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Nasal cytology: towards a discovering of a predictive tool of common airways diseases' onset in pediatric population.

MED 31

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SUMMARY

INTRODUCTION

Pediatric diseases such as Acute Otitis Media (AOM), Upper Respiratory Tract Infections (URTI), bronchitis, asthma, allergy, rhinosinusitis and adenoidal hypertrophy are a daily challenge for clinicians such as otorhinolaryngologists, pediatricians and allergists.

Those pathologies may lead, especially during the growth period, to relevant comorbidities and complications such as obstructive sleep apnea syndrome (OSAS), hearing impairment as well as learning and attention difficulties and it is presumable that such diseases may have important implications in both scholar and social performances[1,2].

The possibility to screen newborns for the risk of developing these diseases would be therefore of enormous value, in order to potentially engage in strict follow-up that could guarantee early diagnosis and hence satisfactory treatment. However, to our knowledge, it is currently difficult to determine predictive factors which may increase the risk of developing any of these clinical issues. Nowadays, family history, smoking of the parents and similar environmental factors, are the only correlations that clinicians may find and which may suggest a predisposition to some of these pathologies such as asthma or allergy. However, no objective measures have been introduced into neonatal screenings.

Nasal cytology (NC) is a simple diagnostic procedure to evaluate the health of the nasal mucosa by recognizing and counting cell types and their morphology[2,3]. NC is able to detect infectious agents such as fungi and bacteria, allowing for example the diagnosis of infectious rhinitis. It also evaluates cellular composition detecting ciliated and muciparous cells, eosinophils, neutrophils, mast cell and other. Specific cytological patterns in NC can help in discriminating among various forms of rhinitis, including Allergic Rhinitis (AR), Non Allergic Rhinitis (NAR), idiopathic rhinitis, and overlapping forms[3].

Since the diagnosis of atopy and allergy in children, especially in the youngest age groups, is a difficult challenge and requires careful and broad analysis, NC may be considered a valid diagnostic tool, thanks to its simple, noninvasive and inexpensive method able to show any sign of local type 2 inflammation in the nasal mucosa[4]. Moreover, following the “united airways” concept, both upper and lower airways tracts share the same mucosal structure and functioning, and nasal secretions are delivered directly into the bronchial airways[5]. Therefore, diagnosis and treatment of nasal airways is essential to also improve and/or prevent lower respiratory tract disfunctions.

Previous studies have looked at an array of rhinocytograms in neonates and infants. Assessing healthy newborns or newborns exposed to for e.g cigarette smoke[6,7].

However, these studies are very heterogeneous in their enrollment of subjects and leave many questions unanswered. We therefore thought to extend this concept to other common pediatric pathologies, and hence to uncover if there is a link between nasal cellular composition at birth and the chance of developing such diseases in the first 3 years of life. Literature already explored the ability of nasal cytology in predicting the onset of various diseases, but to our knowledge no longitudinal prospective studies have been performed correlating nasal cytology at birth and the later onset of any disease[8].

The study aims to collect and analyze the nasal mucosa cytological composition, on a bigger scale as previously reported, at birth (in the first 24 hours of life) and therefore obtaining an uncontaminated and representative sample; collection of nasal mucosa cytology at 1 and 3 years of life in order to analyze any variation on its composition. Moreover, we set a longitudinal prospective study until 3 years of life of the child in order to evaluate the association of nasal cytology with the development of diseases like asthma, rhinitis, allergy, bronchitis, otitis and URTI and to assess whether nasal cytology is influenced by selected external factors. Finally, we would like to provide a baseline of cytological composition as well as of any external influencing factors that could interfere with the nasal cell composition in order to further investigate the relationship between genetic and environmental components in the pathogenesis of these diseases, since no literature sheds light on this topic.

MICROSCOPIC ANATOMY

Nasal Mucosa

The walls of the nasal cavity are lined with two types of mucosal layers:

- Respiratory mucosa, which occupies the majority of the surface.
- Olfactory mucosa, which contains the olfactory receptors and is limited to the roof of the nasal cavities and the cranial ends of the lateral and medial walls[10].

The nasal cavities can be divided into two distinct portions: the vestibule and the nasal cavity proper, which are connected to the paranasal sinuses through ostia. A part of the nasal cavity, dedicated to odor recognition, is referred to as the olfactory region. The respiratory portion of the nasal cavities, including large segments of the olfactory region, is lined with smooth and pink-colored mucosa, approximately 2 μm thick. The thickness of the mucosa increases at the level of the inferior turbinate, where abundant cavernous tissue is present, especially on its convex portion, as well as on the free margin of the head and the tail of the middle turbinate[1].

The respiratory mucosa appears shiny pink due to the presence of a mucus layer. This mucous lining is called "Schneiderian" after the anatomist Schneider, who first demonstrated that nasal secretions originated from the nasal mucosa rather than the brain, as previously believed[10]. (Fig. 5)

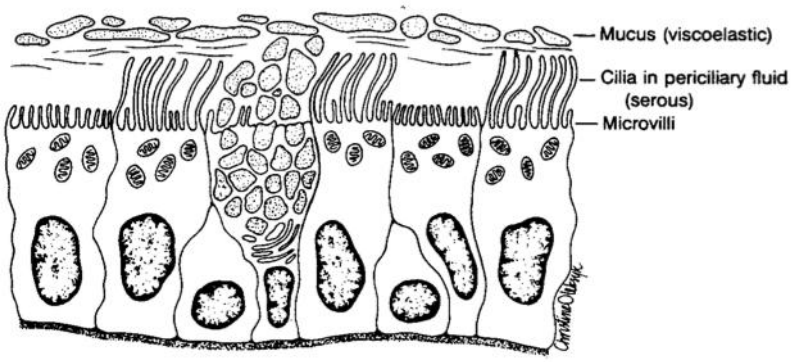

Figure 5 "Mucociliary transport" (Cole, Physiology of the Nose and Paranasal Sinuses, Clinical Reviews in Allergy and Immunology, 1998.)

From a microscopic perspective, the nasal cavity mucosa consists of an epithelium resting on a thin basal lamina, which separates it from the underlying lamina propria rich in blood vessels and serous and mucous glands. The pseudostratified columnar epithelium of the nasal mucosa includes the

following cell types: ciliated columnar cells, non-ciliated columnar cells (also called "brush cells" or "striated cells"), goblet cells, and basal cells. These cells are adhered to each other through desmosomal and hemidesmosomal anchoring systems. Lymphocytes and polymorphonuclear cells are occasionally observed in the intercellular spaces.

Within the nasal cavity, depending on the region considered, the lining epithelium exhibits histological variations. The nasal vestibule is covered by a thin layer of stratified squamous keratinized epithelium. This region also contains robust and rigid hairs called vibrissae, large sebaceous glands, and small sweat glands known as vestibular glands. The vestibular epithelium transitions to a stratified columnar epithelium without the cornified layer as it proceeds towards the nasal cavity proper. In this portion, vibrissae, sebaceous glands, and sweat glands are absent, while compound tubuloacinar glands appear in the lamina propria. Finally, the transitional epithelium continues as a pseudostratified ciliated columnar epithelium that covers the remaining surface of the nasal mucosa, composed of ciliated columnar cells, non-ciliated columnar cells (or striated cells), goblet cells, and basal cells.

The respiratory epithelium, as mentioned before, rests above the basal lamina, which separates it from the underlying lamina propria. The basal lamina is a thin, resilient, and highly adherent hyaline membrane, approximately 0.2 μm thick, perforated by openings through which leukocytes migrate to the epithelial surface. The lamina propria, also known as stroma or corium, consists of fibroelastic connective tissue that continues deeply with the periosteum or perichondrium. Within the lamina propria, three layers can be distinguished: the subepithelial or lymphoid layer, the intermediate or glandular layer, and the deep or vascular zone. The lamina propria contains serous and mucous glands responsible for mucus production and a network of blood vessels.

The respiratory mucosa is entirely covered by mucus, which is microscopically arranged in two superimposed layers (sol-gel):

- The aqueous layer (sol phase), in direct contact with the epithelial surface, with a thickness of approximately 3 μm, covers almost the entire length of the cilia on the epithelial cells.
- The dense layer (gel phase), located above the sol phase, is about 5 μm thick and consists mainly of mucin glycoproteins.

The double-layered mucus traps small particles inhaled from the air, which deposit in the gel layer and are pushed toward the pharynx by the coordinated movement of the cilia, beating at a frequency of approximately 800 beats per minute. The glandular secretions of the mucus also have antibacterial properties due to their content of immunoglobulins (IgA, IgG, IgM, IgE), lysozyme, lactoferrin, and complement proteins. Thus, the mucosal epithelium not only acts as a

physical barrier protecting the body from irritants but also plays an active metabolic role in regulating immune and inflammatory responses, as it is responsible for the production of proteases and the secretory portion of IgA.

The vascularization is abundant throughout the respiratory mucosa, particularly in the inferior meatus and the middle part of the septum. Arteries, originating from deep branches located near the periosteum, run perpendicular to the mucosal surface, branching into a dense subepithelial capillary network. From this network, venules branch out, their walls rich in smooth muscle that, in deeper regions, exhibit a nearly sphincter-like arrangement. By contracting, these fibrocellular elements can block venous flow and congest the mucosa. Finally, the mucosal vascular system is influenced by adrenergic fibers from the superior cervical ganglion, which exert constrictor action, and cholinergic fibers from the pterygopalatine ganglion, which have dilating and excitosecretory effects.

The inspired air's temperature is adjusted to match the body's temperature through heat exchange with the extensive network of thin-walled blood vessels in the lamina propria. The air is also humidified by contact with glandular secretions, especially the serous glands.

Finally, the olfactory mucosa, which lines the roof of the nasal cavities, consists of a covering epithelium composed of olfactory cells, supporting cells, and basal cells, as well as a lamina propria.

NASAL CYTOLOGY

Physiological Cellular Population

The nasal cellular population of a healthy individual includes the presence of ciliated cells, mucous goblet cells, striated cells, and basal cells.

Ciliated Cell

The ciliated columnar cell is the most differentiated and abundant cytotype in the nasal mucosa, constituting approximately 80% of it.
The numerical ratio between ciliated cells and mucous goblet cells is 5:1, which tends to increase as it progresses towards the distal parts of the lower airways, where it can reach a peak of 200:1.
The ciliated cell is interspersed with non-ciliated columnar cells ("striated cells") and submucosal gland ducts. It has an elongated polygonal shape, with a height ranging from 15 to 20 μm.
On its apical surface, there are approximately 100-250 cilia of varying height and numerous microvilli, around 300. In a rhinocytogram, with a microscopic magnification of at least 400x, the ciliated cell has a typical morphology, where the following can be recognized:

- The apical part, the site of the important ciliary apparatus.
- The cell body, which includes a significant portion of the cytoplasm and the nucleus, which is round-shaped and located at a variable height relative to the basement membrane (giving the pseudostratified appearance of the mucosal epithelium).
- The basal region, which can be defined as the base of the ciliated cell, as it is the narrowest part of the cell located below the nucleus and in contact with the basement membrane.

The ciliated cell is not anchored to the basement membrane in any way, and its adherence to the lamina propria is ensured through desmosomal connections with basal cells.
Sometimes, in some ciliated cells stained with the May-Grunwald-Giemsa (MGG) technique, it is possible to distinguish within the cytoplasm, just above the nucleus, a hyperchromatic stripe arranged perpendicular to the major axis of the cell, called the supranuclear hyperchromatic stripe (SIS), as shown in Figure 6.
The SIS is a direct indicator of cellular integrity and is absent in ciliated cells that have undergone alterations in the nucleus, cytoplasm, or ciliary apparatus.

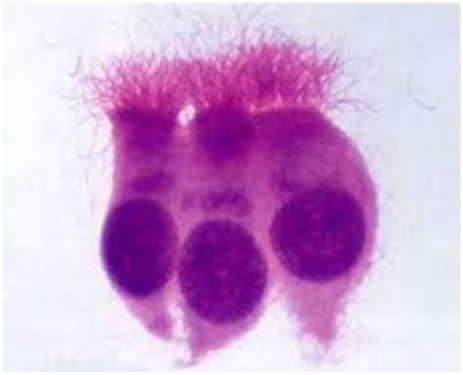

Figure 6: Ciliated cells stained with MGG at a magnification of 1000X. The supranuclear hyperchromatic stripe (SIS) is clearly visible above the nucleus. From "Atlas of Nasal Cytology," second edition, Matteo Gelardi, Edi.Ermes Editore 2012.

In vasomotor, inflammatory, and infectious rhinopathies, the percentage of cells that exhibit the hyperchromatic stripe (SIS+) is significantly reduced. The more severe the rhinopathy, the lower the percentage. In light of this, the SIS can also be used as a marker of therapeutic efficacy during topical or systemic treatments. During an infection, the ratio between ciliated cells and mucous goblet cells changes in favor of the latter, which increase in number and begin to secrete abundant mucus[11]. This represents the main cause of intranasal catarrhal stasis, which promotes bacterial superinfection.

Mucous Goblet Cell

The mucous goblet cell, also known as the goblet cell, is a unicellular mucous gland located between the cells of the pseudostratified respiratory epithelium but absent in the squamous, transitional, and olfactory epithelia.

It is responsible for the secretion of mucin, a polysaccharide protein that, upon contact with water, forms mucus.

Structurally, mucous goblet cells have numerous microvilli on the apical surface, whose size can vary depending on the stage of cellular secretion, and a small opening called a stoma through which mucin granules are exocytosed.

The nucleus is always located in a basal position, at the lower pole of the cell, while the vacuoles containing mucinogen granules (the biochemical precursor of mucin) are situated above the nucleus, giving the mature cell its characteristic goblet shape, from which it derives its name.

When the quantity of mucus is considerable, the nucleus is pushed further downward, creating a characteristic zone of chromatin reinforcement, which facilitates the identification of these cells under light microscopy (Figure 7).

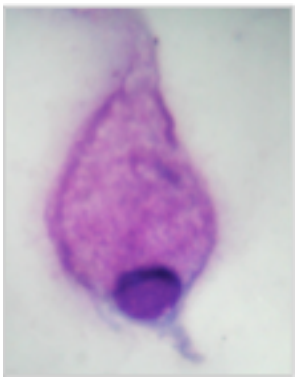

Figure 7: Mucous goblet cell viewed in nasal cytology, highlighted with MGG staining at a magnification of 1000X. The black arrow indicates the zone of chromatin reinforcement. From "Atlas of Nasal Cytology," second edition, Matteo Gelardi, Edi.Ermes Editore 2012[1].

Sometimes, the intracytoplasmic content may be absent since the most common fixation methods degrade the mucus.
Although this cytotype constitutes less than 1% of the total cells present in the respiratory-type mucosa (percentage varies according to chronic exposure to irritants), the role of mucous goblet cells is essential for maintaining proper airway clearance and preventing infections.
In neonates, the density of mucous goblet cells is not very high but increases with age until reaching normal density during adolescence[12]. In the septal region, their density is lower compared to the turbinates, and the turbinate with the lowest density of mucous goblet cells is the middle turbinate. In the septum, the mucous goblet cell population decreases as it moves in a superoinferior and anteroposterior direction[1]

Striated Cell

The striated cell is a columnar-shaped cell with its nucleus located in the basal part of the cell.
The apical surface has a regular series of microvilli, which function to increase the cellular surface area and prevent dehydration of the mucosal surface, maintaining the necessary moisture for cellular function.
Microfilaments are present in the cytoplasm of the striated cell, giving it a striated appearance.

The role of non-ciliated columnar cells has not yet been fully elucidated: they are thought to be absorptive cells with chemoreceptor function or progenitors of ciliated cells. However, there are currently no studies that can prove either hypothesis.

Basal Cell

The basal cell is a small-sized cell that adheres to the basement membrane without reaching the superficial portion of the mucosal epithelium of the airways.

It has electron-dense cytoplasm with bundles of tonofilaments, few mitochondria, scant rough endoplasmic reticulum, and a relatively large hyperchromatic nucleus compared to the cytoplasm. Basal cells have long been considered progenitors of mucous goblet cells and ciliated cells, although there were no studies supporting this hypothesis. It is more likely that the function of these cells is to facilitate the adhesion of columnar cells to the basement membrane. Supporting this hypothesis is the fact that ciliated columnar cells do not have their own hemidesmosomes but adhere to the basement membrane through the hemidesmosomes expressed at the base of basal cells (Fig. 8)

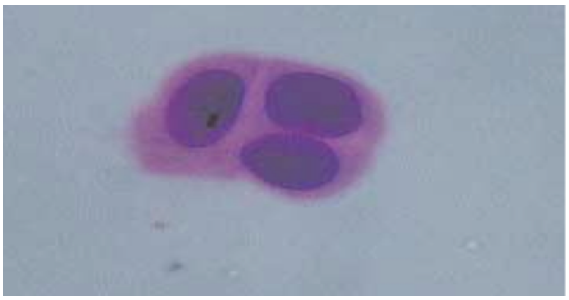

Figure 8: basal cell.

Cells of Inflammation

In inflammatory conditions, various types of inflammatory cells can be found in the nasal mucosa, which vary depending on the rhinopathy. The most represented cells are neutrophil granulocytes, eosinophil granulocytes, mast cells, lymphocytes, plasma cells, and macrophages.

Neutrophil Granulocyte

The neutrophil granulocyte is a round cell with a diameter ranging from 12 to 14 μm. Structurally, neutrophils have a characteristic multi-lobed nucleus, with a typical shape, which is arranged in the cytoplasm without a specific order, hence the name polymorphonuclear (Fig. 9).

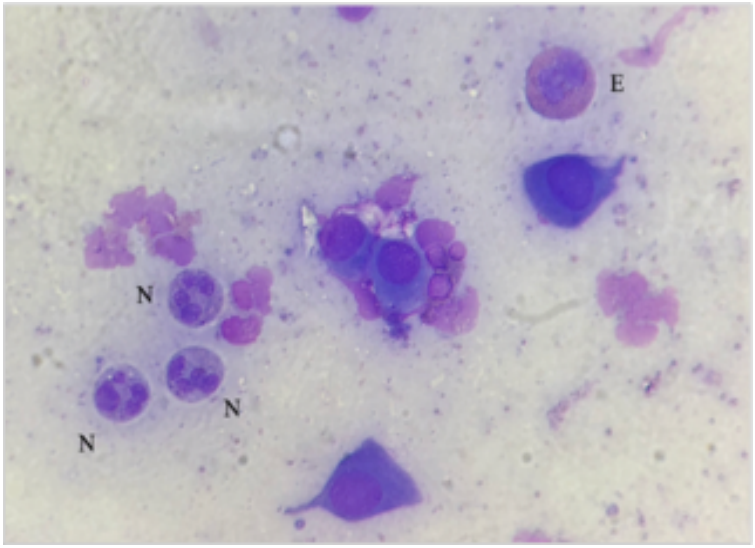

Figure 9: Cytological examination highlighting the presence of three neutrophil granulocytes (N) with their typical multi-lobed nucleus. An eosinophil (E) can also be distinguished. MGG staining, magnification 1000X. From "Atlante di citologia nasale, seconda edizione" by Matteo Gelardi, Edi.Ermes Editore 2012.

The number of lobes is believed to depend on the age of the neutrophil: in young neutrophils, the nucleus is usually kidney-shaped, elongated, and unsegmented, while in more mature ones, there are two or more constrictions that divide it into multiple nuclear lobes connected by slender nuclear bridges. Therefore, based on the characteristics of the nucleus, six types of granulocytes can be distinguished according to Arneth (Figure 9).

At the cytoplasmic level, neutrophils have mixed neutrophilic granules and some basophilic granules, although the number of these granules varies. Among the main functions of neutrophils is phagocytosis: these cells are responsible for ingesting and digesting harmful elements such as spores, bacteria, and dust. In the rhinocytograms of healthy individuals, the sporadic presence of neutrophils is a completely physiological condition, while an increase in their number should be monitored and is commonly associated with infectious and allergic rhinitis. They even constitute the predominant cellular element in both perennial and seasonal forms of allergic rhinitis. In these cases, the neutrophil's phagolysosomes will contain immune complexes formed by antigen-

antibody. Finally, an increase in polymorphonuclear cells is also observed in rhinocytograms of individuals living in industrialized areas or those exposed to specific substances or particulates.

Eosinophil Granulocyte

Eosinophil granulocytes, named for the presence of cytoplasmic granules that avidly bind to the eosin dye, are produced in the bone marrow, where they reside for about 4 days before being released into the bloodstream and eventually migrating to the tissues. In the blood of a healthy individual, eosinophils represent between 1% and 6% of the total leukocytes. In allergic pathology, this percentage can reach 15-30%. Morphologically, eosinophils are slightly larger than neutrophils, and they have a lobed nucleus (2-3 lobes) but less polymorphic than that of neutrophils (Fig 10).

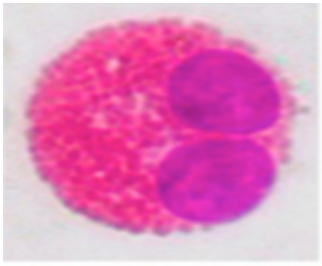

Figure 10: Eosinophil granulocyte, MGG staining. From "Atlante di citologia nasale, seconda edizione" by Matteo Gelardi, Edi.Ermes Editore 2012.

Their characteristic appearance is due to intracytoplasmic granulations (primary and secondary granules), which have a spherical or diamond shape. These granules are larger than those found in neutrophils and consist of protein macromolecules organized in concentric lamellae that strongly bind to eosin, a dye that gives these cells their typical reddish-purple color (Figure 10). In some cases, these cells may not appear with the typical appearance (i.e., bilobed cell with eosinophilic granules) but may have a single or trilobed nucleus. Moreover, in the acute phases of allergic rhinitis, especially in pollen-induced rhinitis, partial or complete degranulation can occur. In this case, numerous granules scattered from eosinophilic staining will be found in cytological examinations.

Mast Cell

Mast cells are distributed in all tissues, including mucosal and serosal surfaces, lymphoid and connective tissues, and associated with nervous tissue, blood vessels, and neoplastic tissue. In the bronchial and nasal mucosa, mast cells are located at the interface with the external environment

and are the first cells to come into contact with inhaled allergens. Unlike basophilic leukocytes that invade tissues only during inflammatory events, mast cells are commonly distributed on mucosal and serosal surfaces and in other tissues. Mast cells have an oval-shaped nucleus and numerous basophilic granules, as they stain with basic dyes such as toluidine blue, which partly obscures the nucleus (Figure 11).

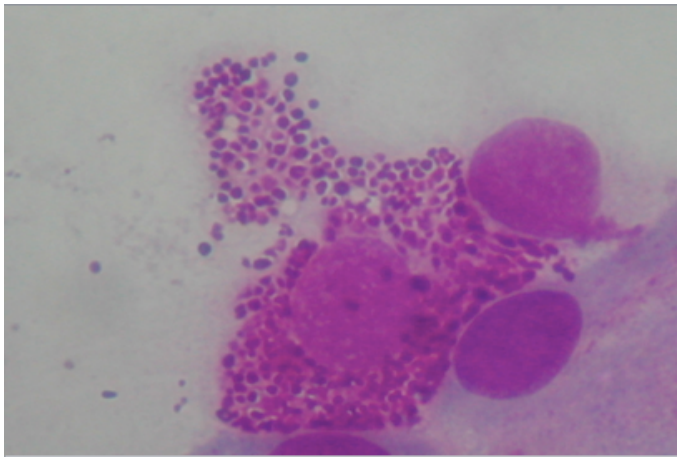

Figure 11: Mast cell in the process of degranulation. From "Atlante di citologia nasale, seconda edizione" by Matteo Gelardi, Edi.Ermes Editore 2012.

Both mast cells and basophils are characterized by the presence of high-affinity IgE receptors on the cell membrane, which remain on the surface of mast cells after initial exposure to the antigen. Mast cells have long been considered cells with a fundamental role in the acute phase of allergic inflammation, during which the release of mediators such as histamine, tryptase, PGD2, and LTC4, through their action on microvessels and sensory nerve endings, is responsible for allergic symptoms (rhinorrhea, nasal obstruction, and sneezing). Mast cells can thus be considered one of the main cells responsible for the induction of both immediate-type allergic reactions and chronic allergic inflammation, with the terminal phase being characterized by extensive degranulation (Figure 11) and release of primary and secondary chemical mediators.

Lymphocyte

Lymphocytes are circulating elements responsible for both humoral and cell-mediated immune responses. In the context of nasal pathology, they are characteristic of chronic inflammation but are often present in inflammatory conditions with an allergic basis. From a functional perspective,

based on the type of immune response they are dedicated to, lymphocytes can be classified as T lymphocytes, B lymphocytes, and non-T non-B lymphocytes. From a morphological point of view, they can be differentiated into small lymphocytes, large lymphocytes, and activated lymphocytes (Fig.12).

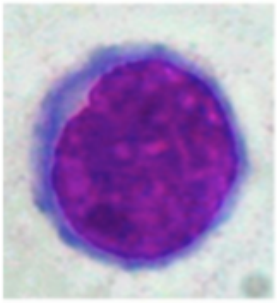

Figure 12: Lymphocyte, MMG staining. From "Atlante di citologia nasale, seconda edizione" by Matteo Gelardi, Edi.Ermes Editore 2012.

Small lymphocytes have a diameter ranging from 7 to 10 μm, a violet round nucleus, and a thin rim of cytoplasm that stains blue. They are normally in a quiescent phase, ready to activate in case of immune system stimulation. Large lymphocytes have a diameter 2-3 times larger than small lymphocytes. The shape, staining, and chromatin structure in the nucleus are similar to small lymphocytes, although the cytoplasm has a basophilic tinge. Activated lymphocytes, on the other hand, have further increased in size; the round nucleus is indented or lobulated with more dispersed chromatin. The basophilic and ample cytoplasm exhibits granules, vacuoles, and pseudopods. These cells assume heterogeneous morphological characteristics and are therefore referred to as atypical.

Plasma Cell

In the rhinocytogram, it appears as an oval or rounded cell with irregular margins and dimensions ranging from 15 to 25 μm. Typically, its nucleus, also oval in shape, is located at one pole of the cell, and the chromatin has a characteristic "turtle shell" or "wheel" appearance due to the arrangement in polygonal-shaped chromocenters. The cytoplasm of plasma cells is intensely basophilic, giving them an intense bluish coloration that makes them easily recognizable under the optical microscope. Sometimes identification can be difficult due to intracytoplasmic accumulation of immunoglobulins, which can cause variations in both the shape and staining characteristics of the cell.

Macrophage

Macrophages originate in the bone marrow as monocytes, which are then released into the bloodstream and ultimately migrate to the tissues where they mature into macrophages. Tissue macrophages are usually in a quiescent state and become activated when needed, particularly in chronic inflammatory conditions where microorganisms cannot be digested by neutrophils. In such cases, there is a significant recruitment of circulating monocytes by the tissues. Macrophages have a rounded shape and a kidney-shaped nucleus. The nucleolus is usually visible, and the cytoplasm is abundant and filled with digestive vacuoles containing bacteria or cellular debris (Figure 13). The population of macrophages in the nasal cavity is relatively scarce compared to that found in the alveoli, which can make their detection challenging in rhinocytograms.

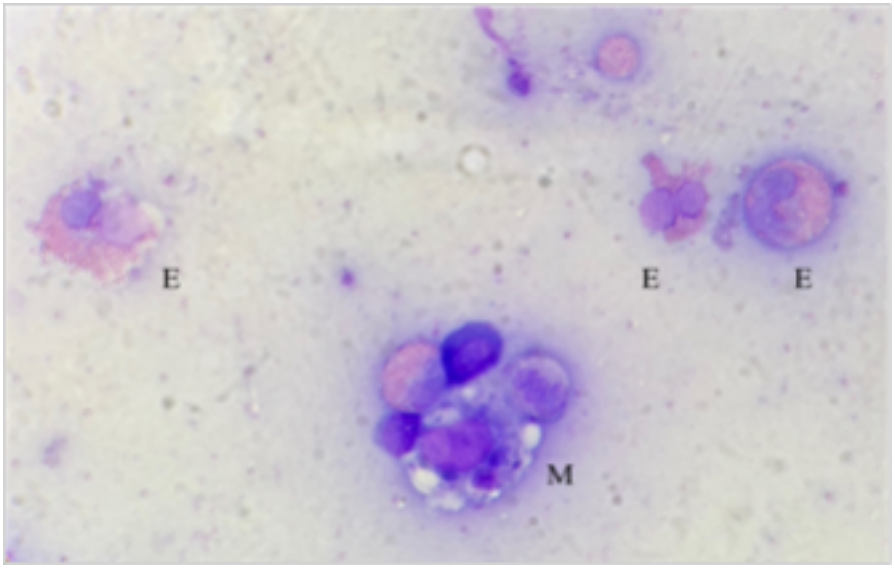

Figure 13: Macrophage (M) highlighted with MGG staining. Three eosinophilic granulocytes (E) are also indicated. Magnification 1000X. From "Atlante di citologia nasale, seconda edizione" by Matteo Gelardi, Edi.Ermes Editore 2012[1].

PHYSIOLOGY OF THE NOSE AND PARANASAL SINUSES

The anatomical and functional unit consisting of the nose and paranasal sinuses performs several functions, some primary (respiratory and olfactory function) and others complementary but no less important (defensive, phonatory, aesthetic).

Respiratory Function

The main function of the nose is respiratory in nature.
Nasal respiration, in addition to ensuring proper lung ventilation, allows for quantitative and qualitative modifications of air during its passage from the external environment to the nasopharynx.
The respiratory mucosa, with its secretory, ciliary, and vasomotor activities, ensures the filtration (purification) and conditioning (humidification and warming) of inhaled air. Additionally, it participates in defense mechanisms against infectious agents[13].

Ventilation

Nasal respiration ensures proper lung ventilation, resulting in slower and deeper respiratory movements compared to oral respiration. As a result, there is better distribution of air in the alveoli. Physiologically, nasal ventilation precedes oral ventilation, which is acquired and used later as needed. In fact, during the first weeks of life, nasal breathing is exclusively used, leading to the term "obligate nasal breather" for newborns.
Approximately 10,000 liters of air pass through the nasal passages each day, with a respiratory rate ranging from 12 to 24 breaths per minute[14].
On the other hand, the paranasal sinuses do not play a significant role in the respiratory process. The nose serves as the entrance for inhaled air and contributes, on its own, to 70% of the total resistance of the respiratory tract. This resistance is much higher than that offered by the mouth and is regulated by the muscles of the nasal wing and the degree of vascular congestion of the mucosa, which has the characteristics of erectile tissue due to the presence of a large number of muscle cells in the venous vessels of the lamina propria.

Conditioning of Inhaled Air and Nasal Cycle

Another important function of the nose is conditioning of inhaled air, which involves the processes of warming and humidifying the air during its passage through the nasal cavities. Through contact with the nasal mucosa and the presence of turbinates, the air reaches the pharynx at a constant temperature of 34°C with 95% humidity.

The humidification of inhaled air is related to the evaporation of water from the mucous layer.

The warming of inhaled air is achieved through thermal action by the superficial arteriole-capillary network. The passage of cold and dry air through the nasal cavities causes vasodilation and congestion of the cavernous tissue, resulting in slowed airflow that facilitates heat exchange between the air and the mucosa.

The degree of congestion of the turbinates is essential for regulating the conditioning of inhaled air. The vascular structures of the turbinates have both sympathetic innervation, which causes vasoconstriction, and parasympathetic innervation, which causes vasodilation.

These two systems alternate dominance, resulting in alternating phases of vasoconstriction and vasodilation in the two nasal cavities. This mechanism, which can last for 2 to 5 hours, is known as the nasal cycle.

Although the degree of resistance can fluctuate between severe obstruction and optimal opening for each side, healthy individuals are usually unaware of these variations. This is because the phenomenon is reciprocal between the two sides (one side congested, the other decongested), minimizing the overall variation[13].

Purification and Defense Function

The nasal cavities have both specific and nonspecific purification and defense mechanisms. Nonspecific mechanisms include filtration, mucociliary clearance, the action of phagocytic elements, and biological substances with antibacterial and antiviral activities.

Specific defense mechanisms are carried out by the immune system, particularly secretory IgA.

Airborne particles deposit on the mucus layer lining the nasal cavities (producing 20-40 ml of mucus per day), and the ciliated epithelium's sweeping action transports the mucus and particles toward the nasopharynx for removal. The cilia of the epithelial cells move at a frequency of 150 to 1500 beats per minute in a coordinated, continuous, and unidirectional manner[15].

This mechanism is known as mucociliary clearance, which transports mucus, particles, and various pathogens inhaled with the air to the digestive tract, thereby maintaining the health and immunological defenses of the upper airways[16]. Inspiratory airflow predominantly passes between

the inferior turbinate and the middle turbinate, resulting in the deposition of most particulate matter inhaled in this area.

Studies have not reached a consensus on whether mucociliary clearance increases upon contact with inhaled allergens. However, in patients with positive skin allergy tests and positive methacholine challenge tests, an increase in mucociliary clearance time is observed[15].

Reflex Function

The nose provides additional effective protection through reflexes originating from it. These are the nose-bronchial and nose-pulmonary reflexes, with the trigeminal nerve as the afferent pathway and the vagus nerve as the efferent pathway. These reflexes control bronchial dilation and respiratory rhythm, respectively. Sneezing and coughing involve the same neural pathways (closing at the bulbopontine level) and consist of a forced expiration through the nose or mouth, respectively.

Phonatory Function (Resonance)

The nose also serves a phonatory function: the passage of air through the nasal cavities allows certain phonemes to acquire nasal resonance. The nasal and paranasal cavities contribute to this function by amplifying the sound produced by the sound production system, thereby altering its tone and pitch.

The nasal cavities directly enable the pronunciation of nasally sounding phonemes, such as "m," "n," and "gn." They also indirectly contribute to resonance through the vibration of air contained within them, even if there has been no airflow through the nasal cavities.

Aesthetic Function

Lastly, the nasal pyramid is essential for facial harmony, giving the nose an aesthetic function as well. This aspect justifies the increasing demand for surgical interventions aimed at correcting nasal imperfections.

Regarding the functions of the paranasal sinuses, they are not yet fully understood. It seems that these structures contribute to lightening the cranial skeleton and play a role in phonation by acting as a resonance chamber for the voice.1

Olfactory Function

The olfactory function is carried out through the contact between the neuroreceptors of the olfactory mucosa and odor particles, which are concentrated toward the olfactory area upon entering with the inhaled air.

The olfactory mucosa lines the roof of the nasal cavities and has a total surface area of approximately 5 cm2, containing olfactory receptors. The olfactory nerve originates from these neurosensory cells and carries stimuli centrally, allowing for the perception of odors.

DIAGNOSTIC TOOLS

Nasal Cytology

In the field of rhinology, among the various diagnostic methods available, nasal cytology is also utilized. Nasal cytology is a simple and safe diagnostic procedure for the evaluation of normal and pathological characteristics of the nasal mucosa by analyzing different types of cells and their morphology[17].

The foundations of this practice were laid in the late 1800s when Gollash and Von Mihalkovics first described the microscopic aspects of nasal mucosa. However, their descriptions remained purely anatomical and morphological[18].

It was Eyermann in 1927 who first identified eosinophils in the nasal secretions of patients with hay fever[19]. Although the pathogenesis of allergic reactions was still far from being identified, these evaluations helped understand the correlation between the presence of a particular cell population in the nasal mucosa and a specific clinical pathology.

After decades of limited interest, the study of nasal cytology experienced rapid and progressive development starting in the 1970s when the method was used to assess pharmacological therapy and the mucosa's response to exogenous stimuli[11].

However, it was only from 2006 that the procedures for nasal cytological sampling and diagnostic criteria were systematized, becoming a field of great interest for otorhinolaryngologists, allergologists, and pediatricians[12].

Over the years, nasal cytology has been reevaluated as it has proven to be a decisive technique in diagnosing various rhinopathies, such as allergic rhinitis, NARES (non-allergic rhinitis with eosinophilia syndrome), NARNE (non-allergic rhinitis with neutrophilia), NARMA (non-allergic rhinitis with mast cells), and, more recently discovered, the new nosological entity called NARESMA (non-allergic rhinitis with eosinophils and mast cells)[12].

In the rhinocytogram of a healthy individual without rhinopathies (as shown in Figure 27), the four cytotypes that make up the ciliated pseudostratified epithelium of the nasal mucosa are normally identified:

- Ciliated cells
- Mucous-secreting goblet cells
- Basal cells
- Brush cells

In addition, occasional neutrophils and bacteria may be observed.

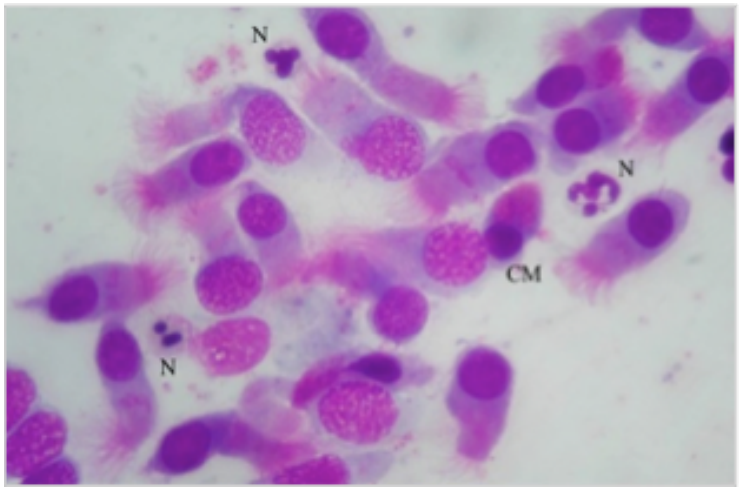

Figure 27: Example of a rhinocytogram from a healthy individual. Numerous ciliated cells, some neutrophils (N), and a mucous-secreting goblet cell (CM) can be seen, highlighted with MGG staining at 1000X magnification. From "Atlas of Nasal Cytology," second edition, Matteo Gelardi, Edi.Ermes Publisher 2012.

Therefore, based on the knowledge of a normal rhinocytogram, the presence of other cytotypes, such as eosinophils, mast cells, numerous bacteria, fungal hyphae, indicates a pathological condition[12]. This is a fundamental concept that helps understand the importance of this simple diagnostic technique, which allows for differentiation between inflammatory and infectious rhinopathies (and within infectious rhinopathies, bacterial rhinitis from viral and fungal rhinitis), allergic from non-allergic vasomotor rhinitis, and even diagnosing overlapping rhinopathies in the same patient (e.g., allergic rhinitis associated with NARES).

Nasal cytology is a valuable tool, not only for targeted diagnosis but also for disease follow-up and assessing the response to pharmacological treatment. Of course, cytological evaluation should always be accompanied by a comprehensive clinical assessment, considering the patient's signs and symptoms, medical history, nasal endoscopy, and the presence of any IgE sensitization, demonstrated by skin prick tests.

Other advantages of this technique should not be overlooked: It is highly sensitive, specific, and repeatable during clinical and therapeutic follow-up[11].

Nasal cytological sampling is easy, non-invasive, painless, and cost-effective.

The cytological sampling procedure includes:

- Collection of the sample;
- Fixation and preparation of the sample;
- Observation under the optical microscope at 1000X magnification, following immersion in oil.

Various sampling techniques exist, including nasal blowing, nasal lavage, nasal swabs, nasal brushing, and nasal scraping.
Each method has its advantages and disadvantages, and the choice of one method over another is made by the physician based on specific needs.
In this study, the nasal scraping technique was chosen, which has proven to be the best method for obtaining valid cellular sampling of the nasal mucosa, not only in adults but also in children.
In particular, as demonstrated in the study conducted by Pipolo et al. in 2017 on a sample of 208 children, nasal scraping (NSC - nasal scraping) has significantly higher diagnostic accuracy than nasal cotton swabs (NSW), while the two techniques do not differ in terms of tolerability[20].
Therefore, nasal scraping is the method of choice for obtaining nasal cytological samples in pediatric patients as well.
This technique uses a plastic curette type Rhino-probe® (Arlington Scientific, Springville, UT, USA) as the sampling instrument. The sampling is performed using anterior rhinoscopy with a nasal speculum and adequate illumination; the collection site is the medial surface of the inferior turbinate (middle third) where there is an appropriate ratio of ciliated and goblet cells, approximately 4:1.
It is a non-invasive method and, therefore, does not require any anesthesia.
Once the sample is collected, the material is evenly spread on a slide and allowed to air dry. Subsequently, it will be fixed and stained. The staining can be performed using the May-Grunwald-Giemsa (MGG) method, which allows for the easy identification of the different cellular components of the collected sample, as well as any bacteria, fungal hyphae, or spores. This procedure would traditionally take 30 minutes, but today, there are MGG QUICK STAIN kits (Bio Optica®, Milan, Italy) available that allow for satisfactory slide preparation in less than 30 seconds.
Finally, the slide is examined under an optical microscope at 1000X magnification with immersion oil. For a meaningful evaluation of the different cellular components, at least 50 fields should be read (Figure 29).

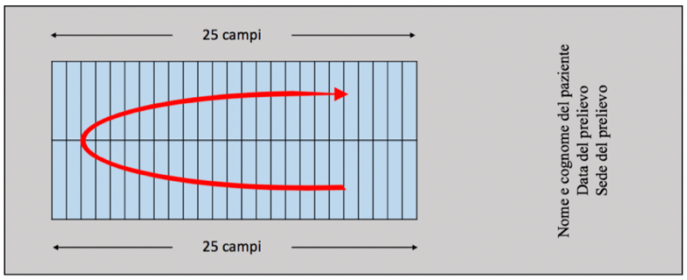

Figure 29: Diagram of a slide's structure. The red line indicates how the evaluation of 50 microscopic fields at 1000X magnification with immersion should be conducted to obtain a meaningful assessment of the different cellular components.

The count of each cell type can be expressed as a percentage of the total (quantitative method), as an absolute value (descriptive method), or through a semiquantitative grading. In this study, the rhinocytograms were interpreted using a semiquantitative evaluation.

SEMIQUANTITATIVE EVALUATION OF NASAL CYTOLOGY (evaluation of 1000X microscopic fields)					
	Absent	Rare (+)	Some (++)	Numerous (+++)	Very numerous (++++)
Ciliated cells	0	1-100	101-200	210-300	>300
Muciparous cells	0	1-100	101-200	210-300	>300
Neutrophils	0	1-20	21-40	41-100	>100
Eosinophils	0	1-5	6-10	11-30	>30
Mast cells	0	1-5	6-10	11-30	>30
Lymphocytes	0	1-5	6-10	11-30	>30
Bacteria	0	I I I	II II	III III III	IIIIIIIIIIIIIIIIIIIII
Spores	0	I I I	II II	III III III	IIIIIIIIIIIIIIIIIIIII

Figure 30: Table of semiquantitative evaluation of nasal cytology (adapted from "Atlas of Nasal Cytology," Matteo Gelardi, Centro Scientifico Editore, 2012 edition).

In conclusion, nasal cytology, adequately integrated with clinical findings and allergologicAL tests, has proven to be a very useful tool for differential diagnosis in the field of rhinopathies, allowing for the distinction between various forms: infectious, allergic, and non-allergic.
In particular, only nasal cytology allows for the recognition and characterization of different types of non-allergic rhinitis, distinguishing the specific inflammatory infiltrate.
Furthermore, the accurate cytological diagnosis provides valuable assistance in choosing the appropriate therapeutic treatment (e.g., between an anti-inflammatory drug or specific immunotherapy)[21].

Skin Prick Test

The skin prick test is an allergological investigation that, due to its simplicity, safety, and low cost, is used as a first-level examination in the diagnosis of allergies.
This test is primarily used for screening allergens of food and inhalant origin (pollens, grasses, dust mites, etc.). For the identification of skin allergies, such as nickel allergy, which typically causes contact dermatitis, the so-called patch test is used instead.
The purpose of the test is to evaluate whether a specific substance, to which the subject has previously been sensitized, causes allergic inflammation through an immediate mechanism mediated by IgE (immediate-type hypersensitivity or Type I reaction according to the Coombs and Gell classification). It thus highlights the presence of IgE antibodies responsible for allergy symptoms.
The skin prick test is a rapid and painless examination, suitable for both adults and children. In pediatrics, it can be performed starting from one year of age, while in younger children, it may not be very useful due to limited skin reactivity. For this reason, within the study, the test was conducted during the third visit, when the children had turned three years old.
The prick test is performed on the volar surface of the forearm; the points under investigation are marked with a pen, or a grid with different cells, each corresponding to an allergen, can be drawn. The test involves applying a drop of each sensitizing substance (allergen) to the skin of the forearm (in children, it can also be done on the upper back). Then, the skin is lightly pricked with a sterile lancet at each drop, allowing the allergen components to penetrate about 1 mm into the epidermis (prick in English means to puncture).
After one minute, the drop is removed with a piece of absorbent paper. The lancet must be replaced for each puncture to avoid contamination between different allergens. The same applies to the gauze strip used to remove the allergen drop.
To increase the reliability of the prick test, the same procedure is performed with two control drops: one containing saline solution and the other containing 1% histamine. Unlike the saline drop, the response to histamine should always be positive; conversely, the response to histamine should always be negative. If this does not happen, the prick test is unreliable, and other allergological investigations are necessary.
After 15-30 minutes, the skin response to the prick test is observed. The test is considered positive if a wheal with a diameter equal to or greater than 3 mm appears, surrounded by an erythematous (redness) halo, which is usually itchy.

The interpretation of the results is performed by a doctor who, in the presence of skin reactions, assesses their intensity and identifies the allergenic substances responsible.
The prick test is a safe, painless, and well-tolerated diagnostic examination that, in case of positivity, may cause slight itching due to the allergic reaction, which is short-lived. Severe adverse reactions are rare; therefore, the test is not recommended for patients with a history of anaphylaxis.
The prick test is a highly sensitive examination (> 90%); hence, a negative result can exclude allergic sensitization to the allergen. However, it should be noted that if the patient is on antihistamine or corticosteroid medications, the test may yield falsely negative results.
On the other hand, the prick test is burdened with low specificity (50-85%): this means that a positive test result cannot ensure the actual presence of hypersensitivity in the patient, and the final diagnosis of allergy should always rely on other tests.

COMMON PEDIATRIC PATHOLOGIES

Children are commonly affected by upper and lower airways diseases as rhinits, allergies, asthma, bronchitis etc, that are one of the main reasons for consultation with the general practitioner, pediatrician, and otorhinolaryngologist.
These conditions constitute a broad and heterogeneous group of pathological conditions with different etiologies:

- Allergic;
- Infectious;
- Non-allergic and non-infectious inflammatory.

"UNITED AIRWAY DISEASE" CONCEPT

There is an intimate anatomical and functional correlation between the upper and lower airways. Over the last 15 years, scientific interest in the nose-lung-bronchus connection has increased, leading to the term "United Airway Disease"[22]. This concept is based on the presence of numerous similarities between the upper and lower respiratory tracts, which include:

- Epidemiological: many studies confirm the association between allergic and non-allergic rhinitis and asthma.
- Anatomical: the respiratory epithelium shares common features from the nasal cavity to the bronchioles (ciliated and muciparous cells, basement membrane, and lamina propria, submucosal glands), although some differences exist (nasal mucosa rests on bone, while bronchial mucosa rests on cartilage; nasal mucosa is rich in blood vessels, while bronchial mucosa contains smooth muscle cells).
- Physiological: the nose and bronchi have the same adrenergic and vagal innervation.
- Immunopathological: mast cells, T lymphocytes, and eosinophils can infiltrate both the upper and lower airway mucosa.
- Pathophysiological: reduced airflow is the most relevant functional consequence of both allergic rhinitis and asthma.
- Therapeutic: antihistamines, leukotriene antagonists, corticosteroids, and specific immunotherapy can be prescribed for both allergic rhinitis and asthma.

"United Airway Disease" presents two main phenotypes: allergic (also called atopic or extrinsic) and non-allergic (or non-atopic or intrinsic). Both are associated with an increased risk of

developing asthma. In pediatric clinical practice, special attention is given to the clinical and therapeutic management of atopic patients who, in the early years of life, may develop dermatitis and eczema, allergic rhinitis, asthma, or food allergies. This wide range of pathologies, sharing the same allergic substrate, tends to develop progressively during childhood.

Classic atopic manifestations in the first year of life include dermatitis and food allergies, while asthma and allergic rhinitis are more likely to develop between 3 and 6 years of age.

The association between atopy, rhinitis, and asthma exemplifies the theory of "United Airway Disease." Asthma and allergic rhinitis can be viewed as different clinical manifestations of the same atopic entity.

Allergic rhinitis promotes, triggers, maintains, and worsens bronchial asthma through multiple mechanisms, such as the rinobronchial reflex, direct infiltration of inflammatory mediators, systemic release of inflammatory cytokines, irritation from posterior nasal drip, and oral breathing due to nasal obstruction, promoting bronchial hyper-reactivity and allowing the entry of dry and cold air into the bronchi.

Numerous studies have highlighted the correlation between nasal inflammation, particularly eosinophilic inflammation, and bronchial asthma. Moreover, the onset of asthma is more frequent in subjects with perennial allergic rhinitis compared to seasonal forms. Even patients with allergic rhinitis who do not report bronchial asthma symptoms may have bronchial hyper-reactivity, indicating subclinical inflammation of the lower airways. Therefore, nasal eosinophilia is associated with bronchial hyper-reactivity in subjects with perennial allergic rhinitis (who do not yet have asthma symptoms) and in patients with bronchial asthma.

The nose acts as a window to the lower airways: in children with allergic nasal symptoms, the inflammatory changes observed in the nasal mucosa reflect the degree of bronchial asthma involvement in the lower airways. Additionally, nasal provocation tests performed on subjects with allergic rhinitis without an asthma diagnosis have allowed for the evaluation of hypersensitivity accompanied by obstruction and the presence of bronchial eosinophilia. Non-asthmatic children, especially those with persistent allergic rhinitis, show bronchial hyper-reactivity to methacholine and adenosine, especially when evaluated during or immediately after the pollen season.

Based on these premises, a retrospective Polish study in 2010 aimed to determine the usefulness of nasal cytology as a prognostic tool in pediatric patients with suggestive clinical features of allergic disease and candidates for atopic evolution. The study divided children into three groups: those with atopic dermatitis/eczema, those with recurrent respiratory infections and bronchial obstruction, and those with gastrointestinal symptoms. At the beginning of the observation, nasal cytology with exfoliative technique and Papanicolaou staining was performed. After 4 years of follow-up, it was

observed that dermatological manifestations typically precede the development of allergic rhinitis and asthma, and that the development of food allergy or cutaneous atopy significantly increases the risk of allergic rhinitis or asthma. Concerning the cytological correlation, the data showed an increased incidence of allergic rhinitis in children under 4 years with baseline rhinocytograms containing at least 8% eosinophils.

In conclusion, a high count of eosinophils in the nasal cytology of infants may represent a predictive marker for the risk of the allergic march and thus be useful in identifying children with hyper-allergic traits of the respiratory tract. Given this information, one may wonder if the allergic march can be interrupted through early and appropriate treatment of upper respiratory tract conditions.

Rhinitis

Rhinitis represents one of the main otorhinolaryngological issues in pediatric and adolescent age. Its prevalence is increasing around 10% of the population, with an overall prevalence of self-reported lifetime rhinitis of 19.93%[23].

Rhinitis is a clinical syndrome characterized by an acute or chronic nasal mucosa's inflammation leading to rhinorrhea, sneezing, nasal obstruction, nasal or ocular itching, and tearing, present for at least one hour a day and for at least two consecutive days.

Its etiological classification is resumed is Table 1.

	Description
IgE-mediated (allergic)	• IgE-mediated inflammation of the nasal mucosa, resulting in eosinophilic and Th2-cell infiltration of the nasal lining • Further classified as intermittent or persistent
Autonomic	• Vasomotor • Drug-induced (rhinitis medicamentosa) • Hypothyroidism • Hormonal • Non-allergic rhinitis with eosinophilia syndrome (NARES)
Infectious	• Precipitated by viral (most common), bacterial, or fungal infection
Idiopathic	• Etiology cannot be determined

Table 1. Etiological classification of rhinitis[24].

Infectious rhinitis

Any infectious agent capable of overcoming the nasal mucosa barrier and causing an inflammatory process can lead to infectious rhinitis. The most common etiological agents are viruses, followed by bacteria and fungi.

Infectious rhinitis can be further classified as:

- Acute epidemic (common cold): is the most frequent rhinosinusal condition, characterized by an acute inflammatory process of viral origin, localized in the nasal mucosa and associated with secondary involvement, mostly mild, of the paranasal sinuses. Sometimes a bacterial superinfection may be encounter, with purulent rhinorrea and prolonged fever.
- Acute during systemic infectious diseases (symptomatic rhinitis): in many systemic infectious diseases (measles, chickenpox, scarlet fever, rubella, influenza, meningitis), there is, in the initial phase, an acute rhinitis that is entirely similar to the common cold.
- Chronic: a clinical condition characterized by nasal obstruction, mucopurulent rhinorrhea, sneezing, and nasal itching that persists over time. In children, this condition is characteristic of adenoid hypertrophy, in which relative immune defenses deficiency is associated with continuous nasal cavity obstruction by hypertrophic adenoid tissue. Chronic rhinitis often leads to secondary involvement of the paranasal sinuses (chronic rhinosinusitis).

Allergic Rhinitis

Allergic rhinitis is the most common chronic pathology in pediatric age, with a prevalence of up to 40%[25]. Currently, the prevalence of allergic rhinitis is constantly increasing, and the causes responsible for this phenomenon have not yet been fully clarified [26]. Several hypotheses have been formulated, considering exposure to environmental factors such as urbanization and pollution, or the reduced incidence of infectious diseases in infants (hygiene hypothesis). Allergic rhinitis commonly develops at a young age, with approximately 80% of cases manifesting before the age of 20. Additionally, allergic pathologies, including asthma, often have a family history, as they tend to be heritable.
From a pathogenic point of view, allergic rhinitis (AR) is an immune-mediated inflammatory disease, specifically a type I hypersensitivity reaction. It occurs when the subject, previously sensitized to specific allergens, is exposed to them, leading to the production of a large amount of IgE antibodies or reagents. These antibodies bind to the surface of certain cells, which release inflammatory mediators (such as histamine). These mediators trigger an inflammatory reaction in the nasal mucosa, resulting in the onset of symptoms.

The immune response that develops during allergic rhinitis consists of two phases: an immediate or early phase and a late phase[27]. Microscopically, both responses are characterized by the infiltration of inflammatory cells (eosinophils, mast cells, neutrophils, and lymphocytes) and the presence of numerous chemical mediators in nasal secretions, responsible for the main symptoms of this condition (nasal congestion, sneezing, itching, rhinorrhea, and tearing)[28]. In perennial allergic rhinitis, where allergen exposure is continuous, albeit low, a condition of minimal persistent inflammation is established, characterized by infiltration of neutrophils, sporadic eosinophils, and rare mast cells[29]. Moreover, studies have shown that patients with perennial rhinitis have a higher number of mucous goblet cells compared to those suffering from seasonal rhinitis.

According to the ARIA guidelines, allergic rhinitis can be classified as intermittent (seasonal) or persistent (perennial) based on the duration of symptoms over time, and as mild-moderate or severe based on the severity of symptoms[22].
In this regard, it is worth mentioning that allergic rhinitis, although not presenting severe clinical manifestations, can significantly impact the quality of life, sleep, and school performance of the child[14]. Intermittent forms are typically seasonal and affect individuals sensitized to allergens present only during specific times of the year (pollens). On the other hand, persistent forms tend to occur in patients sensitive to allergens constantly present in the environment (dust mites).
The typical clinical symptoms of allergic rhinitis include:

- Sneezing
- Watery rhinorrhea
- Nasal itching
- Nasal congestion
- Hyposmia (reduced sense of smell)
- Ocular symptoms due to allergic conjunctivitis

In addition to the typical signs and symptoms, atypical symptoms may also occur, including unilateral symptoms, isolated nasal obstruction, isolated mucopurulent or posterior rhinorrhea, anosmia (loss of sense of smell), recurrent pain, and epistaxis (nosebleeds).

In recent years, allergic rhinitis has been considered not only as an inflammation limited to the nasal cavities but also as one of the sites where allergic reactions can occur. These reactions usually involve both the upper and lower airways, leading to the concept of "united airway

disease." According to this concept, allergic rhinitis should be considered as part of a unique underlying immune-mediated pathological state that predisposes to allergic manifestations of the airways. This principle explains why allergic rhinitis is often associated with allergic conjunctivitis and asthma. Even in individuals with allergic rhinitis, especially if persistent, nonspecific bronchial hyperreactivity may be detected before asthma is fully manifested.

For diagnosis, skin prick tests (prick tests) are essential. Specific IgE titration (RAST) or total serum IgE tests are only performed as second-level investigations.

Otitis

The term otitis refers to an inflammation of the ear, which can have an acute or chronic course. Acute otitis can involve the external ear (external otitis) or the middle ear (middle ear otitis); whereas chronic otitis usually affects the middle ear.
From a clinical point of view, the following types are distinguished:

- Acute Otitis Media (AOM) is an acute infection of the middle ear, caused by pathogens, mainly bacteria but also viruses. It is characterized by the presence of purulent fluid in the middle ear, accompanied by signs and symptoms of infection. Clinically, it presents with ear pain (otalgia), ear discharge (otorrhea), conductive hearing loss, fever, and irritability. In the early stages of the infectious process, otoscopy shows a hyperemic and bulging tympanic membrane, a critical finding for the diagnosis of AOM. If not promptly treated with appropriate therapy, the tympanic membrane may undergo spontaneous perforation, leading to the accumulation of purulent secretions in the external auditory canal. AOM often develops on the basis of Otitis Media with Effusion (OME).

- Otitis Media with Effusion (OME), also known as serous otitis media or glue ear, is a form of chronic middle ear inflammation characterized by the presence of serous fluid in the tympanic cavity, causing conductive hearing loss due to the attenuation of sound transmission from the air to the inner ear. In this case, unlike AOM, the tympanic membrane is intact and opaque, and there are no signs or symptoms of acute infection. The diagnosis of OME is based on the detection of fluid in the middle ear during otoscopy.

Middle ear infections are among the most common pathologies in pediatric patients, with approximately 80% of children experiencing at least one episode of middle ear otitis in the first three years of life[30]. This high frequency can be attributed to the anatomy of the Eustachian tubes. The middle ear, together with the nasal cavities and the nasopharynx, forms an anatomical continuum thanks to the Eustachian tubes, which connect the middle ear to the nasopharynx through the tubal orifices. These tubes protect the middle ear through ventilation, drainage of secretions (thanks to the presence of respiratory epithelium and mucociliary clearance), and protection from physiological pressure variations.

In children, Eustachian tubes are shorter, more horizontal, and wider compared to adults, making them more susceptible to blockage. As a result, the middle ear loses its natural protection from the nasopharyngeal environment. The most common causes of Eustachian tube dysfunction and middle ear otitis in children are adenoid hypertrophy, upper respiratory tract infections, and cleft palate. Other common risk factors for OME in childhood include mucociliary dysfunction, lack of breastfeeding, immunodeficiency, climatic conditions, passive smoking, and genetic factors.

The scientific community also debates the possible role of rhinitis in the development of middle ear infections. The influence of allergic rhinitis in the development of middle ear otitis is now well established and supported by numerous studies. Recently, the hypothesis that non-allergic rhinitis may also play a role as a predisposing factor for middle ear infections has been proposed. Although the discussion is ongoing, the general consensus is that there is an increased risk of OME in children with allergic rhinitis, probably related to nasal obstruction and allergic response mediators that alter Eustachian tube function.

A recent study by Quaranta et al. investigated the correlation between allergic and non-allergic rhinitis and the development of OME. The results confirmed a 2 to 4.5-fold increase in the relative risk of OME in allergic patients, while subjects with non-allergic rhinitis showed a positive association between mast cells in the nasal mucosa and the risk of evolving into OME[31]. The role of eosinophils in this context still needs to be further clarified, as it has led to conflicting results in different studies.

Allergy

Allergies are abnormal or exaggerated immune reactions to external antigens, induced by contact, inhalation, and/or exposure to foreign substances normally harmless to the body, called allergens. This condition is called hypersensitivity[32].
Hypersensitivity reactions that underlie allergies can be Type 1 (IgE-mediated) or Type 2 (cell-mediated) according to the Gell and Coombs classification. Therefore, it is possible to distinguish two forms of allergies: IgE-mediated and non-IgE-mediated. We will focus on the IgE-mediated forms, which are based on a condition called "atopy," defined as a familial or individual tendency to produce IgE antibodies in response to low doses of allergens. Type 1 hypersensitivity reactions are the basis of all atopic diseases, including atopic dermatitis, allergic rhinitis, allergic conjunctivitis, asthma, food allergies, and anaphylaxis.
The most common allergens include tree and grass pollen (grasses, olive, beech, birch, hazel, cypress, wall pellitory, mugwort, ragweed), dust mites, foods (nuts, shellfish, eggs, cow's milk, red fruits), pet dander, drugs, and insects.
Allergies are the most common chronic diseases in children and young adults. In developed countries, about 20% of people have allergic rhinitis, approximately 6% have at least one food allergy, and about 20% have atopic dermatitis.

Allergies are mainly due to:

- Genetic factors: Allergy is strongly influenced by genetic factors, as suggested by the familiar nature of the disease and the association between atopy and specific HLA (human leukocyte antigen) loci and polymorphisms of various genes, including those of the beta chain of the high-affinity receptor for IgE, the alpha chain of the IL-4 receptor, IL-13, and CD14.

- Environmental factors: pollution, tobacco smoke, allergen exposure, reduced circulation of infectious agents, defined as the "hygiene hypothesis." Environmental factors interact with genetic factors in maintaining immune responses mediated by type 2 helper T cells (TH2).

The allergic reaction is a specific immune response mediated by class E immunoglobulins (IgE) produced by B lymphocytes and exposed on the surface of mast cells[32].

Allergic diseases include allergic rhinitis, allergic conjunctivitis, food allergies, atopic dermatitis, allergic asthma, and anaphylaxis. The most common symptoms include serous rhinorrhea, sneezing, red and swollen eyes, itching. When the allergic disease affects the respiratory system, there will be a sense of chest tightness, shortness of breath, and wheezing, accompanied by a dry cough. The skin can also be involved in allergic diseases, which can manifest with hives and edema, associated with itching, or with swelling and burning of the lips. The diagnosis of allergies is performed through skin tests (Prick Test or Patch Test in contact dermatitis) and blood tests (Prist and Rast).

Asthma

Asthma is a chronic respiratory condition that commonly affects children, characterized by inflammation and narrowing of the airways, leading to recurrent episodes of wheezing, coughing, chest tightness, and shortness of breath. It is one of the most prevalent chronic diseases in childhood. The prevalence of pediatric asthma varies across different regions and is influenced by genetic, environmental, and lifestyle factors. In many developed countries, asthma affects approximately 5-10% of children[33].

The pathogenesis of asthma is believed to result from a complex interplay of genetic susceptibility and environmental triggers. Children with a family history of asthma or allergies are at a higher risk of developing the condition. Common triggers include respiratory infections, allergens (such as pollen, pet dander, dust mites), air pollutants, tobacco smoke, and cold air.

Clinical manifestations of asthma in children can vary from mild to severe and may include coughing, wheezing or whistling sounds during breathing, shortness of breath, chest tightness, and difficulty in performing physical activities. Asthma symptoms can be triggered or exacerbated by exposure to allergens, viral infections, cold air, exercise, or irritants.

The diagnosis of asthma in children involves a thorough medical history, physical examination, and lung function tests such as spirometry or peak flow measurement. Doctors may also use allergy testing to identify specific triggers. Monitoring asthma symptoms and lung function over time is crucial for accurate diagnosis and management.

The treatment of pediatric asthma aims to control symptoms, prevent exacerbations, and improve the child's quality of life. This typically involves two types of medications: quick-relief medications (short-acting bronchodilators) to provide immediate relief during asthma attacks, and long-term control medications (inhaled corticosteroids, leukotriene modifiers, long-acting bronchodilators) to reduce airway inflammation and prevent future attacks.

Bronchitis

Bronchitis in the pediatric population is a respiratory condition characterized by inflammation of the bronchial airways. It can occur in acute, recurrent, or chronic forms, with acute bronchitis being more prevalent in children. This condition is most commonly seen during the colder months, when viral infections tend to spread more easily. Viruses like respiratory syncytial virus (RSV), influenza, and parainfluenza are the primary culprits behind pediatric bronchitis. These viruses invade the bronchial lining, causing irritation and swelling, which leads to the narrowing of the airways and increased mucus production. As a result, children with bronchitis experience persistent coughing, wheezing, and difficulty breathing.

The prevalence of pediatric bronchitis is significant, as it accounts for a considerable number of doctor visits and hospitalizations in children each year, affecting up to 40% of children newly referred for specialist management of persistent wet cough[34]. While most cases of acute bronchitis resolve on their own with supportive care, children with recurrent or chronic bronchitis may require more attention and monitoring.

The clinical manifestations of pediatric bronchitis may range from mild to severe. Besides the characteristic cough, children may present with fever, fatigue, chest discomfort, and nasal congestion. In more severe cases, the cough may persist for several weeks, affecting sleep and daily activities. Some children may also develop a secondary bacterial infection, leading to more severe symptoms and complications.

Diagnosing pediatric bronchitis usually relies on a thorough medical history, physical examination, and evaluation of symptoms. In some cases, additional tests like chest X-rays or respiratory secretions analysis may be necessary to rule out other respiratory conditions and determine the specific pathogen responsible for the infection.

The treatment of pediatric bronchitis mainly focuses on supportive care. Rest, hydration, and fever management are essential components of the treatment plan. In viral cases, antibiotics are not effective, as bronchitis is typically caused by viral infections. However, if the child develops a secondary bacterial infection, antibiotics may be prescribed. Respiratory treatments, such as bronchodilators and inhaled corticosteroids, may be used to alleviate airway inflammation and improve breathing in more severe cases or in children with underlying respiratory conditions.

EXTERNAL FACTORS

Breastfeeding

Exclusive breastfeeding for the first 6 months of an infant's life, with continued breastfeeding for up to 2 years or longer, is recognized as the best option because of its nutritional content and bioactivity[35].

The human milk has a complex composition, containing allergens, cytokines, immunoglobulins, polyunsaturated fatty acids and chemokines that interact with the infant immune system and intestinal milieu. Instead, formula-fed infants had lower bacterial diversity and an altered intestinal microbiota in the first few weeks of life.

Breastfeeding up to 6 months from birth is proved as a protective factor on allergic outcomes[35]. Moreover, a recent systematic review stated that never being fed with human milk is associated with higher risk of childhood asthma on lower airways tract infections[36].

Nevertheless, in current literature there is insufficient evidence to determine the relationship of formula milk feeding with the onset of allergic rhinitis throughout the lifespan, but also of asthma in adolescence or in adulthood[37].

Maternal smoking during pregnancy

Tobacco exposure is a known environmental factor. It had been reported as an important factor involved in allergic rhinitis of general population. In fact, long-term effects lead to damage of the ciliary cells of the nasal mucosa, becoming the inducement of allergic rhinitis and/or aggravating the condition itself[38].

Substantial evidence suggests that environmental factors exposure in early life not only influence prenatal development, but also may produce structural and functional alteration, leading to increased risks of metabolic, cardiovascular, and neuroendocrine disorders in offspring. Tobacco exposure is found to be the most important toxic factor in utero and in early life, which has been implicated in the aetiology of asthma and some allergic disease in offspring, according to a recent review[39]. However, a meta-analysis showed that maternal smoking exposure during pregnancy is not associated with the risk of allergic rhinitis in the offspring[40].

Type of delivery

Rates of cesarean delivery continue to rise worldwide, with rates of 24.5% in Western Europe. Noteworthy, the risks of short-term and long-term complications are known for both mother and child.

As shown in a meta-analysis, children delivered by cesarean delivery had increased risk of asthma up to the age of 12 years[41]. Another interesting study divided evidences between emergency cesarean delivery and programmed delivery: the former in associated with an higher risk of developing wheeze and food allergy, while the latter shows no statistical association[42]. An association exists also between cesarean section and the increased risk of pediatric allergic rhinitis[43].

Nasogastric tube

A nasogastric tube can be passed at birth through each nostril in all newborns with breathing difficulties or anomalies which may depose a suspect for choanal atresia.

The insertion of a foreign body made us hypnotize that there could be some sort of reaction in the newborn, especially at the level of nasal mucosa.

To our knowledge, no study investigated any possible reaction nor any correlation with the onset of further diseases during development.

Kindergarten

The school environment can be an important size of exposure to indoor allergens detected in settled school dust. From a clinical perspective, the school environment in industrialized nations has a lower potential for exposure than the home environment, but it becomes significant for allergic individuals whose home environment has been addressed to their needs[44].

An interesting study evaluated external factors which may influence the detection of specific IgE in children's blood and their positive anamnesis for allergies: an early beginning to attend kindergarten or nursery increases the frequency of specific IgE detection from various allergens, which more strongly stimulates an immune system to allergic reactions, in humans exposed to these allergens in the first year of their life[45].

Aurrecoechea et al. also evidenced in 975 children who attended kindergarten before 24 months , that the activity increases the risk of recurrent wheezing by 69%, bronchitis by 57%, and otitis media by 64%[46].

Siblings

Siblings are always been of interest for research: shared genetic background and common environmental exposure may be extremely important to better understand diseases and their etiology.

What is known is that between siblings we can observe a similar scenario: the positive rates of different allergens are similar between siblings. Elder siblings with dust mites, fungi, weed pollen, or food allergen positivity will have younger siblings sensitive to the same types of allergens[47].

A recent study showed that having 3 or more older siblings increases the risk of adult onset of asthma, and this might reflect their childhood infection burden[48].

On the contrary, a review of 2023 evidenced that being second- or later-born child is protective against allergic rhinitis. Also having siblings, regardless of birth order, is associated with a decreased risk of current allergic rhinitis[49].

CLINICAL STUDY

BACKGROUND

Pediatric respiratory diseases, such as otitis, rhinitis, asthma, and bronchitis, are common afflictions among children worldwide. These conditions pose significant challenges for the healthcare system, affect social life, and impact school attendance. Despite various risk factors known to be associated with their onset, there is currently no foolproof method to predict these pathologies in children[50]. Given the high incidence of these respiratory diseases, their implications on the healthcare system and social life, nasal cytology may be considered as a diagnostic tool to predict their onset, supported by existing literature[51].

The prevalence of common pediatric respiratory diseases is alarming. Otitis media, characterized by inflammation of the middle ear, affects a substantial number of children annually. Upper respiratory tract infections, like rhinitis, are frequent and often recurrent, leading to considerable morbidity in pediatric populations. Also, asthma affects millions of children globally, while bronchitis, an inflammation of the bronchial tubes, is also prevalent among young patients[23,35,51].

The high incidence of pediatric respiratory diseases exerts a considerable burden on healthcare systems. Frequent doctor visits, diagnostic tests, medication prescriptions, and potential hospitalizations significantly contribute to the escalating healthcare costs. Treatment and management of these conditions can place a substantial strain on families and government healthcare budgets alike[52].

Pediatric respiratory diseases can have profound effects on children's social life and school attendance. Frequent illness episodes may lead to reduced physical activity, social isolation, and missed opportunities for skill development and education. Children with chronic respiratory conditions like asthma often face restrictions in participating in physical activities and may experience stigmatization from their peers.

Currently, predicting the onset of pediatric respiratory diseases remains a challenge. While certain risk factors such as family history of allergies, exposure to smoke during pregnancy and after, and air pollution have been associated with an increased likelihood of developing these conditions, there is no definitive tool to predict their occurrence in individual children. This limitation hinders early intervention and preventative strategies[53].

Nasal cytology, a diagnostic tool commonly used in the ENT (Ear, Nose, and Throat) field, shows promise as a potential predictor of the onset of pediatric respiratory diseases[2,54]. The technique involves the examination of nasal secretions and cells obtained through scraping of the inferior turbinate. By analyzing cellular patterns and inflammatory markers, nasal cytology may offer valuable insights into the early stages of respiratory diseases in children[54, 55].

Recent studies have explored the potential link between nasal cytology and the onset of respiratory diseases[8]. Furthermore, a polish research team started to study nasal cytology at birth and its connection with the exposure to smoke during fetal life[7,56]

We thought to extend this concept to other common pediatric pathologies, and hence to uncover if there is a link between nasal cellular composition at birth and the chance of developing such diseases in the first 3 years of life.

The study aims to collect and analyze the nasal mucosa cytological composition, on a bigger scale as previously reported, at birth (in the first 24 hours of life) and therefore obtaining an uncontaminated and representative sample; collection of nasal mucosa cytology at 1 and 3 years of life in order to analyze any variation on its composition. Moreover, we set a longitudinal prospective study until 3 years of life of the child in order to evaluate the association of nasal cytology with the development of diseases like asthma, rhinitis, allergy, bronchitis, otitis, positive prick test and URTI, and to assess whether nasal cytology is influenced by selected external factors. Finally, we would like to provide a baseline of cytological composition as well as of any external influencing factors that could interfere with the nasal cell composition in order to further investigate the relationship between genetic and environmental components in the pathogenesis of these diseases.

Materials and Methods

A longitudinal prospective study was carried out. We enrolled 241 consecutive newborns whose mothers were admitted for delivery at San Paolo Hospital, Milan from February to December 2016. Patient underwent nasal cytology at birth, 1 year and at 3 years of life.

Inclusion and exclusion criteria and data collection

We included newborns of both sexes born after the 34th pregnancy week, with both parents Italian, with the rational to minimize genetic differences due to diverse ethnic backgrounds. Newborns with any pathology and/or alterations of the head and neck area were excluded.

All participating newborns received nasal scraping within their first 24 hours. According to standardized criteria, samples have been collected by means of a Rhino-Probe™ curette (Arlington Scientific Inc. Springville, Utah, USA) from the middle portion of the inferior turbinate where the rate ciliate/mucinous cells are expected to be well balanced[17]. The procedure was performed under anterior rhinoscopy, with an appropriate light source. No application of anaesthetic is required. The curette was immediately smeared on a glass slide paying attention to properly distribute the collected material on the slide and to dissipate the possible clots of material. Then, samples are stained with May-Grunwald-Giemsa (MGG) Quick Stain coloring (Bio -Optica)[1]. The slides were examined by a Zeiss Axio Lab A1 and 50 microscopic fields were read at a magnification of 1000x, to assess the presence of normal and abnormal cellular elements. A minimum of fifty fields is considered necessary to identify a sufficient number of cells. A qualitative and quantitative grading was used[20].

The process was repeated at 1 and 3 years of life. A skin prick test for frequent allergens was performed during the last sampling (3y) in order to discern AR from NAR and to objectify allergic sensitization.

Structured questionnaires were administered face to face to newborns' parents at birth, 1 year and at 3 years of life. At birth, we collected socio-economic factors, anamnestic data of parents, pregnancy exposures and delivery characteristics (e.g., pregnancy complication, drugs and supplementations use, mother alcohol use, type of delivery, induction and delivery complications), parents smoking (including mother passive smoking during pregnancy), and children characteristics at birth. At 1 and 3 years of age we collected data on siblings, breastfeeding and solid foods, children exposure to passive smoking and kindergarten attendance. In addition, children's history of AOM, URTI, bronchitis, and bronchial asthma/wheezing during, respectively, the first and the second/third years of life were reported by their parents.

Since there is no validated questionnaire evaluating the incidence of asthma, URTI, allergy, bronchitis and AOM all together, colleagues built a questionnaire including questions taken from ISAAC study for asthma and allergies, and The Acute Respiratory Tract Infection Questionnaire for URTI, AOM and bronchitis[57,58]. Questionnaires were administered in Italian language, since an inclusion criteria for patients' selection was having Italian parents. Moreover, parents were asked to bring to the 1 and 3-year evaluation all documentation relating to primary care visits, hospital stays and specialists' visits.

At year 3, NC was performed only on 118 patients due to SARS-COV2 pandemic limitations at our hospital; 204 underwent questionnaire completion (parents and their children who could not come to the hospital because of the aforementioned limitations underwent telephonic questionnaire completion).

Statistical analysis

Descriptive statistics were used to describe data on newborns', parents' and nasal mucosa cytological composition.

Sex-adjusted log-binomial regression models were used to evaluate the association of selected exposures (in pregnancy, at birth, and during the first 3 years of children life) with nasal mucosa cytological composition at birth, 1 and 3 years. Relative risks (RR), with their corresponding 95% confidence intervals (CI) were calculated. In case of cross-sectional associations, we calculated the odds ratios (OR) and the corresponding 95% CI by sex-adjusted logistic regression models.

Log-binomial regression models adjusted for sex were also used to assess the association between nasal mucosa cytological composition at birth (exposure) and the development of AOM, URTI, bronchitis, and bronchial asthma/wheezing (outcomes) during the first year of life and during the first 3 years of life. Similarly, we estimated the RR of positivity to the skin prick test at the 3-year evaluation according to NS at birth.

In supplementary analyses, we evaluated the association between the presence of selected diseases during the first year of life and nasal mucosa cytological composition at 1 year the chi-square test or the Fisher exact test.

All the analyses were performed using the SAS software, version 9.4 (SAS Institute, Inc., Cary, NC, USA).

Results

We included 241 newborns in our study; among them, 233 had sufficient material to evaluate NC at birth, at 1 year, questionnaire data were available for 222 children; of them, 218 had sufficient material to evaluate NC. At 3 years, 204 children had questionnaire data and 126 NC data. Overall, a group of 118 children completed the follow up period up to 3 years fulfilling all the evaluated fields, including NC, while 86 patients were evaluated up to 3 years without performing NC due to pandemic restrictions, with a total case series of 204 children who completed the study.

A description of newborns and their parents' characteristics as well as pregnancy and delivery characteristics is shown in Table 2. Overall, 50.6% newborns were males; the mean gestational age at birth was 39.2 weeks (SD 1.3). Standard delivery was seen in 77.1% of children, while 22.9% were born through cesarian delivery.

	N[a] (%)
Male sex	122 (50.6)
Gestational age (week), mean (SD)	39.2 (1.3)
Birth weight (g), mean (SD)	3266 (413)
Choanal exploration with nasogastric tube	84 (35.1)
Siblings	118 (53.4)
Kindergarten[b]	78 (35.1)
Exclusive breastfeeding[c]	136 (61.3)
Maternal age at birth (yr), mean (SD)	33.7 (5.0)
Paternal age at birth (yr), mean (SD)	36.5 (5.7)
University maternal education	103 (42.9)
University paternal education	82 (34.3)
Mother passive smoking during pregnancy	39 (16.3)
Any pregnancy complication	78 (32.5)
Delivery	
Vaginal	186 (77.1)
Caesarean section	55 (22.9)
Elective	37 (67.9)
Emergency	18 (32.1)

Table 2. Characteristics of 241 newborns included in the study and their parents [a] Numbers in the Table are frequencies (percentages), unless otherwise specified. The sum may do not add up to the total because of missing values. Percentages are calculated over the number of subjects with non-missing information.[b] During the first year of life. [c] Until solid food introduction.

Nasal mucosal composition

Cellular composition of nasal mucosa at birth, 1 and 3 years is synthetized in Table 3. At birth, there was a prevalent cellular composition of ciliated cells and rare neutrophils. At 1 year, ciliated cells started reducing in favor of muciparous cells and neutrophils; rare bacteria were detectable. Evaluation at 3 years shows a stable composition with a progressive decrease per field of ciliated cells and increasing of bacteria.

	N[c] (%)		
	Birth	1 year[a]	3 year[b]
Ciliated cells			
0	7 (3.0)	2 (1.0)	5 (4.2)
+	15 (6.4)	35 (16.7)	21 (17.8)
++	25 (10.7)	55 (26.2)	29 (24.6)
+++	54 (23.2)	50 (23.8)	38 (32.2)
++++	132 (56.7)	68 (32.4)	25 (21.2)
Muciparous cells			
0	152 (65.2)	80 (38.1)	50 (42.4)
+	52 (22.3)	63 (30.0)	47 (39.8)
++	21 (9.0)	47 (22.4)	18 (15.3)
+++	6 (2.6)	14 (6.7)	2 (1.7)
++++	2 (0.9)	6 (2.9)	1 (0.9)
Neutrophils			
0	36 (15.5)	25 (11.9)	14 (11.9)
+	80 (34.5)	40 (19.1)	30 (25.4)
++	56 (24.1)	46 (21.9)	26 (22.0)
+++	27 (11.6)	52 (24.8)	28 (23.7)
++++	33 (14.2)	47 (22.4)	20 (17.0)
Eosinophils			
0	223 (95.7)	195 (93.3)	110 (93.2)
+	10 (4.3)	10 (4.8)	6 (5.1)
++	0	3 (1.4)	2 (1.7)
+++	0	1 (0.5)	0
Lymphocytes			
0	204 (88.3)	165 (78.6)	110 (85.6)
+	25 (10.8)	32 (15.2)	15 (12.7)
++	2 (0.9)	13 (6.2)	2 (1.7)
Mast-cells			
0	232 (99.6)	208 (99.0)	118 (100.0)
+	1 (0.4)	2 (1.0)	0
Macrophages			
0	231 (99.6)	191 (100.0)	118 (100.0)
+	1 (0.4)	0	0
Bacteria			
0	190 (81.9)	88 (41.9)	50 (42.4)

+	30 (12.9)	42 (20.0)	38 (32.2)
++	8 (3.5)	42 (20.0)	14 (11.9)
+++	3 (1.3)	26 (12.4)	11 (9.3)
++++	1 (0.4)	12 (5.7)	5 (4.2)

Table 3. Nasal mucosa cytological composition at birth (N=233), 1 year (N=210[a]) and 3 years (118[b]). Semiquantitavie evaluation of cellular composition is based on Evaluation table of "Atlante di citologia nasale. M. Gelardi."[1] [a] The analysis included only children with nasal cytology data both at birth and 1 year. [b] The analysis included only children with nasal cytology data at birth, 1 year and 3 years. [c]The sum may do not add up to the total because of missing values. Percentages are calculated over the number of subjects with non-missing information.

Factors influencing nasal mucosal composition

Table 4 gives results for the association between selected exposures and nasal mucosa composition at each time point. Caesarian delivery significantly impacted the presence of eosinophils at birth: 11.5% of children born by caesarean delivery vs. 2.2% of those born by vaginal delivery had eosinophils at birth, for a corresponding RR of 5.17 (95% CI: 1.52-17.6). No association was found between delivery modus and presence of bacteria at rhinocytogram.

Evaluation of choanal patency at birth with a nasogastric tube when a stenosis was suspected was associated with the presence of eosinophils (8.6% vs. 2.0%, RR: 4.66, 95% CI: 1.23-17.64) and abundant neutrophils (61.7% vs. 43.6%, RR: 1.37, 95% CI: 1.07-1.77) at birth; the use of nasogastric tube was also associated with a lower risk of abundant muciparous cells at birth (25.9% vs. 36.7%, RR: 0.66, 95% CI: 0.44-1.01) (Table 4). No other factor was significantly associated with nasal mucosa composition at the various time points.

	Muciparous cells		Neutrophils		Eosinophils		Lymphocytes		Bacteria	
	Abundant[e] n (%)	RR[f] (95% CI)	Abundant[e] n (%)	RR[f] (95% CI)	Present n (%)	RR[f] (95% CI)	Present n (%)	RR[f] (95% CI)	Present n (%)	RR[f] (95% CI)
NC at birth										
Maternal smoking in pregnancy[a]										
No	66 (35.7)	1	92 (49.7)	1	10 (5.4)		23 (12.6)	1	34 (18.4)	1
Yes	14 (29.8)	0.83 (0.52-1.35)	24 (52.2)	1.05 (0.77-1.42)	0 (0)	ne	4 (8.5)	0.68 (0.25-1.87)	8 (17.4)	0.95 (0.47-1.90)
Delivery										
Vaginal	63 (35.0)	1	87 (48.6)	1	4 (2.2)		19 (10.6)	1	34 (19.0)	1
Caesarean section	17 (32.7)	0.94 (0.61-1.46)	29 (55.8)	1.15 (0.87-1.52)	6 (11.5)	5.17 (1.52-17.6)	8 (15.7)	1.48 (0.69-3.18)	8 (15.4)	0.81 (0.40-1.64)
Choanal exploration with Nasogastric tube										
No	58 (36.7)	1	65 (43.6)	1	3 (2.0)	1	14 (9.4)	1	30 (20.1)	1
Yes	21 (25.9)	0.66 (0.44-1.01)	50 (61.7)	1.37 (1.07-1.77)	7 (8.6)	4.66 (1.23-17.64)	12 (15.0)	1.65 (0.79-3.42)	10 (12.4)	0.61 (0.32-1.20)
NC at 1 year										
Smoking exposure[b]										
No	48 (32.2)		104 (69.8)	1	12 (8.1)	1	34 (22.8)	1	92 (61.7)	1
Yes	21 (30.9)	0.96 (0.63-1.47)	47 (69.1)	0.98 (0.81-1.18)	2 (3.0)	0.37 (0.09-1.61)	13 (19.1)	0.84 (0.47-1.48)	34 (50.0)	0.81 (0.62-1.06)
Delivery										
Vaginal	52 (31.1)	1	119 (71.3)	1	11 (6.6)	1	38 (22.8)	1	95 (56.9)	1
Caesarean section	17 (34.0)	1.08 (0.69-1.69)	32 (64.0)	0.89 (0.71-1.11)	3 (6.0)	0.90 (0.26-3.12)	9 (18.0)	0.79 (0.41-1.51)	31 (62.0)	1.09 (0.85-1.41)
Nasogastric tube										
No	42 (30.2)		101 (72.7)	1	10 (7.3)	1	30 (21.6)	1	84 (60.4)	1
Yes	25 (32.5)	1.10 (0.73-1.65)	50 (64.9)	0.91 (0.75-1.08)	4 (5.2)	0.72 (0.23-2.21)	17 (22.1)	1.04 (0.61-1.76)	42 (54.6)	0.91 (0.71-1.16)
Exclusive breastfeeding[c]										
No	28 (32.9)		57 (67.1)	1	4 (4.7)	1	19 (22.4)		52 (61.2)	1
Yes	41 (30.8)	0.95 (0.64-1.41)	95 (71.4)	1.05 (0.87-1.26)	10 (7.6)	1.61 (0.52-4.98)	28 (21.1)	0.94 (0.56-1.58)	75 (56.4)	0.92 (0.74-1.16)
Breastfeeding at 1-year										
No	50 (36.0)		98 (70.5)	1	10 (7.3)		33 (23.7)	1	80 (57.6)	1
Yes	19 (24.4)	0.57[g] (0.31-1.07)	54 (69.2)	0.95[g] (0.52-1.74)	4 (5.1)	0.69[g] (0.21-2.29)	14 (18.0)	0.70[h] (0.35-1.42)	47 (60.3)	1.12[h] (0.64-1.70)
NC at 3years										
Smoking exposure[d]										
No	17 (20.2)	1	52 (61.9)	1	8 (9.5)	1	13 (15.5)	1	50 (59.5)	1
Yes	6 (14.6)	0.71 (0.30-1.65)	23 (56.1)	0.92 (0.67-1.27)	1 (2.4)	0.26 (0.03-1.99)	4 (9.8)	0.63 (0.22-1.81)	21 (51.2)	0.90 (0.64-1.26)
Delivery										
Vaginal	19 (18.6)	1	63 (61.8)	1	9 (8.8)		16 (15.7)	1	59 (57.8)	1
Caesarean section	5 (20.8)	1.03 (0.43-2.46)	13 (54.2)	0.91 (0.61-1.36)	0 (0)	ne	1 (4.2)	0.27 (0.04-1.91)	13 (54.2)	0.96 (0.65-1.42)
Nasogastric tube										
No	19 (23.5)	1	52 (64.2)	1	8 (9.9)	1	12 (14.8)	1	44 (54.3)	1
Yes	5 (11.1)	0.40 (0.16-0.99)	24 (53.3)	0.85 (0.62-1.17)	1 (2.2)	0.22 (0.03-1.74)	5 (11.1)	0.75 (0.28-2.03)	28 (62.2)	1.24 (0.92-1.66)
Exclusive breastfeeding[c]										
No	8 (17.0)	1	30 (63.8)	1	4 (8.5)	1	9 (19.2)	1	26 (55.3)	1
Yes	15 (19.2)	1.14 (0.53-2.46)	45 (57.7)	0.91 (0.68-1.21)	5 (6.4)	0.75 (0.21-2.66)	8 (10.3)	0.54 (0.22-1.29)	45 (57.7)	1.02 (0.75-1.39)
Breastfeeding at 1-year										
No	15 (20.0)	1	45 (60.0)	1	7 (9.3)	1	8 (10.7)	1	40 (53.3)	1
Yes	8 (16.0)	0.82 (0.38-1.78)	30 (60.0)	1.02 (0.76-1.36)	2 (4.0)	0.42 (0.09-1.96)	9 (18.0)	1.69 (0.70-4.08)	31 (62.0)	1.13 (0.84-1.52)

Table 4. Association of type of delivery, nasogastric tube, exposure to smoking, breastfeeding with nasal mucosa cytological composition at birth, 1 and 3 years of life. CI: confidence interval; ne: not estimable; RR: relative risk.[a] Maternal smoking during pregnancy or maternal passive smoking

exposure during pregnancy. [b] Maternal smoking during pregnancy, or maternal passive smoking exposure during pregnancy or children passive smoking exposure during the first year of life. [c]Until solid food introduction. [d]Maternal smoking during pregnancy, or maternal passive smoking exposure during pregnancy, or children passive smoking exposure during the first 3 years of life. [e] Abundant muciparous cells definition: +/++/+++/++++ at birth, and ++/+++/++++ at 1 and 3 years. [f]Adjusted for sex

Nasal mucosal composition and disease development

Results for the association between nasal cytology at birth and the development of AOM, URTI, bronchitis, and bronchial asthma/wheezing during the first year and during the first 3 years of life are summarized in Table 4.

Among the 214 children who had both nasal cytology data at birth and questionnaire data at 1 year, 32 were reported to have had AOM during the first year of life (15.0%), 196 URTI (91.6%), 58 bronchitis (27.1%), 14 allergy (6.5%) and 23 bronchial asthma/wheezing (10.7%). Presence of eosinophils at birth significantly increased the risk of AOM during the first year of life (RR=3.4, 95% CI: 1.55-7.62). No other significant association was found between the various cellular types in the nasal mucosa at birth and diseases occurrence during the first year.

Within three years of life, AOM developed in 43.5% of children (87 out of 200 with available information on nasal cytology at birth and outcome), URTI in 94.4% (201 out of 213), bronchitis in 46.5% (93 put of 200), and bronchial asthma/wheezing in 19.9% (39 out of 196); 17 out of the 152 children who underwent skin prick testing at the 3 years visit were positive for at least one allergen (18.2%).

Nasal cytology at birth did not significantly affect the risk of the various diseases during the first three years of life: in particular, the direct association between the presence of eosinophils at birth and the risk of AOM was no longer detectable (RR: 1.32, 95% CI: 0.72-2.44) (Tab. 5).

	AOM		URTI		Bronchitis		Allergy (at 1 year)/Atopy (at 3 years)		Bronchial asthma/wheezing	
	Cases/tot (%)	**RR[a]**	**Cases/tot (%)**	**RR[a]**	**Cases/tot (%)**	**RR[a]**	**Cases/tot (%)**	**RR[a]**	**Cases/tot (%)**	**RR[a]**
					1 year					
Muciparous cells										
0	22/139 (15.8)	1	129/139 (92.8)	1	29/139 (28.1)	1	10/138 (7.3)	1	15/139 (10.8)	1
+/2+/3+/4+	10/75 (13.3)	0.82 (0.41-1.62)	67/75 (10.7)	0.96 (0.88-1.05)	19/56 (74.7)	0.90 (0.56-1.44)	4/75 (5.3)	0.74 (0.24-2.27)	8/75 (10.7)	0.99 (0.44-2.22)
Neutrophils										
0/+	19/110 (17.3)	1	99/110 (90.0)	1	30/110 (27.3)	1	5/110 (4.6)	1	11/110 (10.0)	1
2+/3+/4+	13/103 (12.6)	0.70 (0.36-1.33)	97/201 (94.2)	1.04 (0.96-1.13)	28/103 (27.2)	1.02 (0.65-1.59)	9/102 (8.8)	1.96 (0.68-5.68)	12/103 (11.7)	1.08 (0.50-2.33)
Eosinophils										
0	28/204 (13.7)	1	186/204 (91.2)		56/204 (27.5)	1	13/203 (6.4)	1	21/204 (10.3)	1
+	4/10 (40.0)	3.44 (1.55-7.62)	10/10 (100.0)	ne	2/10 (20.0)	0.70 (0.20-2.48)	1/10 (10.0)	1.56 (0.22-10.9)	2/10 (20.0)	2.34 (0.66-8.33)
Lymphocytes										
0	29/187 (15.5)	1	169/187 (90.4)		52/187 (27.8)	1	13/186 (7.0)	1	21/187 (11.2)	1
+/2+	3/25 (12.0)	0.75 (0.25-2.26)	25/25 (100.0)	ne	5/25 (20.0)	0.71 (0.32-1.61)	1/25 (4.0)	0.57 (0.08-4.19)	1/25 (4.0)	0.34 (0.04-2.42)
Bacteria										
0	25/174 (14.4)	1	162/174 (93.1)	1	49/174 (28.2)	1	13/174 (7.5)	1	19/174 (10.9)	1
+/2+/3+/4+	7/39 (18.0)	1.23 (0.57-2.62)	33/39 (84.6)	0.91 (0.79-1.04)	9/39 (23.1)	0.82 (0.44-1.52)	1/38 (2.6)	0.35 (0.05-2.61)	4/39 (10.3)	0.93 (0.34-2.55)
					3 years					
Muciparous cells										
0	59/130 (45.4)	1	133/139 (95.7)	1	64/131 (48.9)	1	25/128 (19.5)	1	12/95 (12.6)	1
+/2+/3+/4+	28/70 (40.0)	0.88 (0.62-1.24)	68/74 (91.9)	0.96 (0.90-1.03)	29/69 (42.0)	0.86 (0.62-1.19)	14/68 (20.6)	1.02 (0.57-1.82)	5/57 (8.8)	0.69 (0.26-1.85)
Neutrophils										
0/+	45/100 (45.0)	1	102/110 (92.7)	1	48/101 (47.5)	1	19/99 (19.2)	1	10/75 (13.3)	1

2+/3+/4+	41/99 (41.4)	0.91 (0.66-1.25)	98/102 (96.1)	1.03 (0.97-1.09)	45/98 (45.9)	0.98 (0.73-1.32)	20/96 (20.8)	1.05 (0.60-1.84)	6/76 (7.9)	0.56 (0.21-1.49)
Eosinophils										
0	82/191 (42.9)	1	191/203 (94.1)	1	89/191 (46.6)	1	36/187 (19.3)	1	17/145 (11.7)	
+	5/9 (55.6)	1.32 (0.72-2.44)	10/10 (100)	n.e.	4/9 (44.4)	0.93 (0.44-1.97)	3/9 (33.3)	2.03 (0.77-5.32)	0/7 (0)	ne
Lymphocytes										
0	79/176 (44.9)	1	174/186 (93.4)	1	83/176 (47.2)	1	34/138 (19.8)	1	13/133 (9.8)	1
+/2+	7/22 (31.8)	0.69 (0.37-1.31)	25/25 (100)	n.e..	9/22 (40.9)	0.87 (0.51-1.47)	4/22 (18.2)	0.89 (0.35-2.27)	2/17 (11.8)	1.18 (0.29-4.83)
Bacteria										
0	74/163 (45.4)	1	166/174 (95.4)	1	79/163 (48.5)	1	31/160 (19.4)	1	15/122 (12.3)	1
+/2+/3+/4+	13/36 (36.1)	0.79 (0.50-1.26)	34/38 (89.5)	0.95 (0.85-1.06)	14/36 (38.9)	0.80 (0.52-1.25)	8/35 (22.9)	1.18 (0.60-2.32)	2/29 (6.9)	0.56 (0.14-2.31)

Table 5. Association between nasal mucosa cytological composition at birth and development of acute otitis media (AOM), upper respiratory tract infections (URTI), bronchitis, allergy, and bronchial asthma/wheezing during the first year of life (top part od the table) and the first 3 year of life (bottom part of the table).[a] Adjusted for sex. ne: not estimable.

Influence of diseases on nasal mucosal composition

Children with URTI or allergy during the first year if life had more frequently lymphocytes at nasal cytology evaluation at 1 year (URTI: 23.5% vs. 0%, p=0.015; allergy: 42.9% vs. 20.1%, p=0.084). Children with allergy during the first year of life tended also to have more frequently abundant (++/+++/++++) neutrophils (100% vs. 67.7%, p=0.084) and those with bronchial asthma/wheezing to have less frequently abundant muciparous cells (16% vs. 33.7%, p=0.074) (Supplementary Table 1).

Discussion

The cytological expression of nasal mucosa in newborns and its association with the onset of ENT and other disorders is barely investigated. Nowadays, we can consider the rhinocytogram in adults well studied: normal nasal mucosa is composed by ciliated and muciparous cells, with a ratio of 4/5:1, plus straited and basal cells. Other cellular type shouldn't be present, except for rare neutrophils. The evidence of eosinophils, mastcells, bacteria and macrophages has to be considered sign of nasal pathology[1].

Rhinocytogram

Our study shows how, at birth, nasal cellular composition appears to have a preponderance of ciliated cells and almost absence of muciparous cells, with some neutrophils. At 1 year of life cellular composition seems to shift towards the normal adult composition, with a slight decrease of ciliated cells in favor of muciparous cells (ratio 4:1); neutrophils, however, are the only pathological cells which remain higher than in adult life. This could be explained by the higher incidence of infectious rhinitis and adenoiditis in children of 1-3 years and the consequent activation of neutrophils in the fight for bacterial growth. Evaluation at 3 years of life shows a stability of cytological asset.

As early as in 1985 Cohen et al. used nasal cytology to study the nasal mucosa of 22 infants, concluding that the technique is as safe and effective in infants as in children and adults and that the majority of healthy young infants do not have nasal eosinophilia[59].

Similarly to our study Tarchalska-Kryńska et al. evaluated nasal mucosa with cytology in 105 healthy newborns and cytograms of the nasal mucosa of 1-7 days old infants and showed how those differ from those of adults: both columnar cells or neutrophils are prevalent in cytograms of these children[6]. Despite the significance of the study, inclusion criteria allowed newborns up to 7 days of life, whose nasal mucosa was already exposed to a multitude of external influxes of the environment. This could possibly alter the evidences of normality and the concept of "baseline"-finding .

Another study evaluated premature newborns, which seem to respect the same cellular composition[56]: we did not assess this aspect since original inclusion criteria allowed only to term infants, nevertheless it would have been an interesting factor to consider.

Our study therefore is the first to show a large-scale presentation of newborn's cellular nasal composition when still uncontaminated from any external factor.

External factors

The influence of external factors during pregnancy and after birth related to the onset of altered nasal cytology at birth is of intense interest, as it could be an alarm bell for the development of further diseases later in life. We therefore analyzed whether there is an association with external and parental factors (e.g., breastfeeding at birth and at 1 year, smoking). Analysis revealed that active and passive smoking during pregnancy do not influence the cytological picture of the nasal mucosa of neonates as shown in table 3: it remains comparable to the one of the whole case series. Literature still lacks about this topic. Only an interesting study by Krol and colleagues evaluated nasal cytology of newborns who were exposed to tobacco smoke during fetal life: the most common type of cytogram contained neutrophils, columnar cells, and squamous cells[7].

Perinatal influencing factors

Analysis of pregnancy and delivery aspects revealed how caesarian delivery impacts the presence of eosinophils at birth, carrying the possibility of developing eosinophil-driven diseases. This may be also due to different handling of the child in the first minutes to hours after birth as often those deliveries are done for more high-risk situations and/or the absence of the passage of the birth canal. Literature reinforced our evidences: a study by Jensen et al. proved how caesarian delivery is associated with a higher risk of developing eosinophilic esophagitis[60]. Also Ren and his group discovered how cesarian delivery aggravates the nasal symptoms of AR in mices[61]. Although we did not investigate esophagitis and AR, our analysis did not find any significant association between cesarian delivery and the incidence of atopic diseases such as asthma, allergies or rhynocytograms that could be those of allergic rhinitis patients.

Further investigation about this association in a wider population and with a longer follow up would be very important to validate or rebut our results.

Moreover, evaluation of choanal patency at birth with nasogastric tube increased the chance to have eosinophils and abundant neutrophils in nasal mucosa; to our knowledge, no studies evaluated this aspect previously. We suppose that the stimulus given by nasogastric tube at birth may lead to an inflammatory reaction with a recruitment of eosinophils and neutrophils in nasal mucosa even right after birth, showing the quick response extra-utero towards mechanic stimuli; nevertheless, it does not carry any further consequences in the evaluated time period. Interestingly, delivery modus (vaginal vs. cesarian) did not influence presence and quantity of bacteria at rhinocytogram at birth; however, microscopic analysis lacks the potential to differentiate distinctive types of bacteria, fact

that we would expect to differ between the two populations. Microbiome analysis could shed light on this.

Association of rhinocytogram and ENT diseases

Alteration of the rhinocytogram appears to be associated with the onset of a spectrum of ENT diseases.

Otitis

Our analysis revealed that the presence of eosinophils in nasal mucosa at birth is associated with a higher risk of developing AOM at 1 year; among these patients, only one dropped out of the study at three-years follow up, all others corroborated how that risk disappears at 3 years. Literature does not provide any study about AOM incidence associated to newborn's nasal cytology; therefore, further studies would be interesting to validate our finding and hence to investigate how the growth process up to 3 years may improve this predisposition.

Atopy

When correlating nasal cytology to atopy, literature seems divided. Borres analyzed cytograms of infants with a positive family history of allergies and then repeated the study at 18 months of age. In children with symptoms of atopy mast cells were more common. Eosinophils were in turn found in both groups of children without significant association[62]. Other authors maintain that eosinophils in nasal mucosa are the predictors of occurrence of varying atopic diseases (atopic dermatitis, asthma, and AR)[53,55,63].

Another interesting report about the development of mast cells and nasal eosinophils from age 4 months through 4 years in children of atopic parents reported that both eosinophils and mastcells were rare at 4 months in all infants, increased in atopic children from 1 to 4 years, and remained infrequent in nonatopic children [63]. Also Kajosaari et al. proved the higher percentage of mastcell and eosinophils in atopic children[64].

Surprisingly, we did not find any significant association of nasal cytology with the onset of atopy. In fact, none of the patients who were positive to skin prick test at 3 years presented eosinophils at nasal cytology at birth or at 3 years of age, while only one child with a history of allergy presented eosinophils at birth.

On the other hand, this is the first study finding an association between allergy during the first year of life and the presence of lymphocytes in nasal cytology at 1 year. It would be interesting to further investigate this finding expanding the observation of our study to a longer follow-up and to a wider cohort.

URTI

Also, the occurrence of URTI during the first year of life appeared to be related with the presence of lymphocytes in nasal cytology at 1 year. Literature does not provide any evidence about this association. Other association of the rhinocytogram and URTI at different timepoints was not seen.

Asthma

When evaluating lower airways, we found an association between asthma and wheezing during the first year of life and the number of muciparous cells in nasal mucosa at 1 year. A higher number of muciparous cells at 1 year could be interpretated either as a mechanism of protection of upper airways for lungs towards external factors, or maybe it may be seen as an expression of the United AirwayDisease concept, where the expression of muciparous cells in the lower tract may be seen also in the upper part. In fact, literature shows how the nose might be seen as an open window to the lower respiratory tract [64] and, among children, there is evidence that nasal epithelial cells are a valid surrogate for bronchial epithelial cells[22]. In any case, the association was not statistically significantly.

Our study has shown interesting findings: we could assess the normal cellular composition of newborns' nasal mucosa, which was before only poorly explored. This may be of enormous relevance in order to retain a baseline for further studies about nasal cytology modification from a healthy set to a developed pathological condition. On the other hand, it led us to depart from the diffuse thought that nasal eosinophils usually correlate with atopy and are already present at birth: in our study neither nasal cytology at birth or at 3 years was associated with the onset of allergy, URTI, bronchitis or asthma; only presence of eosinophils at birth increased the risk of AOM during the first year of life.

Wider studies with longer follow-up are needed to confirm our results. Our limited number of patients, also due to the unforeseeable advent of the SARS-COV2 pandemic, and the often low

prevalence of specific cells at rhinocytogram resulted in a low statistical power to detect many of the associations.
Also, inclusion of premature newborns could have provided interesting findings, as well as the evaluation of nasal mucosa in association with the onset of AR, adenoidal hypertrophy or obstructive sleep disorders.

Conclusions

Nasal cytology may be considered to become a promising tool of risk stratification, although we need further evidences. In our study, we found that caesarian delivery and nasogastric tube use for testing choanal patency are significantly related with the development of an inflammatory cellular nasal composition. Also, some specific nasal cytologies showed to be statistically significant in the prediction of the onset of many airways' diseases such as Upper Respiratory Tract Infections, AOM and allergy.
However, in our study no clearcut associations could be shown, further studies that take into account children especially older then 3 years, are essential to validate NC risk-evaluation role in the pediatric population.

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