (well-distinguished but less branching arborization) fern pattern. Patients suffering from rhinopaties present with "pathologic" type III (scarce or single ferns) or type IV (absence of ferns) fern patterns[70].

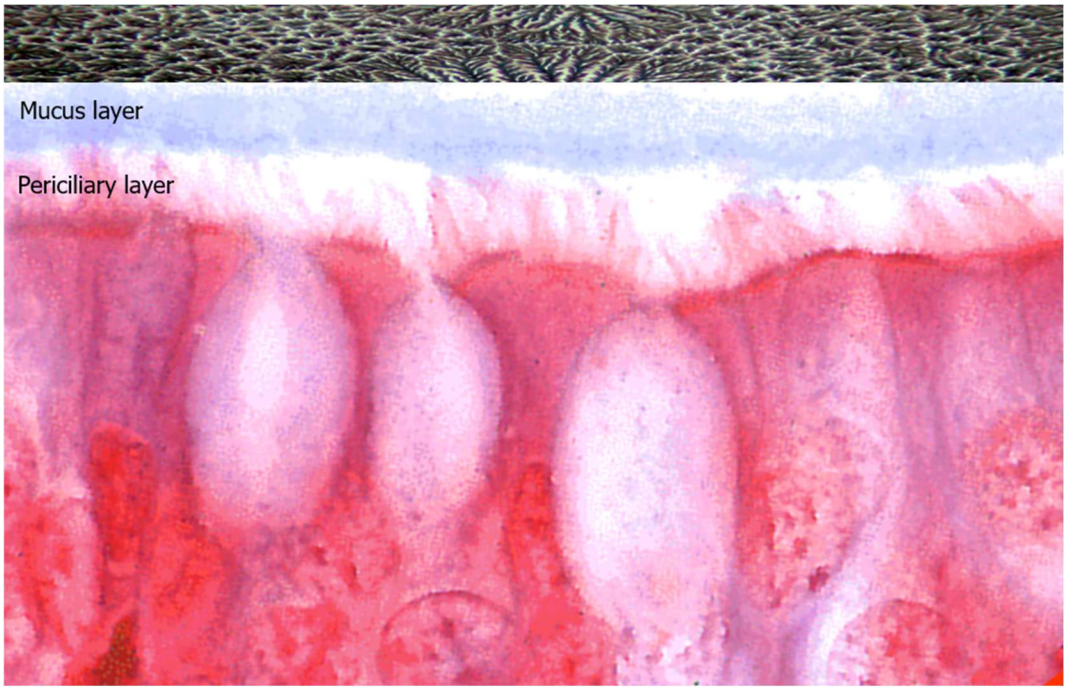

Figure. 5 Representation of ciliated epithelial cells covered by two distinct mucin distribution layers: the mucus layer and the periciliary layer. Above, an example of nasal ferning, observed under phase-contrast microscopy at 1000x magnification.

Under physiological conditions, mucins serve various functions, including cell proliferation, inflammation, immune response, and cell-cell adhesion. However, abnormal mucin expression, decreased clearance, and excessive mucus formation can lead to airway obstruction and exacerbation of preexisting airway disease, and turn a powerful innate cleaning defense system into a detrimental mechanism[71].

Currently, research on mucin primarily focuses on MUC5AC and MUC5B. In particular, MUC5AC has been shown to play a key role in the inflammatory response of the respiratory tract in general, while MUC5B appears to have a specific role in the development of CRS[72,73]. Nevertheless, the chronic inflammatory pathways underlying CRS result in cellular alterations and structural remodeling of the nasal mucosa that led to a change in the rheological properties of mucus, with altered expression of other mucins, including MUC1[74].

MUC1 is a highly glycosylated transmembrane protein expressed mainly in the apical surface of most glandular epithelial cells. Interestingly, MUC1 is known to display a regulatory role in mucosal immunity and is primarily involved in the formation of a physical barrier to lubricate and protect normal epithelial tissues, limiting infection and colonization, and mediate signal transduction. Several studies have shown that MUC1 acts as an anti-inflammatory molecule in several airway infections and mediates the expression of anti-inflammatory genes in lung diseases such as chronic rhinosinusitis, chronic obstructive pulmonary disease and severe asthma[75]. However, dysregulated and inappropriately glycosylated MUC1 have been correlated with chronic epithelial diseases and cancers, suggesting that the perturbation of the general function of this glycoprotein can alter tissue homeostasis[76].

Since the role of MUC1 in CRSwNP remains poorly understood, we decided to conduct an immunofluorescence study to evaluate MUC1 expression in nasal polyps' samples and assess its potential correlation with disease severity.

As shown in the results, the tissue expression of MUC1 in the nasal mucosa samples increased when the low, medium and high CCG tissues were examined, showing a correlation with the severity of CRSwNP. Interestingly, previous studies had shown the anti-inflammatory function of MUC1 in asthma, by reducing the NLRP3

inflammasome-mediated pyroptosis trough the inhibition of inhibiting TLR4/MyD88/NF-κB pathway thereby suppressing neutrophilic airway inflammation in patients with asthma[77]. Moreover, MUC1 downregulation was associated with TNF-α-induced necroptosis in human bronchial epithelial cells by mediating the RIPK1/RIPK3 pathway[78]. Precisely because MUC1 has traditionally been attributed anti-inflammatory properties both in the respiratory tract and in extrapulmonary tissues, so much so that it has been defined as a "peacemaker", we hypothesized that it would inversely correlate with the severity of CRSwNP[79].

However, contrary to our initial hypothesis, our results showed a direct correlation between MUC1 expression and the severity of CRSwNP. This finding could be explained by the close colocalization of MUC1 with CD15+ eosinophils (***Fig. 2***). In fact, a recent study demonstrated that MUC1 promotes eosinophil survival through the release of its N-terminal domain, mediated by CCL4. This mechanism may contribute to the accumulation of eosinophils in the nasal mucosa, amplifying the inflammatory pathways underlying CRSwNP and explaining the paradox of our observation[80]. As a matter of fact, CRSwNP is well known to be characterized by Type 2 inflammation with pronounced eosinophilia and the presence of high levels of Type 2 cytokines, such as IL-4, IL-5 and IL-13[20]. Therefore, if MUC1 is involved in the regulation of eosinophil survival and promotes Type 2 inflammation, it could play a key role in the pathogenesis of CRSwNP. Since eosinophil is a widely recognized biomarker of disease severity, the correlation between MUC1 and eosinophilia suggests that MUC1 should also be considered a potential marker of CRSwNP severity[81].

Also mast cells, are now widely recognized as key players in the pathogenesis of the more severe forms of CRSwNP, contributing to chronic inflammation and tissue remodeling[82]. Since previous oncological studies had shown a strong correlation between MUC1 expression and abundant mast cell infiltration in the context of renal and breast cancer, and considering the finding of increasing MUC1 in severe forms of CRSwNP, we expected to observe a colocalization of mast cells with MUC1 in nasal polyps as well[83,84]. Contrary to what was expected, both MUC1 and mast cells showed a close correlation with the severity of the disease, but not colocalization. Probably, in the context of the tumor microenvironment, among the

different carcinogenic mechanisms, a sialylated glycoform associated with tumor-associated MUC1 could induce the differentiation of monocytes into tumor-associated macrophages (TAMs), which in turn become mast cells[85,86].

In CRSwNP, both MUC1 and mast cells appear to be independent factors of disease severity, although the common proportional increase with increasing CCG would suggest distinct but potentially complementary mechanisms in driving the disease's progression. As already known, most severe forms of CRSwNP are characterized by a mixed eosinophilic-mast cell inflammatory infiltrate and the two cytotypes appear to influence each other[87,88]. It is still unclear whether MUC1 plays a role in the interaction between eosinophils and mast cells or whether it acts independently, contributing separately to disease progression and influencing the underlying inflammatory mechanisms in CRSwNP.

CHAPTER 7

CONCLUSIONS

In conclusion, the role of MUC1 in the pathogenesis of CRSwNP represents an intriguing but still partially incomplete field of research. The correlation of MUC1 with the severity of CRSwNP, assessed according to CCG, offers a promising perspective for the use of MUC1 as a biomarker of CRSwNP. However, the variability in data regarding the function of MUC1, sometimes described as pro-inflammatory and other times as anti-inflammatory, raises important questions. This discrepancy highlights the need for further studies to clarify how different forms of MUC1 and its interactions with other immune cells may influence the inflammatory response in CRSwNP. Understanding these mechanisms underlying the interactions between MUC1, eosinophils and mast cells could have significant implications for the development of more targeted and personalized therapies.

Looking forward, it will be crucial to resolve the discrepancies regarding the different functions attributed to MUC1, as a deeper understanding of its biology could not only clarify its role in CRSwNP but also allow for the design of personalized therapeutic treatments. Furthermore, research should focus on identifying specific biomarkers that, along with MUC1, could improve early diagnosis and management of CRSwNP, thus reducing the risk of progression to more severe forms and improving the Quality of Life (QoL) for patients.

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