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KU LEUVEN

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EXPLORING THE NEURO-IMMUNE MECHANISMS UNDERLYING NASAL HYPERREACTIVITY IN RHINITIS AND RHINOSINUSITIS

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List of abbreviations

ACh	Acetylcholine
ACTB	ß-actin
AIT	Allergen immunotherapy
AR	Allergic rhinitis
CC16	Clara cell protein 16
CDA	Cold, dry air
CLDN1	Claudin 1
CGRP	Calcitonin gene-related peptide
CRS	Chronic rhinosinusitis
CRSsNP	Chronic rhinosinusitis without nasal polyps
CRSwNP	Chronic rhinosinusitis with nasal polyps
CT	Computed tomography
FESS	Functional endoscopic sinus surgery
GAD-7	Generalized anxiety disorder-7
GNB2L1	Guanine nucleotide-binding protein subunit beta-2-like 1
HC	Healthy control
IL	Interleukin
ILC	Innate lymphoid cell
IQR	Interquartile range
LPS	Lipopolysaccharide
LT	Leukotriene
NA	Noradrenaline
NANC	Non-adrenergic, non-cholinergic
NAR	Non-allergic rhinitis
NHR	Nasal hyperreactivity
NKA	Neurokinin A
OCLN	Occludin
oNHR	Objectively diagnosed nasal hyperreactivity
PG	Prostaglandin
PGP9.5	Protein gene product 9.5

PHQ-9	Patient health questionnaire-9
PHQ-15	Patient health questionnaire-15
PNIF	Peak nasal inspiratory flow
PSS	Perceived stress scale
SCIT	Subcutaneous immunotherapy
sIgE	Allergen-specific immunoglobulin E
SLIT	Sublingual immunotherapy
sNHR	Self-reported nasal hyperreactivity
SP	Substance P
TAC1	Tachykinin precursor 1
TRP	Transient receptor potential
TRPA1	Transient receptor potential channel ankyrin 1
TRPM8	Transient receptor potential channel melastatin 8
TRPV1	Transient receptor potential channel vanilloid 1
TSLP	Thymic stromal lymphopoietin
VAS	Visual analogue scale
VIP	Vasoactive intestinal peptide
ZO-1	Zonula occludens 1

CHAPTER 1

Introduction

Adapted in part from: Backaert W *et al.* A TRiP Through the Roles of Transient Receptor Potential Cation Channels in Type 2 Upper Airway Inflammation. Curr Allergy Asthma Rep. 2021;21(3):20.

1. Chronic upper airway inflammation

Upper airway inflammation is an umbrella term covering multiple inflammatory diseases of the nasal mucosa – termed *rhinitis* – with possible expansion to the mucosa of the paranasal sinuses, in which case it is termed *rhinosinusitis*.[1,2] Typical rhinitis symptoms are nasal congestion, increased nasal secretions resulting in rhinorrhea or post-nasal drip, nasal itch, and sneezing. In case of rhinosinusitis, patients can also suffer loss of smell and/or facial pressure. Symptoms are present at least one hour per day for at least two consecutive days. Upper airway inflammation is arbitrarily defined as "acute" when symptoms are short-lasting, and "chronic" or "persistent" in case symptoms persist for 12 weeks or longer.[1,2]

About 10 % of the primary care consultations is related to upper airway inflammation, illustrating their high prevalence and incidence.[3] Indeed, 10-30 % of the European population suffers from chronic rhinitis or rhinosinusitis.[1,2,4,5] Chronic upper airway inflammation greatly impacts the quality of life of patients by inducing for example emotional stress, impairment of social functioning, or disturbed sleep, and it might even trigger clinical depression or anxiety disorders.[1,4,6–8] On top, it poses an enormous economic burden, estimated to cost tens of billions per year in the United States of America and Western European countries.[1,2,9–11] A substantial amount of this burden is due to loss of productivity (*i.e.* presenteeism) and loss of work days (*i.e.* absenteeism).[12,13] Given all these data, upper airway inflammation clearly poses a significant individual and societal burden.

Both persistent rhinitis and chronic rhinosinusitis encompass multiple clinical subgroups, called *phenotypes*. Considering persistent rhinitis, three major phenotypes can be recognized and distinguished: allergic rhinitis (AR), infectious rhinitis, and the heterogenous group of non-allergic rhinitis (NAR).[14] In case of chronic rhinosinusitis (CRS), a phenotype with and without nasal polyps can be distinguished.[2,15]

Allergic rhinitis is present when rhinological symptoms are caused by a type 1 hypersensitivity reaction to one or more airborne allergens.[1] It can be present in individuals who are priorly sensitized to the encountered allergen. Common examples are hay fever, house dust mite allergy, or allergy to animal dander. AR will be discussed more in detail in section 2.1 of this chapter.

Acute ***infectious rhinitis***, known as the "common cold", is the most common form of rhinitis. It covers all acute mucosal inflammatory diseases due to infection.[16] Most commonly, the infection has a viral origin, often caused by rhinoviruses, coronaviruses, adenoviruses, (para)influenzavirus, enterovirus, or respiratory syncytial virus. Typical symptoms are excessive, sticky nasal secretions and nasal obstruction. A viral infection is nearly always self-limiting and patients recover spontaneously, often without even consulting a physician.[16] It is estimated that an average adult suffers 2-5 episodes per year, while children suffer 7-10 episodes per year.[2] In some cases, bacterial surinfection can prolong the disease course, or the infection can spread to the mucosa of the paranasal sinuses.[17] This *post-viral rhinosinusitis* affects about 18 % of the adult population per year and is characterized by discolored nasal secretions, severe local pain, and fever.[2,18] Symptoms last for more than 10 days or there is "double sickening", with deterioration of symptoms after 5 days.[2] Post-viral rhinosinusitis is responsible for 1-2 % of primary care consultations and is reported to be the 13th most common cause for physician consultation by patients.[2]

Non-allergic rhinitis – previously known as non-allergic, non-infectious rhinitis (NANIR); non-allergic, non-infectious perennial rhinitis (NANIPER); or vasomotor rhinitis – is defined when no sensitization or signs of nasal infection can be observed (diagnosis '*per exclusionem'*).[14] Due to the lack of a clear consensus on the diagnostic criteria, epidemiological data is scarce. However, it has been reported that 5.5-23.5 % and 9.6 % of the European and Belgian population respectively suffer from NAR.[19–21] NAR is a heterogeneous group of several inflammatory phenotypes comprising occupational/irritant-induced rhinitis, drug-induced rhinitis, hormonal rhinitis, rhinitis of the elderly/senile rhinitis, gustatory rhinitis, smoking rhinitis, and – by exclusion – idiopathic rhinitis.[16] About 40-50 % of the patients with NAR suffer from idiopathic rhinitis.[22,23] When allergic sensitization and viral/bacterial infection are ruled out (by skin prick testing/determination of allergen-specific IgE levels in serum and nasal endoscopy respectively), further diagnostic work-up to determine the sub-phenotype of NAR is heavily dependent on patient history. Given the heterogeneity of the group of NAR patients, achieving good disease control can be challenging. In addition, for many subtypes of NAR the pathophysiology remains unclear. In practice, treatment of a patient with NAR remains therefore a matter of trial-and-error.[14]

Chronic rhinosinusitis is characterized by inflammation of the mucosal lining of the paranasal sinuses. It is diagnosed in case of at least 2 sinonasal symptoms such as nasal obstruction and/or rhinorrhea with possibly also facial pressure and loss of smell for more than 12 weeks.[2] On clinical examination, mucosal edema and/or mucopurulent discharge may be seen, and mucosal changes in the paranasal sinuses may be seen on computed tomography.[2] Although studies on prevalence report a wide variation of 6.9-27.1 %, it is clear that CRS is a very prevalent disease.[2] CRS can be further classified as *chronic rhinosinusitis without nasal polyps* (CRSsNP) and *chronic rhinosinusitis with nasal* polyps (CRSwNP), depending on whether or not nasal polyps can be visualised on nasal endoscopy. Studies based on electronic health records reported a 3-10 times higher prevalence of CRSsNP compared to CRSwNP.[24,25] Nevertheless, many studies focus on CRSwNP since presence of polyps allows clear-cut patient characterization.

Several challenges are present when taking care for patients with upper airway inflammatory symptoms.

Firstly, there is great overlap in possible symptoms for several phenotypes. Similar symptoms may result from various pathologies.[26]

Secondly, multiple phenotypes may be present simultaneously, resulting in a mixed phenotype.[27]

Thirdly, diagnosis can be further complicated by presence of structural pathology, such as septal deviation, alar valve insufficiency, or nasal turbinate hypertrophy which also result in nasal obstruction.[28,29]

Lastly, the underlying mechanisms causing the nasal symptoms, called *endotypes*, may differ between patients. In general, four endotypes are described.[27,30,31] *Type 1 inflammation* is characterized by increased interferon-ɣ levels and Th1-mediated immune responses.[32] *Type 2 inflammation* is reflected by increased levels of typical Th2-cytokines such as IL-4, IL-5, and IL-13, and eosinophilia.[33] In case of increased levels of mainly the Th17-cytokines IL-17A, IL-17F, and IL-22 one can speak of *type 3 inflammation*.[34] Sometimes cytokine levels are normal but increased levels of neuropeptides such as substance P (SP) or calcitonin gene-related peptide (CGRP) are observed without clear infiltration of inflammatory cells.[35,36] In this case, the term *neurogenic inflammation* is used. On top, an extra mechanistic feature is the

presence of epithelial barrier defects, which results from any type of aggression towards the mucosa, often caused by the underlying endotypes. Type 1 inflammation is classically seen in CRSsNP, while type 2 inflammation is a feature of AR and CRSwNP. Infectious phenotypes are thought to be related to type 1 and/or type 3 inflammation, while idiopathic rhinitis and more specifically the phenomenon of nasal hyperreactivity (NHR) are considered to result from mainly neurogenic inflammatory mechanisms. Importantly, the contribution of different endotypes to the clinical picture may vary amongst different patients. There is no exclusive 1-to-1 relationship between clinical phenotypes and pathophysiological endotypes (Figure 1).[15,37,38]

Nevertheless, successful treatment of chronic upper airway inflammatory diseases highly depends on knowledge of the underlying mechanisms.[27]

In this doctoral thesis, I will focus on AR and CRSwNP since they feature some remarkable endotypic similarities.

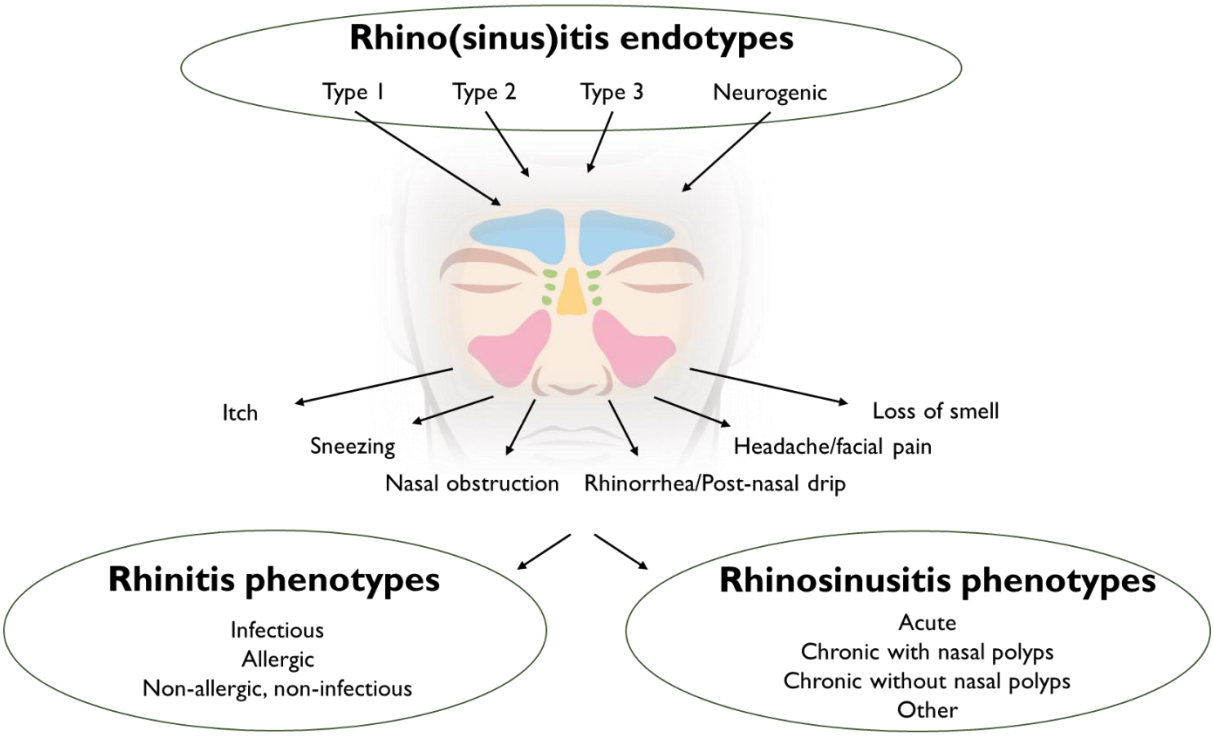

Figure 1: Rhinitis and rhinosinusitis endotypes and phenotypes. Different pathological mechanisms or endotypes may lead to similar rhinological symptoms. Based on the clinical picture, various phenotypes can be distinguished. There is no 1-to-1 relationship between phenotypes and underlying endotypes.

2. Type 2-predominated chronic upper airway inflammatory diseases

AR and CRS are common disorders, with a prevalence of 28.5 and 10.9 % respectively and huge economic costs.[1,2,13,39] Both AR and CRSwNP are historically considered to be mainly mediated by type 2 inflammatory pathways.

2.1. Allergic rhinitis

AR can be defined as a type 1-hypersensitivity reaction in response to contact with allergens in sensitized individuals. This leads to inflammation of the nasal mucosal lining, finally resulting in nasal symptoms.[1]

2.1.1. Pathophysiology of allergic rhinitis

The pathophysiology of AR can be divided in two phases: a predisposing sensitization phase and a subsequent exposure.[40]

When an aero-allergen, such as house dust mite, grass-/tree-pollen, or animal dander, enters the nasal cavity of predisposed atopic individuals for the first time, it is picked up by antigen presenting cells (mostly dendritic cells).[41,42] The antigen presenting cell migrates to the draining lymph node, where it presents the allergen via major histocompatibility complex class II to naive CD4+ Th0 cells.[41,43] In presence of interleukin 4 (IL-4), the naive CD4+ Th0 cell is subsequently primed into a Th2 cell.[44,45] Next, the Th2 cell travels back to the submucosal space and produces typical Th2-inflammatory cytokines such as IL-4, IL-5, and IL-13.[37,40,46] IL-5 mainly attracts and activates eosinophils, while IL-4 and IL-13 induce B-cell isotype class switching to IgE. This allergen-specific IgE (sIgE) can subsequently bind its high-affinity receptor FcεRI on mast cells and basophils.[37,39,47]

Upon subsequent exposure, aero-allergens will bind to the allergen-specific IgE. This induces cross-linking of the high-affinity receptors FcεRI on mast cells and basophils, activating them and inducing release of mediators such as leukotrienes (LT), prostaglandins (PG), tryptase, and histamine.[40,41,48] Within minutes, these mediators induce mucus production by epithelial goblet cells, vasodilation, and plasma extravasation, leading to nasal congestion and edema.[37,41] These mechanisms ultimately result in nasal obstruction, rhinorrhea, and post-nasal drip.[49,50] Moreover, histamine can activate sensory nerve endings, resulting in nasal itch

and sneezing via a central reflex arch.[48,51] In the following hours, symptoms may persist or even deteriorate when T lymphocytes, basophils, and eosinophils are recruited due to upregulation of adhesion molecules, growth factors, and chemokines.[40,48] Indeed, histamine can also be released by basophils and eosinophilic mediators (such as IL-3, IL-5, eosinophilic cationic protein, and major basic protein) result in epithelial damage.[48] Th2 cells further maintain the **type 2 inflammation**.[48]

Moreover, epithelial barrier defects are observed in AR, which can be caused by Th2-cytokines, histamine, or direct protease-activity of allergens themselves.[52–55] Epithelial damage by allergens, but also viruses, toxins, lipopolysaccharides (LPS), smoke, air pollution, and others induces release of thymic stromal lymphopoietin (TSLP), IL-25, and IL-33 from the nasal epithelium.[56,57] These cytokines can activate dendritic cells and shift towards a Th2-inflammation.[58,59]

Lastly, due to impaired mucociliary clearance, environmental triggers such as pathogens, viruses, and allergens, but also inflammatory mediators might remain present for a longer time in AR.[60] Indeed, it has been reported that a more severely reduced mucociliary clearance correlates with higher disease severity and an increased risk for rhinosinusitis.[61,62]

In summary, as far as is currently known, the pathophysiology of AR is mainly driven by type 2 inflammation with also epithelial damage and impaired mucociliary clearance as maintaining factors (Figure 2).

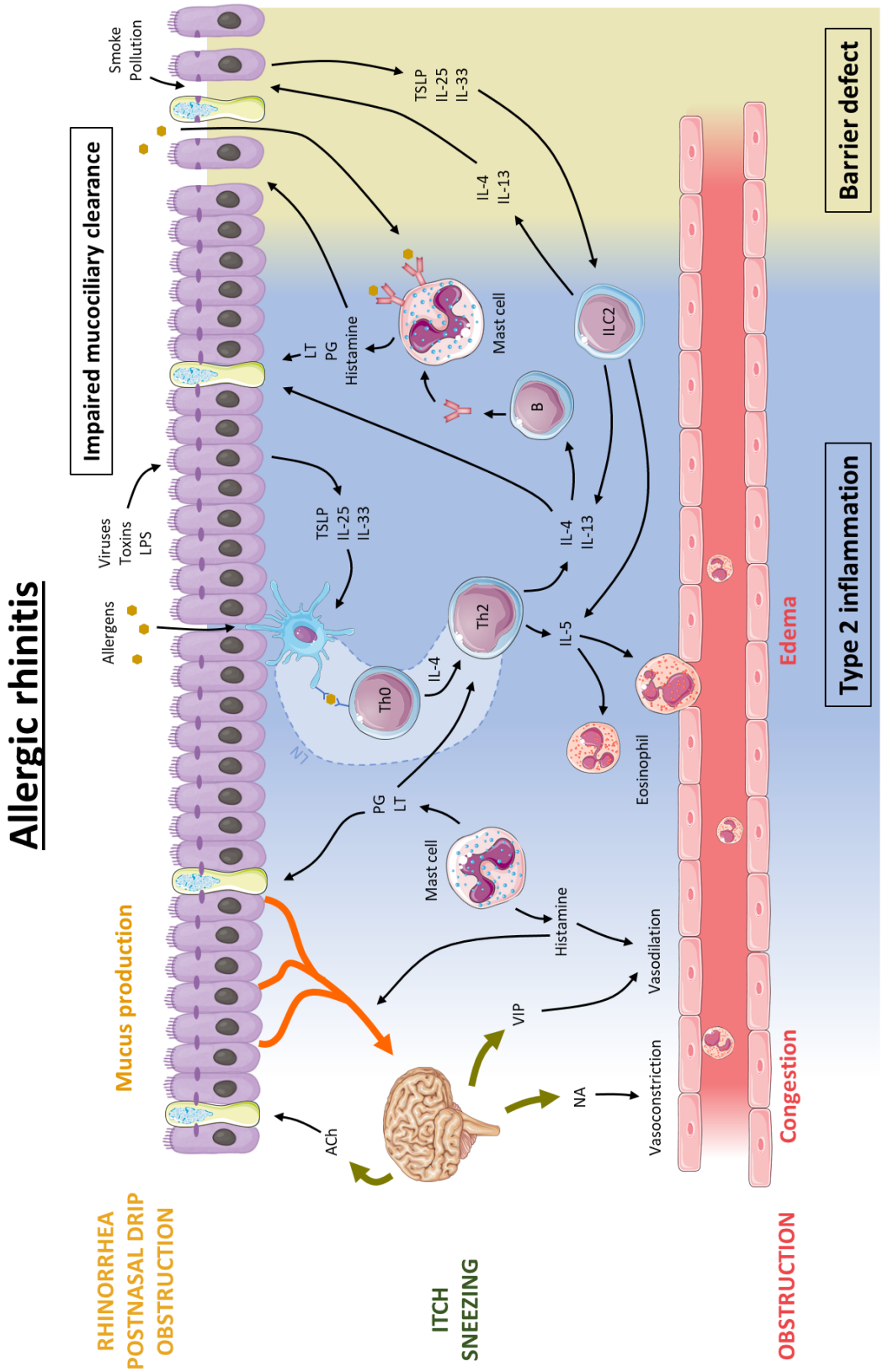

Figure 2: Main pathophysiological mechanisms in allergic rhinitis. Adapted from [37]. Allergic rhinitis is generally characterized by a type 2 inflammatory endotype and barrier defects. Ach: acetylcholine, NA: noradrenaline, VIP: vasoactive intestinal peptide, ILC2: type 2 innate lymphoid cell, PG: prostaglandins, LT: leukotrienes, LN: lymph node, IL: interleukin, LPS: lipopolysaccharide, TSLP: thymic stromal lymphopoietin.

2.1.2. Diagnosis of allergic rhinitis

Based on patient history, AR can often be suspected.[4] For example, patients might suffer from nasal obstruction, rhinorrhea, post-nasal drip, nasal itch, and sneezing upon encounter with known allergens such as pollen or dander from pet animals.

In children, so-called Dennie-Morgan lines may be seen underneath the eyes as a sign of allergic diathesis.[39] Nasal endoscopy can reveal white-blueish nasal mucosa with congestion and clear nasal secretions, but this is not mandatory for diagnosis.[39,63]

Measurement of allergen-specific IgE in serum via ImmunoCAP can give an idea about the allergens an individual is sensitized to, yet does not allow distinction between sensitization (= presence of sIgE) and allergy (= presence of sIgE + symptoms concomitant with the allergen sensitized to).[63,64] Diagnosis of AR is made in case of sensitization to specific allergens and presence of symptoms relevant to the identified sensitizations. An allergen skin prick test or nasal allergen provocation test can detect systemic or local sensitization, and are currently considered as the gold standard in detecting sensitization.[4,39,63] These are provocation tests where small amounts of allergens are administered intracutaneously or via a nasal spray respectively before evaluating reaction to the allergens by a wheal-and-flare reaction or increase in nasal symptoms.[65] In a basophil activation test, a type 1 hypersensitivity reaction experienced by a patient *in vivo* is replicated *in vitro* using peripheral blood. This test has a high specificity yet lower sensitivity compared with a skin prick test and is currently not implemented in daily clinical practice due to its higher complexity.[63,66] Lastly, cytologic analysis of nasal samples collected by nasal lavage or blown secretions may show increased tryptase-, eosinophilic cationic protein-, and sIgE-levels.[63,67]

2.1.3. Treatment of allergic rhinitis

After diagnosis of AR and identification of the causative allergen, patient counseling is of primordial importance.

The first step should be avoidance of allergen exposure.[39] However, allergen reduction is not always easy to achieve. For example, in case of house dust mite allergy, removal of carpets, washing sheets in hot water, use of allergen-impermeable bedding covers, or weekly mopping are all measurements that will decrease presence of house dust mites, yet full

eradication is not achievable.[68] Another example is the environmental pollen concentration, which is obviously impossible to control completely.

When exposure cannot be avoided or insufficiently reduces symptoms, pharmacotherapy is needed. Several categories exist, of which H_1-antihistamines and corticosteroids are the most important.

H_1-antihistamines bind the H_1-receptor. By doing so, they competitively inhibit binding of endogenous histamine, hence preventing histamine-induced vasodilation and mucus secretion.[69] The first antihistamine, antargan, was implemented in clinical practice in 1942.[70] The first-generation antihistamines could pass the blood-brain barrier and inhibit central histamine neurons, exerting important sedative effects and leading to reduced work-productivity, drowsiness, and potential traffic accidents.[69,71] Many molecules have been developed since, reducing sedative effects with each generation while maintaining good clinical efficacy in reducing nasal symptom scores.[72–74] Most currently available preparations should be administered orally once daily, yet topical formulations such as eye drops or nasal drops are available as well with equal effectivity compared with systemic administrations.[39,75]

Corticosteroids can strongly improve nasal symptoms by anti-inflammatory and immune-suppressive functions.[76] After binding to the glucocorticoid receptor, the receptor is transported to the nucleus where it binds glucocorticoid response elements and consequently suppresses transcriptional activity of the pro-inflammatory NF-κB transcription factor.[77,78] Hence, synthesis of multiple pro-inflammatory cytokines, such as IL-4, IL-5, IL-6, IL-8, or tumor necrosis factor α, is reduced.[76] Also, mast cell recruitment is inhibited by reduced expression of adhesion molecules, and their cytokine production, FcεRI expression, and mediator release is reduced.[76,79] On top, influx and activity of basophils, eosinophils, and Th2 cells is reduced.[79] Lastly, corticosteroids restore epithelial barrier function by increasing tight junction mRNA and proteins.[80] Corticosteroids are typically applied via a nasal spray or nasal drops to reach maximal local effect while minimizing side effects after systemic administration.[39] Corticosteroids are more effective in improving nasal symptoms – especially nasal obstruction – and quality of life compared with antihistamines in patients with AR.[81,82]

Several other drugs are available for use in specific indications: Leukotriene receptor antagonists are EMA-approved for AR patients with comorbid asthma. It has generally al lower

effect on nasal symptoms compared with antihistamines or corticosteroids, but supports control of concomitant asthma.[83] Anticholinergic drugs can reduce rhinorrhea. Mast cell stabilizers are safe, but often lack efficacy in reducing nasal symptoms.[39] Topical or systemically administered nasal decongestants are effective in short-term relief of nasal obstruction, but are not intended for prolonged use due to the risk of developing turbinate hypertrophy and tachyphylaxis, a condition commonly known as *rhinitis medicamentosa*, a subgroup of NAR.[39,84,85]

It should be noted that the above described pharmacotherapeutic interventions do not have a curative goal, *i.e.*, they do not cure the allergy itself. Rather, they just relieve patients of their symptoms.

The last decades, research has focused on developing monoclonal antibodies which target specific mediators or receptors that play important roles in AR.[86] Omalizumab, a recombinant humanized monoclonal antibody binds circulating IgE, preventing binding to its high-affinity receptor. A recent meta-analysis showed that omalizumab effectively reduces nasal and ocular symptoms, reduces the need for rescue medication, and increases the disease-related quality of life.[87] Dupilumab binds IL-4Rα and hence targets both IL-4 and IL-13 since their receptors both consist of an IL-4Rα-subunit.[88] Currently, it is mainly studied in the context of atopic dermatitis and asthma, but it was shown to reduce AR-associated symptoms and improve AR-related quality of life in asthmatics.[89,90] Mepolizumab and reslizumab bind IL-5 and benralizumab blocks the IL-5 receptor. Collectively, all three molecules target mainly eosinophil recruitment and activation. This effectively reduces disease severity in asthma, illustrating the therapeutic potential in allergic diseases.[91]

Lastly, patients with AR may benefit from allergen immunotherapy (AIT). In contrast to antihistamines and corticosteroids, AIT has disease-modifying effects. In AIT, the causative allergen is administered in increasing doses. This elicits an increase in allergen-specific IgG4, which can bind allergens and therefore competitively inhibit binding to sIgE. In later phases, generation and activation of regulatory T cells ultimately suppress Th2 cells.[91,92] The allergens can be administered subcutaneously (SCIT) or sublingually (SLIT). Therapy schemes typically comprise a dose-escalating phase in the first weeks of the treatment, followed by a maintenance phase where doses remain stable. Treatment should be continued for 3-5 years.[92] SCIT is about 2-3 times cheaper than SLIT, but it requires a doctor consultation for

injection. SLIT has the advantage of at-home administration after a first in-hospital dose. Both SCIT and SLIT are effective in treating perennial AR, while SLIT is generally safer and more convenient in treatment of seasonal AR.[40,93]

Finally, rinsing the nasal and paranasal cavities with saline lavages is a cheap, easy, and effective way to wash away all mucus secretions along with entrapped irritants, allergens, and inflammatory mediators, reducing allergenic and inflammatory load. Moreover, it hydrates the mucous blanket, enhancing mucociliary clearance.[94] On top, rinsing away mucus allows intranasally administered medication to reach the mucosa better. In conclusion, saline lavages help to reduce patient-reported disease severity.[95]

2.2. Chronic rhinosinusitis with nasal polyps

CRSwNP is featured by inflammation of the mucosa of the nose and paranasal sinuses and presence of saggy, polypoid mucosa.

2.2.1. Pathophysiology of chronic rhinosinusitis with nasal polyps

The pathophysiological mechanisms of CRSwNP remain incompletely understood and are greatly multifactorial. Many immune cells and cytokines are involved and interact with each other, resulting in a chaotic inflammatory soup, as extensively reviewed by Schleimer[96] and Stevens *et al.*[97]. I will here only focus on the most important pathways needed to understand this doctoral thesis (Figure 3).

In the western population, CRSwNP is mainly characterized by a **type 2 inflammatory** response, like AR.[27,97] Various pathways lead to or maintain activation of Th2 cells. Firstly, irritants, pathogens, proteases, and antigens can damage epithelial cells, which then release TSLP, IL-25, and IL-33 to prime dendritic cells.[37,96–98] Antigens and pathogen-associated molecular patterns are picked up by dendritic cells and subsequently presented to naïve CD4+ Th0 cells.[99] In presence of IL-4, this results in Th2 formation, similar to the sensitization phase of allergic disease.[96,100] Secondly, moreover, epithelial cytokines drive type 2 inflammation by stimulating Th2 cells.[2,98,101] Thirdly, in Caucasian patients with CRSwNP, there is often colonization by *Staphylococcus aureus*, which can embed themselves in a biofilm and produce

enterotoxins that act as superantigens leading to massive polyclonal T cell proliferation and activation.[2,102,103]

Hence, several mechanisms lead to activation of Th2 cells, reflected by release of signature cytokines IL-4, IL-5, and IL-13. *IL-4* is suspected to be the main driver for disease, maintaining the inflammatory cycle by supporting proliferation of Th0 cells to Th2 cells. *IL-4 and IL-13* impair barrier integrity and stimulate B cells to produce *Staphylococcus aureus* enterotoxin-specific IgE which binds mast cells and basophils.[53,96,100,103] This forms the basis for a type 2 inflammatory reaction against bacterial antigens instead of protective mucosal immunity.[104] B cells also produce autoantibodies against the basement membrane, leading to local complement activation.[53,96] *IL-5* recruits and activates eosinophils, which release several barrier-impairing mediators like eosinophilic cation protein, but also chemokine (C-C motif) ligand 23 (CCL23).[96] Monocytes are attracted by eosinophil-produced CCL23, and differentiate to M2 macrophages under influence of IL-13.[96] Factor XIIIa, secreted by these macrophages, results in fibrin crosslinking and tissue remodeling.[37,96,100] Polyp formation is further enhanced by decreased levels of regulatory T cell-derived transforming growth factor-ß, which normally drives fibrosis.[105,106] Increased viscosity of mucus and impaired mucociliary clearance results in continuous and longer exposure to inflammatory mediators and environmental aggressors.[107,108]

Like in AR, activation of goblet cells by inflammatory mediators induces increased mucus production, leading to rhinorrhea and postnasal drip. Polyp formation, mucus hypersecretion, nasal congestion, and edema contribute to nasal obstruction. Facial pain is mediated by congestion, which impairs the pressure-equalizing properties of the ostia to the paranasal sinuses, and irritation of trigeminal afferent nerves.[49] Lastly, nasal obstruction, but also inflammation in the olfactory cleft can induce conductive and/or perceptive loss of smell.[49,109]

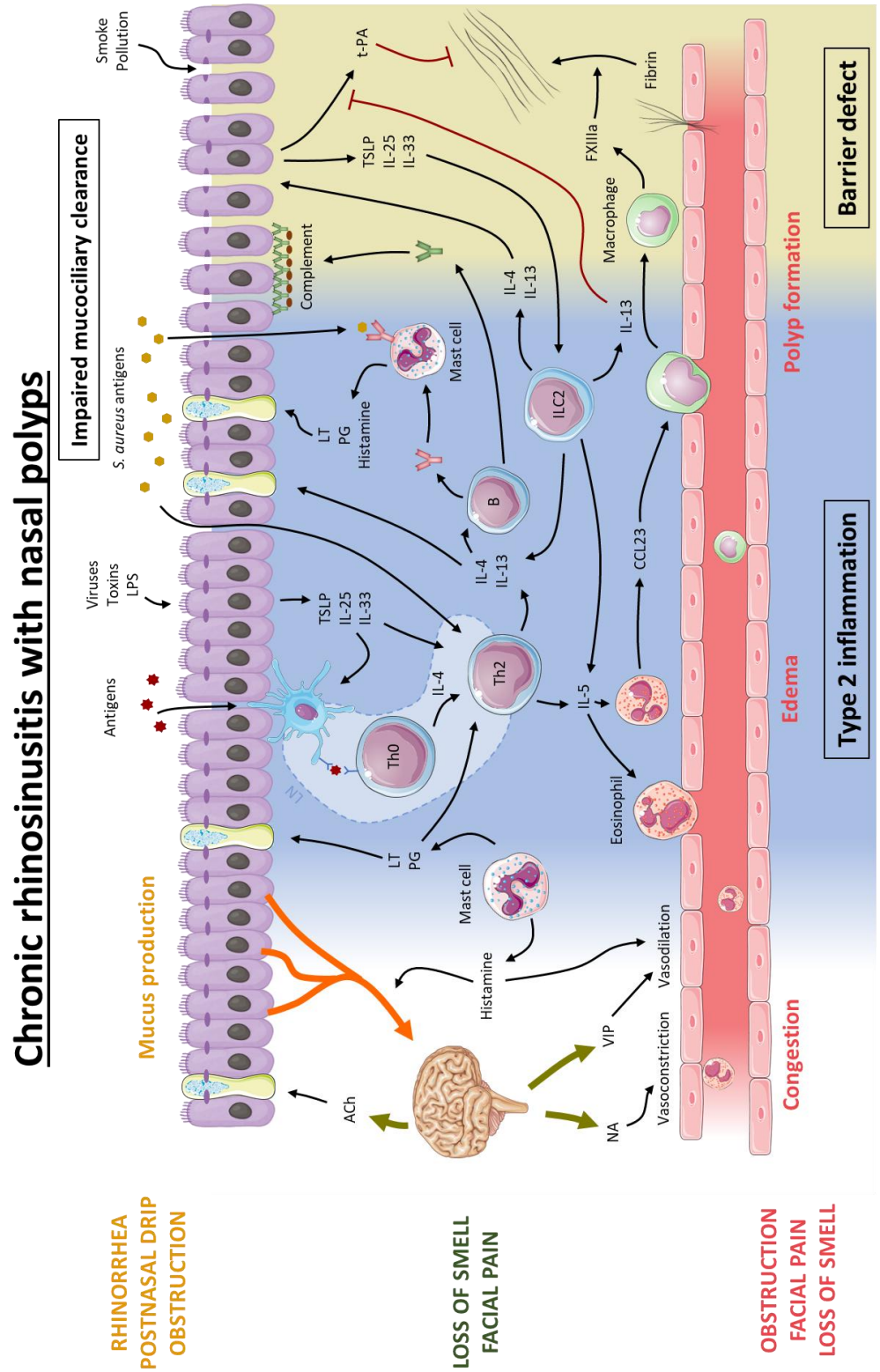

Figure 3: Main pathophysiological mechanisms in chronic rhinosinusitis with nasal polyps. Adapted from [37]. Chronic rhinosinusitis is characterized by a predominantly type 2 inflammatory endotype in presence of epithelial barrier defects. Ach: acetylcholine, NA: noradrenaline, VIP: vasoactive intestinal peptide, ILC2: type 2 innate lymphoid cell, PG: prostaglandins, LT: leukotrienes, LN: lymph node, IL: interleukin, LPS: lipopolysaccharide, TSLP: thymic stromal lymphopoietin, CCL23: chemokine (C-C motif) ligand 23, FXIIIa: activated coagulation factor 13, t-PA: tissue plasminogen activator.

Historically, type 1 inflammation was typically related with CRSsNP, while type 2 inflammation was found in patients with CRSwNP and asthma. Recent studies increasingly nuance this solely type 2 inflammatory response in CRSwNP. In the Asian population, more patients with underlying neutrophilic inflammation suffer from CRSwNP, and mixed endotypes are possible.[110,111] More specifically, several clusters could be defined between type 1 and type 2 inflammation based on their inflammatory signature.[15,112] Nevertheless, more pronounced type 2 inflammation is associated with presence of nasal polyps and asthma.[15]

2.2.2. Diagnosis of chronic rhinosinusitis with nasal polyps

CRS is diagnosed in case of two or more nasal symptoms – *i.e.* nasal obstruction and/or rhinorrhea/postnasal drip, ± facial pain/pressure, ± loss of smell – are present for ≥ 12 weeks.[2] Nasal endoscopy shows mucopurulent discharge and edema or mucosal obstruction primarily in the middle meatus; and signs of nasal polyps in case of CRSwNP.[2] A computed tomography (CT) scan can be performed in case of diagnostic doubt, showing mucosal changes within the ostiomeatal complex and/or paranasal sinuses, the severity of which can arbitrarily be quantified by the Lund-Mackay score.[2,113] A CT scan allows to differentiate from possible other entities causing the symptoms like fungus balls, mucocoeles, or tumors. Scans are also necessary for pre-operative surgical planning.[100] Endotyping of the pathophysiological mechanisms is an emerging concept in the diagnostic work-up of CRS. For example, comorbid asthma, blood and tissue eosinophilia, increased serum IgE, and presence of *Staphylococcus aureus* enterotoxin-sIgE are indicators of underlying type 2 inflammation.[100]

2.2.3. Treatment of chronic rhinosinusitis with nasal polyps

Given the endotypic similarities, management of CRSwNP and AR unsurprisingly shows considerable overlap.

Since CRSwNP is characterized by type 2 inflammation and barrier defects, use of corticosteroids is extensively studied.[2] Over the years, compelling evidence has been gathered supporting their therapeutic efficacy by restoring the epithelial barrier and tempering type 2 inflammation just as in AR.[2,78] Many preparations are available for local, intranasal application.

In case of severe symptoms or in a peri-operative phase, a short course of oral corticosteroids may be considered.[2,76,78]

In addition to corticosteroids, high-volume nasal irrigations flush away nasal secretions and crusts, together with the pro-inflammatory mediators, but also *Staphylococcus aureus* enterotoxins and antigens it contains.[2,114,115] Moreover, it enhances ciliary beat activity and hydrates the mucosal sol layer.[2,116] Xylitol can be added to break down a possible biofilm.[117] Pharmacological compounds can be added to the lavage fluid to deliver them deep in the nasal and paranasal cavities.[2]

In the past decade, a new emerging pharmacotherapeutic concept is the use of monoclonal antibodies against mainly type 2 inflammatory mediators: IL-4Rα (dupilumab), IgE (omalizumab), IL-5 (mepolizumab, reslizumab), and IL-5Rα (benralizumab).[89,118,119] Omalizumab reduces nasal congestion and increases quality of life in patients with CRSwNP.[120] Mepolizumab was shown to effectively reduce the need for corticosteroids in patients with eosinophilic CRS and asthma, and to reduce polyp size and nasal obstruction in patients with CRSwNP.[121,122] Studies on benralizumab report a reduced nasal polyp score, reduced nasal obstruction, and reduced sense of smell when compared with placebo.[123] Dupilumab appears to be the most effective in reducing disease severity and polyp score and increasing quality of life, but head-to-head comparison studies are to still be enrolled.[124,125] Tezepelumab binds TSLP, preventing binding to its receptor. In asthmatics with concomitant CRS, it reduced blood eosinophilia and IL-5 and IL-13 levels in serum.[126] Its safety and efficacy in patients with CRSwNP with or without asthma is currently under investigation (NCT04851964). These monoclonal therapies show promising results, but come with a considerable price of thousands of euros per year. Only Xolair® (omalizumab) and Nucala® (mepolizumab) are currently reimbursed in Belgium. Treatment with monoclonal antibodies poses a significant cost for society and the individual patient.

Finally, the nasal and paranasal microbiome has gained increasing attention. Studies on microbiome-related therapies are emerging but remain scarce. In CRSwNP, a decreased microbial diversity has been observed.[127] Some mainly preclinical evidence suggests potential a beneficial effect of probiotics on this microbial dysbiosis and epithelial barrier function.[128,129] In cases of recalcitrant CRS, bacteriophage therapy has high therapeutic potential yet requires further studies.[130,131] Lastly, macrolide antibiotics and doxycycline exert some anti-

inflammatory and immune modulatory effects aside from their antibacterial effects.[132,133] Long-term use is sometimes prescribed, though the evidence is weak and potential mainly cardiovascular side effects are not to be underestimated.[2]

If pharmacological treatment fails, the only remaining option is Functional Endoscopic Sinus Surgery (FESS).[2,100] During this procedure, diseased mucosa is endoscopically removed and the ostia to the paranasal sinuses are surgically widened. Since it does not intervene with the underlying pathophysiological mechanisms, polyps are likely to recur, especially in patients with a pronounced type 2 inflammatory endotype.[100] By opening the ostia, however, nasal lavages and topical medications are more likely to reach the site of required action (*i.e.* the entire nasal and paranasal cavities), making them more effective. Indeed, FESS improves olfaction and quality of life, and leads to better asthma control.[134]

3. Nasal hyperreactivity

3.1. Definition and prevalence

Increasing studies indicate that patients with various upper airway inflammatory phenotypes experience one or more nasal symptoms upon encounter with not only allergens, but also environmental stimuli, such as temperature or humidity changes, air conditioning, cigarette smoke, or strong odors. This phenomenon is known as nasal hyperreactivity (NHR).[36]

NHR was studied for the first time in 1960 by Van Lier.[135] He noted that patients with NAR exhibited mucosal thickening and increased nasal secretions after being intranasally challenged with pepper extracts compared with healthy control subjects.[135] Since the 1990's, NHR is described by Lindberg *et al.* to be subjectively present in 60.9 % of NAR patients and 55.1 % of AR patients.[136] Segboer *et al.* found a prevalence of 66.9 and 63.4 % in NAR and AR respectively. Diagnosis was based on questionnaires, but was confirmed by an objective provocation test in a sample of 18 patients with AR and 21 patients with NAR.[137] One pathophysiological study in very well-characterized patients with idiopathic rhinitis, a subgroup of NAR, found a questionnaire-based prevalence of 57.6 %.[138] In 2020, Doulaptsi *et al.* found 64.8 % of patients with CRS subjectively reporting presence of NHR.[139] Lastly, objective NHR was present in about 80 % of patients with infectious rhinitis.[140]

So far, no clear difference could be observed across the different phenotypes in terms of triggers, albeit physical (temperature changes, humidity changes…) or chemical (cigarette smoke, strong odors…).[137] This suggests NHR is a general feature of inflamed upper airway mucosa, analogous to what has been described for bronchial hyperreactivity which is present in multiple lower airway diseases.[36,141] Remarkably, 69 % of asthma patients report NHR and bronchial hyperreactivity and NHR often co-exist.[142]

3.2. Pathophysiology

The pathophysiology of NHR is mostly studied in idiopathic rhinitis, where it seems to be mainly neurogenically mediated with an important role for the **Transient Receptor Potential (TRP) channels**.[36] Six subfamilies of mammalian TRP proteins comprising a total of 28 members have been identified so far: TRPC (Canonical 1-7), TRPV (Vanilloid 1-6), TRPM (Melastatin 1-8), TRPA (Ankyrin 1), TRPP (Polycystic 1-3), and TRPML (Mucolopin 1-3).[143,144] TRP channels are built by homo- or hetero- arrangements of four monomers, each with 6 putative transmembrane segments, with the C- and N- termini located in the cytoplasm.[143,145,146] They can be activated directly or are part of intracellular signaling pathways.[147–151] TRP channels are amongst others expressed on sensory neurons where they can be directly activated by mechanical and thermal stimuli, by a wide variety of potentially noxious exogenous chemicals, and by endogenous molecules that signal tissue damage.[22,144,146,150,152–156] TRP channel activation, *i.e.* the transition from a closed to open pore conformation, leads to cation entry at physiological resting membrane potentials, resulting in the increase of intracellular Na^+ and Ca^{2+} concentrations and therefore membrane depolarization.[146] To summarize, TRP channels are polymodal, nociceptive, cation-permeable channels, directly sensitive to a plethora of endogenous and exogenous stimuli and indirectly controlled by various intracellular regulatory mechanisms.

Most studies on NHR are performed in patients with idiopathic rhinitis and indicate an important role for the nervous system in the nasal mucosa. Nasal sensory neurons arise from the olfactory nerve for olfaction, and from the ethmoidal and maxillary branches of the trigeminal nerve for sensing respectively.[51] The latter comprises mainly fast-conducting Aδ fibers and unmyelinated C fibers.[51] In **physiological conditions**, trigeminal afferent neurons

sense the nasal lumen by expression of the polymodal and nociceptive TRP channels. Activation of these sensory C fibers generates an action potential which propagates to the central nervous system where an efferent response is initiated.[36] The efferent nervous system consists of two main parallel and counter-acting systems.[157] The sympathetic nervous system on one hand has overweight in normal conditions. Upon activation, noradrenaline and neuropeptide Y are released, resulting in vasoconstriction and decreased mucus secretions.[158,159] On the other hand, parasympathetic nerve fibers release vasoactive intestinal peptide (VIP) and acetylcholine (ACh), inducing vasodilation and mucus secretion.[157] This orthodromic pathway of neurogenic propagation exists mainly as a protective reflex, where entry of potentially harmful or noxious triggers in the nasal cavity induces nasal obstruction, rhinorrhea, and sneezing, preventing further progression of the noxious substance to the lower airways.[36,51]

From an **anatomical point of view**, the signal travels via the trigeminal ganglion to the main trigeminal nucleus and the spinal trigeminal nucleus.[153] Here, it synapses onto second order neurons and the information is relayed via the trigeminothalamic tract to the ventral posteromedial nucleus of the thalamus, before reaching the primary somatosensory cortex.[153,160] A possible sneezing reflex center is believed to be situated in the *medulla oblongata*, though the exact anatomic correlates remain unknown.[161,162] Paraympathetic fibers are thought to originate from the *nucleus salivatorius superior* and travel via the facial nerve, greater petrosal nerve, and Vidian nerve of the pterygoid canal to the pterygopalatine ganglion where it synapses on postganglionic neurons that reach the respective end-organs.[159] Sympathetic fibers originate in the intermediolateral nucleus of the cervicothoracal spinal cord and synapse in the superior cervical ganglion with postganglionic neurons that travel via the deep petrosal nerve and Vidian nerve of the pterygoid canal to the nasal mucosa.[51]

It is long known that mainly unmyelinated C fibers contain neuropeptides, hence forming a so-called non-adrenergic, non-cholinergic (NANC) nervous system.[163] Upon activation, these sensory neurons are suspected to release neuropeptides such as Substance P (SP), neurokinin A (NKA), and calcitonin gene-related peptide (CGRP). Subsequently, these neuropeptides bind their receptors on blood vessels, glands, goblet cells, and also immune cells, mediating nasal symptoms.[36] This **neurogenic inflammation** is currently thought to underly NHR.[36,164] Indeed, in patients with idiopathic rhinitis, in whom NHR is a key feature,

increased levels of SP in nasal secretions and *TRPV1* mRNA in nasal mucosal biopsies were observed.[138,165] Moreover, these increased SP levels, increased *TRPV1* mRNA, the panneuronal marker protein gene product 9.5 (PGP9.5), and *TRPM8* mRNA are reduced after treatment with capsaicin (a specific TRPV1-agonist), along with reduced reactivity to cold, dry air provocation.[165,166] On top, patients with self-reported NHR had lower thresholds for neurologic reactivity to the TRPA1- and TRPV1-agonist allyl isothiocyanate, which was reset to normal levels after treatment with capsaicin.[138] In concordance with these findings, a combination of fluticasone and azelastine, a H_1-receptor-antagonist with presumed TRPV1-inhibitory effects, reduced NHR in patient with AR.[167,168] Furthermore, it was shown that TRPV1, TRPA1, and TRPM8 are upregulated in neuronal cells after rhinoviral infections, which could explain post-infectious NHR.[169] Similarly, TRPV1 and TRPA1 seem to be associated with the development and maintenance of bronchial hyperreactivity.[170–175] Lastly, a similar pathway is found in patients with irritable bowel syndrome and neurogenic detrusor overactivity, showing enhanced TRPV1/A1 expression and hypersensitivity of the bowel and bladder respectively.[176–179]

3.3. Diagnosis

Definite and clear diagnosis of NHR remains challenging, impeding studies on its pathophysiology and therapeutic options.[36] In general, distinction should be made between diagnosis based on subjective and objective tests.

Subjective NHR (sNHR) can be diagnosed by means of questionnaires.[137,139] Questionnaires allow for easy, quick, and extensive data collection and are of main importance in epidemiologic studies. The major drawback of questionnaire-based diagnosis of NHR, apart from its inherent subjective nature, is the lack of correct and clearly-reported definitions of NHR across different studies.

Nasal provocation tests form the basis of **objective diagnosis of NHR** (oNHR).[36] Over the past decades, hyperosmolar solutions (hypertonic saline, mannitol), methacholine, histamine, capsaicin, adenosine 5'-monophosphate, distilled water, and phentolamine have all been used as challenge to diagnose oNHR with varying success.[36,180] The key to successful implementation in daily practice comprises a combination of a high sensitivity and specificity

and patient- and investigator-friendliness.[36] This was reached with the development of a Cold, Dry Air (CDA) provocation test. Indeed, it was shown in 1998 that dose-escalating CDA provocation is superior to histamine provocations in discriminating idiopathic rhinitis patients from healthy controls with good reproducibility in terms of CDA-induced nasal obstruction and mucus secretion.[181] The protocol was later shortened and optimized for clinical use without major loss of sensitivity (83.3 %) or specificity (100 %) in discriminating idiopathic rhinitis patients, where NHR is a main feature, from healthy control subjects.[182] Today, the best-founded test diagnosing oNHR consists of exposure to cold (< -10°C), dry (< 10 % relative humidity) air at a flow of 25 L/min for 15 minutes. Before and after, the Peak Nasal Inspiratory Flow (PNIF) is determined as a measure for nasal patency. A CDA-induced decrease in PNIF of ≥ 20 % is considered diagnostic for oNHR, extrapolated from the cutoff of a ≥ 20 % drop in the forced expiratory volume in 1 second after a methacholine or histamine provocation test for bronchial hyperreactivity.[36,182,183]

Taken together, questionnaire-based diagnosis of sNHR is quicker and easier compared with CDA provocation-based diagnosis of oNHR, yet inherently remains subjective. CDA provocation on its turn is more robust and less prone to subjective variability but is still more time-consuming (15 minutes) and no CDA device is currently commercially available. Therefore, objective diagnosis of NHR is mostly limited to research setting in specialized institutions. Given these difficulties in characterization of patients with or without NHR, literature on the pathophysiology and treatment options of NHR is scarce.

3.4. Treatment

Despite many efforts, correct diagnosis of NHR remains challenging, complicating studies on possible treatment options.[36]

So far, NHR is mainly treated by intranasal administration of capsaicin, a specific TRPV1-agonist. As stated higher, this treatment reduces NHR, *TRPV1* and *TRPM8* expression levels, expression of the panneuronal marker PGP9.5, and SP in nasal secretions of patients with NAR.[36,165,184] Capsaicin works via several mechanisms. First, it induces a short-lasting refractory period where TRPV1 does not respond to any stimulus. On the longer term, strong activation of TPRV1 interferes with mitochondrial respiration and causes a Ca^{2+} overload in

the sensory nerve endings, defunctionalizing the nerve endings.[185] Following initial protocols, in-hospital administration of capsaicin 0.1 mM 5 times on a single day is an effective treatment for patients for idiopathic rhinitis and NHR.[165] Recently, daily at-home administration of low-dose (0.01 mM) for 2 weeks was proven to be equally effective.[166]

One study showed an additive beneficial effect of azelastine topically administered in combination with fluticasone to reduce NHR in patients with AR.[167] Interestingly, aside from its original function as antihistamine, azelastine also seems to desensitize TRPV1. A short exposure of *in vitro* cultured murine neuronal cells to azelastine induced a TRPV1-dependent increase in intracellular Ca^{2+} concentration. Continuous exposure, however, desensitized the neurons for TRPV1-agonist capsaicin.[168] Similar to these murine experiments, levels of SP in nasal fluid of NAR patients were increased after nasal lavage with hypertonic saline, but less so when intranasal azelastine was administered.[186] Taken together, azelastine might possibly reduce reactivity of nociceptive neurons to environmental stimuli.

4. Unclarities on nasal hyperreactivity in allergic rhinitis and chronic rhinosinusitis with nasal polyps

Despite the complex insights in the pathophysiology of the main predominantly type 2 inflammation-driven chronic upper airway diseases, AR and CRSwNP, a complete understanding is still lacking. About 20 % of the patients remains uncontrolled, even though the type 2 inflammatory endotype and epithelial barrier defects are well targeted with current treatments.[187]

Recently, NHR was described in AR and patients with seasonal AR reported more symptoms and pain upon capsaicin challenge during the allergy season compared to challenges outside of the season, suggesting sensitization to capsaicin during allergic inflammation.[137,167,188,189] On the other hand, sole inhibition of TRPV1 did not reduce symptoms triggered by allergen challenge, nor did it affect the total nasal symptom score in seasonal AR patients.[190,191] Also, azelastine was shown to have an additional effect to intranasal corticosteroids and capsaicin therapy could have beneficial effects in patients with a mixed rhinitis phenotype.[167,192] Both therapies are thought to exert their effect by interfering

with neurogenic pathways. Moreover, about one third of the patients with AR suffers comorbid asthma, which is often associated with bronchial hyperreactivity.[39] In 2020, NHR was reported for the first time in CRS, but mechanistic studies on its pathophysiology in this patient group are currently non-existing.[139] Lastly, histamine – an important mediator in type 2 inflammation – is shown to sensitize sensory neurons in the context of irritable bowel syndrome and detrusor overactivity.[179,193–195]

In summary, there is increasing evidence for presence of NHR, which is supposed to be mainly neurogenically mediated, in the classical type 2-predominated chronic upper airway inflammatory diseases, AR and CRSwNP.

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CHAPTER 2

Objectives

1. General hypothesis

In contrast to the abundant clinical presentation and impact of NHR on quality of life and disease control in patients with chronic upper airway disease, little is known on the pathophysiology of NHR so far.[1,2] TRP channels play a major role in "sensing" the nasal environment, but if and how they are influenced by various mediators present in the nasal mucosa or secretions of patients with type 2 chronic upper airway disease remains unexplored. As mentioned in the introduction, it is currently thought that neurogenic pathways underly NHR in patients with idiopathic rhinitis.[2] More specifically, upregulation of the nociceptive TRP-channels is suspected to sensitize sensory neurons for environmental stimuli. Upon activation, these sensory neurons may release neuropeptides, which can subsequently induce vasodilation and increased mucus secretion.[2] AR and CRSwNP on the other hand feature a predominant type 2 inflammatory endotype.[3] Nevertheless, there is increasing evidence of presence of NHR in AR and CRSwNP and the underlying mechanisms are yet to be discovered.[4,5] The main clinical need is to decipher if and how NHR can be cured or prevented, possibly by therapeutically targeting key players in the nociceptive signaling pathway, which is incompletely understood. Therefore, the general goal of this PhD project was to investigate the pathophysiology of NHR in AR and CRSwNP.

2. Specific objectives

Three research objectives were formulated. Firstly, we investigated the prevalence of NHR in chronic upper airway inflammation. Secondly, we searched for mediators in nasal secretions and tissue potentially involved in the pathophysiology of NHR in type 2 chronic upper airway inflammation. Lastly, a potential interaction between the type 2 mediator histamine and TRP channels expressed on sensory neurons was studied.

2.1. To investigate the prevalence of nasal hyperreactivity in various phenotypes of persistent rhinitis and chronic rhinosinusitis

NHR was previously reported in 60.9-66.9 % of NAR patients and in 55.1-63.4 % of AR patients.[4,6] In 2020, the first study on NHR in CRS reported a prevalence of 64.8 %, though without distinction between CRSwNP and CRSsNP.[5] It was reported that 49.4 % and 50.6 % of the patients with NHR suffered CRSwNP and CRSsNP respectively.[5] The definition of NHR used in each of these studies is not clearly reported and seems to vary slightly. Lindberg *et al.* (1993) report:

> "*There was no difference between the two groups…"* (AR and NAR) *"…in frequency of reported aggravation of symptoms by airway irritants such as cigarette smoke or strong perfume."*[6]

In 2013, Segboer *et al.* report:

> "*Patients were asked to report sensitivity to temperature change, tobacco smoke or scents, exercise, emotional stress and humidity."*[4]

Lastly, Doulaptsi *et al.* report in 2020:

> "*Patients were asked to evaluate if their nasal symptoms were worsening upon one or more triggers, i.e. changes in humidity or temperature, emotional stress, exercise, and chemical pollutants like perfumes and tobacco smoke, responsible for provoking NHR*."[5]

These studies diagnosed patients with NHR in case of patient-reported sensitivity to temperature changes, tobacco smoke, exercise, emotional stress, chemical irritants, and humidity. By using a single question, with no information on the duration of the symptoms, patients with normal physiologic, protective reflexes could have been potentially included in those studies, overestimating the prevalence of NHR. Moreover, no study investigated the prevalence of NHR in multiple phenotypes at the same time, hindering comparative analysis. In conclusion, no universal definition of NHR across multiple phenotypes currently exists.

Therefore, in this first objective, we wanted to study the prevalence of clearly defined, self-reported NHR in a large cohort of patients with any phenotype of chronic upper airway inflammation (AR, NAR, CRSwNP, CRSsNP, and mixed phenotypes). We defined self-reported

NHR as a positive answer to both of the questions "*Are your nasal complaints triggered or exacerbated by any of the following triggers: (…)?*" and "*In this case, do they last longer than 10 minutes?*". This second question was added to discriminate from physiologic, protective reflexes that take place when one is exposed to environmental triggers. Participants could tick multiple of the following triggers: temperature changes, humidity changes, physical exercise or sports, (cigarette) smoke, air conditioning, strong odors, or others.

2.2. To identify mediators underlying nasal hyperreactivity in type 2 chronic upper airway inflammation

The mechanisms underlying NHR are almost exclusively studied in NAR, where an upregulated TRPV1 – SP axis has been observed.[2] In these patients, increased expression of *TRPV1* mRNA in nasal mucosal biopsies and increased levels of SP in nasal secretions are present, along with an increased sensitivity to the TRPV1 and TRPA1 agonist allyl isothiocyanate.[7,8] Here, activation of sensory neurons via stimulation of nociceptive TRP channels is suspected to induce release of neuropeptides which can lead to nasal symptoms.[2] This hyperreactivity is abolished after treatment with the TRPV1-agonist capsaicin.[7] Capsaicin defunctionalizes sensory neurons by inducing a refractory period and inhibition of voltage-gated Na-channels on short term, and by inducing a massive Ca^{2+} overload with long-lasting effects interfering with mitochondrial respiration and activation of Ca^{2+}-dependent proteases.[9]

Nevertheless, NHR is reported to be present in patients with other chronic upper airway inflammatory phenotypes such as AR, CRSwNP, and CRSsNP.[4,5,10] Rather than being classified as neurogenic inflammatory endotypes, AR or CRSwNP are classically seen as type 2-inflammatory diseases. In these diseases, knowledge on the underlying mechanisms is lacking.[4,5] In AR patients, but not in patients with NAR, there was an increased mast cell degranulation after CDA provocation.[11] Also, endonasal application of a specific TRPV1-inhibitor in these patients reduced reactivity to a capsaicin challenge, but not to allergen challenge.[12] Lastly, one study reported an additional benefit of adding azelastine, an old antihistamine with presumed TRPV1-inhibiting properties, to an intranasal corticoid spray in AR, reducing SP-levels in nasal secretions and reducing reactivity to CDA-provocation.[13]

Therefore, we wanted to investigate a potential neurogenic contribution to NHR in AR and CRSwNP.

2.3. To study the interaction between histamine and TRP channels on trigeminal afferent neurons

As indicated in the introduction, histamine is extensively studied in AR. In CRSwNP there is production of *Staphylococcus aureus* enterotoxin-specific IgE which can probably bind the FcεRI present on mast cells.[14,15] Moreover, increased levels of histamine are described in polyp tissue of patients with CRSwNP.[16] Lastly, presence of NHR has been described in AR and CRS.[4,5,10] NHR is associated with increased levels of SP, which is known to induce mast cell degranulation and release of histamine via the mas-related g-protein coupled receptor member X2.[7,17]

The antihistamine azelastine was already shown to desensitize TRPV1 *in vitro.*[18] In AR, it was proven to be beneficial in treatment of NHR when added to baseline corticosteroid therapy *in vivo*.[13] Moreover, histamine is shown to sensitize TRPV1 and TRPA1 in dorsal root ganglionic neurons projecting from the bladder and gut.[19–21] More specifically, increased histamine levels are found in the gut of patients with irritable bowel syndrome and it can sensitize nociceptive neurons for TRPV1- and TRPA1-agonists. This effect can be blocked by inhibition of the H_1-receptor.[20,22,23] In the bladder, histamine induces sensitization of TRPV1+ afferent nerves and hypersensitivity to bladder distension via the same H_1-receptor.[19,24] Lastly, histamine injection in skin induces thermal and mechanical hypersensitivity depending on TRPV1/A1.[25]

Hence, we wanted to investigate potential sensitizing properties of histamine on trigeminal TRPV1/A1.

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CHAPTER 3

Self-reported nasal hyperreactivity is a common feature of chronic upper airway inflammatory phenotypes and is not related to general mental or physical well-being

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Introduction

Chronic upper airway inflammation is a prevalent condition, with allergic rhinitis (AR) affecting around 25 % of the European population and over 500 million people worldwide.[1] Chronic rhinosinusitis (CRS) affects about 10 % of all Europeans.[2] The estimated economic burden of chronic upper airway inflammatory disorders is enormous, with 500-1000 USD per patient per year, and billions per year in total in Western countries.[3–5] A substantial amount of this burden is due to loss of productivity (*i.e.* presenteeism) and loss of work days (*i.e.* absenteeism).[6]

Symptoms include rhinorrhea or postnasal drip, nasal obstruction, sneezing, and itch in rhinitis, and extend to loss of smell and/or facial pain in CRS. They have a major impact on the quality of life of patients.[1] AR, non-allergic rhinitis (NAR), and CRS have all been linked to increased levels of stress, anxiety, and depression.[6–17] This relationship is probably bi-directional: psychiatric illness results in a chronic pro-inflammatory state via the hypothalamic-pituitary-adrenal axis on one hand, and chronic upper airway inflammation induces increased expression of inflammatory cytokines in the central nervous system and more specifically in the prefrontal cortex on the other hand.[18,19] Major progress has been made in the past years in terms of therapeutic options, such as allergen immunotherapy for AR and use of biologicals for CRS.[20,21] However, many patients remain uncontrolled with current treatment modalities, illustrating our incomplete understanding of disease pathogenesis.

Similar to the lower airways, upper airway symptoms can worsen upon exposure to daily environmental triggers, such as temperature or humidity changes, smoke, and strong odors.[22–25] There is increasing evidence that this nasal hyperreactivity (NHR) is involved in a range of chronic upper airway inflammatory conditions.[23,24] Indeed, two studies reported a prevalence of 55.1-63.4 % and 60.9-66.9 % in AR and NAR patients respectively.[23,26] Recently, the prevalence of NHR in CRS has been reported to be 64.8 %.[24] Interestingly, no differences in triggers of NHR could be observed between phenotypes.[23]

Until now, NHR has been largely neglected in clinical trials on chronic upper respiratory inflammation, apart from one study in house dust mite allergic rhinitis patients.[27] This is partly due to the lack of a good, commercially available diagnostic test, like the histamine

provocation test for bronchial hyperreactivity.[28] Recently, a user-friendly and accurate diagnostic test has been validated: a short provocation test with cold, dry air has been shown to diagnose idiopathic rhinitis patients – in whom NHR is a key feature – with good sensitivity (83 %) and specificity (100 %).[28] Unfortunately, no cold, dry air-device is commercially available and its use is restricted for research purposes in specialized centers using custom-made devices. Consequently, the majority of the studies on NHR were questionnaire-based. Several studies reported NHR in specific phenotypes of upper airway inflammation with varying incidence, mainly due to lack of a standardized questionnaire. So far, no large comparative studies investigating various groups at the same time have been published.

Therefore, we aimed to investigate the prevalence of clearly defined NHR, as well as the triggers and relation with disease severity, and to explore a possible association with general mental and physical well-being in a large cohort of patients with various phenotypes of chronic upper airway inflammation. In this explorative case-control study, we included well-characterized subjects with physician-based diagnosis, including patient history, nasal endoscopy, skin prick test and CT scanning. This allowed further subgroup analyses based on phenotype and comorbidities to screen for factors associated with the presence of NHR.

Methods

Study design

From January 2019 until September 2020, patients and healthy volunteers (patients' companions) were recruited from the outpatient clinic of the Department for Otorhinolaryngology of University Hospitals Leuven (Leuven, Belgium). All participants were asked to fill out a questionnaire on nasal symptoms, presence of NHR, and general demographic data. General mental and physical well-being were assessed using the Perceived Stress Scale (PSS)[29] for stress, General Anxiety Disorder-7 (GAD-7)[30] for anxiety, Patient Health Questionnaire-9 (PHQ-9)[31] for depression, and Patient Health Questionnaire-15 (PHQ-15)[32] for somatic symptom severity. The study was approved by the Ethical Committee Research of University Hospitals Leuven (S62213) and registered on clinicaltrials.gov (NCT03893227).

Study participants

All participants were aged 18-65 years old at the moment of inclusion and were Dutch-speaking. Participants were excluded in case of relevant nasal structural abnormalities, such as major septal deviation, and in case of ear-/nose-/throat-surgery or intranasal capsaicin therapy in the past three months. All participants gave written informed consent.

Participants were included as patients in case of physician-diagnosed chronic upper airway inflammation. This could be either AR, NAR, CRS without nasal polyps (CRSsNP), CRS with nasal polyps (CRSwNP), or a mixed phenotype.

The AR group consists of patients with a positive skin prick test to any of the most frequent aero-allergens in Belgium (*i.e.* house dust mite, timothy grass, English ryegrass, rye, stinging nettle, plantago, ragweed, mugwort, alder, birch, hazel, horse, cat, dog, rabbit, Alternaria, Aspergillus, Cladosporium) and concomitant nasal symptoms relevant to their sensitization.

The NAR group consist of patients with persistent symptoms of upper airway inflammation (*i.e.* rhinorrhea/postnasal drip, nasal obstruction, sneezing, itch) where

inflammation was limited to the nasal cavity as seen on nasal endoscopy and/or CT scan and with negative skin prick tests.

CRS patients were defined according to the EPOS-guidelines[2]: inflammation of the nose and paranasal sinuses characterized by two or more symptoms of which at least one is nasal obstruction or rhinorrhea, with or without facial pain or loss of smell, together with endoscopic signs of sinonasal inflammation and/or mucosal changes within the ostiomeatal complex or sinuses. If nasal polyps could be observed on nasal endoscopy, patients were included in the CRSwNP group; if not, they were included in the CRSsNP group.

Lastly, patients with two or more of the abovementioned phenotypes were defined as patients having a mixed phenotype (*e.g.* patients with presence of both nasal polyps and house dust mite allergy).

Healthy control subjects had no known sinonasal symptoms nor (history of) sinonasal pathology, no proven nor suspected allergy, and a mean Visual Analogue Scale (VAS) score for nasal obstruction, postnasal drip, rhinorrhea, itch, sneezing, facial pain, and loss of smell of 20 mm or less.

Outcome parameters

The primary outcome parameter was the prevalence of self-reported NHR (sNHR), defined as a positive answer to both of these questions: "*Are your nasal complaints triggered or exacerbated by any of the following triggers: (…)?*" and "*In this case, do they last longer than 10 minutes?*". Participants could tick multiple of the following triggers: temperature changes, humidity changes, physical exercise or sports, (cigarette) smoke, air conditioning, strong odors, or others. Nasal symptom severity and the subjective improvement with medication were assessed using Visual Analogue Scale (VAS) scores, where participants indicated severity and improvement respectively on a 100 mm long line.

For stress, a PSS score of 0-13, 14-26, and 27-40 correlate with low, moderate and high stress levels respectively.[29] For anxiety, cutoff values were 5, 10, and 15 on the GAD-7 for mild, moderate and severe anxiety. A cutoff value of 10 is used for screening purposes for generalized anxiety disorder with a sensitivity of 89 % and specificity of 82 %.[30] For depression,

cutoff values were 5, 10, 15, and 20 on the PHQ-9 for mild, moderate, moderately severe, and severe depression respectively. Patients scoring 10 or higher screen positive for major depression with a sensitivity and specificity of 88 %.[31] Lastly, somatic symptom severity could be considered to be negligible (PHQ-15 score of 0-4), low (PHQ-15 score of 5-9), medium (PHQ-15 score of 10-14), or high (PHQ-15 score of 15-30).[32]

Statistical methods

Data were analyzed using GraphPad Prism 8 (GraphPad Software, San Diego, Calif, USA). For comparison of proportions, chi-square or Fisher's exact test was used. For continuous variables, normality was tested with Shapiro-Wilk test. Differences were analyzed using a two-tailed unpaired t-test or Mann-Whitney test, depending on normality, and Holm-Sidak correction for multiple testing was applied when multiple outcomes were being compared between groups. Data are presented as mean ± SD in case of normally distributed data, or median and interquartile range (IQR) in case of non-normally distributed data. Values were considered significantly different when $p < 0.05$.

Results

Participants

A total of 605 patients and 151 controls were included in the study (Table 1). Patients and controls were age and sex matched. In the group of patients with mixed phenotype, 134 (81.2 %) suffer from CRS with concomitant allergy.

	Controls (N=151)	AR (N=144)	NAR (N=97)	CRSwNP (N=111)	CRSsNP (N=88)	Mixed phenotype (N=165)	Total patients (N=605)	P-value (controls *vs* patients)
Mean age (years ± SD)	44 ± 14	36 ± 12	43 ± 14	51 ± 12	44 ± 14	43 ± 13	43 ± 14	NS†
Male/female	74/77	77/67	44/53	82/27	50/38	94/71	347/258	NS‡
Current smokers (%)	14.7	15.3	16.1	17.3	18.2	12.8	15.3	NS‡
Previous rhinological surgery/trauma (%)	10.6	23.6	38.1	80.2	55.7	60.0	50.9	<0.0001‡
Asthma or COPD (%)	4.0	12.6	10.3	19.1	9.1	28.0	17.1	<0.0001‡
Cystic fibrosis (%)	0.0	0.7	0.0	6.4	1.1	3.0	2.3	NS‡

Table 1: Demographic data. (†Mann-Whitney test, ‡ Fisher's exact test). SD: standard deviation, COPD: chronic obstructive pulmonary disease, AR: allergic rhinitis, NAR: non-allergic rhinitis, CRSwNP: chronic rhinosinusitis with nasal polyps, CRSsNP: chronic rhinosinusitis without nasal polyps.

Prevalence of nasal hyperreactivity

Nearly half of the patients (46.9 %) reported NHR (Figure 1). Prevalence of sNHR was the highest in patients with a mixed phenotype (52.1 %), followed by patients with AR (48.6 %), NAR (47.4 %), CRSsNP (42.1 %) and CRSwNP (40.5 %), without significant differences between groups ($p = 0.33$). Notably, also 8.6 % of the healthy control subjects reported NHR, which was significantly lower than in the patient group ($p < 0.0001$). NHR was reported equally frequently by rhinitis patients and by rhinosinusitis patients (48.1 %, N = 266 *vs* 41.2 %, N = 199, $p = 0.16$). In case of CRS, prevalence of sNHR tended to be higher in case of concomitant allergy, though this did not reach statistical significance (41.2 %, N = 199 *vs* 52.2 %, N = 134, $p = 0.08$). Prevalence of sNHR did not differ when comparing patient groups based on gender, current tobacco-use, history of rhinological surgery or severe nasal trauma, history of allergen immunotherapy, or presence of lower airway disease (data not shown).

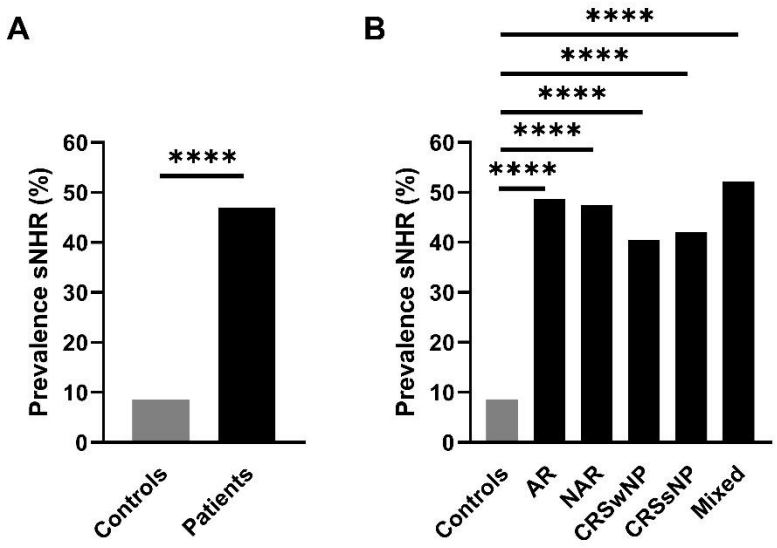

Figure 1: Prevalence of self-reported nasal hyperreactivity (sNHR) in all patients (A) and in specific phenotypes (B). Self-reported NHR is prevalent in all phenotypes of chronic upper airway inflammation. (Two-tailed Fisher's exact test with Holm-Sidak correction for multiple testing, **** $p < 0.0001$). AR: allergic rhinitis, NAR: non-allergic rhinitis, CRSwNP: chronic rhinosinusitis with nasal polyps, CRSsNP: chronic rhinosinusitis without nasal polyps.

Disease severity and nasal hyperreactivity

In patients with NAR, VAS scores for total nasal symptoms were higher in subjects with sNHR compared with those without sNHR (median 59.5, IQR 37-73 mm *vs* median 71, IQR 51-84 mm, $p = 0.0287$) and patients with more severe disease reported NHR more frequently ($p = 0.0431$) (Figure 2). This effect could not be observed in the other subgroups, and was lost when patients with various phenotypes were pooled together (Figure 3).

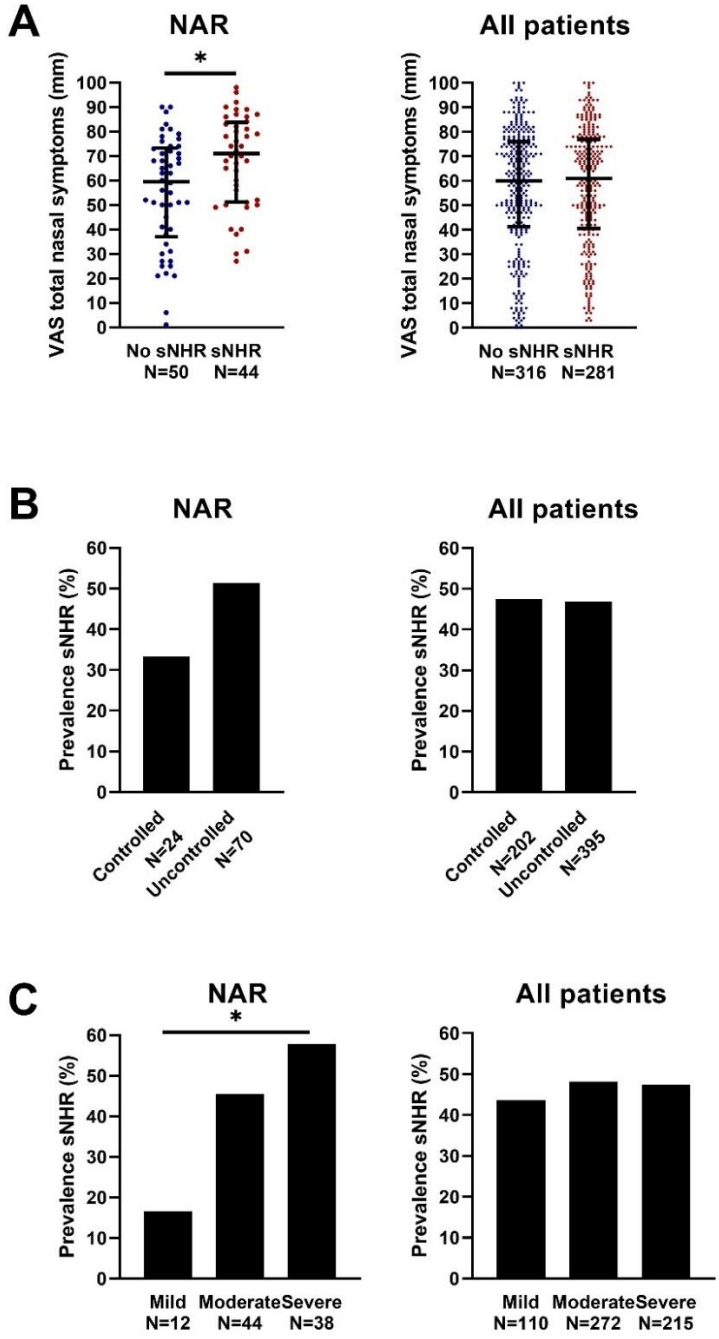

Figure 2: Relationship between the prevalence of self-reported nasal hyperreactivity (sNHR) and disease severity in non-allergic rhinitis (NAR) patients specifically and in all phenotypes pooled together. A: Visual analogue scale (VAS) score of total nasal symptoms in patients with and without sNHR. B: Prevalence of sNHR in controlled (VAS total nasal symptoms < 50 mm) and uncontrolled (VAS total nasal symptoms ≥ 50 mm) patients.[2] C: Prevalence of sNHR in mild (VAS total nasal symptoms 0-30 mm), moderate (VAS total nasal symptoms 31-70 mm), and severe (VAS total nasal symptoms 71-100 mm) disease.[2] In NAR patients, there is a relationship between VAS scores and prevalence of sNHR, which is lost when pooling all phenotypes together. (A: data presented as median and interquartile range, Mann-Whitney test with Holm-Sidak correction for multiple testing, * $p < 0.05$. B: Fisher's exact test with Holm-Sidak correction for multiple testing, not significant. C: chi-square test with Holm-Sidak correction for multiple testing, * $p < 0.05$).

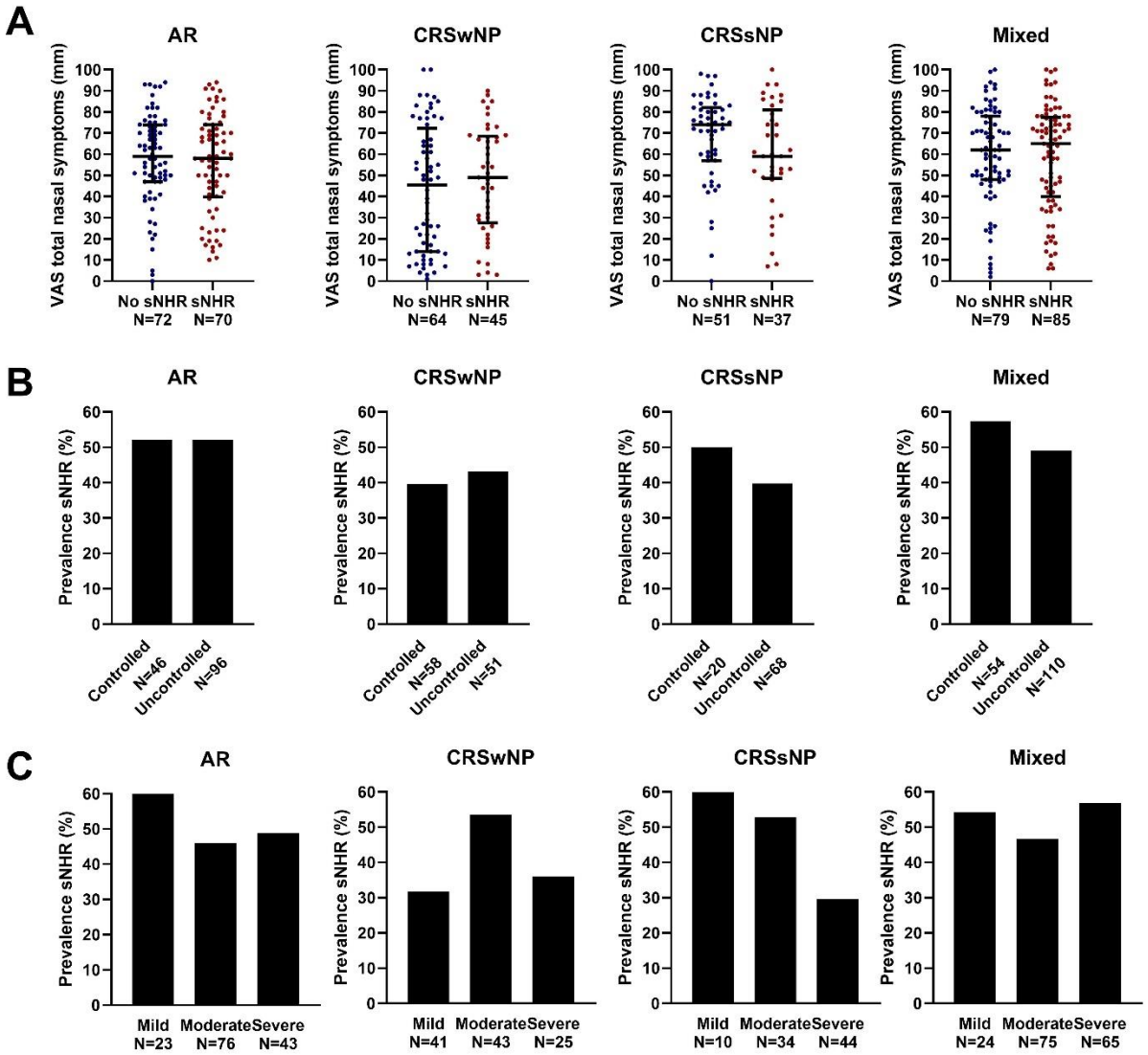

Figure 3: Relationship between the prevalence of self-reported nasal hyperreactivity (sNHR) and disease severity in allergic rhinitis (AR), chronic rhinosinusitis with (CRSwNP) and without nasal polyps (CRSsNP), and patients with mixed phenotype. A: Visual analogue scale (VAS) score of total nasal symptoms in patients with and without sNHR. B: Prevalence of sNHR in controlled (VAS total nasal symptoms < 50 mm) and uncontrolled (VAS total nasal symptoms ≥ 50 mm) patients.[2] C: Prevalence of sNHR in mild (VAS total nasal symptoms 0-30 mm), moderate (VAS total nasal symptoms 31-70 mm) and severe (VAS total nasal symptoms 71-100 mm) disease.[2] (A: data presented as median and interquartile range, Mann-Whitney test with Holm-Sidak correction for multiple testing, not significant. B: Fisher's exact test with Holm-Sidak correction for multiple testing, not significant. C: chi-square test with Holm-Sidak correction for multiple testing, not significant).

NHR was equally reported by patients using medication for their sinonasal symptoms and by patients not using rhinological medication (47.2 %, N = 557 *vs* 42.6 %, N = 47, p = 0.65)

(Figure 4). Patients without sNHR did not report a better effect of the medication on reduction of symptoms compared with those with sNHR (median 48, IQR 25-72 mm, N = 287 *vs* median 53, IQR 25-74 mm, N = 255, p = 0.24). In patients, the reported effect of medication correlated negatively with VAS scores of most (sino)nasal symptoms, but not with VAS scores of sneezing and itch (Table 2). Intranasal corticosteroids were used by 63 %, 69 %, 88 %, 75 %, and 77 % of patients with AR, NAR, CRSwNP, CRSsNP, or mixed phenotype respectively. No difference in the prevalence of self-reported NHR could be observed in subgroups based on type of medication used (nasal lavage, short-term nasal spray, intranasal corticosteroids, antihistamines, or others) (data not shown).

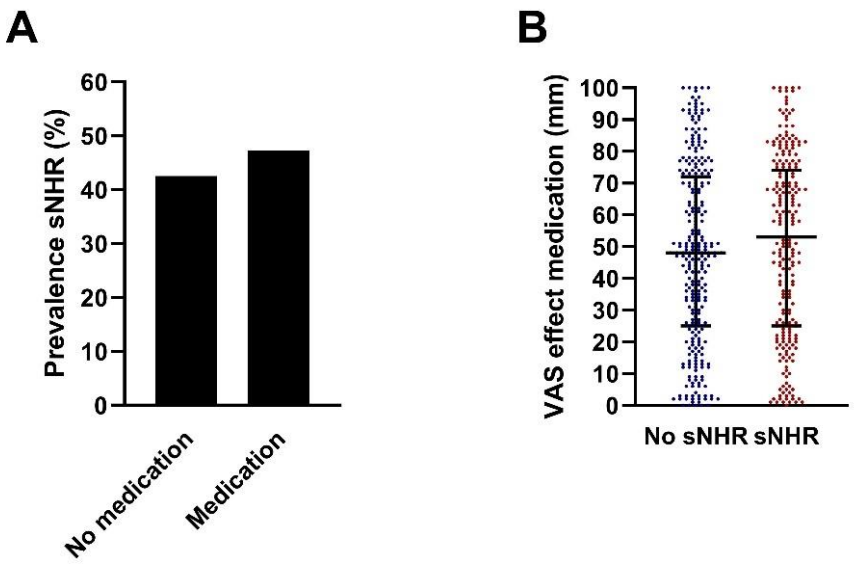

Figure 4: Effect of medication on prevalence of self-reported nasal hyperreactivity (sNHR). A: Prevalence of sNHR in patients having used medication for their sinonasal symptoms in the past three months and those who did not. B: Visual analogue scale (VAS) scores of the effect of medication in patients with and without sNHR. Medication does not affect prevalence of sNHR. (A: Fisher's exact test, not significant. B: data presented as median and interquartile range, Mann-Whitney test, not significant).

Correlation with VAS effect of medication			
	r	**Significance**	**p**
VAS total nasal symptoms	-0.2517	****	<0.0001
VAS nasal obstruction	-0.1796	****	<0.0001
VAS postnasal drip	-0.1673	****	<0.0001
VAS rhinorrhea	-0.1163	***	0.0069
VAS nasal itch	-0.09361	*	0.0302
VAS sneezing	-0.07919	NS	0.0677
VAS headache/facial pain	-0.1937	****	<0.0001
VAS loss of smell	-0.1637	****	<0.0001
VAS ocular itch	-0.02118	NS	0.6234

Table 2: Correlation between the visual analogue scale (VAS) scores on the effect of medication and on various (sino)nasal symptoms. There is a good correlation between the effect of medication and severity of most symptoms, but not for sneezing and (ocular) itch. (Spearman r test).

Triggers and induced symptoms of nasal hyperreactivity

We investigated which triggers provoked sNHR. Temperature changes (66.9 %), air conditioning (53.2 %) and humidity changes (51.1 %) were the most frequently reported triggers for sNHR symptoms (Figure 5). Less frequently reported triggers were physical exercise or sports (31.7 %), (cigarette) smoke (28.9 %) and strong odors (19.4 %) ($p < 0.0001$). Less than 5 % of patients reported other triggers which could not be grouped around a particular theme. All patients who reported other triggers, also reacted to at least one of the predefined triggers. Similar findings were observed in the different phenotypic subgroups.

Patients with sNHR reported most frequently both nasal obstruction and rhinorrhea/postnasal drip to be evoked after exposure to environmental triggers (63.7 % and 65.1 % respectively). Headache or facial pain was reported secondly in 49.0 %, followed by sneezing (38.4 %), itch (26.4 %), and loss of smell (25.0 %) ($p < 0.0001$). Headache/facial pain and loss of smell were reported more frequently in CRS patients compared with rhinitis patients (59.8 %, N = 82 *vs* 39.1 %, N = 128, p = 0.0045 and 32.9 %, N = 82 *vs* 15.6 %, N = 128, p = 0.0040 respectively).

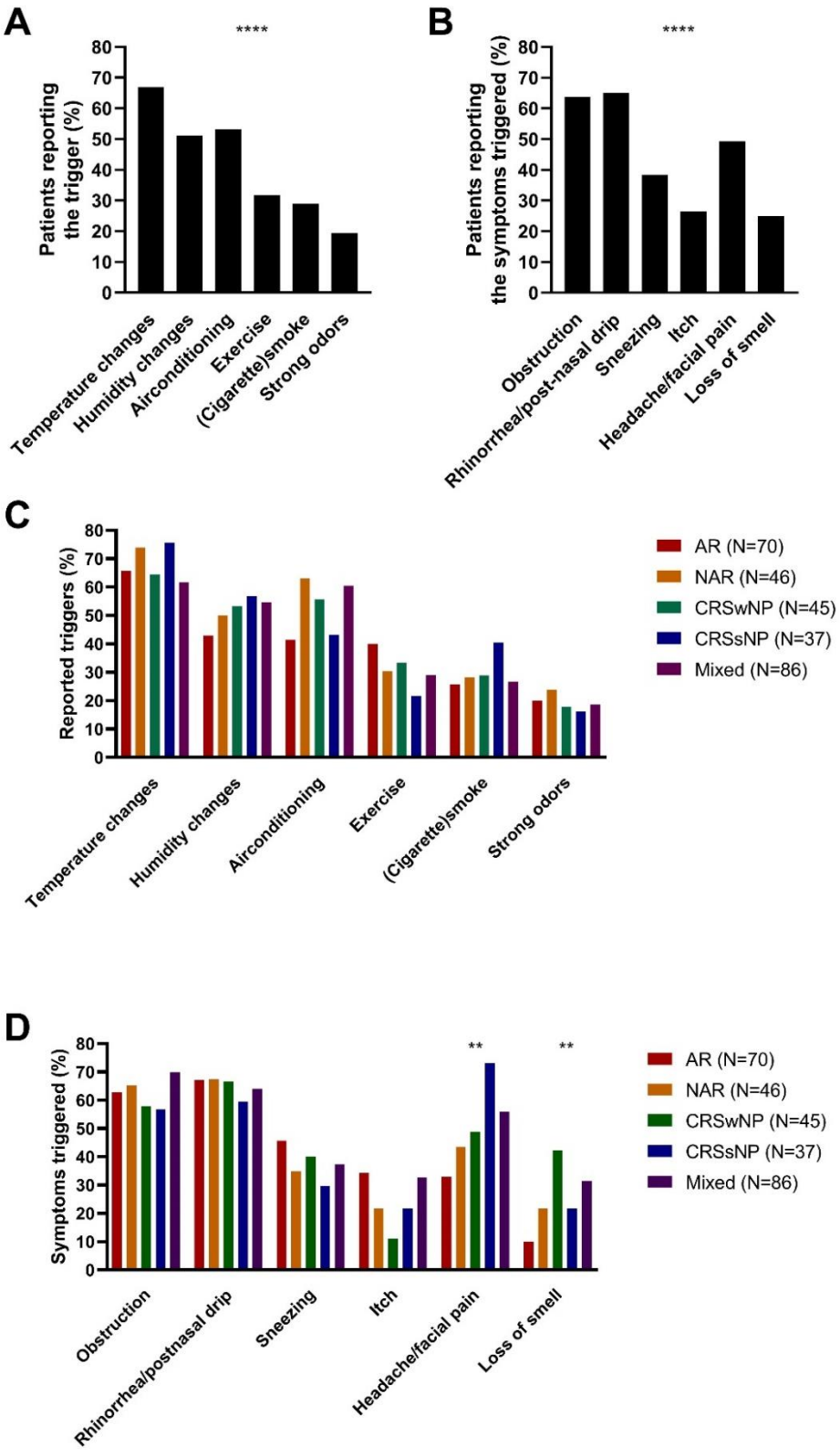

Figure 5: Percentage of patients with self-reported nasal hyperreactivity reacting to each trigger (A and C) and reporting each symptom to be triggered (B and D). (A and B: chi-square test, **** $p < 0.0001$. C and D: chi-square test with Holm-Sidak correction for multiple testing, ** $p < 0.01$). AR: allergic rhinitis, NAR: non-allergic rhinitis, CRSwNP: chronic rhinosinusitis with nasal polyps, CRSsNP: chronic rhinosinusitis without nasal polyps.

Chronic upper airway inflammation, nasal hyperreactivity and general well-being

When pooling all patients with chronic upper airway inflammation together, higher scores were found on the PSS, GAD-7, PHQ-9, and PHQ-15 compared to healthy controls (Figure 6). This was also the case for most of the subgroup-comparisons, but not for patients with CRSwNP. In the control group, 11.3 % of the participants screened positive for generalized anxiety disorder, compared with 24.3 %, 19.8 %, 10.8 %, 20.6 %, and 20.7 % of patients with AR, NAR, CRSwNP, CRSsNP, or mixed phenotype respectively ($p = 0.0171$). Regarding depression, 6.8 % of the control subjects screened positive for major depression, compared with 16.0 % of AR patients, 13.5 % of NAR patients, 10.9 % of CRSwNP patients, 23.0 % of CRSsNP patients, and 18.4 % of patients with mixed phenotype ($p = 0.0076$). PSS, GAD-7, PHQ-9, nor PHQ-15 scores were significantly associated with presence of sNHR, not in the overall cohort of patients (Figure 6), nor in phenotypic subgroups (data not shown). Lastly, there was generally a positive correlation between VAS scores of nasal symptoms and mental/physical well-being in the patient group (Figure 7).

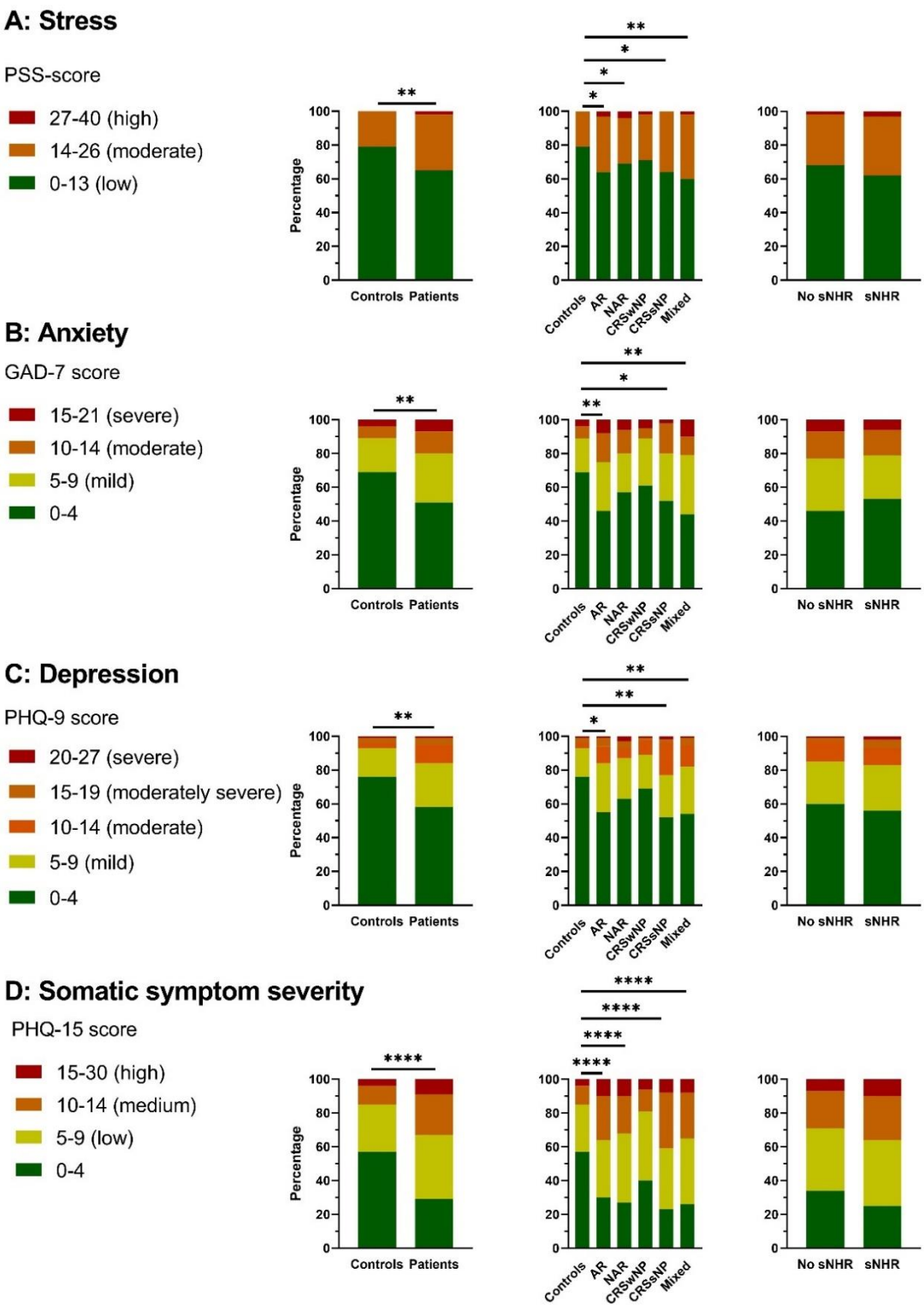

Figure 6: Evaluation of stress levels (A), anxiety levels (B), depressive symptoms (C) and somatic symptom severity (D). (Chi-square test with Holm-Sidak correction for multiple testing, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$). PSS: perceived stress scale, GAD-7: general anxiety disorder 7, PHQ-9: patient health questionnaire 9, PHQ-15: patient health questionnaire 15, AR: allergic rhinitis; NAR: non-allergic rhinitis, CRSwNP: chronic rhinosinusitis with nasal polyps, CRSsNP: chronic rhinosinusitis without nasal polyps, NHR: nasal hyperreactivity.

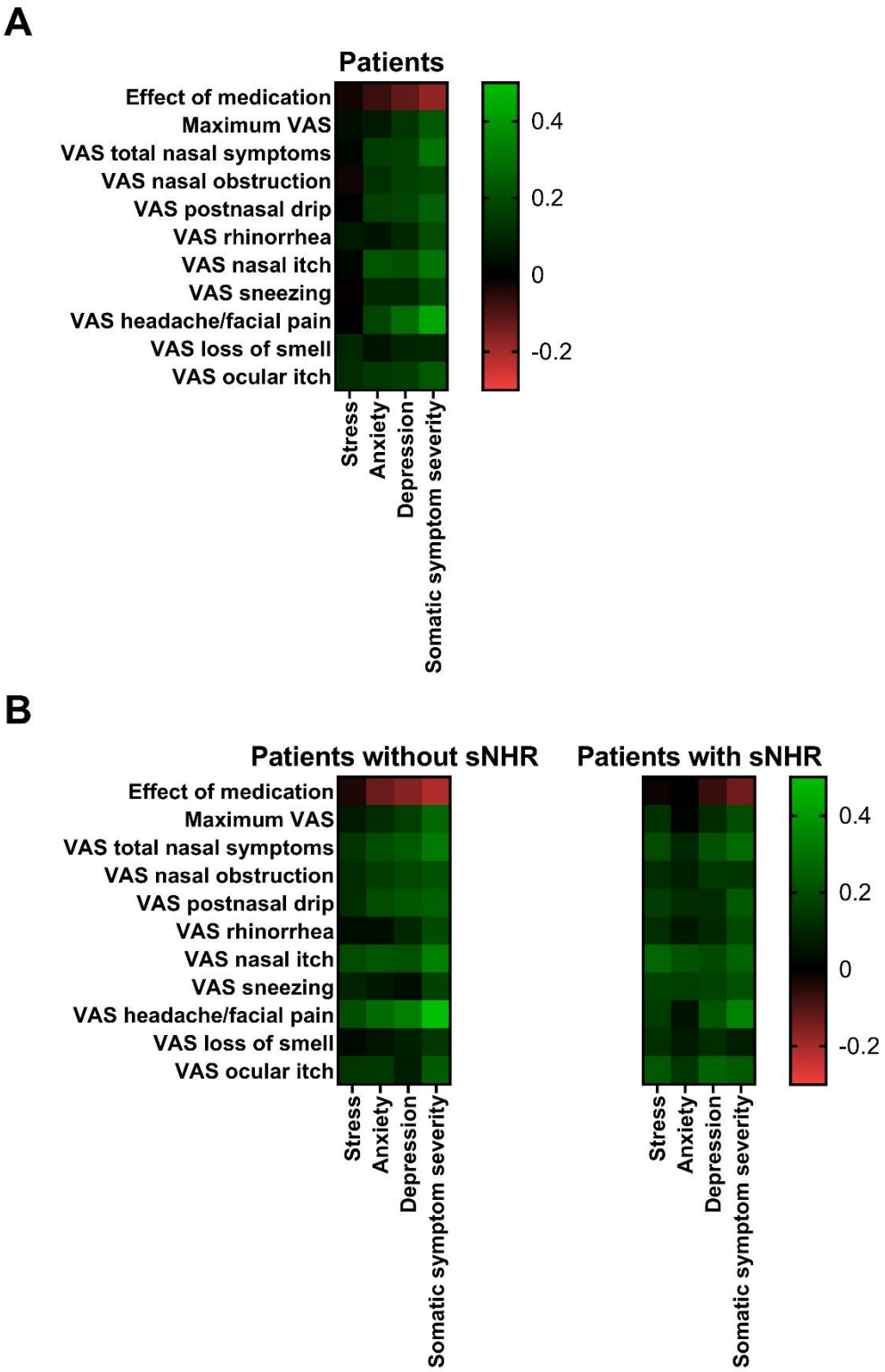

Figure 7: Heat map of the r values showing the correlation between age, effect of medication, or symptom severity on one hand and mental/physical well-being on the other. Correlations were analyzed in the entire cohort of patients (A), and in patients without and with self-reported nasal hyperreactivity (NHR) (B). (Spearman r test). VAS: visual analogue scale.

Discussion

The current study is the first to register the high prevalence of sNHR in all phenotypes of chronic upper airway inflammation. Compared to previous studies, we found a lower prevalence of sNHR in AR (48.6 % *vs* 55.1-63.4 %), NAR (47.4 % *vs* 60.9-66.9 %), and CRS (42.1 % and 40.5 % in CRSsNP and CRSwNP respectively *vs* 64.8 %) (Figure 1).[23,24,26] This discrepancy might be related to variations in patient selection and/or characterization, or – more importantly – to differences in the definitions being used. In contrast to previous studies, symptoms were required to last at least 10 minutes to diagnose sNHR in our study, in order to differentiate from normal physiological reactions to, for example, cold. This illustrates the urgent need for an accurate and universal definition of sNHR. Another factor that could influence prevalence of sNHR is the presence or recent history of acute upper airway infection or exacerbation of the chronic disease.[22]

In line with what was reported previously, we could not observe a higher prevalence of sNHR in both our CRSwNP and CRSsNP patients with concomitant allergy compared with their non-allergic counterparts.[24] Similar to what is observed in the lower airways, we found sNHR to be a common feature in all types of chronic upper airway inflammation.[33,34] This underlines the idea of hyperreactivity being a generic outcome of upper respiratory mucosal inflammation.

Interestingly, we observed a small proportion of the general population reporting NHR (Figure 1). Since NHR can often be the sole symptom of NAR, these participants might suffer a mild and undiagnosed form of NAR.[22] On top, the question whether someone has NHR is not always easy to answer: instead of seeing presence of NHR as an on/off-phenomenon, reactivity to environmental triggers might be part of a continuous spectrum, leaving room for error in subjectively placing oneself above or below the threshold for NHR. Lastly, NHR could be present in a post-infectious phase, even though other symptoms of upper respiratory tract infection are long gone.[22,35]

Recruitment of the participants partly took place during the Coronavirus Disease 2019 (COVID-19) pandemic. This raises the possibility that reports of loss of smell were due to a hidden SARS-CoV-2 infection.[36] However, less than 10 % of the patients/controls were included after March 2020 when the pandemic reached Belgium. Also, as a preventive

measure, patients were routinely asked for symptoms suspicious for COVID-19 before coming to the hospital, in which case the consultation was postponed.

Prevalence of sNHR showed to be related to VAS scores of total nasal symptoms in NAR patients, which was not observed in other rhinitis/rhinosinusitis phenotypes (Figure 2 and 3). Hence, as opposed to NAR, sNHR seems not to be related to disease severity in patients with AR, CRSwNP, CRSsNP, or a mixed phenotype. This discrepancy could be due to the complexity of the underlying mechanisms. Until now, NHR is mainly studied in patients with NAR, where the pathophysiology is mainly mediated by neurogenic inflammation.[22,23] However, in other phenotypes the situation is more complex, with also type 1 and type 2 inflammatory mechanisms and impairment of the epithelial barrier.[2,37–39] sNHR is not limited to those with uncontrolled disease, in contrast to what is often stated.[40] Hence, all patients could benefit from effective treatment strategies targeting NHR and not only those with severe disease.

Prevalence of sNHR did not differ between patients who used medication for their sinonasal symptoms in the 3 previous months compared with those who did not (Figure 4). This highlights the lack of sufficiently satisfying treatment of NHR. On top, the subjective effect of medication correlated not/poorly with the symptom scores of nasal/ocular itch and sneezing (Table 2). Since these symptoms are mainly neurogenically mediated, this illustrates that the entire neurogenic component of upper airway inflammation remains untargeted with current treatment modalities such as nasal douching and (intranasal) corticosteroids.[41,42]

Temperature and humidity changes, together with air-conditioning were reported as the major triggers of NHR (Figure 5). This observation confirms a previous report and is in line with the concept of using a cold, dry air provocation test for objective diagnosis of NHR.[23] Aside from nasal obstruction, patients also reported rhinorrhea as one of the main nasal symptoms being provoked by environmental triggers. Currently, only nasal obstruction reflected by a reduction in peak nasal inspiratory flow of 20 % or more is considered for objective diagnosis of NHR.[28] Consequently, we postulate that extending the diagnostic criteria of a cold, dry air provocation test with parameters measuring rhinorrhea or postnasal drip could be useful, as is already the case for nasal allergen provocation tests.[43]

Presence of lower airway inflammation did not affect prevalence of sNHR in our study. However, where upper airway disease was diagnosed by an otorhinolaryngologist, patients

themselves indicated whether they suffered from asthma or chronic obstructive pulmonary disease. It is known that up to 70 % of asthmatic patients remain undiagnosed.[44] Therefore, it is plausible that undiagnosed asthmatics were considered as participants without lower airway disease.

We found increased levels of stress, anxiety, depression, and somatic symptom severity in our patients with chronic upper airway inflammation (Figure 6). According to questionnaire-based cutoffs, generalized anxiety disorder was observed in 11 % of healthy controls, 24 % of AR patients, 11 % of CRSwNP patients, and 21 % of CRSsNP patients, which is in line with previous reports (10 % in healthy controls, 25 % of AR patients, and 14-21 % of CRS patients).[10,13,14,16] One study focused on anxiety in NAR and reports a prevalence of nearly 53 %.[16] However, this same study also reported a far higher prevalence of anxiety in AR patients (46 %) and depression in AR (39%) and NAR (48 %) patients compared with other studies.[16] This could partly be explained by the fact that other screening tests than GAD-7 and PHQ-9 were used in the latter study.

Depression is reported to be present in 5-10 % of the general population, 17-19 % of AR patients, and 9-27 % of CRS patients.[6,10,11,13–15,18] Similarly, in our study a prevalence of 7.3 %, 16.6 %, 10.9 %, and 23.9 % was found in healthy controls, AR patients, and CRS patients with and without nasal polyps respectively (Figure 6). Moreover, in the CRS group, depression scores are higher in the group without than the group with nasal polyps. This is suggested to be linked to more severe headache in the former group, which is linked with depression.[6,7] Indeed, the median VAS score for headache/facial pain was 73 mm in the CRSsNP patients compared with 21 mm in the CRSwNP group (data not shown), and there is a positive correlation between VAS headache/facial pain and PHQ-9 scores (Figure 7). Our results show that depression in chronic upper airway inflammation is at least as prevalent as in other chronic diseases such as type 2 diabetes mellitus (7.4-18 %)[45,46], pulmonary hypertension (15.9 %)[47], angina pectoris (15.0 %)[48], or asthma (18.1 %)[48].

It has previously been shown that mental well-being correlates well with subjective sinonasal symptom severity in CRS patients, but not with objective measurements of disease severity such as the Lund-Mackay score.[10] Patients tend to report more severe symptoms for similar objective disease severity in case of impaired mental well-being.[10] In line with these previous findings, we found a correlation between VAS sinonasal symptoms and anxiety levels,

depressive symptoms, or somatic symptom severity (Figure 7). No statistically significant difference in PSS, GAD-7, PHQ-9, and PHQ-15 scores could be observed between patients with and without sNHR (Figure 6). Hence, our finding of an increased prevalence of sNHR in patients cannot be explained by impaired mental well-being.

The major strength of this large cohort questionnaire-based study is the physician-based diagnosis of upper airway inflammation by extensive patient history, nasal endoscopy, skin prick test, and CT scan in case of doubt on presence of sinus-involvement in the disease, allowing excellent patient characterization and allocation to the correct group. Also, having used the same questionnaire and definitions for all participants allows for comparison of the different subgroups. Good characterization of participants and use of clear definitions are the key to successful use of questionnaires and to future inter-study comparison.

An inherent limitation of the current questionnaire-based study lies in its subjective nature. Segboer *et al*. found a good correlation between self-reported NHR and objectively diagnosed NHR in patients with AR or NAR.[23] Nevertheless, only a small sample size was studied and hence further confirmation is needed. Previous studies indicate that 19.8-24.7 % of patients with NAR suffer from a local allergic rhinitis.[49,50] Since the vast majority of our patients with NAR did not undergo a nasal allergen provocation test, we were not able to discriminate patients with a possible local allergic rhinitis. However, prevalence of NHR was in our study similar between our patients with AR and patients with NAR.

In conclusion, we found a high prevalence of self-reported NHR in all patients with chronic upper airway inflammation. Temperature and humidity changes are the main triggers for both nasal obstruction and rhinorrhea. When looking at all patients with chronic upper airway inflammation, self-reported NHR is not correlated with level of disease control, nor with general well-being. Consequently, specific treatment of NHR targeting its presumed neurogenic pathophysiology may have therapeutic potential in all patients across the severity spectrum and all phenotypes of chronic upper airway inflammation.

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APPENDIX TO CHAPTER 3

Published Letter to the Editor:

Self-reported nasal hyperreactivity is a common feature of chronic upper airway inflammatory phenotypes and is not related to general mental or physical well-being

Wout Backaert, Brecht Steelant, Mark Jorissen, Lukas Van Oudenhove, Karel Talavera, Peter W. Hellings, Laura Van Gerven

Backaert W *et al.* Self-reported nasal hyperreactivity is common in all chronic upper airway inflammatory phenotypes and not related to general well-being. Allergy. 2021 Aug 21;76(12):3806–9.

To the editor

Chronic upper airway inflammatory diseases like allergic rhinitis (AR), non-allergic rhinitis (NAR), and chronic rhinosinusitis (CRS) are prevalent and relate to higher stress, anxiety, and depression, impacting life quality and raising a large economic burden.[1–3]

Nasal hyperreactivity (NHR) – defined as worsening of upper airway symptoms upon exposure to environmental triggers such as temperature/humidity changes – can be diagnosed objectively by a cold, dry air (CDA) provocation test.[4] However, most studies are questionnaire-based with varying definitions of NHR poorly discriminating between physiologic and pathologic responses to environmental triggers. Moreover, no studies investigated NHR in various phenotypes simultaneously.

We investigated the prevalence and triggers of clearly-defined NHR, its relationship with disease severity and general well-being in well-characterized patients with chronic upper airway inflammation.

To swiftly gain a general overview of the prevalence of NHR in chronic upper airway inflammation, we performed a questionnaire-based study in 605 otorhinolaryngologist-diagnosed patients with various phenotypes and 151 healthy controls (HC) (Table S1). Diagnosis of the specific chronic upper airway inflammatory phenotype was based on patient history, nasal endoscopy, skin prick test, and CT-scanning when necessary. Self-reported NHR (sNHR) was defined by a positive answer to the questions "*Are your nasal complaints triggered or exacerbated by any of the following triggers: (…)?*" and "*If so, do they last longer than 10 minutes?*". Visual Analogue Scales (VAS) assessed symptom severity and improvement with medication. Stress, anxiety, depression, and somatic symptom severity were assessed using the questionnaires Perceived Stress Scale (PSS), General Anxiety Disorder-7 (GAD-7), Patient Health Questionnaire-9 (PHQ-9), and Patient Health Questionnaire-15 (PHQ-15) respectively. Methods are elaborated in the Supplement.

Prevalence of sNHR in upper airway inflammatory phenotypes was between 40.5 and 52.1 %, without differences between groups ($p = 0.33$) (Figure 1A). Other studies reported higher prevalences of sNHR in AR (55.1-63.4 %[4,5]), NAR (60.9-66.9 %[4,5]), and CRS (64.8 %[6]), supposedly related to variations in patient-characterization or, chiefly, to differences in NHR-definitions. To diagnose sNHR we required symptoms to last at least 10 minutes to discard

normal physiological reactions to, e.g., cold. Interestingly, 8.6 % of HC-subjects reported NHR. These participants might suffer mild/undiagnosed NAR.

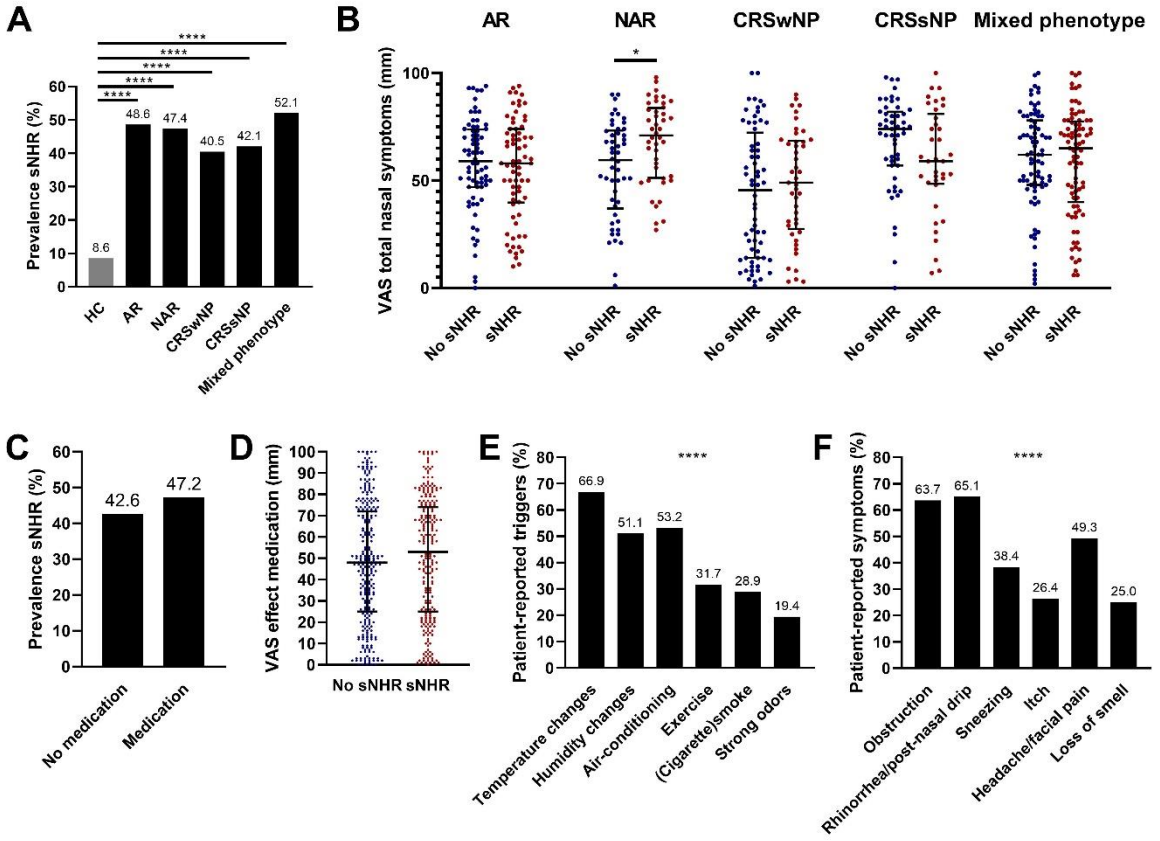

Figure 1: A) Prevalence of self-reported nasal hyperreactivity (sNHR) in healthy controls (HC) and various phenotypes of chronic upper airway inflammation. **B)** Visual analogue scale (VAS) score of total nasal symptoms of patients with and without sNHR in the phenotypic subgroups. **C)** Prevalence of sNHR in patients under medication for their (sino)nasal symptoms and patients not under medication for their (sino)nasal symptoms. **D)** VAS scores of the effect of medication in patients with and without sNHR. **E)** Percentage of patients with sNHR reacting to each trigger. **F)** Percentage of patients with sNHR reporting each symptom to be triggered. (A and C: Two-tailed Fisher's exact test with Holm-Sidak correction for multiple testing, **** p<0.0001. B and D: Data presented as median and interquartile range, Mann-Whitney test with Holm-Sidak correction for multiple testing, * p<0.05. E and F: Chi-square test, **** p<0.0001.) AR: allergic rhinitis, NAR: non-allergic rhinitis, CRSwNP: chronic rhinosinusitis with nasal polyps, CRSsNP: chronic rhinosinusitis without nasal polyps.

VAS scores for total nasal symptoms were higher in sNHR-positive NAR-subjects, but not in other subgroups (Figure 1B/S1). This discrepancy is probably related to the complexity of underlying mechanisms. In NAR, symptoms are presumably mainly neurogenically mediated whereas other phenotypes exhibit also type 1 or 2 inflammatory mechanisms and

barrier defects. NHR, but also for example sneezing and itch, are mainly neurogenically mediated and respond poorly to available treatments (Figure 1C/D, Table S2).

sNHR-positive patients mainly reported temperature/humidity changes and air-conditioning as triggers (Figure 1E), supporting use of CDA provocation for objectification. In a CDA provocation test, objective diagnosis of NHR is purely based on decreased peak nasal inspiratory flow reflecting nasal obstruction. However, also rhinorrhea is triggered in patients with sNHR (Figure 1F). Consequently, we propose extending the diagnostic criteria of a CDA provocation test with rhinorrhea measurements.

Generalized anxiety disorder and major depression were observed in 11.3 and 7.3 % (HC), 24.4 and 16.6 % (AR), 19.8 and 13.5 % (NAR), 10.8 and 10.9 % CRS with nasal polyps (CRSwNP), and 20.7 and 23.0 % CRS without nasal polyps (CRSsNP) respectively (Figure 2). PSS, GAD-7, PHQ-9, and PHQ-15 scores were similar in patients with and without sNHR. Since mental well-being was previously shown to correlate with subjective sinonasal symptom severity but not with objective measurements of disease severity, increased prevalence of sNHR in patients cannot be explained by impaired mental well-being.[3]

This study's strength is the excellent otorhinolaryngologist-based patient-characterization and solid definition of sNHR discriminating physiological from pathological responses to environmental triggers. Questionnaires remain limited by their subjective nature, although sNHR is previously shown to be well correlated with objectively diagnosed NHR in patients with AR or NAR.[4]

We conclude that sNHR is prevalent in all phenotypes of chronic upper airway inflammation (AR, NAR, CRSwNP, CRSsNP, and mixed phenotype) regardless of disease severity, medication use, and general well-being.

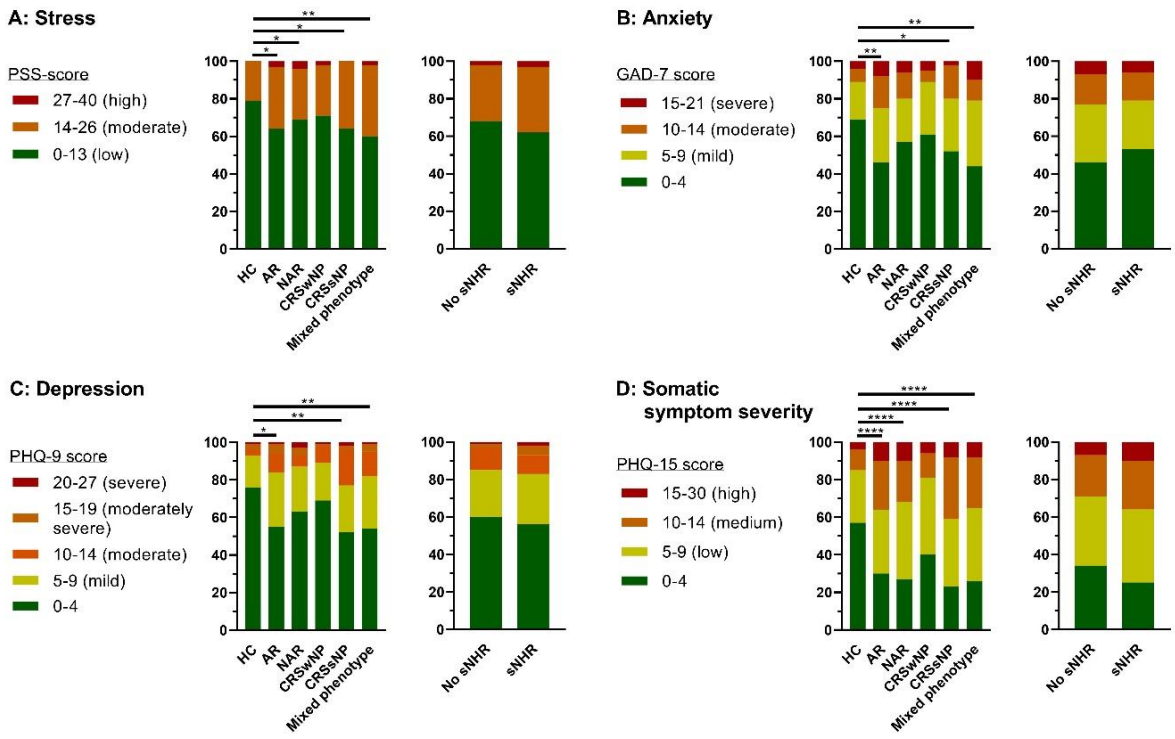

Figure 2: Evaluation of stress levels (A), anxiety levels (B), depressive symptoms (C) and somatic symptom severity (D) in various phenotypes of chronic upper airway inflammation and in patients with and without self-reported nasal hyperreactivity (sNHR). (Chi-square test with Holm-Sidak correction for multiple testing, * p<0.05, ** p<0.01, **** p<0.0001.) PSS: perceived stress scale, GAD-7: general anxiety disorder 7, PHQ-9: patient health questionnaire 9, PHQ-15: patient health questionnaire 15, HC: healthy control, AR: allergic rhinitis, NAR: non-allergic rhinitis, CRSwNP: chronic rhinosinusitis with nasal polyps, CRSsNP: chronic rhinosinusitis without nasal polyps.

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Supplement

Supplementary methods

Study design

From January 2019 until September 2020, patients and healthy volunteers (patients' companions) were recruited from the outpatient clinic of the Department for Otorhinolaryngology of University Hospitals Leuven (Leuven, Belgium) and asked to fill out a questionnaire. General mental and physical well-being were assessed using the Perceived Stress Scale (PSS) for stress[1], General Anxiety Disorder-7 (GAD-7)[2] for anxiety, Patient Health Questionnaire-9 (PHQ-9)[3] for depression, and Patient Health Questionnaire-15 (PHQ-15)[4] for somatic symptom severity. The study was approved by the Ethical Committee Research of University Hospitals Leuven (S62213) and registered on clinicaltrials.gov (NCT03893227).

Study participants

All participants were aged 18-65 years old at the moment of inclusion and were Dutch-speaking. Participants were excluded in case of relevant nasal structural abnormalities, such as major septal deviation, and in case of ear-/nose-/throat-surgery or intranasal capsaicin therapy in the past three months. All participants gave written informed consent.

Participants were included as patients in case of physician-diagnosed chronic upper airway inflammation. This could be either AR, NAR, CRS without nasal polyps (CRSsNP), CRS with nasal polyps (CRSwNP), or a mixed phenotype.

The AR group consists of patients with a positive skin prick test to any of the most frequent aero-allergens in Belgium (*i.e.* house dust mite, timothy grass, English ryegrass, rye, stinging nettle, plantago, ragweed, mugwort, alder, birch, hazel, horse, cat, dog, rabbit, Alternaria, Aspergillus, Cladosporium) and a pattern of nasal symptoms compatible with the atopic sensitizations identified.

The NAR group consist of patients with persistent symptoms of upper airway inflammation (*i.e.* rhinorrhea/postnasal drip, nasal obstruction, sneezing, itch) where

inflammation was limited to the nasal cavity as seen on nasal endoscopy and/or CT scan and with negative skin prick tests.

CRS patients were defined according to the EPOS-guidelines[5]: inflammation of the nose and paranasal sinuses characterized by two or more symptoms of which at least one is nasal obstruction or rhinorrhea, with or without facial pain or loss of smell, together with endoscopic signs of sinonasal inflammation and/or mucosal changes within the ostiomeatal complex or sinuses. If nasal polyps could be observed on nasal endoscopy, patients were included in the CRSwNP group; if not, they were included in the CRSsNP group.

Lastly, patients with two or more of the abovementioned phenotypes were defined as patients having a mixed phenotype (e.g. patients with presence of both nasal polyps and house dust mite allergy).

Healthy control subjects had no known sinonasal symptoms nor (history of) sinonasal pathology, no proven nor suspected allergy, and a mean Visual Analogue Scale (VAS) score for nasal obstruction, postnasal drip, rhinorrhea, itch, sneezing, facial pain, and loss of smell of 20 mm or less.

Outcome parameters

The primary outcome parameter was the prevalence of sNHR, defined as mentioned in the text. Participants could tick multiple of the following triggers: temperature changes, humidity changes, physical exercise or sports, (cigarette) smoke, air conditioning, strong odors, or others. For the VAS scores, participants indicated symptom severity and improvement with medication on a 100 mm long line.

For stress, a PSS score of 0-13, 14-26, and 27-40 correlate with low, moderate and high stress levels respectively.[1] For anxiety, cutoff values were 5, 10, and 15 for mild, moderate and severe anxiety. A cutoff value of 10 is used for screening purposes for generalized anxiety disorder with a sensitivity of 89% and specificity of 82%.[2] For depression, cutoff values were 5, 10, 15, and 20 for mild, moderate, moderately severe, and severe depression respectively. Patients scoring 10 or higher screen positive for major depression with a sensitivity and specificity of 88%.[3] Lastly, somatic symptom severity could be considered to be negligible

(PHQ-15 score of 0-4), low (PHQ-15 score of 5-9), medium (PHQ-15 score of 10-14), or high (PHQ-15 score of 15-30).[4]

Statistical methods

Data were analyzed using GraphPad Prism 8 (GraphPad Software, San Diego, Calif, USA). For comparison of proportions, chi-square or Fisher's exact test was used. For continuous variables, normality was tested with Shapiro-Wilk test. Differences were analyzed using two-tailed unpaired t-test or Mann-Whitney test, depending on normality, and Holm-Sidak correction for multiple testing was applied when multiple outcomes were being compared between groups. Data are presented as mean ± SD in case of normally distributed data, or median and interquartile range in case of non-normally distributed data. Values were considered significantly different when $p<0.05$.

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Supplementary tables

	Controls (N=151)	AR (N=144)	NAR (N=97)	CRSwNP (N=111)	CRSsNP (N=88)	Mixed phenotype (N=165)	Total patients (N=605)	P-value (controls *vs* patients)
Mean age (years ± SD)	44 ± 14	36 ± 12	43 ± 14	51 ± 12	44 ± 14	43 ± 13	43 ± 14	NS†
Male/female	74/77	77/67	44/53	82/27	50/38	94/71	347/258	NS‡
Current smokers (%)	14.7	15.3	16.1	17.3	18.2	12.8	15.3	NS‡
Previous rhinological surgery/trauma (%)	10.6	23.6	38.1	80.2	55.7	60.0	50.9	<0.0001‡
Asthma or COPD (%)	4.0	12.6	10.3	19.1	9.1	28.0	17.1	<0.0001‡
Cystic fibrosis (%)	0.0	0.7	0.0	6.4	1.1	3.0	2.3	NS‡

Table S1: Demographic data. (†Mann-Whitney test, ‡ Fisher's exact test.) AR: allergic rhinitis, NAR: non-allergic rhinitis, CRSwNP: chronic rhinosinusitis with nasal polyps, CRSsNP: chronic rhinosinusitis without nasal polyps.

Correlation with VAS effect of medication

	r	Significance	p
VAS total nasal symptoms	-0.2517	****	<0.0001
VAS nasal obstruction	-0.1796	****	<0.0001
VAS postnasal drip	-0.1673	****	<0.0001
VAS rhinorrhea	-0.1163	***	0.0069
VAS nasal itch	-0.09361	*	0.0302
VAS sneezing	-0.07919	NS	0.0677
VAS headache/facial pain	-0.1937	****	<0.0001
VAS loss of smell	-0.1637	****	<0.0001
VAS ocular itch	-0.02118	NS	0.6234

Table S2: Correlation between the visual analogue scale (VAS) scores on the effect of medication and VAS scores of various (sino)nasal symptoms. (Spearman r test.)

Correlation with number of triggers

	r	Significance	p
VAS total nasal symptoms	0.05480	NS	0.3601
VAS nasal obstruction	-0.01943	NS	0.7458
VAS postnasal drip	0.1485	*	0.0124
VAS rhinorrhea	0.1604	**	0.0069
VAS nasal itch	0.2676	****	<0.0001
VAS sneezing	0.2179	***	0.0003
VAS headache/facial pain	0.1487	*	0.0126
VAS loss of smell	0.1457	*	0.0143
VAS ocular itch	0.1697	**	0.0042

Table S3: Correlation between the number of triggers of symptoms in patients with self-reported nasal hyperreactivity and visual analogue scale (VAS) scores of various (sino)nasal symptoms. There is a correlation between the number of triggers and many (sino)nasal symptoms, most significantly with the neurogenically mediated symptoms nasal/ocular itch and sneezing. (Spearman r test.)

Supplementary figures

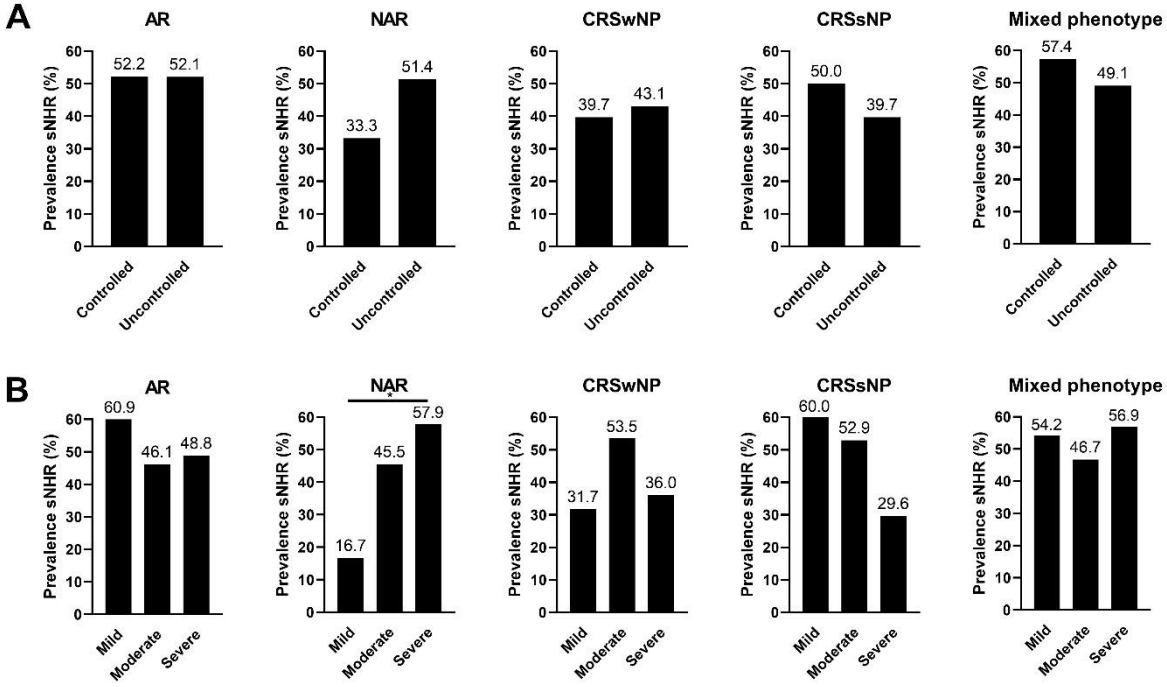

Figure S1: Relationship between the prevalence of self-reported nasal hyperreactivity (sNHR) and disease severity. A) Prevalence of self-reported NHR in controlled (visual analogue scale (VAS) total nasal symptoms <50 mm) and uncontrolled (VAS total nasal symptoms ≥50 mm) patients.[5] **B)** Prevalence of self-reported NHR in patients with mild (VAS total nasal symptoms 0-30 mm), moderate (VAS total nasal symptoms 31-70 mm) and severe (VAS total nasal symptoms 71-100 mm) disease.[5] (A: two-tailed Fisher's exact test with Holm-Sidak correction for multiple testing, not significant; B: chi-square test with Holm-Sidak correction for multiple testing, * p<0.05.) AR: allergic rhinitiss NAR: non-allergic rhinitis, CRSwNP: chronic rhinosinusitis with nasal polyps, CRSsNP: chronic rhinosinusitis without nasal polyps.

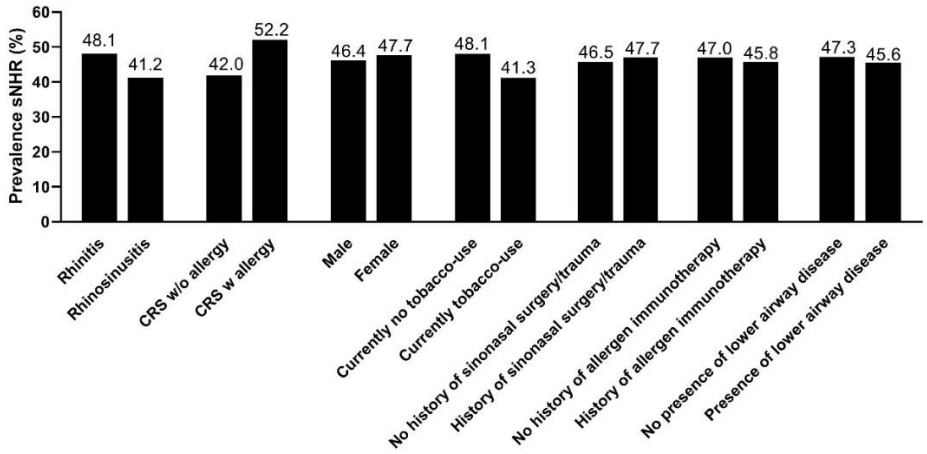

Figure S2: Prevalence of self-reported nasal hyperreactivity (sNHR) in specific subgroups. Prevalence of sNHR did not differ when comparing patient groups based on sinusal involvement, presence of concomitant allergy in CRS patients, gender, current tobacco-use, history of rhinological surgery or severe nasal trauma, history of allergen immunotherapy, or presence of lower airway disease. (Fisher's exact test, not significant.) CRS: chronic rhinosinusitis.

CHAPTER 4

Determination of the chronic upper airway inflammatory phenotype using a symptom-score-based algorithm

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To the Editor

Allergic rhinitis (AR), non-allergic rhinitis (NAR), and chronic rhinosinusitis (CRS) are distinct yet prevalent phenotypes of chronic upper airway inflammation.[1] The gold standard for diagnosis currently consists of history taking, clinical (endoscopic) examination, skin prick testing, and – if indicated – computed tomography.

Ten percent of primary care consultations is about upper airway symptoms.[2] Diagnostic differentiation is important for targeted treatment. However, general practitioners are not trained in performing nasal endoscopy and patients often need referral for specialized investigations. Practical diagnostic tools that can be used in primary care would therefore be useful in daily practice. Patient questionnaires, often used in epidemiologic studies, are useful tools to collect patient data and to assess disease-severity[3] and/or quality of life[4]. Unfortunately, only few questionnaires, mainly for AR, are developed to assess upper airway pathology.[5–7] No diagnostic questionnaires have been developed for NAR or CRS.

In our recently published study on prevalence of nasal hyperreactivity, patients with otorhinolaryngologist-diagnosed chronic upper airway inflammation scored symptom-severity on a 100 mm long visual analogue scale (VAS).[8] Patients were excluded in case of relevant nasal structural abnormalities, such as major septal deviation. AR was diagnosed in case of a positive skin prick test with nasal symptoms compatible with the identified sensitization (284 patients), NAR in case of persistent symptoms in absence of allergy or endoscopic signs of rhinosinusitis (112 patients), and CRS in case of long-lasting nasal obstruction and/or rhinorrhea, with or without facial pain or loss of smell, together with endoscopic signs of sinonasal inflammation (328 patients) (Table S1). Multiple conditions were present simultaneously in 147 patients. In this large cohort, VAS-score profiles were visually distinct across the different phenotypes, with for example more severe itch and sneezing in AR and more olfactory dysfunction and headache/facial pressure in CRS (Figure 1A). A similar symptom-profile in patients with CRS was already described in the past with increased loss of smell, headache, and postnasal drip, without pronounced sneezing or rhinorrhea.[9]

We hypothesized that symptom-specific VAS-scores could be used to predict the chronic upper airway inflammatory phenotype.

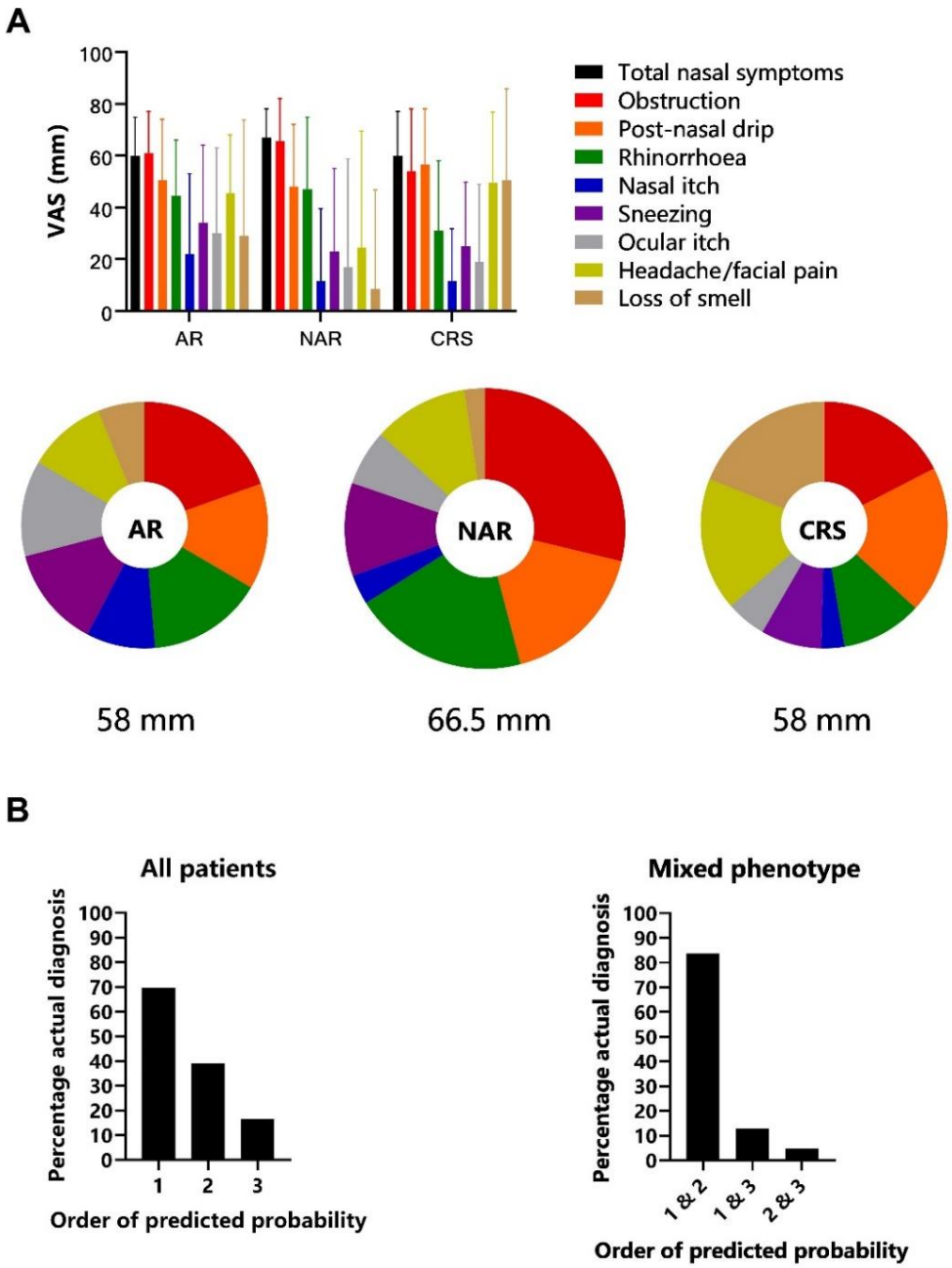

Figure 1: Weight of each symptom in different phenotypic patient groups and diagnostic accuracy of the model. A) Bar chart (median and interquartile range) and pie charts of the VAS scores across different phenotypic groups. The pie charts are built up from the median VAS-scores for every symptom. One hundred percent of the pie equals the sum of the symptom-specific medians. Or, 100 % of the pie = median($VAS_{obstruction}$) + median($VAS_{post\text{-}nasal\ drip}$) + median($VAS_{rhinorrhoea}$) + median($VAS_{nasal\ itch}$) + median($VAS_{sneezing}$) + median($VAS_{ocular\ itch}$) + median($VAS_{headache/facial\ pain}$) + median($VAS_{loss\ of\ smell}$). The median VAS-score for total nasal symptoms is indicated below each pie chart. **B)** The percentage of cases where the actual clinical diagnosis had the highest (1), middle (2), or lowest (3) predicted probability according to the algorithm in all patients and specifically in patients with mixed phenotype. For example, if a patient has the clinical diagnosis of allergic rhinitis (AR) and the predicted probability of AR is higher than the predicted probability of non-allergic rhinitis (NAR) but lower than the predicted probability of chronic rhinosinusitis (CRS), this patient would fall in category 2.

The reported VAS-scores and clinical diagnoses – based on history taking, clinical examination, skin prick test, and computed tomography – were used to develop a diagnostic tool. The least absolute shrinkage and selection operator model was used to select the optimal set of VAS-scores for diagnosis classification. Methods are detailed in the online supplement.

Scores for AR, NAR, and CRS were calculated for each patient by the formulas 1, 2, and 3.

$$\begin{aligned}\mathbf{ARscore} = {} & -\mathbf{0.3369} + \mathbf{0.0001} * \mathbf{VAS}_{\mathbf{Nasal\ obstruction}} + \mathbf{0.0092} * \mathbf{VAS}_{\mathbf{Nasal\ itch}} + \mathbf{0.002} \\ & * \mathbf{VAS}_{\mathbf{Sneezing}} + \mathbf{0.0047} * \mathbf{VAS}_{\mathbf{Itchy\ eyes}} - \mathbf{0.0039} \\ & * \mathbf{VAS}_{\mathbf{Headache/facial\ pressure}}\end{aligned}$$

(Formula 1)

$$\begin{aligned}\mathbf{NARscore} = {} & -\mathbf{1.8797} + \mathbf{0.023} * \mathbf{VAS}_{\mathbf{Total\ nasal\ symptoms}} + \mathbf{0.0088} * \mathbf{VAS}_{\mathbf{Nasal\ obstruction}} \\ & + \mathbf{0.0119} * \mathbf{VAS}_{\mathbf{Rhinorrhea}} - \mathbf{0.0076} * \mathbf{VAS}_{\mathbf{Postnasal\ drip}} - \mathbf{0.0039} \\ & * \mathbf{VAS}_{\mathbf{Nasal\ itch}} - \mathbf{0.0113} * \mathbf{VAS}_{\mathbf{Sneezing}} - \mathbf{0.0075} \\ & * \mathbf{VAS}_{\mathbf{Headache/facial\ pressure}} - \mathbf{0.0218} * \mathbf{VAS}_{\mathbf{Loss\ of\ smell}}\end{aligned}$$

(Formula 2)

$$\begin{aligned}\mathbf{CRSscore} = {} & \mathbf{0.0359} + \mathbf{0.0123} * \mathbf{VAS}_{\mathbf{Postnasal\ drip}} + \mathbf{0.0161} * \mathbf{VAS}_{\mathbf{Headache/facial\ pressure}} \\ & + \mathbf{0.0258} * \mathbf{VAS}_{\mathbf{Loss\ of\ smell}} - \mathbf{0.005} * \mathbf{VAS}_{\mathbf{Total\ nasal\ symptoms}} - \mathbf{0.0188} \\ & * \mathbf{VAS}_{\mathbf{Nasal\ obstruction}} - \mathbf{0.0029} * \mathbf{VAS}_{\mathbf{Rhinorrhea}} - \mathbf{0.008} * \mathbf{VAS}_{\mathbf{Nasal\ itch}} \\ & - \mathbf{0.0017} * \mathbf{VAS}_{\mathbf{Sneezing}} - \mathbf{0.0131} * \mathbf{VAS}_{\mathbf{Itchy\ eyes}}\end{aligned}$$

(Formula 3)

The predicted probability (P) of a specific patient having a particular diagnosis was calculated by formula 4.

$$\mathbf{P} = \frac{\mathbf{exp(score)}}{\mathbf{1} + \mathbf{exp(score)}}$$

(Formula 4)

In our cohort, the diagnosis (AR/NAR/CRS) with the highest predicted probability correlated with the clinical diagnosis in 69.6 % of the cases. In patients with a mixed phenotype, the diagnosed phenotypes had the highest and second highest predicted probability in 83.7 % of the cases (Figure 1B). Nasal hyperreactivity did not aid differentiation between AR, NAR, and CRS.

Although it was no objective of the initial study and patients with (anatomical) pathologies contributing to nasal symptoms were excluded, the interesting observation that a model could be created based on just 9 VAS-scores illustrates the power of well-targeted questions. This observation opens doors for future studies where models with an even higher predictive accuracy could be obtained by carefully selecting and attributing weight to the correct questions. Indeed, our questionnaire did not include questions on, for example, the seasonal variation, previous personal or familial diagnosis of atopy, or the effect of medications already used.

Such practical tools could facilitate diagnosing patients when clinical/technical examination of the patient is limited, such as in tele-consultation. Additionally, they could be used by non-ENT clinicians, who often are not trained in performing a rhinological examination or lack access to required tools (e.g. endoscopy/skin prick test). To this end, the currently presented model illustrates the concept of symptom-score-based algorithmic differentiation of disease phenotypes yet requires further validation. Production of a clear-cut, validated, and ready-for-use algorithm was beyond the scope of our report. Rather, we here present a new concept as illustration and inspiration for future studies where such use of statistics and automated computation is the primary goal (Figure S1). Lastly, implementation of such questionnaires in mobile e-health applications could generate large data sets, serving to develop more potent algorithms based on machine learning.[10]

In conclusion, based on symptom-specific VAS-scores and clinical diagnosis by thorough clinical and technical examination, we developed an illustrative diagnostic algorithm which helps to differentiate patients with chronic upper airway inflammation in various phenotypic subgroups.

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Supplement

Methods

Study design, participants and outcome parameters

Six hundred and five otorhinolaryngologist-diagnosed patients filled-out a questionnaire encompassing questions on symptom severity. The study was approved by the Ethical Committee Research of University Hospitals Leuven (S62213) and registered on clinicaltrials.gov (NCT03893227).

Patients with chronic upper airway inflammation were defined as having upper airway symptoms persisting for at least 1 hour per day for 12 weeks or longer in absence of anatomical causes. Patients were aged 18-65 years old and were diagnosed with *allergic rhinitis* (in case of a positive skin prick test and a pattern of nasal symptoms compatible with the atopic sensitizations identified), *non-allergic rhinitis* (in case of persistent symptoms of upper airway inflammation and inflammation limited to the nasal cavity and negative skin prick tests or symptoms not compatible with the atopic sensitization), or *chronic rhinosinusitis* according to the EPOS-guidelines (inflammation of the nose and paranasal sinuses characterized by two or more symptoms of which at least one is nasal obstruction or rhinorrhea, with or without facial pain or loss of smell, together with endoscopic signs of sinonasal inflammation and/or mucosal changes within the ostiomeatal complex or sinuses).[1]

The severity of various rhinological symptoms were indicated on a 100 mm long line, resulting in a visual analogue scale (VAS)-score ranging from 0 to 100. Twenty-nine patients of whom one or more VAS-scores were missing were excluded from analysis.

Statistical methods

To describe and compare patient characteristics, continuous variables were tested with Kruskal-Wallis test with post-hoc Dunn's multiple comparisons test and proportions were compared with a chi-square test. P-values were considered significant if $p < 0.05$.

The least absolute shrinkage and selection (LASSO) models were used in order to select the optimal set of VAS scores for diagnosis classification. The LASSO model is a shrinkage method which minimizes the regression coefficients in order to avoid overfitting, forcing the coefficients towards 0 and select the non-zero variables as the optimal predictors. In this way, the potential multicollinearity is avoided and a variable selection is performed including the more relevant predictors.

The LASSO models were performed for each diagnosis including all VAS scores by using GLMNET package in R version 4.0.2 (R-Studio, Boston, MA).[11] We used 10-fold cross-validation step for hyper-parameter tuning for the shrinkage parameter for LASSO model. Subsequently, the logistic LASSO regression coefficients of selected covariates were used to calculate the risk score as a measure of the probability of having diagnosis for each patient. Twenty-six percent of the patients exhibited a mixed phenotype, restricting to fit a multinomial model with three diagnoses.

Supplementary table

	AR (N=141)	NAR (N=93)	CRS (N=195)	Mixed phenotype (N=147)				P-value
				AR+NAR (N=14)	AR+CRS (N=128)	NAR+CRS (N=4)	AR+NAR +CRS (N=1)	
Median age (years) (IQR)	32 (25-44)	43 (31-55)	51 (36-59)	51 (36-58)	41 (33-52)	44 (26-59)	49	<0.0001†
Male/female	76/65	43/50	130/65	7/7	78/50	0/4	0/1	0.0028‡
Smokers (%)	23 (16.3)	11 (11.8)	35 (17.9)	0 (0)	17 (13.3)	1 (25.0)	0 (0)	NS‡
Allergy (%)								
House dust mite	92 (65.2)	0 (0)	0 (0)	6 (42.9)	88 (68.8)	0 (0)	0 (0)	<0.0001‡
Tree-/grass pollen	103 (73.0)	0 (0)	0 (0)	9 (64.3)	92 (71.9)	0 (0)	1 (100)	<0.0001‡
Animals	56 (39.7)	0 (0)	0 (0)	4 (28.6)	42 (32.8)	0 (0)	0 (0)	<0.0001‡
Fungi	11 (7.8)	0 (0)	0 (0)	5 (35.7)	10 (7.8)	0 (0)	0 (0)	<0.0001‡
Nasal polyps (%)	0 (0)	0 (0)	107 (54.9)	0 (0)	81 (63.3)	0 (0)	0 (0)	<0.0001‡
Median VAS total nasal symptoms (mm) (IQR)	58 (42-74)	65 (48-78)	59 (30-77)	70 (46-77)	62 (42-78)	73 (67-79)	93	NS†
Medication use last 3 months (%)	131 (92.9)	80 (86.0)	184 (94.4)	14 (100)	124 (96.9)	4 (100)	1 (100)	NS‡
History of rhinological surgery or trauma (%)	35 (24.8)	38 (40.9)	106 (54.4)	5 (35.7)	66 (51.6)	1 (25.0)	0 (0)	<0.0001‡

Table S1: Patient characteristics. († Mann-Whitney test, ‡ Chi square test). AR: allergic rhinitis, NAR: non-allergic rhinitis, CRS: chronic rhinosinusitis, IQR: interquartile range, VAS: visual analogue scale.

Supplementary figure

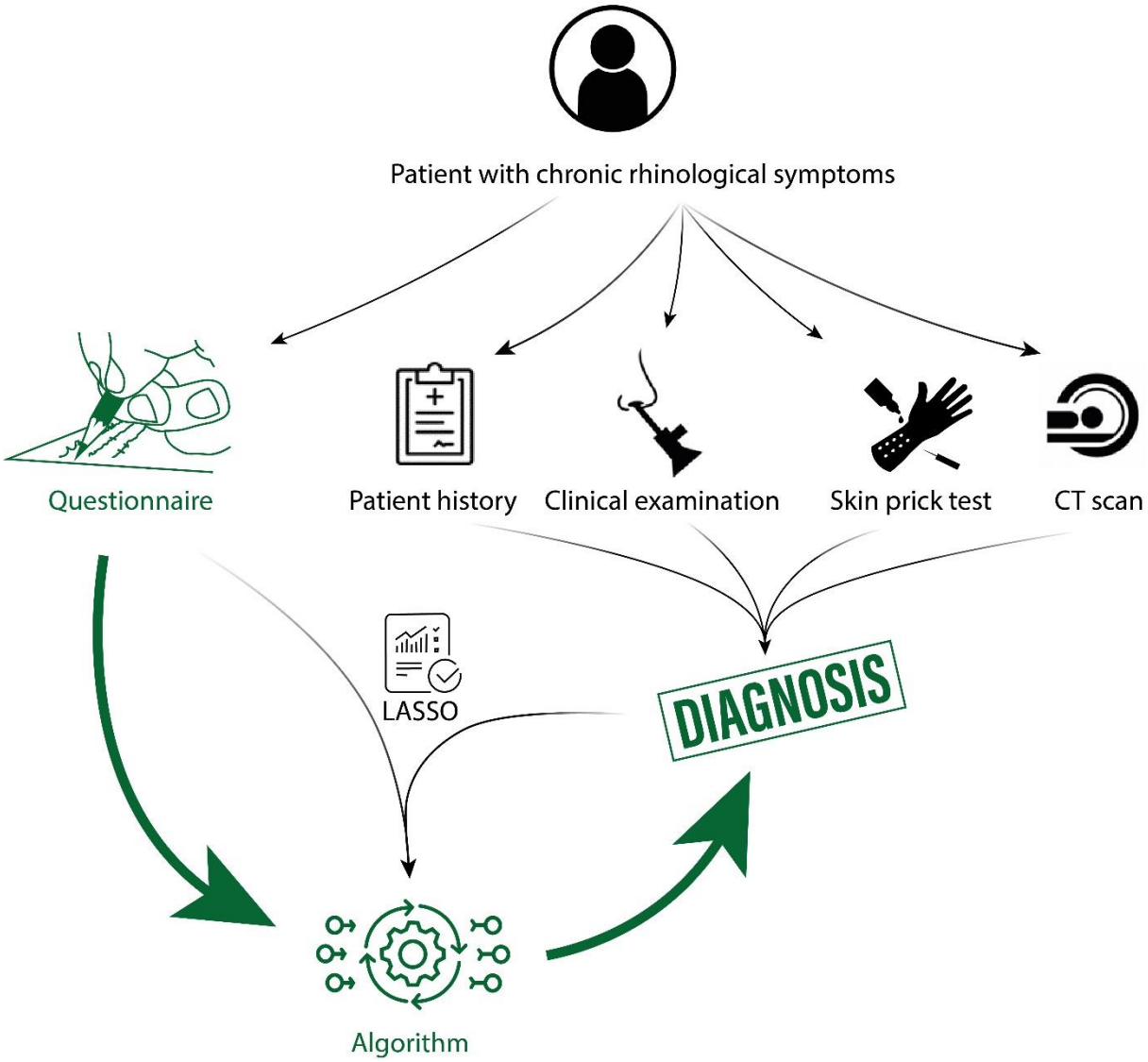

Figure S1: Overview of the methodological principle. From each patient, we collected the symptom-specific VAS-scores by means of a questionnaire. Clinical diagnosis was made by an otorhinolaryngologist based on patient history, clinical examination including nasal endoscopy, skin prick testing, and computed tomography. We then developed a diagnostic algorithm based on symptom-specific VAS-scores only, bypassing the need for full clinical work-up.

CHAPTER 5

Neurogenic pathways underly nasal hyperreactivity in type 2 chronic upper airway inflammatory disorders

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Introduction

Nasal hyperreactivity (NHR) is defined as the induction of nasal symptoms upon exposure to particular environmental stimuli such as temperature or humidity changes, air-conditioning, (cigarette) smoke, or strong odors.[1] In contrast to symptoms that are induced as a physiological, protective reflex, symptoms due to NHR last for more than 10 minutes.[2]

NHR is a prevalent phenomenon mostly studied in patients with idiopathic rhinitis, a specific subgroup of non-allergic rhinitis (NAR).[1] In this group, its presence is described in 47.4-66.9 %.[2–5] In prevalence studies, NHR is often diagnosed by means of a questionnaire. Objective diagnosis can be obtained by a cold (< -10°C), dry (< 10 % relative humidity) air (CDA) provocation test, where CDA is inhaled via the nose for 15 minutes. A CDA-induced decrease in peak nasal inspiratory flow (PNIF) of at least 20 % is considered diagnostic for NHR.[6]

The pathophysiology of NHR is suspected to be mainly neurogenically mediated with an important role for the non-adrenergic, non-cholinergic (NANC) nervous system.[1,7] Afferent, sensory NANC fibers contain neuropeptides such as substance P (SP), calcitonin gene-related peptide (CGRP), and neurokinin A (NKA).[8] From pathophysiological point of view, it is currently thought that strong activation of these NANC fibers results in the release of these neuropeptides, which consequently induce vasodilation and increased mucus secretions, ultimately resulting in nasal symptoms like nasal obstruction and rhinorrhea; a situation termed “neurogenic inflammation”.[1,8]

Multiple studies indicate a contribution of the nociceptive and cation-permeable transient receptor potential (TRP) channels to activation of sensory neurons.[9,10] In patients with idiopathic rhinitis, an increased nasal mucosal sensitivity has been observed for the TRP Vanilloid 1 (TRPV1) and TRP Ankyrin 1 (TRPA1) channel agonist allyl isothiocyanate.[5] This hypersensitivity was abrogated by treatment with the potent and highly specific TRPV1-agonist capsaicin, along with decreased NHR.[5] Capsaicin, the pungent component of hot peppers, is thought to defunctionalize afferent nerve endings by inducing a toxic Ca^{2+} overload.[11] Moreover, patients with idiopathic rhinitis exhibited a baseline overexpression of TRPV1 and increased levels of SP in nasal secretions.[12] In addition, increased levels of TRPV1, TRPA1, and TRP melastatin 8 (TRPM8) were described after rhinovirus infection, potentially

contributing to postinfectious NHR.[13] Lastly, TRPV1, TRPA1, and TRPM8 were shown to contribute to bronchial hyperreactivity in patients with

asthma.[14–16]

The last years, NHR is increasingly being associated with other chronic upper airway inflammatory phenotypes. Patients with allergic rhinitis (AR) were shown to display more nasal obstruction, rhinorrhea, itch, sneezing, pain, and plasma extravasation when challenged with capsaicin, which was even more outspoken during the pollen season in patients with seasonal AR.[17–21] Other studies reported a high prevalence of NHR in AR, chronic rhinosinusitis (CRS) without (sNP) and with nasal polyps (wNP), and mixed phenotypes.[2,4,22–24]

The pathophysiology of both AR and CRSwNP is characterized by upregulation of type 2 inflammatory responses, with interleukins (IL) 4, 5, and 13 produced by Th2 cells and type 2 innate lymphoid cells, eosinophilic infiltrates, mast cells, and increased IgE levels.[25–27] Moreover, both phenotypes exhibit barrier defects, exposing the submucosal immune cells to external allergens, pathogens, and noxious substances.[28,29]

Despite the well-founded insight in these pathophysiological mechanisms, about 20 % of patients with AR or CRS remain uncontrolled with current treatment modalities that mainly target type 2 inflammatory pathways, possibly due to lack of treatment of NHR in these groups.[30] Moreover, the mast cell mediator histamine was already shown to sensitize TRPV1 and TRPA1 in dorsal root ganglion neurons, channels which are shown to play an important role in NHR.[31–33] We therefore investigated the mediators involved in NHR in AR and CRSwNP. We designed a prospective interventional study to investigate the (concomitant) presence of neurogenic inflammation in classical type 2 inflammatory diseases, AR and CRSwNP, and searched for key mediators contributing to the interplay between neurogenic and type 2 inflammation.

Methods

Study participants

From February 2020 until December 2021, patients with predominated type 2 chronic upper airway inflammation (persistent AR or CRSwNP) and healthy controls (patients' companions) were recruited from the outpatient rhinology clinic of University Hospitals Leuven.

All patients had a Visual Analogue Scale (VAS) score of ≥ 40/100 for any nasal symptom for at least 12 weeks. Patients with persistent AR were included in the months October-December. House dust mite allergy was confirmed on study visit 1 by skin prick test and presence of relevant nasal symptoms. Patients with CRSwNP were included in case of bilateral presence of nasal polyps on nasal endoscopy during visit 1. Patients with a mixed phenotype as identified by nasal endoscopy or skin prick test were excluded.

	Healthy control	**Allergic rhinitis**	**Chronic rhinosinusitis with nasal polyps**
Inclusion	• 18-65 years old • VAS score < 20/100 for any nasal symptom*	• 18-65 years old • House dust mite-allergy based on patient history and skin prick test • VAS score of ≥ 40/100 for any nasal symptom*	• 18-65 years old • Chronic rhinosinusitis with nasal polyps based on nasal endoscopy • VAS score of ≥ 40/100 for any nasal symptom*
Exclusion	• Aero-allergy and/or (history of) use of AIT • Chronic rhinosinusitis without/with nasal polyps	• Chronic rhinosinusitis without/with nasal polyps • (History of) use of AIT	• Aero-allergy and/or (history of) use of AIT
	• Relevant anatomical abnormalities in the nose • Acute upper airway infection in the past 2 weeks • Recent (7 days) use of nasal medication • Alcohol consumption in the past 24 hours • Use of tricyclic antidepressants • Intranasal drug-abuse in the past 12 months • Currently participating in other clinical studies • Pregnancy or breastfeeding • Active malignancy		

Table 1: In- and exclusion criteria. *Individual VAS scores for total nasal symptoms, nasal obstruction, rhinorrhea/post-nasal drip, sneezing, nasal itch, headache/facial pain, and loss of smell. VAS: visual analogue scale (mm), AIT: allergen immunotherapy.

Healthy control subjects suffered no sinonasal symptoms, had no evidence of atopy to aero-allergens on skin prick test, and had no signs of rhinosinusitis on nasal endoscopy.

All participants were 18-65 years old, never used allergen immunotherapy, used no nasal medication 1 week before the first study visit, had no relevant anatomic abnormalities in the nose contributing to nasal symptoms, and were free of acute upper airway infection. In- and exclusion criteria are detailed in Table 1.

Study design

Healthy controls and patients with AR or CRSwNP were screened for eligibility and willingness to participate by phone call. The study consisted of two study visits at the Department of Otorhinolaryngology at University Hospitals Leuven at least 3 weeks apart (Figure 1A). Participants did not use any nasal medication, corticosteroids, antihistamines, or saline lavages during the study, starting 1 week prior to visit 1.

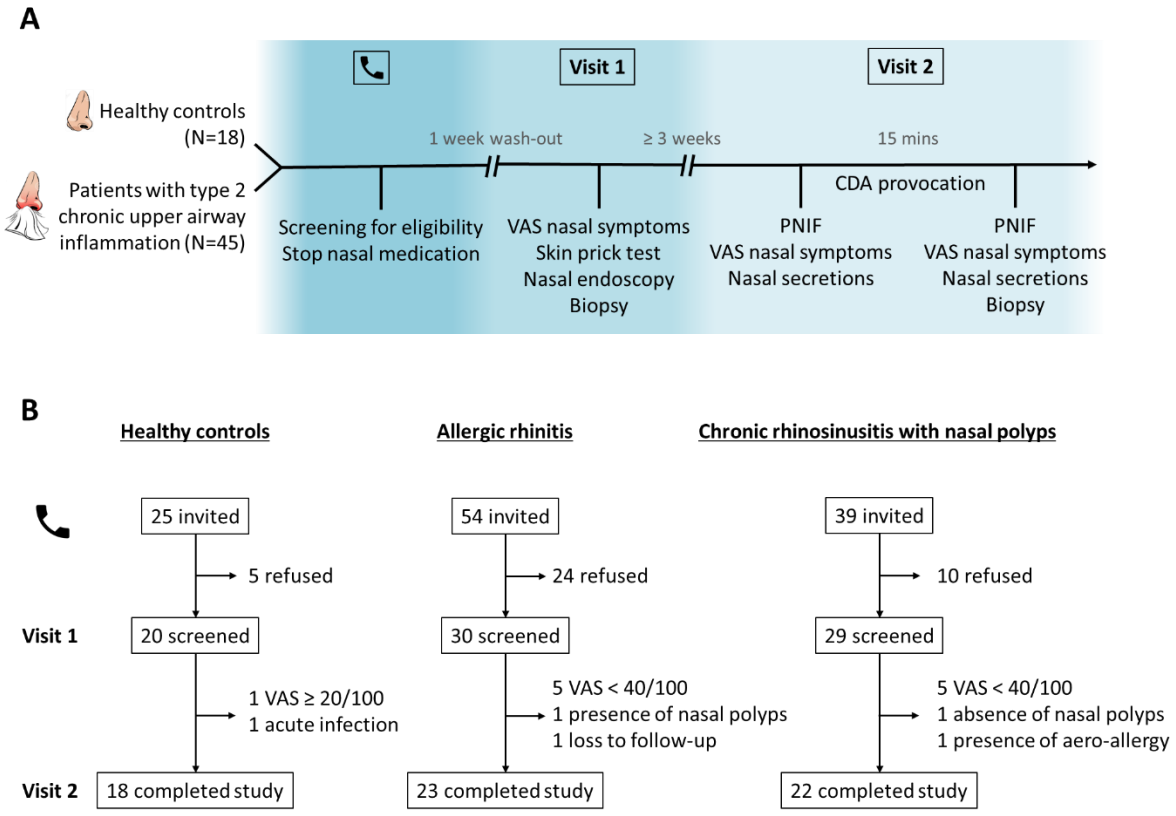


Figure 1: Study design (A) and in-/exclusion chart (B). PNIF: peak nasal inspiratory flow, VAS: visual analogue scale.

At the first study visit, participants scored nasal symptom severity on VAS scales and baseline biopsies were taken bilaterally from the inferior turbinate. On study visit 2, symptom severity was scored on VAS scales, PNIF was measured, and nasal secretions were harvested before and after CDA provocation. Biopsies were taken bilaterally from the inferior turbinate after provocation.

Informed consent was obtained from all participants. The study was approved by the Ethical Committee Research of University Hospitals Leuven (S63139) and registered on clinicaltrials.gov (NCT04286542).

Assessment of symptom severity and nasal hyperreactivity

Patient-reported symptom scores for total nasal symptoms, nasal obstruction, rhinorrhea or post-nasal drip, nasal itch, sneezing, facial pressure, and loss of smell were indicated on 100 mm-long VAS scales.

Participants were considered to suffer from self-reported NHR (sNHR) in a binary way (yes/no) in case of a patient-reported increase in nasal symptoms upon encounter to specific environmental triggers (temperature/humidity changes, air-conditioning, (cigarette) smoke, strong odors, fragrances) which lasts for more than 10 minutes.[2] After a CDA provocation test, participants indicated to what degree the CDA exposure exacerbated their symptoms on a VAS scale, allowing to study subjective NHR as a continuous parameter (Figure 3A).

For diagnosis of objective NHR (oNHR), participants underwent a CDA provocation test as described previously.[6] For 15 minutes, cold (< -10°C), dry (< 10 % relative humidity) air was delivered by a custom-made CDA-device at a flow of 25 L/min via an anesthetic mask. Drilled holes in the mask assured escape of redundant air, preventing overpressure. Stability of temperature and humidity was continuously checked with a custom-made device calibrated using a 176H datalogger (0572 1765, Testo, Ternat, Belgium). Participants inhaled strictly via the nose. PNIF was measured immediately before and after provocation with a PNIF-device (In-Check Nasal Inspiratory Flow Meter, Clement Clarke International, Harlow, UK). At each time point, the median of three measurements ≤ 10 % apart was used. oNHR was diagnosed (yes/no) in case PNIF decreased ≥ 20 % during CDA provocation.[6] The relative change in PNIF during CDA was used to objectively study reactivity to CDA in a continuous manner.

Collection of nasal secretions and biopsies of nasal mucosa

To collect nasal secretions, nasal sponges (Post-Op Sinus Pack K9, Q770532, Ivalon Surgical Products, Fabco, New London, Connecticut, USA) were weighed and inserted in both nostrils for 10 minutes. Afterwards, the sponges were weighed again, and saline was added to reach a 1:3 dilution. The sponges were squeezed with a syringe and centrifuged at 500 g at 4 °C for 15 minutes. Nasal secretions were stored at -80 °C until further analysis.

At the end of visit 1 and 2, cotton balls soaked in cocaine 1 % (obtained, stored, and used according to the Belgian Royal Decree regulating the use of anesthetics and psychotropic substances (2019-12-09/21)) were placed next to the inferior turbinates and left in place for 15 minutes to achieve local anesthesia. Next, a biopsy was taken from the inferior turbinate bilaterally with a Fokkens forceps. Biopsies were snap frozen in liquid nitrogen and stored at -80 °C until further analysis.

Measurement of SP, NKA, CGRP, IL-4, IL-5, IL-13, IL-33, and histamine in nasal secretions

Protein levels were measured in nasal secretions. Using commercially available ELISA-kits, levels of neuropeptides – SP (583751, Cayman Chemical, Ann Arbor, Michigan, USA), NKA (abx152497, Abbexa, Cambridge, UK), and CGRP (RD-CGRP-Hu, Reddot Biotech, Kelowa, British Columbia, Canada) – and histamine (LS-F39267, Lifespan Biosciences, Seattle, Washington, USA) were measured according to manufacturer's instructions. Levels of type 2 inflammatory markers IL-4, IL-5, and IL-13, and epithelial marker IL-33 were determined using the U-plex platform of Mesoscale Discovery (Mesoscale Diagnostics, Rockville, Maryland, USA). Total protein concentration was determined with a bicinchoninic acid assay (23225, Thermo Fisher Scientific, Waltham, Massachusetts, USA) according to manufacturer's instructions. The insertion of a nasal sponge could induce nasal secretions, diluting the total protein concentration (Figure 2). Therefore, protein levels in nasal secretions were corrected for total protein concentration ([concentration of protein of interest]/[total protein concentration]).

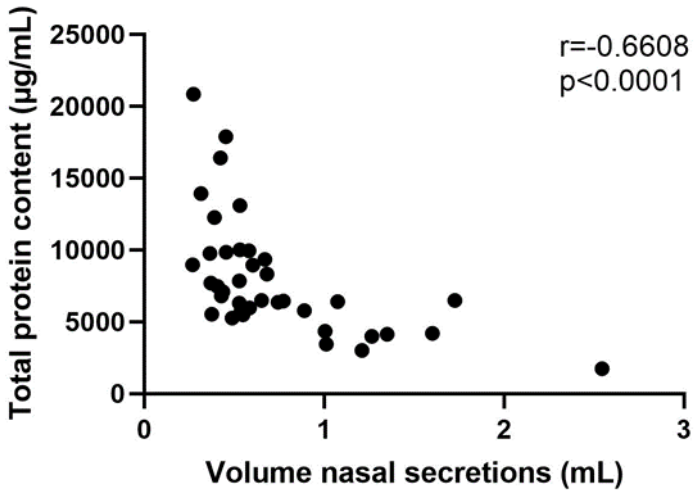


Figure 2: Correlation between the harvested volume of nasal secretions and total protein concentration. (Spearman r test).

RT-q-PCR for TRPV1, TRPA1, TRPM8, TAC1, PGP9.5, ZO-1, OCLN, and CLDN1 on nasal mucosal biopsies

Biopsies were homogenized in lysis buffer using Lysing Matrix D and a FastPrep-24-device and RNA was extracted using the RNeasy Mini Kit (74106, Qiagen, Hilden, Germany). cDNA was obtained using a High-Capacity cDNA Reverse Transcription kit (4368814, Thermo Fisher Scientific, Waltham, Massachusetts, USA) starting from 500 ng RNA. Real time quantitative PCR was performed for *TRPV1*, *TRPA1*, *TRPM8*, protein gene product 9.5 (*PGP9.5*), tachykinin precursor 1 (*TAC1*), zonula occludens 1 (*ZO-1*), occludin (*OCLN*), and claudin 1 (*CLDN1*) (Table 2) with the CFX Connect Real-Time PCR Detection System (Bio-rad, Hercules, California, USA). Expression levels were normalized to the geometric mean of reference genes ß-actin (*ACTB*) and guanine nucleotide-binding protein subunit beta-2-like 1 (*GNB2L1*). cDNA plasmid standards of each specific target gene were used to quantify the amount of target genes in unknown samples.[34]

ACTB	FW	gga cat ccg caa aga cct gt
	RV	ctc agg agg agc aat gat ctt gat
	TP	ctg gcg gca cca cca tgt acc ct
GNB2L1	FW	cac tgt cca gga tga gag cca
	RV	cat acc ttg acc agc ttg tcc c
	TP	tcc gct tct cgc cca aca gca g
TRPV1	FW	aag cca tgc tca acc tgc ac
	RV	tgt ctg gcc ctt gta gta gct g
	TP	cgg aca gcc tga agg agc ttg tca a
TRPA1	FW	tcc tgc cga gac tat tat atc gag tat
	RV	gct cta tgc ggt tat ttt gta cca t
	TP	tat gaa ccg ctt aca gcc ctc aac gc
TRPM8	FW	gcc tac gtg ctg ctc atg g
	RV	cat tta cgt acc act gtc tca ctt ca
	TP	ttt cca ttc ggt gcc aca ccc c
PGP9.5	FW	agg cca atg tcg ggt aga tg
	RV	gtt cac cgg aaa agg cat tc
	TP	tgg atg gcc acc tct atg aac ttg atg g
TAC1	FW	gga ctg tcc gtc gca aaa tc
	RV	tcc tat ttc ttc tgc aaa cag ctg
	TP	aac atg aaa atc ctc gtg gcc ttg gc
ZO-1	FW	gtg cct aaa gct att cct gtg agt c
	RV	cta tgg aac tca gca cgc cc
	TP	tgg cca cag ccc gag gca tat t
OCLN	FW	cca atg tcg agg agt ggg tta a
	RV	ttg cca ttg gaa gag tat gcc
	TP	ctg cag gca cac agg acg tgc c
CLDN1	FW	cca gtc aat gcc agg tac gaa t
	RV	ata ggg cct tgg tgt tgg gt
	TP	tca ggc tct ctt cac tgg ctg ggc

Table 2: Primer and probe sequences used for real-time quantitative PCR on human nasal mucosal biopsies. ACTB: ß-actin, GNB2L1: guanine nucleotide-binding protein subunit beta-2-like 1, TRPV1: transient receptor potential channel vanilloid 1, TRPA1: transient receptor potential channel ankyrin 1, TRPM8: transient receptor potential channel melastatin 8, PGP9.5: protein gene-product 9.5, TAC1: tachykinin precursor 1, ZO-1: zonula occludens, OCLN: occludin, CLDN1: claudin 1.

Isolation and culture of murine trigeminal ganglionic neurons

Trigeminal ganglia from wild type C57Bl/6J mice (male, 8 weeks old) were dissected and digested with collagenase and dispase (1 mg/mL and 2.5 mg/mL respectively, Thermo Fisher Scientific, Waltham, Massachusetts, USA) for 40 minutes, similar to what was previously described for dorsal root ganglionic neurons.[35] Next, the ganglia were mechanically disrupted using hollow needles with decreasing diameters. Cells were seeded on poly-ornitin (500 µg/mL) and laminin (100 µg/mL) coated glass bottom dishes (FD35-100, Fluorodish WPI, Hertfordshire, UK) and cultured for 18-24 hours at 37 °C, 5 % CO_2, in Neurobasal A medium

supplemented with B-27 2 %, GlutaMAX 1 %, Penstrep 3 %, neurotrophin 4 10 ng/mL, and glial cell line-derived neurotrophic factor 2 ng/mL (all products bought from Thermo Fisher Scientific, Waltham, Massachusetts, USA). Trigeminal ganglionic neurons of 3-4 mice were used per condition. Experimental procedures were approved by the Ethical Committee for Animal Research at the KU Leuven (P150/2017).

Intracellular calcium imaging experiments

Cultured murine trigeminal ganglionic neurons (mTGNs) were loaded with 2 µM of Fura-2AM ester for 30 minutes at 37 °C prior to the recordings. Alternating illumination at 340 and 380 nm by a Lambda XL illuminator (Sutter Instruments, Mont-Saint-Guibert, Belgium) evoked fluorescent signals that were recorded using an Orca Flash 4.0 camera (Hamamatsu Photonics Belgium, Mont-Saint-Guibert, Belgium) on a Nikon Eclipse TI fluorescence microscope (Nikon Europe, Amsterdam, The Netherlands). The ratio of the fluorescent signals to both excitation wavelengths after correction for background fluorescence was monitored. The intracellular Ca^{2+} concentration was calculated as described previously using a custom-written macro in Igor Pro software (WaveMetrics, Lake Oswego, Oregon, USA).[36]

Dishes were mounted on the microscope under a custom-made, continuously refreshing perfusing system.[32] Cells were exposed to standard Krebs solution (150 mM NaCl, 6 mM KCl, 10 mM HEPES, 1.5 mM CaCl, 1 mM $MgCl_2$, 10 mM glucose, pH adjusted to 7.4 with NaOH). All experiments were performed at 37 °C after a 5-minute acclimatization period. Cells were exposed to TRPV1-agonist capsaicin 10 nM or TRPA1-agonist cinnamaldehyde 10 µM twice and Krebs (control) or histamine 10 µM (1091108, 239968, and H0600000, all products bought from Sigma-Aldrich, Saint Louis, Missouri, USA) was administered in between the exposures for 10 minutes. TRPV1+ or TRPA1+ neurons were identified by applying capsaicin 1 µM or cinnamaldehyde 300 µM respectively at the end of the protocol. Similar experiments were performed in presence of H_1-inhibitor pyrilamine 1 µM (P5514-5G, Sigma-Aldrich, Saint Louis, Missouri, USA).

Statistical methods

GraphPad Prism 9 software was used for data analysis (GraphPad Software, San Diego, California, USA). Fisher's exact test was used to compare proportions. Normality of continuous variables was tested with Shapiro-Wilk test. Differences between two groups were tested using an (un)paired t-test or Mann-Whitney/Wilcoxon matched pairs-signed ranks test depending on normality and whether or not data were paired. Three or more groups were compared using Kruskal-Wallis test with post-hoc Dunn's multiple comparisons test. Correlations were tested with Spearman r test. Data are presented as median and interquartile range (IQR). Values were considered significantly different when $p < 0.05$.

Results

Participants

Eighteen healthy controls and 45 age/sex-matched patients with type 2 chronic upper airway inflammation completed the study (Figure 1B, Table 3). No differences were observed considering smoking status or presence of lower airway disease. The patient group consisted of 23 patients with AR and 22 patients with CRSwNP.

	Controls (N = 18)	Patients (N = 45)	p-value
Median age (years (IQR))	42 (27-53)	41 (30-57)	0.5039†
Male/female	9/9	29/16	0.3938‡
Current smokers (%)	5.6	17.8	0.4258‡
Astma or COPD (%)	0.0	8.9	0.3169‡
NHR			
sNHR (%)	1 (5.6)	19 (42.2)	0.0059‡
oNHR (%)	0 (0)	17 (37.8)	0.0014‡

Table 3: Demographic data. (†Mann-Whitney test, ‡ Fisher's exact test). IQR: interquartile range, oNHR: objective nasal hyperreactivity, sNHR: subjective nasal hyperreactivity.

Clinical evaluation of NHR using objective and subjective parameters

Presence of NHR was evaluated subjectively based on patient-reported reactivity or objectively based on PNIF measurements before and after CDA provocation. We evaluated NHR both as a binary and as a continuous phenomenon (Figure 3A).

In 17/45 patients a decrease in PNIF of ≥ 20 % was observed and was hence higher than the threshold for oNHR. sNHR was found in 5.6 % of the control group and in 42.2 % of patients with type 2 chronic upper airway inflammation. Moreover, in 13/19 patients with sNHR, oNHR was present (Figure 3B). The two-part question to diagnose sNHR had a sensitivity and specificity of 76.5 and 78.6 % respectively. Interestingly, not all patients with sNHR reached the -20 % cutoff for oNHR, though PNIF decreased significantly compared to healthy controls ($p < 0.0001$) or to sNHR-negative patients ($p = 0.0046$) (Figure 3C/4). There was a significant negative correlation between subjective reaction to CDA, *i.e.* VAS effect CDA, and objective reaction (*i.e.* ΔPNIF) ($p < 0.0001$). oNHR-positive patients indicated more severe reaction to CDA ($p = 0.0009$) (Figure 3D). ΔPNIF correlated significantly with the subjective

increase in nasal obstruction (p = 0.0022) or rhinorrhea (p = 0.0292), but not with baseline VAS total nasal symptoms (p = 0.3214) (Figure 3E).

A

NHR	On/off	Continuous
Subjective	sNHR: 2 questions	VAS effect CDA
Objective	oNHR: ΔPNIF ≤ -20 %	ΔPNIF

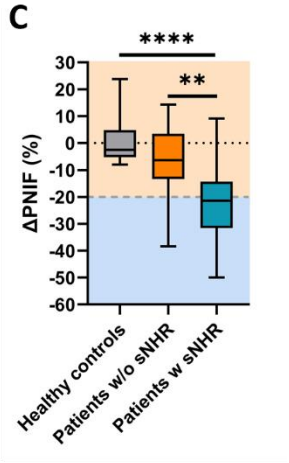
B

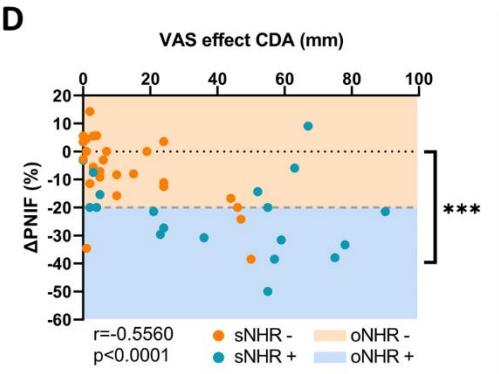
Controls

	No oNHR	oNHR
No sNHR	17	0
sNHR	1	0

Patients

	No oNHR	oNHR
No sNHR	22	4
sNHR	6	13





E

Correlation of ΔPNIF and...	r	p
VAS effect CDA	-0.5560	<0.0001
ΔVAS nasal obstruction	-0.4163	0.0022
ΔVAS rhinorrhea	-0.2843	0.0292
VAS total nasal symptoms	-0.1846	NS

Figure 3: Clinical evaluation of subjective and objective nasal hyperreactivity (NHR). A) Schematic representation of how NHR was evaluated subjectively or objectively and as binary or continuous parameter. **B)** Relation between objective and subjective NHR in controls and patients. **C)** Change in peak nasal inspiratory flow (PNIF) in relation to objective and/or subjective NHR. **D)** Correlation between continuous subjective and objective effect of cold, dry air provocation (CDA) and comparison of Visual Analogue Scale (VAS) scores for subjective effect of CDA in patients with *versus* without objective NHR (oNHR). **E)** Correlations between objective decrease in PNIF and subjective increase in nasal obstruction, subjective increase in rhinorrhea, and subjective baseline disease severity. (C: Kruskal-Wallis test with post-hoc Dunn's multiple comparisons test; D: Spearman r test and Mann-Whitney test; E: Spearman r test; ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$). sNHR: self-reported NHR.

These results suggest that nasal reactivity to environmental stimuli and its underlying mechanisms are part of a continuous spectrum rather than a binary on/off-phenomenon.

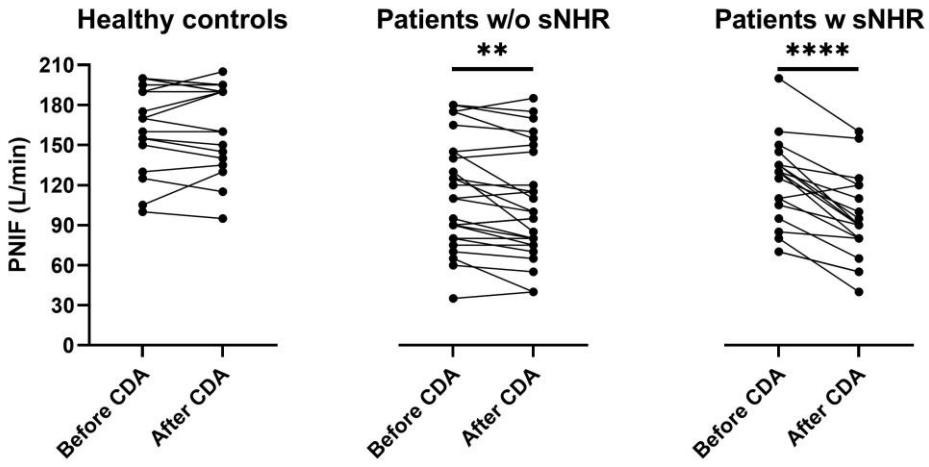
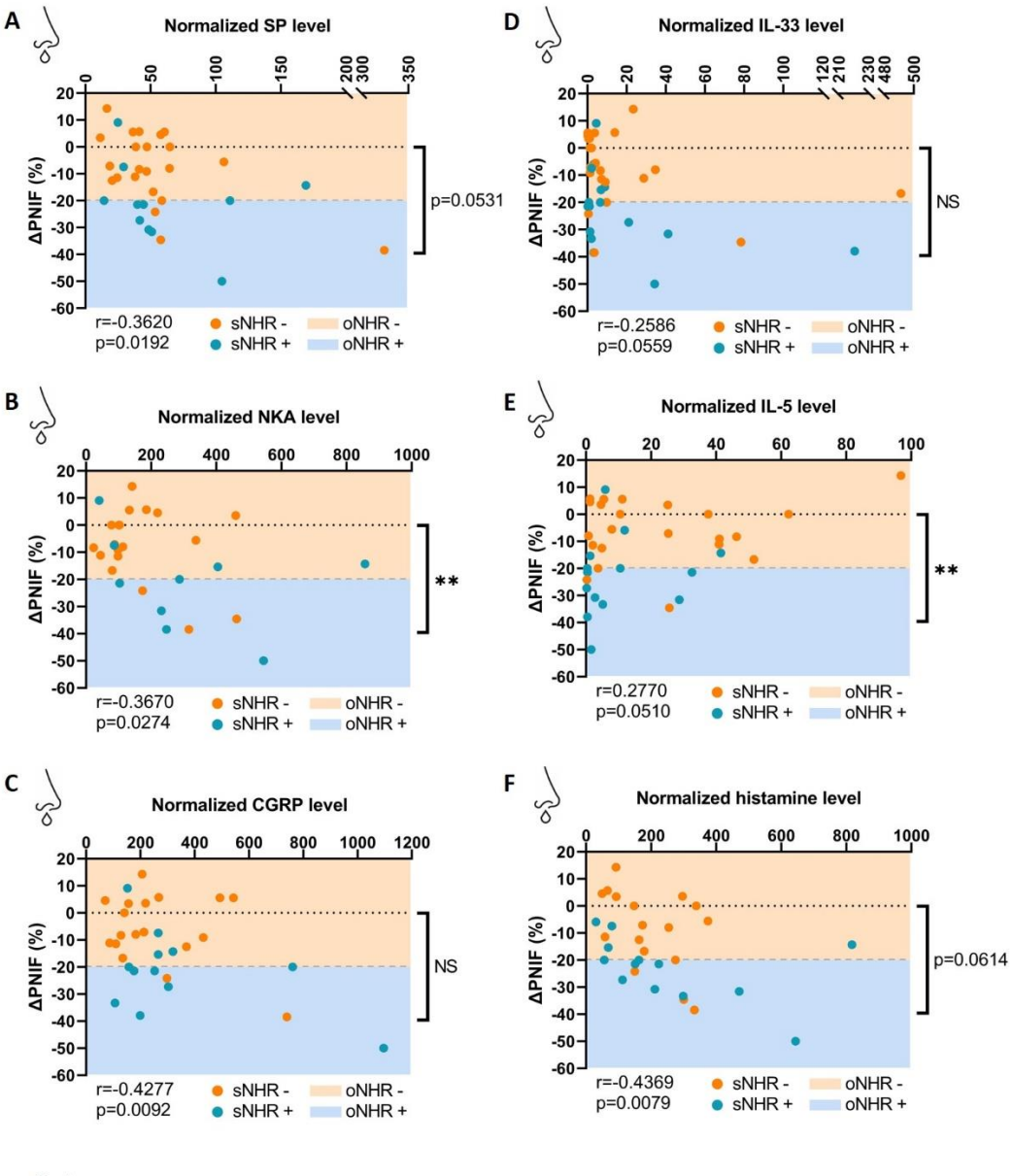


Figure 4: Individual measurements of peak nasal inspiratory flow (PNIF) before and after cold, dry air provocation test (CDA) in healthy controls and patients without or with self-reported nasal hyperreactivity according to the two-part question (sNHR). (Paired t-test, ** $p < 0.01$, **** $p < 0.0001$).

An upregulated neurogenic inflammatory background and increased histamine levels are risk factors for enhanced reactivity to cold, dry air provocation.

Having found that sNHR patients showed stronger decreases in PNIF, we next evaluated which mediators were involved. Firstly, we measured different neuropeptides in nasal secretions. A negative correlation between levels of the neuropeptides SP, NKA, and CGRP in nasal secretions at baseline and objective effects of CDA provocation was observed (Figure 5A-C). In other words, the more neuropeptides measured in nasal secretions, the more severe objective reactivity to CDA provocation. Levels of these neuropeptides correlated strongly with each other (Figure 6). Moreover, a negative correlation considering expression levels of *TRPV1*, *TRPM8*, and *PGP9.5* in nasal biopsies was observed (Figure 5G). Expression levels of *TRPA1* and *TAC1* were below detection limit.



G

Correlation of ΔPNIF and …	*TRPV1*	*TRPM8*	*PGP9.5*	*ZO-1*	*OCLN*	*CLDN1*
r	-0.3390	-0.3263	-0.3188	0.3007	-0.1878	-0.1176
p	0.0187	0.0228	0.0255	0.0353	NS	NS

Figure 5: Endotypic background and reactivity to cold, dry air. A-F) Correlations between baseline protein levels in nasal secretions and objective reactivity to cold, dry air measured by reduction in peak nasal inspiratory flow (PNIF). Subjects situated in the light-blue rectangles are classified as suffering from oNHR (ΔPNIF ≤ -20 %). The color of the dots indicates whether a subject subjectively reported to suffer from sNHR (blue) or not (orange). P-values for the correlations of all patients (regardless of presence of sNHR or oNHR) are found at the bottom-left of each graph, p-values for possible differences between oNHR+ and oNHR- patients are indicated next to the accolade. **G)** Correlation between change in PNIF and baseline expression levels in nasal mucosal biopsies. (A-G: Spearman r test; A-F: Mann-Whitney test; ** $p < 0.01$). sNHR: subjective nasal hyperreactivity, oNHR: objective nasal hyperreactivity, SP: substance P, NKA: neurokinin A, CGRP: calcitonin gene-related peptide, IL: interleukin, TRPV1: transient receptor potential channel vanilloid 1, TRPM8: transient receptor potential channel melastatin 8, PGP9.5: protein gene product 9.5, ZO-1: zonula occludens 1, OCLN: occluding, CLDN1: claudin 1.

Secondly, we studied if CDA was associated with changes in nasal epithelial cell function. No correlation between baseline protein levels of epithelial marker IL-33 in nasal secretions and reactivity to CDA was observed (Figure 5D). Expression of *ZO-1* on nasal biopsies and reactivity to CDA were significantly correlated, which was not observed for other tight junction genes *OCLN* and *CLDN1* (Figure 5G).

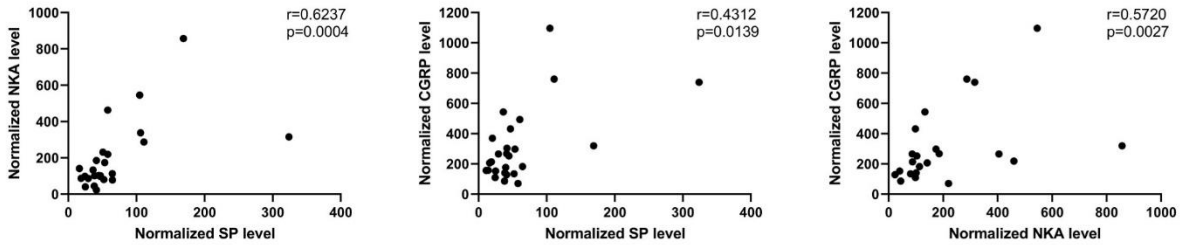


Figure 6: Correlations between the various measured neuropeptides in nasal secretions. (Spearman r test).

Next, we focused on type 2 cytokines and their possible role in NHR. We found a trend towards a positive correlation between IL-5 levels in nasal secretions and ΔPNIF and patients without oNHR had significantly higher levels of IL-5 compared with oNHR-positive patients (median 11.2 and IQR 4.6-41.0 *versus* median 2.8 and IQR 0.4-18.1, p = 0.0081) (Figure 5E). Levels of NKA and IL-5 correlated negatively, but no correlation was observed between levels of SP/CGRP and IL-5 in nasal secretions (Figure 7). Levels of IL-4 and IL-13 were below detection limit. Lastly, a significant correlation was observed between histamine levels in nasal secretions and reactivity to CDA (Figure 5F).

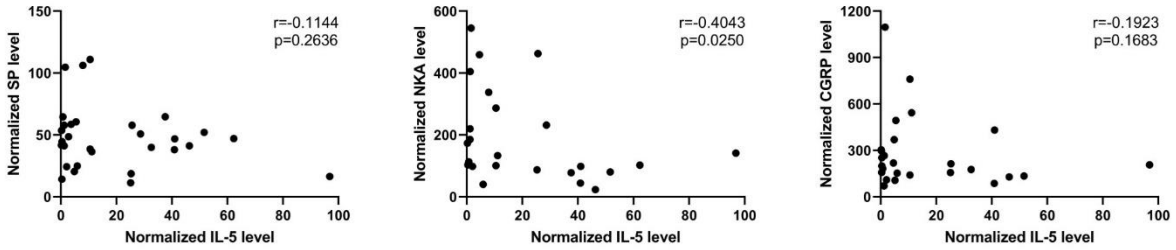


Figure 7: Correlations between level of the neuropeptides substance P (SP), neurokinin A (NKA), and calcitonin gene-related peptide (CGRP) on one side and interleukin 5 (IL-5) on the other side. (Spearman r test).

No differences in baseline protein levels (SP, NKA, CGRP, IL-33, IL-5, or histamine) in nasal secretions or expression levels (*TRPV1*, *TRPM8*, *PGP9.5*, *ZO-1*, *OCLN*, or *CLDN1*) in mucosal biopsies could be observed in patients without *versus* with sNHR.

These results suggest that neurogenic pathways underly reactivity to CDA provocation and that histamine could possibly play a modulating role in it.

Histamine sensitizes murine trigeminal ganglionic neurons to capsaicin and cinnamaldehyde

Given the observation that levels of histamine in nasal secretions correlated with reactivity to CDA, we hypothesized that histamine might lower the activation threshold of trigeminal sensory nerve fibers. For this, trigeminal ganglionic neurons of wild type C57Bl/6J mice were cultured and exposed to the TRPV1-agonist capsaicin 10 nM or the TRPA1-agonist cinnamaldehyde 10 µM twice whilst intracellular Ca^{2+} concentration was monitored. When neurons were exposed to histamine 10 µM in between the two applications, significantly more neurons responded to the second application of capsaicin (15.9 *versus* 27.0 % of TRPV1+ neurons, p = 0.0058) or cinnamaldehyde (8.5 *versus* 16.9 % of TRPA1+ neurons, p = 0.0486) (Figure 8B/D). This increase was not observed in the absence of histamine (19.2 *versus* 11.0 %, p = 0.0226 for capsaicin; 6.2 *versus* 8.7 %, p = 0.5252 for cinnamaldehyde) nor in presence of the H_1-inhibitor pyrilamine (7.5 *versus* 3.8 %, p = 0.0717 for capsaicin; 13.1 *versus* 14.0 %, p = 0.8878 for cinnamaldehyde). These results suggest that histamine can sensitize murine trigeminal ganglionic neurons for capsaicin (TRPV1) and cinnamaldehyde (TRPA1) via an H_1-dependent pathway.

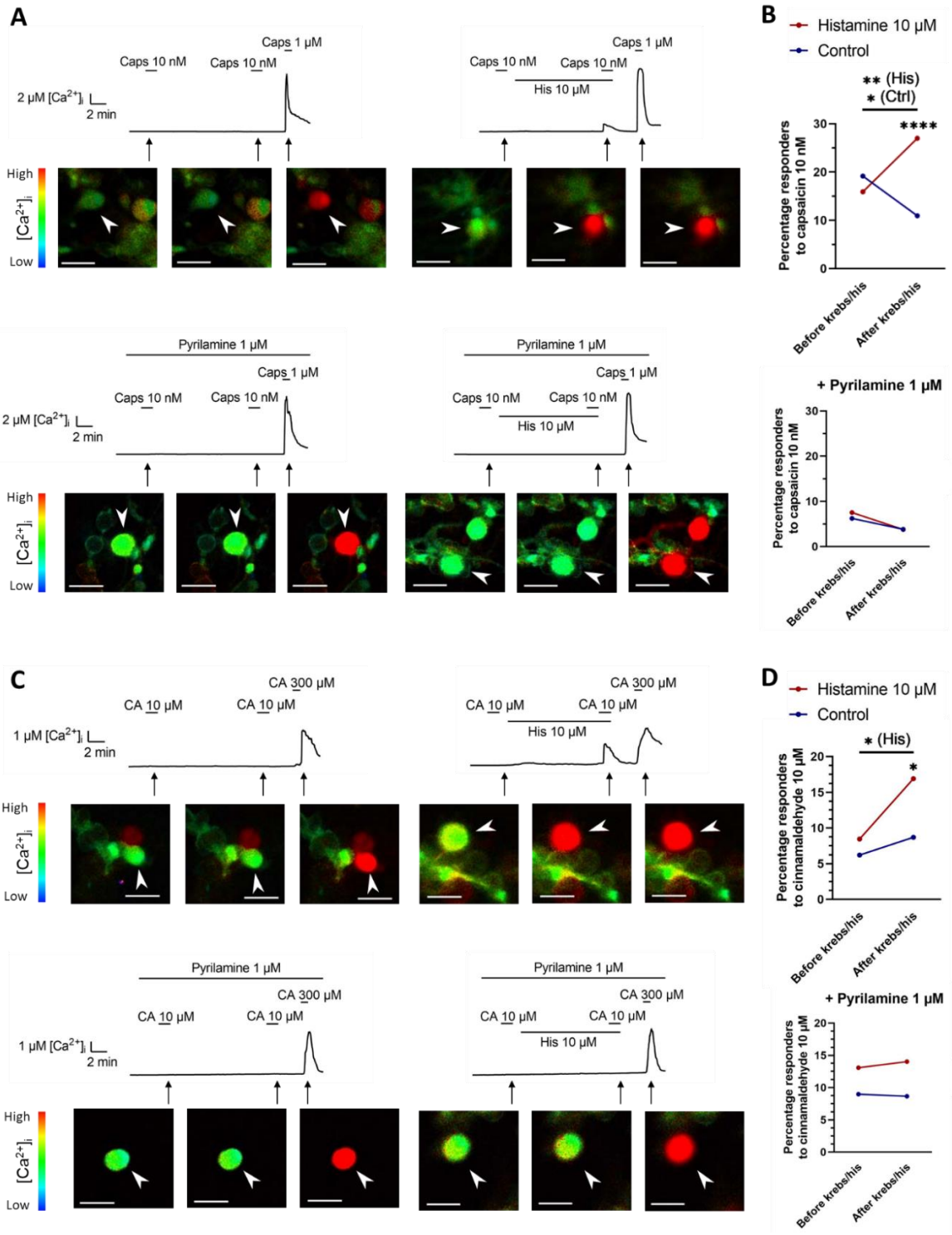


Figure 4: Calcium imaging experiments in murine trigeminal ganglionic neurons. A and C) Representative time courses of intracellular Ca2+ changes in single trigeminal neurons (white arrowheads) (scale bar = 20 µM). **B)** Percentage of neurons responding to application of capsaicin 10 nM (N = 219 neurons (control), N = 226 (histamine 10 µM), N = 209 (control + pyrilamine 1 µM), and N = 292 (histamine 10 µM + pyrilamine 1 µM)). **D)** Percentage of neurons responding to application of cinnamaldehyde 10 µM (N = 161 neurons (control), N = 142 (histamine 10 µM), N = 312 (control + pyrilamine 1 µM), and N = 214 (histamine 10 µM + pyrilamine 1 µM)). (B and D: Fisher's exact test, * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$). Caps: capsaicin, His: histamine, CA: cinnamaldehyde.

Cold, dry air induces an increase in IL-33 in nasal secretions, but does not influence levels of neuropeptides or type 2 inflammatory markers

It is currently proposed that neuropeptides induce nasal symptoms in NHR, yet this has never been studied directly.[1] Therefore, we compared protein levels in nasal secretions of patients before and after CDA provocation. No difference was observed when comparing levels of SP, NKA, CGRP, IL-5, or histamine in nasal secretions before *versus* after CDA provocation (Figure 9A-C, E-F). CDA provocation induced a significant increase in IL-33 levels in nasal secretions (median 3.63 with IQR 1.35-13.95 *versus* median 8.63 with IQR 2.71-47.64, Wilcoxon matched-pairs signed rank test, p = 0.0005) (Figure 9D).

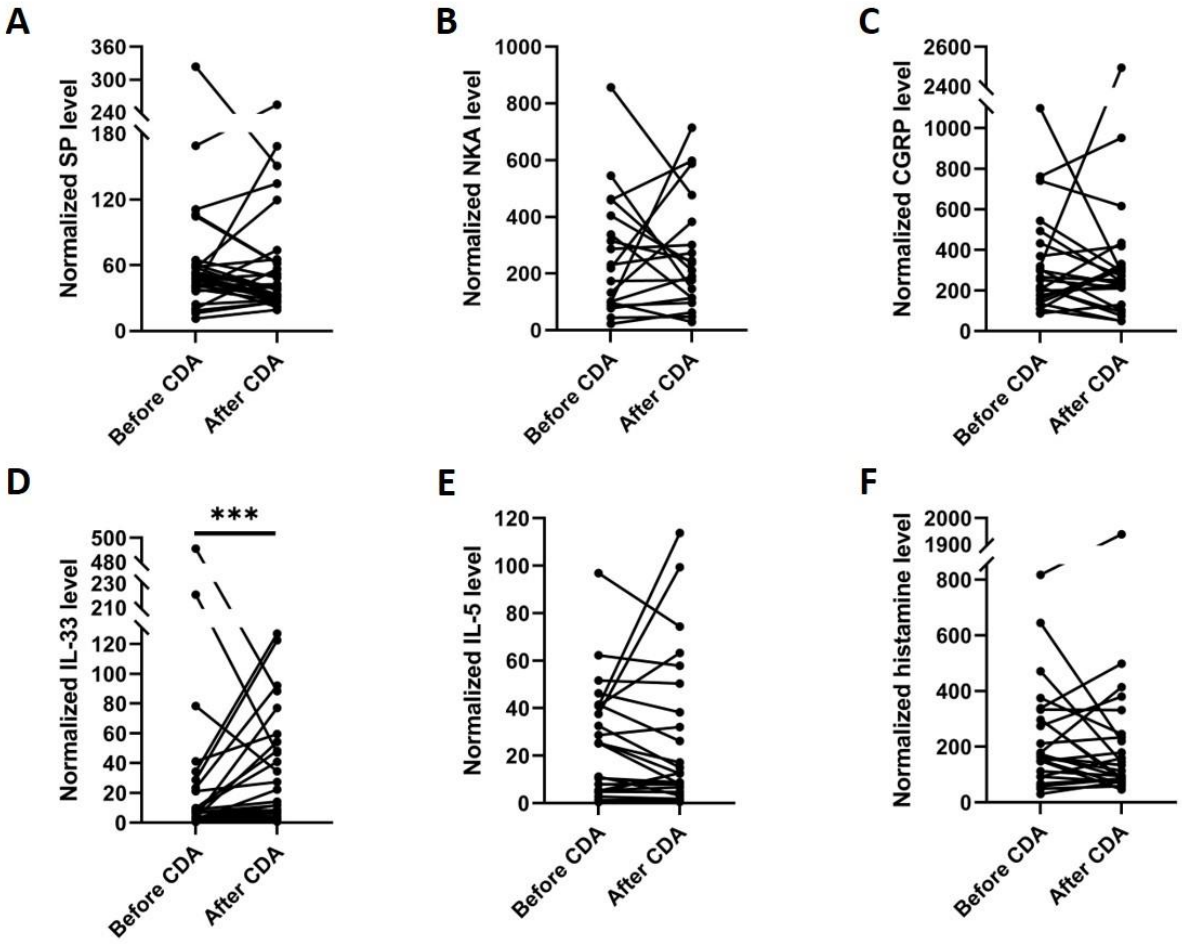


Figure 9: Effect of cold, dry air provocation (CDA) on mediator levels. CDA provocation does not affect levels of neuropeptides, interleukin (IL) 5, or histamine (A-C, E-F), yet induces a significant increase of IL-33 levels (D). (Wilcoxon matched-pairs signed rank test, *** $p < 0.001$). SP: substance P, NKA: neurokinin A, CGRP: calcitonin gene-related peptide, IL: interleukin.

No differences could be observed in expression levels of *TRPV1*, *TRPM8*, *PGP 9.5*, *ZO-1*, *OCLN*, or *CLDN1* in nasal biopsies when comparing visit 1 (baseline) with visit 2 (after CDA provocation) (Wilcoxon matched-pairs signed rank test, data not shown).

Discussion

The mechanisms underlying NHR have been almost exclusively studied in patients with NAR.[1] In the last years, the presence of NHR was shown not to be limited to NAR, but was also observed in other phenotypes of chronic upper airway inflammation, such as AR and CRS.[2,4,23] With this study, we investigated the pathophysiology of NHR in classical type 2-predominated chronic upper airway inflammatory phenotypes, AR and CRSwNP, for the first time.

We report a prevalence of sNHR of 5.6 % in healthy controls and 42.2 % in patients AR or CRSwNP (Figure 3B). This is in line with previous reports using a similar definition of NHR.[2] sNHR is clearly a common and prevalent feature in chronic upper airway inflammation.

NHR is often seen as an on/off phenomenon, which can be assessed in a subjective way by means of the two-part question or in an objective way by a CDA provocation test.[2,6] From a mechanistic point of view, this binary approach is unlikely to sufficiently reflect intercellular interactions and continuous biological responses. Moreover, the reported prevalence of NHR differed over various studies.[2–5,23] Many of these are questionnaire-based studies, providing room for variation due to use of different definitions. Nevertheless, it is possible that NHR is not clearly an on/off phenomenon, making it difficult for patients to subjectively place themselves above or below the threshold for reporting NHR. Even though not all sNHR-positive patients reached the threshold of a decrease in PNIF of ≥ 20 % in the current study, their reaction to CDA provocation is stronger compared to patients without sNHR (Figure 3B-C). This illustrates the continuous aspect of NHR and its underlying mechanisms.

Segboer *et al.* reported a decrease in PNIF after CDA provocation in patients with AR/NAR, while also describing a high prevalence of sNHR in these patient groups.[4] Our study now confirms a correlation between sNHR and oNHR in our patients overall (Figure 3C). Notably, oNHR was observed in only 53.8 % of patients with AR and sNHR, compared with 100 % of patients with CRSwNP and sNHR (Figure 10). This subjective overestimation in the AR-group could be due to the intermittent nature of disease where NHR might be confused with acute allergic reactions.

AR patients

	No oNHR	oNHR
No sNHR	8	2
sNHR	6	7

CRSwNP patients

	No oNHR	oNHR
No sNHR	14	2
sNHR	0	6

Figure 10: Clinical evaluation of subjective (sNHR) and objective nasal hyperreactivity (oNHR) in patients with allergic rhinitis (AR) or chronic rhinosinusitis with nasal polyps (CRSwNP).

At the same time, we observed a correlation between subjective and objective continuous measurements of reactivity to CDA (Figure 3D-E). Indeed, changes in PNIF correlated with the subjective effect of CDA and subjective changes in nasal obstruction, illustrating the validity of the CDA provocation test. Notably, PNIF only reflects nasal obstruction, while we here confirm a previous observation that environmental triggers can induce subjectively increased rhinorrhea or postnasal drip.[2] Increased nasal secretions may contribute to nasal obstruction, but objective measurements of nasal secretions might further improve the diagnostic CDA provocation test. Lastly, we did not observe a correlation between subjective baseline disease severity and reactivity to CDA provocation. Hence, NHR seems to affect patients with various levels of disease control, as was described previously.[2]

In patients with idiopathic rhinitis, NHR is suspected to result from neurogenic inflammation.[1] AR and CRSwNP on the other hand are classically featured by type 2 inflammation and barrier defects.[26,30,37] Our data show a negative correlation between reactivity to CDA and levels of neuropeptides SP, NKA, and CGRP, expression levels of the nociceptors *TRPV1* and *TRPA1*, and expression level of neurogenic marker *PGP9.5* (Figure 5A-C/G). This means that the higher the levels of neurogenic markers are, the stronger the reaction to CDA provocation. With this current study, we show that a background of upregulated neuronal pathways correlates with nasal reactivity to environmental triggers in this group of patients with AR or CRSwNP.

Allergens and antigens can more easily penetrate to the submucosa in AR and CRSwNP due to protease- or immune-induced barrier defects.[38,39] Therefore, it is plausible that epithelial barrier defects might facilitate the exposure of nerve endings to external stimuli, hence contributing to NHR. However, we could not observe a clear relationship between expression levels of tight junction proteins and reactivity to CDA (Figure 5G). Just as an intact

barrier was reported in patients with idiopathic rhinitis, NHR seems to be unrelated to barrier (dys)function in AR and CRSwNP.[40]

Consequently, NHR is presumed to be mainly neurogenically mediated.[1] The effects of type 2 inflammation on reactivity to environmental triggers are currently unknown. Therefore, we also measured the type 2 signature cytokines IL-4, IL-5, and IL-13 in nasal secretions. Patients with oNHR exhibited significantly lower IL-5 levels compared with those without (Figure 5E). Since no differences in subjective disease severity could be observed, we hypothesized that type 2 and neurogenic inflammation are inversely correlated. Only NKA levels negatively correlated with IL-5 levels, and not SP or CGRP (Figure 7). Given the lack of an unambiguous negative correlation between neuropeptides and IL-5, the effects of IL-5 – or by extension type 2 inflammation – on reactivity to CDA remain unclear at this point.

Histamine, another type 2 inflammatory mediator, was already shown to sensitize dorsal root ganglionic neurons for capsaicin and cinnamaldehyde in the context of irritable bowel syndrome and detrusor overactivity.[31–33,41] In this current study we observed a positive correlation between histamine levels in nasal secretions and objective reactivity to CDA provocation (Figure 5F). Moreover, we showed that histamine sensitized murine trigeminal ganglionic neurons for capsaicin and cinnamaldehyde (Figure 8). Thus, considering NHR in type 2 inflammatory phenotypes, one cannot speak only of upregulation of nociceptor expression, but also of sensitization. This can potentially occur by direct sensitization and/or by recruitment of TRP channels to the plasma membrane.[42,43]

Several mediators, such as CGRP or histamine, can exert vasodilatory effects, which consequently lead to nasal obstruction.[9,42,44] Their levels in nasal secretions, however, remained constant over CDA provocation in the current study. In our study, CDA provocation only induced an increase in IL-33 levels in nasal secretions, reflecting potential epithelial activation (Figure 9). IL-33 has been described to induce angiogenesis and increase vascular permeability, which could contribute to nasal congestion.[45] Moreover, as an alarmin, IL-33 is known to interact with many immune cells, such as mast cells or type 2 innate lymphoid cells, which can consequently release their mediators ultimately leading to nasal symptoms.[46,47]

Even though all participants were well-characterized by otorhinolaryngologists, a limitation of this study lies in the small sample size, which restricts the possibility of subgroup

analyses on patients with AR or CRSwNP due to a lack of power. Sampling of nasal secretions using nasal sponges is a widely used technique, but has the limitation that proteins bound to receptors on the mucosal surface are probably not harvested in this way.[48] Moreover, one should keep in mind that many interactions take place at the submucosal level and not in the nasal cavity itself.

In conclusion, we here provide evidence that NHR manifests as a continuum across patients and that it is orchestrated by neurogenic inflammation and modulated by histamine. However, it remains unclear which specific mediators cause nasal symptoms such as nasal obstruction or increased secretions in response to environmental triggers. Future studies should therefore focus on the pathways coupling environmental exposure with induced nasal symptoms and treatment strategies targeting the underlying neurogenic inflammation.

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CHAPTER 6

General discussion and future perspectives

1. Summary of the results

Chronic upper airway inflammatory disorders are various diseases characterized by inflammation of the mucosal lining of the nose (in case of *rhinitis*) and/or the paranasal sinuses (in case of *rhinosinusitis*) with symptoms lasting for ≥ 12 weeks.[1] Several underlying mechanisms, or endotypes, can lead to mucosal inflammation resulting in sinonasal symptoms.[2] A better understanding of these intra- and intercellular mechanisms is of primordial importance since pharmacological interventions can target specific cells or pathways. Unfortunately, there is no 1-to-1 relationship between the chronic upper airway inflammatory phenotype reflected by clinical findings and the underlying endotype.

AR and CRSwNP are historically considered as type 2 inflammatory disorders since they are characterized by upregulation of the type 2-signature cytokines IL-4, IL-5, and IL-13.[1,3] More recently, defective epithelial barriers have been described in both diseases and epithelial cytokines can contribute to and modulate the inflammatory endotype, making the picture even more complicated.[4–6]

The last years, patients with AR or CRSwNP are reported to suffer from NHR.[7–9] NHR is primarily studied in patients with NAR, where it seems to be mainly neurogenically mediated.[10] Upregulation of the nociceptive TRP channels is thought to increase neuronal sensitivity to environmental triggers, such as temperature or humidity changes.[10–12]

Several studies reported a high prevalence of NHR in various types of chronic upper airway inflammation.[7,8,10,12,13] Unfortunately**, lack of a consensus-definition** complicates inter-study comparison. Therefore, a first aim of this doctoral project was to investigate the prevalence of NHR in various phenotypes of chronic upper airway inflammation simultaneously.

Additionally, intranasal capsaicin challenge induces more symptoms in patients with seasonal AR during the pollen season and histamine was shown to sensitize sensory neurons for the TRPV1-agonist capsaicin and the TRPA1-agonist cinnamaldehyde in murine dorsal root ganglionic neurons.[14–17] Conversely, mast cells express more SP-receptors NK1R in the context of atopic dermatitis and neuropeptides stimulate the Th2 response.[18] This suggests an **interaction between the classical type 2 inflammatory endotype and neurogenic inflammation**, which manifests in about 20 % of patients with AR or CRS who reportedly

remain uncontrolled with the current treatment modalities that exclusively target type 2 inflammation.[19] Therefore, a second aim of this project was to investigate the – potentially neurogenic – mechanisms underlying NHR in the classical type 2 inflammation-predominated phenotypes, AR and CRSwNP.

1.1. Prevalence and diagnosis of nasal hyperreactivity in chronic upper airway inflammation

We first studied the prevalence of clearly-defined sNHR in a large cohort of patients with well-characterized, otorhinolaryngologist-diagnosed phenotypes of chronic upper airway inflammation. We found that **NHR is present in all phenotypes** (AR, NAR, CRSwNP, CRSsNP, and mixed phenotypes), without differences between the various groups. This observation supports the idea of NHR being a general feature of diseased mucosa rather than being disease specific. In our study, prevalence of sNHR varied between 40.5 and 52.1 % depending on the phenotype.

After obtaining these results, we wondered how strong the relation is between subjective and objective NHR. In our second study, similar results were found, where sNHR was later objectified by a CDA provocation test. However, these numbers are slightly lower compared with previous studies.[7,8,10,13] This is probably due to use of different definitions of sNHR and/or variations in patient characterization. Indeed, after asking for environmental trigger-induced nasal symptoms, we added a second question on duration of nasal symptoms to **differentiate true NHR from nasal symptoms due to physiological, protective reflexes**. For future studies, we recommend to use our standardized definition of sNHR based on the two-part question, which would allow better inter-study comparison. Nevertheless, with nearly half of the patients suffering from NHR, it remains an important feature in all phenotypes of chronic upper airway inflammation.

In patients with NAR, whom are characterized by solely neurogenic inflammation, presence of NHR was related with subjective disease severity. This phenomenon could not be observed in patients with other phenotypes, nor was a higher prevalence of sNHR in patients with a combination of multiple phenotypes (e.g. CRS + allergy) found. We attributed this

discrepancy to a **more complex underlying mechanism** in these groups, *i.e.*, additional type 1 or 2 inflammation and/or barrier defects, rather than solely neurogenic inflammation.[2]

In our study, **presence of sNHR was not influenced by the use or subjective effects of medication**, even in NAR. Patients were excluded in case of endonasal capsaicin therapy in the last 3 months. Capsaicin is currently the only therapy specifically targeting neurogenic pathways and is currently only used in patients with NAR.[12,20] Consequently, our results illustrate the presence of neurogenically-mediated symptoms in other phenotypes of chronic upper airway inflammation and **highlight the lack of treatment options targeting these mechanisms** in these patients.

A reason for lack of proper medication targeting neurogenically mediated symptoms is the **lack of an accurate diagnosis** of NHR. NHR can be diagnosed subjectively by simply asking the patient for increased and long-lasting symptoms upon encounter with environmental triggers. Unfortunately, this diagnosis remains limited by its inherent subjective nature. With the use of a CDA provocation test, NHR can be diagnosed objectively.[9] The CDA provocation test is the most accurate method currently available, but remains time-consuming and PNIF-measurements are highly dependent on patient participation and motivation. Nevertheless, in our questionnaire-study, temperature/humidity changes and air-conditioning were the most frequently reported triggers of nasal symptoms by patients with NHR as described earlier, **supporting the use of a CDA provocation test for objective diagnosis**.[7] However, where PNIF only reflects nasal patency, patients also indicated increased nasal secretions to be triggered by environmental stimuli. On top, we could see a significant correlation between objective reactivity to CDA and subjective reports of CDA-induced nasal secretions in our second study. It would therefore be useful to implement measurements of nasal secretions as parameter for objective diagnosis of CDA.

Furthermore, binary diagnosis of NHR as “present” or “absent” – as it is currently categorized – is an arbitrary division of the probably more **continuous spectrum** that nasal reactivity spans. Therefore, we included 4 parameters on NHR that covered subjective/objective and binary/continuous measurements. Overall, we noticed a good correlation between subjective and objective NHR in our patients with AR or CRSwNP, with a good sensitivity (76.5 %) and specificity (78.6 %) for the two-part question in diagnosing NHR. In our patients, the CDA-induced reduction in PNIF correlated well with the subjective,

patient-reported effect of CDA provocation. Moreover, we observed a significant CDA-induced decrease in PNIF in patients with AR or CRSwNP, especially in those with sNHR. Lastly, patients with sNHR and/or oNHR reported a stronger subjective reaction to CDA provocation. These results further validate the two-part question for diagnosis of sNHR and a CDA provocation test for diagnosis of oNHR. Simultaneously, it supports the hypothesis of NHR being part of a continuous spectrum rather than being an on/off phenomenon.

1.2. Chronic upper airway inflammation impairs mental well-being

It is long known that chronic upper airway inflammation is related with increased levels of depression, stress, and anxiety, and impairs patients' quality of life.[21–26] Moreover, subjective disease burden is increased in patients with impaired mental well-being in presence of similar objective disease severity as seen on nasal endoscopy or CT scan.[27,28] We therefore studied whether NHR influences general well-being.

In line with previous reports, we found increased scores on PSS, GAD-7, and PHQ-9 in patients with any type of chronic upper airway inflammation, **reflecting higher stress, anxiety, and depression levels**.[21,25,28,29] More specifically, 11-24 % of the patients screened positive for major depressive disorder using a questionnaire-based cut-off. These data confirm previous reports that chronic upper airway inflammation not only comes with a societal burden, but also has a major individual impact for the patients.[30–32] Notably, we found no relationship between presence of NHR and increased depression-, stress-, or anxiety levels. We hence believe that NHR is not a psychosomatic symptom. Conversely, it might presumably not affect mental well-being. Admittedly, we did not distribute validated questionnaires on disease related quality of life (such as the SinoNasal Outcome Test 22), nor was a prospective cohort-study performed, limiting an in-depth analysis of a possible impact of NHR on quality of life.

1.3. Neurogenic pathways underly nasal hyperreactivity in classical type 2 chronic upper airway inflammatory disorders

NHR is often thought of as a result of neurogenic inflammation and is mostly studied in patients with idiopathic rhinitis.[10] In our last study, we investigated which mechanisms

contribute to NHR in classical type 2-predominated chronic upper airway inflammatory disorders, *i.e.*, AR and CRSwNP. To this end, nasal secretions and mucosal biopsies were harvested before and after CDA provocation.

As markers for neurogenic inflammation, we measured levels of SP, NKA, and CGRP in nasal secretions and expression levels of *TRPV1*, *TRPM8*, and *PGP9.5* on nasal mucosal biopsies. Increased levels of SP, *TRPV1*, and *PGP9.5* along with increased sensitivity to the TRPV1- and TRPA1-agonist allyl isothiocyanate have been documented in patients with idiopathic rhinitis.[11,12] In our study, we found a negative correlation between baseline levels of these neurogenic markers and objective reactivity to CDA as measured by ΔPNIF, building further on previous observations. In other words, **the higher the expression of neurogenic markers, the stronger the reactivity to CDA provocation**. A background of upregulated neurogenic pathways is a risk factor for patients with AR and CRSwNP to develop NHR. We could, however, not always observe a significant difference for neurogenic markers when comparing patients with and without oNHR. This could be due to the limited sample size of patients included. Moreover, NKA and CGRP were not measurable in nasal secretions in a previous study.[12] We therefore diluted the nasal secretions of our patients 1:3 instead of 1:5 as is usually done, resulting in measurable values for the majority of the patients. Nevertheless, NKA and CGRP levels were below detection limit in some patients, even further restricting the sample size.

To our knowledge, we are the first to have analyzed typical type 2 inflammatory mediators in the context of NHR. Several studies have described (lower) airway hyperreactivity in presence of type 2 inflammation. It was often suggested that increased levels of type 2 inflammatory mediators are associated with increased airway hyperreactivity, though without a direct, causal relationship.[33–36] Surprisingly, we saw **an trend to an inverse correlation between baseline IL-5 levels in nasal secretions and reactivity to CDA provocation**. At the same time, reactivity to CDA was not correlated with baseline disease severity. We hypothesized that the underlying inflammatory endotype balances between neurogenic and type 2 inflammation. A relative shift towards neurogenic inflammation in patients with more pronounced nasal reactivity to environmental stimuli and, inversely, more pronounced type 2 inflammation in patients without NHR might be a plausible explanation. If true, one would expect that levels of neuropeptides and IL-5 in nasal secretions would be negatively correlated

in patients. We indeed observed an inverse correlation between IL-5 and NKA, but not for SP or CGRP. Therefore, it remains unclear whether such a relationship holds true, even with a higher sample size. In summary, we currently lack clear evidence why patients with relatively lower IL-5 levels seem to react more to CDA provocation.

Histamine, another main mediator in allergic rhinitis and chronic rhinosinusitis with nasal polyps, was previously shown to sensitize dorsal root ganglionic neurons for the TRPV1-agonist capsaicin and the TRPA1-agonist cinnamaldehyde in the context of irritable bowel syndrome or detrusor overactivity.[15–17] Similarly, we here report a **positive correlation between histamine levels in nasal secretions and objective reactivity to CDA provocation**. Consequently, we investigated the potential of histamine to sensitize murine trigeminal ganglionic neurons to capsaicin or cinnamaldehyde by means of calcium imaging. We found a higher number of neurons responding to low doses of capsaicin or cinnamaldehyde in case of pre-stimulus exposure to histamine. Therefore, aside from the described upregulation of nociceptive TRP channels, their sensitivity might also be increased in NHR.

Recent pathophysiological models on NHR suggested a release of neuropeptides after strong activation of sensory afferent neurons.[10] These neuropeptides on their turn would induce mucus production and vasodilation, resulting in nasal symptoms like nasal obstruction and rhinorrhea.[37–40] However, previous studies focused on the inflammatory background rather than on the short-term effects of CDA-exposure.[12] We thus investigated if several mediators in nasal secretions are affected by CDA provocation. Remarkably, we found **no differences in levels of neuropeptides, nor in levels of IL-5 or histamine**. Conversely, we observed a **CDA-induced increase in levels of IL-33**, reflecting epithelial activation. Likewise, a previous study reported increased plasma levels of Clara cell protein 16 (CC16) in athletes who underwent a eucapnic voluntary hyperpnea test for bronchial hyperreactivity. CC16 is released in case of acute lung injury and impaired bronchial epithelial integrity.[41] We also studied expression of tight junctions genes, but observed no major defects. In conclusion, CDA-provocation induces epithelial activation, yet the exact pathway leading to nasal symptoms remains incompletely understood.

1.4. Use of patient-reported symptoms scores to guide phenotyping in chronic upper airway inflammation

In our questionnaire study, we included a large cohort of patients with various phenotypes of chronic upper airway inflammation. The study was designed to evaluate the prevalence of NHR in different phenotypic groups and to identify potential correlated factors. As a serendipity, we had the interesting observation that the symptom-specific VAS-score profiles varied over AR, NAR, and CRS.

Multiple questionnaires predicting the risk for a particular diagnosis or discriminating patients with a specific diagnosis from control subjects have been developed in the past.[42–44] In the context of upper airway inflammation, there is literature on several questionnaires trying to predict presence of AR.[45–47] Questionnaires differentiating between specific phenotypes are however scarce.

Being forced to have tele-consultations by measurements against the COVID-19 pandemic, we realized how dependent we are on clinical and technical investigations to phenotype chronic upper airway inflammatory disorders. Therefore, we thought to use our data to maximize the elements available from taking patients' history and develop a **guiding diagnostic algorithm; a completely new concept in diagnostics**. The developed algorithm predicted the correct diagnosis in nearly 70 % of the patients.

Of course, this algorithm is not validated in daily clinical setting and the accuracy is not 100 %. Nevertheless, we wanted to illustrate this new concept to provide inspiration for future studies. We believe that even better algorithms – maybe even covering structural pathologies – could be developed by studies specifically designed for this purpose, also including questions on, for example, seasonality of the symptoms, history of atopy, unilateral/bilateral presence of symptoms, or history of nasal surgery or trauma.

2. General conclusion

This PhD project focused on the prevalence and mechanisms of NHR beyond the specific field of NAR.

Firstly, we found a high prevalence of NHR in all phenotypes of chronic upper airway inflammation, albeit AR, NAR, CRSsNP, CRSwNP, or mixed phenotypes. We found a generally good correlation between subjective (patient-reported) and objective (PNIF-reduction by CDA provocation) parameters on NHR. Presence of NHR was independent of use/effect of medication, indicating that current treatment modalities do not target the mechanisms underlying NHR. In case of NHR, symptoms were mostly triggered by temperature/humidity changes or air-conditioning, which can be considered a combination of both.

Secondly, we found an increased risk for NHR in AR and CRSwNP, which were historically considered to be mainly mediated by type 2 inflammation (Figure 1). Here, reactivity to CDA provocation correlated with a more pronounced neurogenic background. The interplay between neurogenic and type 2 inflammation is illustrated by the potential of histamine to sensitize trigeminal sensory neurons for capsaicin and cinnamaldehyde. Lastly, CDA provocation had no effects on levels of neuropeptides or type 2 inflammatory mediators. The exact pathway between exposure to CDA and increased nasal obstruction remains incompletely understood.

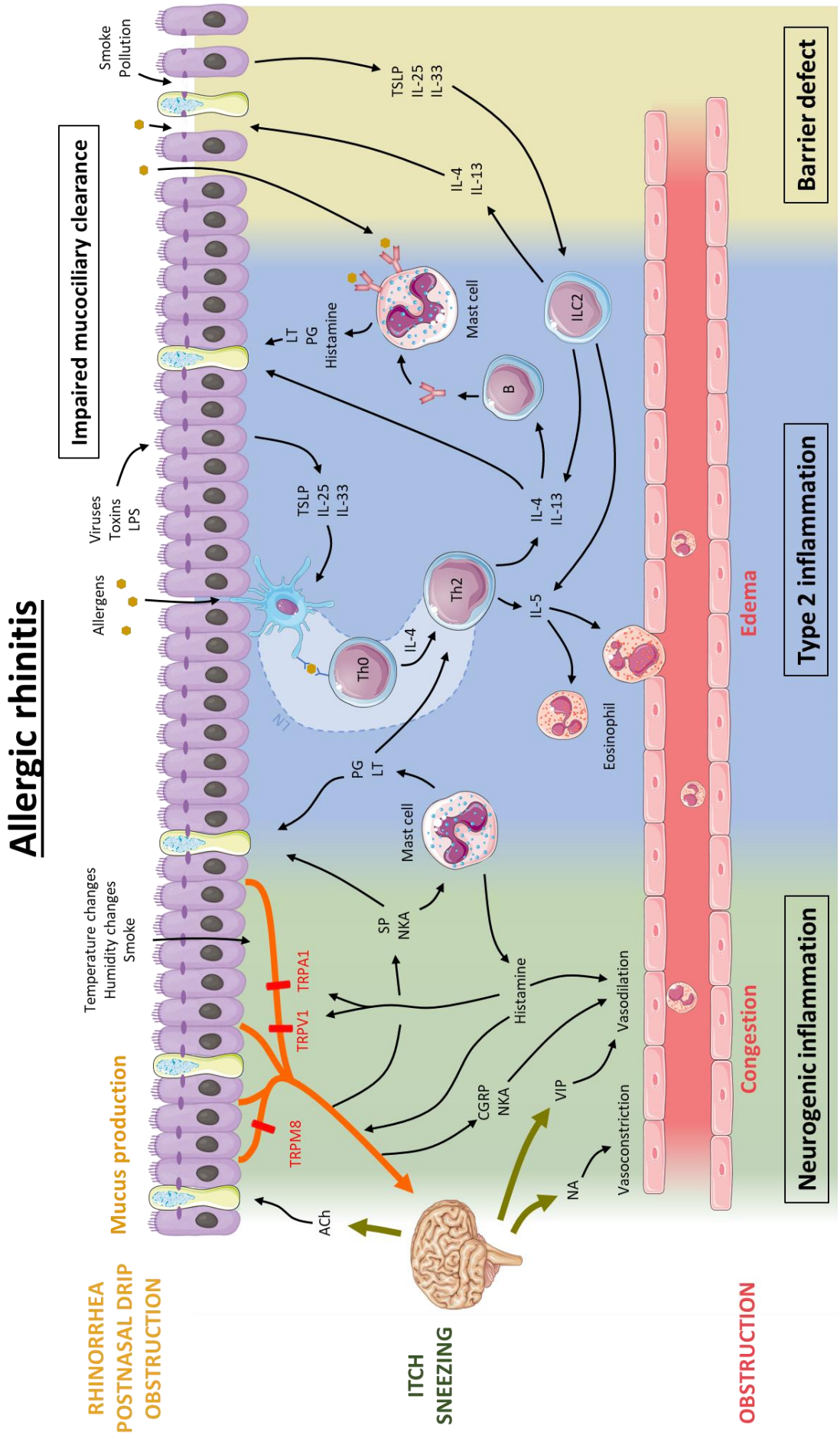
What is now the importance of NHR in chronic upper airway inflammation? We show that NHR is a widespread feature of chronic upper airway inflammation. Admittedly, presence of sNHR did not affect scores on questionnaires assessing presence of depressive symptoms, anxiety, or stress. However, quality of life comprises more than the mere absence of these specific psychologic features. Therefore, considering NHR as a completely irrelevant feature based on these data would be short-sighted.

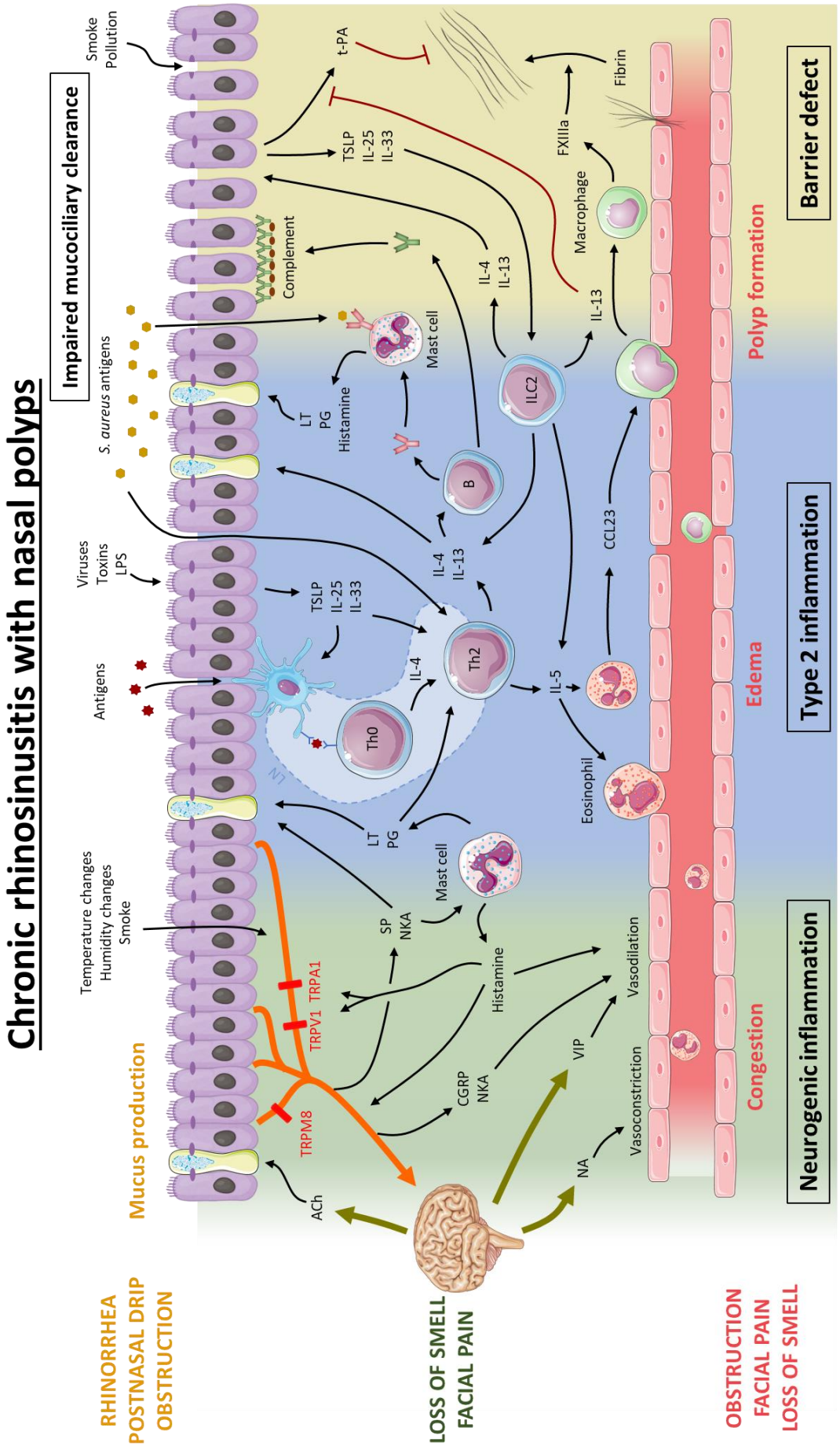
Chronic upper airway inflammation is often classified as being type-2 mediated or non-type 2-mediated. These endotypes are mainly treated by corticosteroids, antihistamines, and nasal lavages. The data in this thesis further support the possibility of a neurogenic inflammatory endotype which should also be considered in patients with chronic upper airway symptoms. Indeed, NHR remains untreated in many cases, due to lack of therapies available targeting neurogenic components and due to lack of studies on the one treatment available

(*i.e.* capsaicin) in patients with a non-NAR-phenotype. Thus, NHR could complicate treatment of patients, regardless of their phenotype, making it harder to achieve "full-time" symptom control.

In daily practice one often tries to make a clear-cut distinction between different endotypic groups, but most probably it is more correct to think about it as a balance with different weights attributed to each endotype in different patients. Additionally, patients with a non-NAR-phenotype could benefit from treatment with capsaicin; though this remains to be investigated. In the end, treatment of chronic upper airway inflammation should be adapted to the underlying endotype. But it is clear that determination of this endotype can of course be challenging when relying on clinical tests.

Figure 1 (see following two pages): Renewed model of the pathophysiology underlying allergic rhinitis and chronic rhinosinusitis with nasal polyps. Adapted from [6]. Both diseases are not only characterized by type 2 inflammation and epithelial barrier defects, but also neurogenic inflammation may be present, contributing to nasal hyperreactivity. Ach: acetylcholine, NA: noradrenaline, VIP: vasoactive intestinal peptide, SP: substance P, NKA: neurokinin A, CGRP: calcitonin gene-related peptide, ILC2: type 2 innate lymphoid cell, PG: prostaglandins, LT: leukotrienes, LN: lymph node, IL: interleukin, LPS: lipopolysaccharide, TSLP: thymic stromal lymphopoietin, CCL23: chemokine (C-C motif) ligand 23, FXIIIa: activated coagulation factor 13, t-PA: tissue plasminogen activator.


Allergic rhinitis
Impaired mucociliary clearance
Barrier defect
Type 2 inflammation
Neurogenic inflammation
Edema
Congestion
Smoke
Pollution
TSLP
IL-25
IL-33
IL-4
IL-13
Viruses
Toxins
LPS
Allergens
LT
PG
Histamine
Mast cell
B
ILC2
Th0
Th2
IL-5
Eosinophil
Temperature changes
Humidity changes
Smoke
SP
NKA
TRPM8
TRPV1
TRPA1
ACh
CGRP
NKA
VIP
NA
Vasodilation
Vasoconstriction
Mucus production
RHINORRHEA
POSTNASAL DRIP
OBSTRUCTION
ITCH
SNEEZING
OBSTRUCTION


Chronic rhinosinusitis with nasal polyps
Impaired mucociliary clearance
Smoke
Pollution
t-PA
Fibrin
FXIIIa
Macrophage
TSLP
IL-25
IL-33
Complement
IL-4
IL-13
Mast cell
LT
PG
Histamine
S. aureus antigens
Viruses
Toxins
LPS
B
ILC2
IL-13
CCL23
Antigens
TSLP
IL-25
IL-33
IL-4
IL-13
Th0
Th2
IL-4
IL-5
N1
Eosinophil
Temperature changes
Humidity changes
Smoke
LT
PG
Mast cell
SP
NKA
Histamine
TRPM8
TRPV1
TRPA1
CGRP
NKA
VIP
Vasodilation
Vasoconstriction
NA
ACh
Mucus production
Edema
Polyp formation
Barrier defect
Type 2 inflammation
Congestion
Neurogenic inflammation
RHINORRHEA
POSTNASAL DRIP
OBSTRUCTION
LOSS OF SMELL
FACIAL PAIN
OBSTRUCTION
FACIAL PAIN
LOSS OF SMELL

3. Future perspectives

With this PhD project, we broadened research on NHR in general and more specifically its mechanisms in chronic upper airway inflammatory phenotypes other than NAR. Although we successfully illustrate the possible presence of neurogenic inflammation in AR and CRSwNP, several questions remain to be resolved. Two major unmet needs are present: (1) is NHR a matter of specific receptors or rather of a general hypersensitive state of neurons achieved by multiple small contributing factors, and (2) which exact molecules induce nasal symptoms in case of hyperreactivity to environmental stimuli?

3.1. Identification of culprit sensors for hyperreactivity

Firstly, it remains unclear whether hypersensitivity is the result of upregulation of specific receptors or whether the entire (neuronal) cell has become generally hypersensitive to any kind of stimulus it expresses a receptor for. Generally, NHR and hyperreactivity/hypersensitivity in other organ systems have often been associated with upregulated neurogenic mechanisms, with an important focus on the nociceptive TRP channels.[10,48–52] Identification of one or few specific receptors as sole culprits in hyperreactivity is challenging. Indeed, TRP channels are polymodal receptors, which can be activated by a broad variety of stimuli, and several TRP channels can be activated by similar compounds. For example, TRPA1 can be activated by many molecules such as isothiocyanates, methyl salicylate, cinnamaldehyde, diallyl disulfide, acrolein, or epoxyeicosatreinoic acids.[53] TRPM8, TRPA1, and TRPV3 can all be activated by menthol, a compound present in mint, and both TRPV1 and TRPA1 can be activated by allicin, the spicy agent of garlic.[54] Moreover, rather than being a phenomenon situated at protein level, hyperreactivity can surely be the result of a general hypersensitive state of sensory neurons. In this situation, neurons would more easily reach the threshold for generating an action potential and thus be more sensitive to any possible trigger.

To study this, development of an animal model on NHR, where lab animals exhibit nasal responses to particular environmental stimuli, would be extremely helpful. This would allow to study effects of gene-specific knock-out or pharmacological inhibition of receptors *in vivo*. Unlike measurement of the PNIF in humans, there is currently no technique available that allows direct measurement of nasal patency in mice. However, in light of the united airways concept, lower airway reactions occur in response to upper airway stimuli.[55–57] Mice could be sensitized for house dust mite and provoked with CDA while breathing parameters are monitored using a double chamber plethysmograph.[58] This allows recording of all ventilatory parameters in conscious mice. An increase in the enhanced pause would serve as a surrogate measurement of airway constriction, whereas the increase of end inspiratory and end expiratory pauses would serve as an indication of irritation signaled by stimulation of sensory afferent fibers innervating the upper and lower airways respectively. Consequently, responses to CDA provocation could be monitored in (*TRP*-)specific knock-out animals or after administration of pharmacological inhibitors of TRP channels, such as SB-705498 (TRPV1-inhibitor). Indeed, in case hyperreactivity is present in these house dust mite-allergic mice, specific knockout or pharmacological inhibition could help to study the role of specific receptors on the reactivity itself. If hyperreactivity to CDA provocation persists while reactivity to TRP-specific agonists (caspsaicin, cinnamaldehyde, menthol) is abolished, this could be an argument for generalized neurological hypersensitivity rather that an increased sensitivity of the specific TRP channels.

In parallel, our findings of the sensitizing potential of histamine for specific TRPV1- and TRPA1 agonists, capsaicin and cinnamaldehyde, at *in vitro* level could be further validated by *in vivo* experiments, where histamine is endonasally administered to wild type mice. Before and after administration, breathing responses to capsaicin/cinnamaldehyde/CDA provocation would be monitored by using a double chamber plethysmograph as described higher. Similarly, since we observed a correlation between elevated levels of neuropeptides and reactivity to CDA provocation in humans, neuropeptides could be administered endonasally to study their effect on CDA provocation. These sets of experiments would allow to study the potential of allergic inflammation and histamine/neuropeptides specifically on developing hyperreactivity.

Alternatively, SB-705498 was previously shown to inhibit the capsaicin-induced increase in nasal secretions in a guinea pig model of allergic rhinitis.[59] Conversely, it did not affect symptom scores from patients with AR, nor did it reduce reactivity to allergen challenge in these patients.[60,61] But indeed, allergen challenge elicits symptoms mainly via type 2 mechanisms, while neurogenic mechanisms and TRPV1 seem to play a role in eliciting symptoms by CDA challenge. Therefore, SB-705498 could be administered endonasally before CDA provocation in NHR-positive subjects to study the effect of TRPV1-inhibition on CDA-induced responses in humans. Similar experiments could be performed with inhibitors of other TRP channels, such as HC-030031 (TRPA1) or AMG9678 (TRPM8). These experiments would give some insight on whether the TRP channels are causative for NHR or rather a side finding in a general hypersensitive neuronal state.

3.2. Studies on pathways linking cold, dry air provocation and nasal symptoms

A second point to be investigated is how exactly exposure to particular environmental stimuli leads to nasal symptoms. We could not identify the mediators and underlying pathways linking CDA provocation and nasal obstruction. In contrast to what was previously hypothesized, levels of neuropeptides in nasal secretions remained stable during CDA provocation in our study.[10] We only observed an increase in levels of IL-33. IL-33 can have various down-stream effects, but it remains unclear whether it is a contributor or driver of CDA-induced nasal obstruction, or if it is just an insignificant bystander. Is IL-33, and maybe also other epithelial cytokines responsible for a signaling cascade resulting in the release of mediators that ultimately lead to nasal symptoms? One important consideration is that we measured peptides in nasal secretions absorbed by a small tampon placed in the nasal lumen. From a biological point of view, mediators released from a specific cell should reach their target-cell expressing its receptor, which is in many cases probably present in the submucosal layer and not in the nasal lumen. Hence, the protein levels measured in nasal secretions may or may not reflect levels in the tissue. Therefore, we plan on measuring levels of the various neurogenic, type 2 inflammatory, and epithelial mediators in biopsies harvested before and after CDA provocation. However, proteins in tissue could also be released from pre-stored granules. In this case, similar absolute numbers of proteins would be found, but due to release

from the intracellular to the extracellular environment, an increased number of proteins could bind their receptor to exert their biological function.

It is possible that epithelial activation and not activation of sensory nerves leads to nasal symptoms. To study this, human subjects could be exposed to CDA while their nasal mucosal potentials are continuously monitored, similar to a previous study where a decreased threshold for neurological reaction to allyl isothiocyanate was observed in patients with NAR.[62] Increased mucosal electrographic responses would be observed if neurogenic activation is the primary event leading to nasal symptoms in individuals responding to CDA provocation.

We surprisingly found increased levels of IL-33 in nasal secretions while levels of other mediators remained stable. Hence, it is possible that IL-33 and not neuropeptides are driving nasal symptoms elicited by CDA provocation. Indeed, IL-33 is known to induce angiogenesis and increase vascular permeability via activation of its receptor ST2 and stimulation of endothelial production of nitric oxide.[63] But also indirect pathways are possible. It is known that epithelium-derived IL-33 can activate tissue mast cells, which subsequently release their mediators among which IL-33, resulting in a positive feedback loop.[64] Moreover, IL-33 stimulates type 2 innate lymphoid cells and Th2 cells to produce pro-inflammatory type 2 cytokines such as IL-5 and IL-13.[65,66] It can activate and attract eosinophils and basophils to produce more pro-inflammatory mediators.[66] Lastly, IL-33 can directly activate and sensitize sensory neurons.[67,68] Future studies should therefore focus on unraveling the various direct and indirect roles IL-33 plays in eliciting symptoms during CDA provocation. In this light, mice could be exposed to IL-33 before being challenged with CDA in a double chamber plethysmography in order to study the potential of IL-33 to induce NHR. Consequently, measurement of breathing parameters while being exposed to IL-33 would be of interest to investigate whether endonasal IL-33 itself can lead to nasal symptoms.

3.3. Barrier function and nasal hyperreactivity

Lastly, we also measured expression levels of several tight junction proteins on nasal mucosal biopsies. Decreased expression of *ZO-1* was correlated with reactivity to CDA provocation, but no such relationship could be observed for *OCLN* or *CLDN1*. A possible effect

of epithelial barrier integrity on presence of NHR could therefore not be concluded. To study this further, barrier integrity of patients with and without NHR could be studied at tissue level with ussing chambers. In parallel, primary nasal epithelial cells could be cultured for measurements of the transepithelial electrical resistance as described previously.[69] In lab animals with and without NHR, passage of endonasally administered fluorescein isothiocyanate dextran 4000 Dalton to the blood could be analyzed.

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CHAPTER 7

Summary - Samenvatting

Summary

Chronic upper airway inflammatory disorders are various diseases characterized by inflammation of the mucosal lining of the nose (in case of *rhinitis*) and/or the paranasal sinuses (in case of *rhinosinusitis*) with symptoms (nasal obstruction, rhinorrhea/post-nasal drip, nasal itch, sneezing, loss of smell, headache/facial pain) lasting for ≥ 12 weeks. Several underlying mechanisms can lead to mucosal inflammation resulting in sinonasal symptoms.

Nasal hyperreactivity is the phenomenon where sinonasal symptoms are triggered by exposure to various specific environmental stimuli – such as temperature/humidity changes, air-conditioning, (cigarette) smoke, or strong odors – that would cause little to no effect in healthy subjects. It is increasingly shown to be present in various clinical subgroups of rhinitis or rhinosinusitis. Diagnosis can be made by means of a questionnaire or by a cold, dry air provocation test. Unfortunately, no consensus-definition for patient-reported diagnosis currently exists. Nasal hyperreactivity is mostly studied in non-allergic rhinitis, where it is suspected to be mainly neurogenically mediated. In these patients, an upregulation of the nociceptive 'transient receptor potential channel vanilloid 1 – substance P' axis is observed, presumably leading to neuronal hypersensitivity. Neuronal activation would consequently lead to release of neuropeptides, which induce nasal symptoms.

Allergic rhinitis and chronic rhinosinusitis with nasal polyps on the other hand are historically considered to be mainly mediated by type 2 inflammation. Nevertheless, there are arguments for presence of nasal hyperreactivity and neurogenic inflammation in these clinical subgroups.

Therefore, the main goals of this doctoral project were:

- To study the prevalence of nasal hyperreactivity in patients with any kind of chronic upper airway inflammation.
- To study the mediators underlying nasal hyperreactivity in classical type 2 chronic upper airway inflammatory disorders.
- To study the interaction between histamine, an important mediator in allergic rhinitis and chronic rhinosinusitis with nasal polyps, and transient receptor potential channels.

In order to gain insight in the prevalence of nasal hyperreactivity, we performed a questionnaire-based study in a large cohort of well-characterized patients with otorhinolaryngologist-diagnosed chronic upper airway inflammation. To avoid false-positive diagnosis of nasal hyperreactivity in patients reporting symptoms upon exposure to environmental triggers due to physiologic, protective reflexes, we defined self-reported nasal hyperreactivity as positive answer to both of the questions "*Are your nasal complaints triggered or exacerbated by any of the following triggers: (…)?*" and "*In this case, do they last longer than 10 minutes?*". In this first study, we observed a prevalence self-reported nasal hyperreactivity in 40.5 – 52.1 % of the patients with chronic upper airway inflammation. Moreover, in groups other than non-allergic rhinitis, prevalence of nasal hyperreactivity was not related with subjective disease severity or use of medication, highlighting the lack of available therapies targeting neurogenic inflammation in these patients. In patients with nasal hyperreactivity, the main elicited symptoms were nasal obstruction and rhinorrhea/postnasal drip, mostly triggered by temperature/humidity changes and air-conditioning.

In a second part, we performed a clinical study in patients with allergic rhinitis or chronic rhinosinusitis with nasal polyps, which are classically considered as type 2 inflammatory disorders. Patients and healthy control subjects underwent a cold, dry air provocation test. Before and after they filled out questionnaires, nasal secretions were collected and nasal mucosal biopsies were harvested. We observed a generally good correlation between presence of subjective, patient-reported nasal hyperreactivity and objective nasal hyperreactivity diagnosed with a cold, dry air provocation test. Even though not all patients with subjective nasal hyperreactivity reached the threshold for objective diagnosis, they clearly had a stronger decrease in nasal patency during cold, dry air provocation. This suggests that nasal reactivity spans a continuous spectrum rather than being an on/off phenomenon. In nasal secretions and biopsies of our patients, we observed a negative correlation between protein levels of neuropeptides, levels of histamine, and expression of neurogenic markers on one hand and reactivity to cold, dry air provocation on the other hand. Hence, neurogenic upregulation and increased levels of histamine are risk factors for nasal hyperreactivity. Lastly, we could not observe an increase in levels of neuropeptides after cold, dry air provocation. Conversely, levels of IL-33 increased after cold,

dry air provocation indicating epithelial activation. It remains currently unclear which mediators cause nasal symptoms in the setting of nasal hyperreactivity.

Since we observed a negative correlation between levels of histamine in nasal secretions and reactivity to cold, dry air provocation, and since nasal hyperreactivity is often linked to transient receptor potential channels, we performed some *in vitro* mouse experiments to investigate the effect of histamine on transient receptor potential channels. Trigeminal ganglionic neurons of wild-type mice were cultured and stimulated with capsaicin (for transient receptor potential channel vanilloid 1) and cinnamaldehyde (for transient receptor potential channel ankyrin 1) before and after exposure to histamine. We observed that more neurons responded to the given stimuli after histamine was administered. This effect was abolished when the histamine 1 receptor-antagonist pyrilamine was added to the medium. This indicates that histamine can sensitize trigeminal sensory neurons for transient receptor potential-specific agonists via a histamine 1 receptor-dependent pathway.

In conclusion, with this doctoral project we showed that presence of nasal hyperreactivity is not limited to patients with uncontrolled non-allergic rhinitis but is a general feature of chronic upper airway inflammation. We provided evidence for presence of neurogenic inflammation along with increased nasal reactivity in classical type 2 inflammatory disorders, allergic rhinitis and chronic rhinosinusitis with nasal polyps. Histamine can enhance the neurogenic pathways by sensitizing sensory neurons for transient receptor potential channel agonists.

Samenvatting

Ziekten met chronische ontsteking van de bovenste luchtwegen worden gekenmerkt door inflammatie van de mucosa van de neus (in geval van *rhinitis*) en/of paranasale sinussen (in geval van *rhinosinusitis*) met symptomen (neusobstructie, rhinorrhee/postnasale drip, jeuk aan de neus, niezen, verlies van geurzin, hoofdpijn/faciale druk) die ≥ 12 weken aanhouden. Verschillende onderliggende mechanismen kunnen leiden tot mucosale inflammatie, hetgeen resulteert in sinonasale symptomen.

Nasale hyperreactiviteit is het fenomeen waarbij sinonasale symptomen uitgelokt worden door blootstelling aan specifieke triggers in de omgeving – zoals veranderingen in temperatuur/luchtvochtigheid, air-conditioning, (sigaretten)rook, of sterke geuren – die in gezonde controle personen geen of weinig klachten zouden uitlokken. Aanwezigheid van nasale hyperreactiviteit wordt steeds meer beschreven in verschillende klinische subgroepen van rhinitis of rhinosinusitis. Het wordt gediagnosticeerd met behulp van vragenlijsten of een koude, droge lucht provocatietest. Helaas bestaat er op dit moment geen consensus-definitie van zelf-gerapporteerde nasale hyperreactiviteit. Nasale hyperreactiviteit is vooral bestudeerd in niet-allergische rhinitis, waar het vermoedelijk neurogeen gemedieerd is. In deze patiënten is een opregulatie van de nociceptieve ‘transient receptor potential kanaal vanilloid 1 – substantie P’-as aanwezig, hetgeen zou leiden tot neuronale overgevoeligheid. Neuronale activatie zou dan leiden tot vrijzetten van neuropeptides, dewelke nasale symptomen uitlokken.

Allergische rhinitis en chronisch rhinosinusitis met neuspoliepen worden historisch gezien dan weer beschouwd als voornamelijk type 2 inflammatie-gemedieerde ziektes. Toch zijn er een aantal argumenten voor aanwezigheid van nasale hyperreactiviteit en neurogene inflammatie in deze klinische fenotypes.

Op deze basis waren de voornaamste doelstellingen van dit doctoraatsproject:

- Bestuderen van de prevalentie van nasale hyperreactiviteit in patiënten met eender welke vorm van bovenste luchtwegontsteking.
- Bestuderen welke mediatoren een rol spelen in nasale hyperreactiviteit bij de klassieke type 2 inflammatoire ziektes van de bovenste luchtwegen.

- Bestuderen van een mogelijke interactie tussen histamine, een belangrijke mediator in allergische rhinitis en chronische rhinosinusitis met neuspoliepen, en transient receptor potential kanalen.

Om inzicht te krijgen in de prevalentie van nasale hyperreactiviteit voerden we een vragenlijst-gebaseerde studie uit in een groot cohort van goed-gekarakteriseerde patiënten met een neus-, keel-, oor-specialist-gebaseerde diagnose van chronische inflammatie van de bovenste luchtwegen. Om vals-positieve diagnose van nasale hyperreactiviteit bij patiënten die symptomen rapporteren bij blootstelling aan omgevingstriggers omwille van fysiologische, protectieve reflexen te vermijden, definieerden we zelf-gerapporteerde nasale hyperreactiviteit als een positief antwoord op volgende twee vragen: "*Worden uw neusklachten uitgelokt of verergerd door blootstelling aan minstens één van de volgende triggers: (…)?*" en "*In dit geval, houden ze dan langer dan 10 minuten aan?*". In deze eerste studie zagen we een prevalentie van zelf-gerapporteerde nasale hyperreactiviteit van 40.5-52.1 % in patiënten met chronische inflammatie van de bovenste luchtwegen. In andere groepen dan de groep van niet-allergische rhinitis was prevalentie van nasale hyperreactiviteit niet gerelateerd met subjectieve ziekte-ernst of gebruik van medicatie, hetgeen het gebrek aan beschikbare medicatie gericht tegen neurogene inflammatie in deze patiënten benadrukt. In patiënten met nasale hyperreactiviteit waren de voornaamste uitgelokte symptomen neusobstructie en neusloop/post-nasale drip, dewelke vooral getriggerd werden door veranderingen in temperatuur/luchtvochtigheid en air-conditioning.

In een tweede deel voerden we een klinische studie uit in patiënten met allergische rhinitis of chronische rhinosinusitis met neuspoliepen, dewelke klassiek beschouwd worden als type 2 inflammatoire ziektes. Patiënten en gezonde controlepersonen ondergingen een koude, droge lucht provocatietest. Voor en na provocatie vulden ze vragenlijsten in en werden nasale secreties en biopten genomen. We zagen een algemeen goede correlatie tussen aanwezigheid van subjectieve, patiënt-gerapporteerde nasale hyperreactiviteit en objectieve nasale hyperreactiviteit gediagnosticeerd met een droge, koude lucht provocatietest. Ondanks dat niet alle patiënten met subjectieve nasale hyperreactiviteit de drempel haalden voor objectieve diagnose, toch hadden ze een duidelijke sterkere daling in neusdoorgankelijkheid tijdens provocatie met koude, droge lucht. Dit suggereert dat nasale hyperreactiviteit een continu spectrum behelst eerder dan dat het een aan/uit-fenomeen is.

In neussecreties en -biopten van onze patiënten zagen we een negatieve correlatie tussen eiwit-niveaus van neuropeptides, niveaus van histamine en expressie van neurogene merkers enerzijds, en reactiviteit bij blootstelling aan koude, droge lucht anderzijds. Neurogene opregulatie en verhoogde niveaus van histamine zijn dus risicofactoren voor nasale hyperreactiviteit. Tot slot konden we geen stijging zien in de niveaus van neuropeptides na koude, droge lucht provocatie. Niveaus van IL-33 daarentegen waren verhoogd na koude, droge lucht provocatie, hetgeen wijst op epitheliale activatie. Momenteel blijft het onduidelijke welke mediatoren nasale symptomen veroorzaken in de context van nasale hyperreactiviteit.

Omdat we een negatieve correlatie zagen tussen niveaus van histamine in neussecreet en reactiviteit op koude, droge lucht provocatie, en omdat nasale hyperreactiviteit vaak gelinkt wordt aan transient receptor potential kanalen, voerden we enkele *in vitro* muisexperimenten uit om het effect van histamine op transient receptor potential kanalen te onderzoeken. Trigeminale ganglionische neuronen van wild-type muizen werden in cultuur gebracht en vervolgens gestimuleerd met capsaicine (voor transient receptor potential kanaal vanilloid 1) en cinnamaldehyde (voor transient receptor potential kanaal ankyrin 1) voor en na blootstelling aan histamine. We zagen dat meer neuronen reageerden op de gegeven stimulus nadat histamine werd toegediend. Dit effect kon verdween wanneer we de histamine 1 receptor-antagonist pyrilamine toevoegden. Deze resultaten tonen aan dat histamine trigeminale sensorische neuronen kan sensitizeren voor transient receptor potential-specifieke agonisten op een histamine 1 receptor-afhankelijke manier.

In conclusie toonden we met dit doctoraatsproject aan dat aanwezigheid van nasale hyperreactiviteit niet beperkt is tot patiënten met ongecontroleerde niet-allergische rhinitis, maar dat het een algemeen kenmerk is van chronische inflammatie van de bovenste luchtwegen. Onze resultaten wijzen op aanwezigheid van neurogene inflammatie samen met verhoogd nasale reactiviteit in de klassieke type 2 inflammatoire ziektes, allergische rhinitis en chronische rhinosinusitis met neuspoliepen. Histamine kan de neurogene activiteit versterken door sensitizatie van de sensorische neuronen voor transient receptor potential kanaal agonisten.

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Personal contribution

The introduction (chapter 1) and objectives (chapter 2) of this thesis were drafted and finalized by Wout Backaert with feedback from prof. dr. Laura Van Gerven, prof. dr. Peter Hellings, prof. dr. Karel Talavera, and dr. Brecht Steelant.

For the first study (chapter 3), Wout Backaert developed the questionnaires with help from prof. dr. Lukas Van Oudenhove, which were later corrected/approved by prof. dr. Laura Van Gerven, prof. dr. Peter Hellings, prof. dr. Karel Talavera, and dr. Brecht Steelant. Questionnaires were collected by Wout Backaert, prof. dr. Mark Jorissen, prof. dr. Laura Van Gerven, prof. dr. Peter Hellings, and the residents from the department of Otorhinolaryngology, Head & Neck Surgery of University Hospitals Leuven. Data processing and analysis as well as drafting the manuscript was performed by Wout Backaert. The manuscript was later critically revised by all authors.

The study on the diagnostic algorithms (chapter 4) was conceptualized by prof. dr. Rik Schrijvers. Development of the diagnostic algorithms was done by prof. dr. Steffen Fieuws and dr. Ipek Guler, based on data provided by Wout Backaert. Wout Backaert drafted the manuscript, which was later critically revised by all authors.

The clinical study (chapter 5) was designed by Wout Backaert, prof. dr. Laura Van Gerven, prof. dr. Peter Hellings, and dr. Brecht Steelant. All patients were contacted by Wout Backaert, who also distributed the questionnaires, collected biological samples, and performed the cold, dry air provocation test at the Department of Otorhinolaryngology, Head & Neck Surgery from University Hospitals Leuven under supervision of prof. dr. Mark Jorissen. Further processing of the samples for storage was done by Wout Backaert and Zhen Qian. Wout Backaert performed all protein assays and RT-q-PCR experiments, with help from Ellen Dilissen, Tine Wils, and Anne-Charlotte Jonckheere. Calcium imaging experiments were performed by Wout Backaert, with help from dr. Brett Boonen. Wout Backaert performed the statistical analysis and drafted the manuscript, which was later critically revised by all authors.

The general discussion and future perspectives (chapter 6) were drafted by Wout Backaert and critically revised by prof. dr. Laura Van Gerven, prof. dr. Peter Hellings, prof. dr. Karel Talavera, and dr. Brecht Steelant.

Conflict of interest

All contributors to this thesis declare no conflicts of interest.

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Curriculum vitae

Wout Backaert was born on June 1st 1994 in Sint-Niklaas, Belgium. After finishing his secondary school at Sint-Jozef-Klein-Seminarie in Sint-Niklaas in 2012, he started his studies in medicine at KU Leuven to graduate in 2018 *magna cum laude*. During his studies, he did an internship in paediatrics and obstetrics/gynaecology in Launceston General Hospital, Launceston, Tasmania, Australia. In 2018, he started his specialist training in Otorhinolaryngology, Head & Neck Surgery at KU Leuven. Currently he is doing residency at the University Hospitals Leuven under supervision of prof. dr. M. Jorissen, while working on a PhD project in the Allergy and Clinical Immunology Research Group of KU Leuven under supervision of prof. dr. L. Van Gerven, prof. dr. P. Hellings, prof. dr. K. Talavera, and dr. B. Steelant. From October 2022, he will continue his clinical training fulltime.

Wout lives in Leuven and is married with Bloemke Van de Wygaert.

List of publications

Submitted peer-reviewed articles

- **Backaert W**, Steelant B, Qian Z, Wils T, Dilissen E, Jonckheere AC, Boonen B, Jorissen M, Schrijvers R, Bullens DMA, Talavera K, Hellings PW, Van Gerven L. Neurogenic pathways underly nasal hyperreactivity in type 2 chronic upper airway inflammation.

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