

# Association between reactive oxygen species and aryl hydrocarbon receptor in chronic rhinosinusitis

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**UNIVERSITY OF ZAGREB  
SCHOOL OF MEDICINE**

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species and aryl hydrocarbon receptor  
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**DISSERTATION**



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The dissertation was made in the Clinical Hospital Center Zagreb's Department of Ear, Nose and Throat and Head and Neck Surgery

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## LIST OF SYMBOLS AND ABBREVIATIONS

AhR - Aryl hydrocarbon Receptor

BSA – Bovine Serum Albumin

CRS - Chronic Rhinosinusitis

CRSsNP - Chronic Rhinosinusitis without Nasal Polyps

CRSwNP - Chronic Rhinosinusitis with Nasal Polyps

DUOX - Dual Oxidase

H<sub>2</sub>O<sub>2</sub> - Hydrogen peroxide

IL - Interleukin

MIG - Monokine induced by Interferon Gamma

NADPH - Nicotinamide Adenine Dinucleotide Phosphate

NOX - Nicotinamide Adenine Dinucleotide Phosphate Oxidase

PBS – Phosphate Buffer Saline

ROS - Reactive Oxygen Species dual oxidase

SHS - Second Hand Smoking

TBST - Tris-Buffered Saline + Tween

Th – T-helper cells

TNF - Tumor Necrosis Factor

IFN- $\gamma$ -interferon gamma

DMSO- dimethyl sulfoxide

ITE-2-(1*H*-Indol-3-ylcarbonyl)-4-thiazolecarboxylic acid methyl ester

TMF - 6,2',4' - trimethoxyflavone

K $\tau$  - Kendal rank

NSAIDs – non steroidal anti inflammatory drugs

EPOS - European Position Paper on Rhinosinusitis

4- HNE- 4 hydroxynonenal

ACR- acrolein

TOC-total oxidative stress capacity

TAC- total antioxidative stress capacity

VAS- visual analogue score



NK –natural killer cells  
MCC- mucociliary clearance  
ILC- innate lymphoid cells  
N-ERD- non-steroidal anti-inflammatory drug exacerbated respiratory disease  
Ig-immunoglobulin  
GA<sup>2</sup>LEN- Global Allergy and Asthma European Network  
NSAID- non-steroidal anti-inflammatory drug  
DC - dendritic cells  
APC - antigen presenting cells  
COX - cyclooxygenase  
ATAD - aspirin treatment after desensitization  
PCD - primary ciliary dyskinesia  
CF - cystic fibrosis  
MDA - malondialdehyde  
LPO - lipid peroxidation  
PMBCs - peripheral monocyte blood cells  
SNEC - sinonasal epithelial cells  
XRE - xenobiotic binding element

# 1. INTRODUCTION

Chronic rhinosinusitis (CRS) is a clinical syndrome, rather than a specific disease, characterized by persistent symptomatic inflammation of the nose and paranasal sinus mucosa for more than 12 weeks. CRS may be used to describe conditions ranging from unilateral single sinus disease to widespread sinonasal airway inflammation.

## 1.1 European Position Paper on Rhinosinusitis (EPOS) guideline.

Based on expert recommendations, criteria for CRS were established in EPOS to sustain uniform epidemiologic studies. EPOS 2012 guideline describes CRS as an inflammatory disorder defined by the presence of two or more cardinal symptoms [obstruction, drainage (anterior or posterior), smell loss, and facial pain or pressure] for at least 12 weeks duration, confirmed by objective evidence using sinus endoscopy or computed tomography (CT) scan [1]. For study inclusion the guideline requires at least two of four symptoms for at least 3 months duration, one of which must be either nasal obstruction or discharge. According to the new EPOS 2020 classification CRS should now be regarded as primary or secondary, and distinction is made between diffuse and localized disease based on anatomic distribution [2]. In primary CRS, the disease is considered by endotype dominance, either type 2 or non-type 2. Clinically localized primary CRS is then subdivided into two phenotypes - allergic fungal rhinosinusitis or an isolated sinusitis. For diffuse CRS, the clinical phenotypes are predominantly eosinophilic CRS (eCRS) and non-eosinophilic CRS (non eCRS), determined by the histologic quantification of the numbers of eosinophilic, i.e. number/high powered field which the EPOS panel agreed to be 10/hpf (400x) or higher. For secondary CRS, again, the division is into localized or diffuse and then considered by four categories dependant on local pathology, mechanical, inflammatory and immunological factors .

## 1.2 Epidemiology of CRS

Epidemiological studies estimated the prevalence of CRS in Europe (10.9%), China (8%), and Brazil (5.5%). CRS is more common in smokers than in non-smokers. The prevalence of self-reported physician-diagnosed CRS is highly correlated with the prevalence of EPOS-diagnosed CRS [3–5]. Studies in the USA, using symptom criteria alone, reported a

prevalence of 12% resembling the European CRS frequency pattern [6]. The overall prevalence of symptom-based CRS in the population has been found to be between 5.5% and 28% [4, 5, 7, 8]. When symptoms are combined with endoscopy or CT scan prevalence is reduced to 3-6% [9-11].

The primary goal of any treatment, especially in chronic diseases, is to achieve and maintain clinical control, which can be defined as a disease state in which the patient does not have symptoms, or the symptoms are not impacting quality of life.

In EPOS 2012 guideline, assessment of CRS symptoms severity can be estimated using many different grading tools. Recorded as such: no symptom, mild, moderate or severe, recorded as visual analogue score (VAS) on a line giving a measurable continuum (0 – 10 cm) [12]. A validation study has shown 'mild disease' to be defined as a VAS score of 0-3 inclusive, moderate as >3-7 inclusive, and severe as  $\geq 7$ . In general, overall quality of life is more likely to be affected with scores of 5 or more. In EPOS 2020 assessment of current clinical control classified as controlled, partially controlled and uncontrolled based on presence or absence of symptoms like nasal blockage, rhinorrhoea, facial pain/ pressure, smell, sleep disturbance, nasal endoscopy view and rescue treatment. At the time of the earlier EPOS review, CRS was divided into two phenotypes: chronic rhinosinusitis without (CRSsNP), a T helper (Th)1 disorder, and chronic rhinosinusitis with nasal polyps (CRSwNP), a Th2 disorder [13]. More recent studies have demonstrated that this paradigm does not apply worldwide, in particular for CRSwNP, as some Asian polyps exhibit Th1 and TH17 cytokine profiles [14].

### **1.3 Immunology of upper airways**

The components of innate immune system include epithelial barrier, sentinel mucosal cells (resident macrophages, dendritic cells, mast cells etc.), circulating and recruited phagocytes (monocytes and neutrophils), innate lymphoid cells, natural killer (NK) cells, as well as non-cellular components (e.g. complement system) [15]. The major functions of the sinonasal innate immune system include phagocytosis, recruiting immune cells to sites of infection, activation of the complement cascade to identify and clear off bacteria, antibody complexes or dead cells, identification and removal of foreign substances, and activation of the adaptive immune system through antigen presentation. Mucociliary clearance (MCC) and

apical junctional complexes between epithelial cells comprise a mechanical barrier between host and environment, which provides the first line of host defence for the nose and sinuses. Innate lymphoid cells (ILCs) are lineage and antigen receptor negative lymphocytes including NK cells. ILCs can act as first-line defenders in the airway epithelial barrier.

#### **1.4 Pathogenesis of CRS**

ILC2 cells are the best studied in CRS, playing a significant role in eosinophilic CRSwNP, and possibly all CRS that exhibits type 2 inflammation [16-17]. ILC2s are likely an important source of type 2 cytokines that drive tissue inflammation. Neutrophils, also known as polymorphonuclear leukocytes, are the most abundant leukocytes in the blood and are important in the early phagocytosis and killing of extracellular mucosal microbes. Neutrophils are driven most strongly by type 3 cytokines and their infiltrates often co-exist with type-2 cytokine-driven eosinophils, suggesting the possibility that this tissue neutrophilia may reflect a superimposed physiologic response against microbiota in CRS patients [18-21]. The role of neutrophils in CRS pathogenesis is uncertain, but these cells are capable of degranulation with tissue damage including some loss of barrier integrity. Basophils are granulocytes found mainly in the circulation and are known to have a role in allergic disease and immunity against parasites. They have been implicated in type 2 responses in general, possibly serving as an early source of Interleukin (IL)-4 driving polarization in the Th2 direction [22]. They are particularly elevated in aspirin tolerant polyps as opposed to non-steroidal anti-inflammatory drug (NSAID)-exacerbated respiratory disease (N-ERD) polyps. Their significance in CRSwNP remains unclear. They can be activated by microbial products binding toll like receptors and by components of the complement system as part of the innate immunity or by an antibody-dependent mechanism in adaptive immunity. Mast cell granules contain vasoactive amines such as histamine that cause vasodilation and increased capillary permeability, as well as proteolytic enzymes, lipid mediators, chemokines and cytokines [23]. In CRS, interest has centred on a role for mast cells in nasal polyposis, in part as a result of the potential to induce, augment and maintain eosinophilic inflammation through immunoglobulin E (IgE)-dependent and IgE-independent processes [24].

Eosinophilic damage to the sinonasal mucosa was believed to be the central pathophysiologic mechanism of CRS and the hallmark of the disorder. Eosinophils are not essential for CRS to exist, they appear to be a biomarker for severe, recalcitrant disease, and may still be the cell that mediates this relatively poor prognosis [25-26].

Systemic or tissue biomarkers for eosinophilic CRS could have, therefore, a significant clinical role. NK cells recognize infected and stressed cells and respond by killing these cells and by secreting the macrophage-activating cytokine interferon  $\gamma$  (IFN- $\gamma$ ) impaired ability of NK cells to degranulate and to produce cytokines such as IFN- $\gamma$  and tumor necrotic factor (TNF)- $\alpha$  was associated with CRSwNP, concomitant asthma and peripheral blood eosinophilia . Humoral immunity is the type of host defence mediated by antibodies, which are produced and secreted by B cells and plasma cells, functioning both in circulation and in peripheral tissues such as sinonasal mucosa [15, 27]. Polyp tissue from CRSwNP patients has been found to contain high levels of B cells, plasma cells, follicles that resemble germinal centres, and high levels of IgA, IgM, IgG and IgE that indicate local production of immunoglobulins [27-32]. Evidence for a dysregulated adaptive B-cell immune response is further suggested by the presence of germinal center like follicles in nasal polyps and the entire process is likely orchestrated by local proliferation and systemic recruitment of B cells [33-34].

### **1.5 T helper immune response into nasal mucosa**

In the immune response of the nose, dendritic cells (DCs) act as the initial antigen presenting cells (APCs) sampling and then presenting antigens to naïve T lymphocytes in draining lymph nodes or local lymph aggregates. Circulating basophils may also enter the tissue and serve along side or instead of resident DCs to function as APCs as well [35]. Following antigen presentation, naïve CD4+ lymphocytes will differentiate into one of several T cell lineages, determining the nature of the adaptive immune response. The subsets include Th1 and Th2 as well as the more recently described Th17 and inducible T regulatory cells; each has distinct molecular, cellular and functional properties. Other subsets have also been recently proposed, including Th9 and Th22, and more are likely to follow. In vitro studies indicate that for the Th1 subset, the key transcription factor is T-bet, the canonical cytokine is IFN- $\gamma$  and the classical cellular infiltrate is macrophage-rich. Th1

responses are particularly effective against viruses and intracellular bacteria, including mycobacteria. For Th2, the transcription factor is GATA-3, the associated cytokines are IL-4, IL-5 and IL-13 and the cellular response eosinophilic. Th2 protective responses are geared against parasites, particularly those too large to undergo phagocytosis. For Th17, the transcription factor is RORc and the associated cytokine IL-17A and the cellular response classically neutrophilic [36-37].

## **1.6 Endotyping of CRS**

Further pathophysiologic research identified different inflammatory patterns leading to the term “endotyping of CRS.” The primary focus of endotyping is to define a dominant inflammatory type allowing for better orientation of therapy especially for severe and recurrent CRS inflammation.

An alternative distinction is the inflammatory type dominance, either type 2 (T2) (eosinophilic) or non-T2 (2). T2 can then be subdivided predominantly via T helper (Th)2/allergy/immunoglobulin E (IgE) mechanisms and via innate mechanisms innate lymphoid cells (ILCs) or a mixture of the two. The emerging linkage between adaptive and innate immune systems has led to the proposal of type 1, 2, and 3 immune responses [38-40]. Type 1 immune responses are characterized by type 1 ILC1 cells, and Tc1 and Th1 cells. The crucial role of this type 1 immune response is to deal with intracellular microbes, protozoa and viruses. The activation of ILC1, Tc1, and Th1 cells will induce the production of type 1 cytokines IFN- $\gamma$  and TNF- $\alpha$  resulting in the activation of mononuclear phagocytes loaded with potent cytotoxic molecules. Type 2 immune responses implicate ILC2s, Tc2, and Th2 cells responsible for the production of IL4, IL-5, and IL-13 cytokines. Type 2 plays an important role in parasite infection and induces allergic diseases with important contribution of eosinophilic cells, IgE production and goblet cell hyperplasia. Type 3 immune responses are currently associated with cytokines IL-17 and IL-22 and controlled by IL3, Tc17 cells, and Th17 cells. Their role is believed to facilitate immune responses opposing extracellular bacteria and fungi. Tomassen et al. [41] used inflammatory biomarker cluster analysis to endotype type 2 and non-type 2 CRS cytokine profiles, revealing a clear distinction between the two immune response. The production of type 2 cytokines is a distinguishing feature of the type 2 inflammatory response (IL-4,IL-5,IL-13

expression). Non-type 2 inflammation in CRS is characterized by a mix of type 1 and type 3 inflammation (as defined by IL-17, IL-22, and IFN-  $\gamma$  expression) and is frequently accompanied by significant neutrophil infiltration. Bachert et al. [42] emphasized the clinical significance of comorbidities and clinical traits in the endotyping process: Non-type 2 inflammation is linked to the CRSsNP phenotype, low asthma risk (<10%), and low recurrence risk; moderate type 2 inflammation is linked to a combination of CRSsNP and CRSwNP, moderate asthma risk (10-40%) , and recurrence risk; and severe type 2 inflammation is linked to the CRSwNP phenotype, high asthma risk(40-70%), and disease recurrence risk. Traditional biomarker-based endotyping attempts to identify patient inflammatory clusters in order to select more tailored therapy options.

Clinical presentation of inflammatory sinonasal disease may be grossly divided in eosinophilic airway inflammation versus non- eosinophilic inflammation. This endotypes show a distinct T2 eosinophilic airway inflammation namely allergy, eosinophilic CRS and CRSwNP versus a non-eosinophilic T1 inflammation pattern also present in the CRSsNP and some of the CRSwNP population. In case of eosinophilic upper airway inflammation, the tissue eosinophilia in CRSwNP is frequently associated with extensive sinus disease [43], higher post-operative symptom scores (72), less improvement in both disease-specific and general quality of life [44]. Patients with a non-eosinophilic airway inflammation may be considered as non-type 2 and are mainly characterized by neutrophils in their nasal mucosa [45,46]. These conditions are present in infectious rhinitis, CRSsNP and the Th17 pathway currently addressed now as type 3 immune response. In Asia, non-eosinophilic CRSwNP is frequently observed and is associated with relatively less oedema and more fibrosis compared with eosinophilic CRSwNP [47]. Of note, the presence of a mixed Th17/Th2 inflammation in CRSwNP is possible as neutrophil biased inflammation may be demonstrated in eosinophilic nasal polyps. The combination of high levels of type 2 inflammation mediators combined with high levels of type 1 or type 3 and/or neutrophilic markers seems to predict a more severe inflammatory burden. Neutrophilic inflammation can be triggered by infections or chronic irritation, environmental toxins, work conditions and air pollution. Very often tissue neutrophilia is not completely controlled by inhaled glucocorticosteroids [48].

## 1.7 Predisposing factors of CRS

As mentioned in EPOS guideline, predisposing factor of CRSwNPs and CRSsNPs could be allergy. Atopy is a genetic predisposition to allergic diseases such as allergic rhinitis, asthma, and atopic dermatitis (eczema). Atopy is commonly associated with increased IgE-mediated immune responses to common allergens, particularly inhaled allergens and food allergens. A more recent non-systematic review points to the fact that different phenotypes/endotypes of CRS may have a variable associations with allergy. The prevalence of allergy in CRS may vary by phenotype, with central compartment atopic disease and allergic fungal rhinosinusitis having a stronger association than CRSwNP and CRSsNP. The prevalence of asthma is around 25% in patients with CRS compared to 5% in the general population [49]. Within the Global Allergy and Asthma European Network (GA<sup>2</sup>LEN) network a multicentre cross-sectional case control study recruited 935 adults (869 eligible for analysis: 237 CRSsNP; 445 CRSwNP; 187 controls). Comorbidities such as asthma, allergy, eczema, food allergy, urticaria, and chronic obstructive pulmonary disease were significantly more frequent in CRS patients [50].

Another predisposing factor is non-steroidal anti-inflammatory drug (NSAID)-exacerbated respiratory disease (N-ERD). N-ERD is a chronic eosinophilic, inflammatory disorder of the respiratory tract occurring in patients with asthma and/or CRS with nasal polyps (CRSwNP), symptoms which are exacerbated by NSAIDs, including aspirin [51]. The pathogenesis of N-ERD is related to dysregulation of eicosanoid synthesis leading to an eosinophilic inflammation of the nasal and sinus membranes and an increased leukotriene production that is further accentuated by cyclooxygenase (COX)-1 inhibitor (aspirin or NSAIDs)[52-53]. The prevalence of N-ERD among patients with CRSwNP in a tertiary referral centre was 16% [54].

A specific phenotype in which asthma and CRSwNP co-occur in a triad, also called as Samter's triad, together with hypersensitivity to acetylsalicylic acid, is non-steroidal anti-inflammatory drug (NSAID) exacerbated respiratory disease (N-ERD). It may be considered a type 2 dominated inflammatory airway disorder. Patients with aspirin-exacerbated respiratory disease (AERD) had undergone two-fold more sinus surgeries ( $p < 0.001$ ) and were significantly younger at the time of their first surgery than were patients with CRSwNP without N-ERD. Diagnosis of N-ERD is mainly based on a patient history of at least



one documented reaction to aspirin or NSAIDs though it is recognized that history alone is not always reliable, especially as asthmatic patients are often warned to avoid these drugs [55]. Thus, aspirin provocation tests are needed when the history is not clear. Accordingly EPOS 2020 guideline at least one documented reaction to aspirin or NSAIDs is required to make the diagnosis of N-ERD though history alone is not always reliable. Nasal challenge with lysine aspirin is a good alternative to oral and bronchial methods, in terms of safety, sensitivity and specificity. Upper airway disease in N-ERD patients is usually CRSwNP. On average, upper respiratory symptoms are worse, extent of opacification on CT scan and recurrence of nasal polyps after surgery are more frequent in N-ERD than in NSAIDs tolerant CRSwNP patients [54, 56-57]. Oral aspirin treatment after desensitization (ATAD) has been shown to be significantly more effective and clinically relevant than placebo in improving quality of life and total nasal symptom score in patients with N-ERD. However, ATAD is associated with significant adverse effects, and the risks of not taking the medication strictly on a daily basis puts a burden on patient and caregiver. Based on these data, the EPOS2020 steering group suggests that ATAD can be a treatment for N-ERD patients with CRSwNP whenever there is confidence in the patient's compliance. Diets, like low salicylate diet were shown to improve endoscopic scores and may improve symptoms compared to a normal diet in patients with N-ERD [58]. However, the quality of the data at this moment is not enough to draw further conclusions.

Immunodeficiency and gastro-oesophageal reflux disease, nasal anatomical variations, bacteria and biofilms , fungal and viral infection have been suggested as a risk factor for developing CRS .

CRS can be manifested in several genetic diseases like primary ciliary dyskinesia (PCD), a rare and under-recognized genetic disease characterized by impaired mucociliary clearance , cystic fibrosis (CF) is a life-shortening genetic condition caused by a mutation in the cystic fibrosis transmembrane conductance regulator gene leading to defective chloride channels.[59-60].

Smoking also should be considered an important risk factor of CRS. The GA2LEN survey showed that current smoking and ex-smoking are significantly associated with CRS [61]. Tobacco is toxic to the nasal mucosa and cigarette smoking plays a significant role in diseases of the upper airway because pollutants and toxins in cigarette smoke are pro-

inflammatory and create oxidative stress of the mucosa, leading to symptoms such as nasal obstruction, increased nasal secretion and mucosal dryness. Recent systematic reviews found a strong correlation between active and passive cigarette smoke with the prevalence of CRS [62].

Recently air pollutants were shown to correlate with CRS symptom severity that may be influenced by exposure levels, with a more pronounced impact on CRSsNP patients [63].

Toxicants such as cigarette smoke, organic air pollutants, exogenous and endogenous compounds, allergens, and other environmental toxins have been shown to cause a persistent inflammatory response in the upper and lower airway epithelium. The significance of smoking as a risk factor for CRS has long been recognized . Passive smoking during childhood increases adult CRS . According to a study on the impact of second-hand smoking (SHS) on ROS generated in CRS patients [64]. SHS raises the levels of ROS in paranasal sinus tissues. ROS levels were found to differ between CRS subtypes.

### **1.8 Reactive oxygen species (ROS) and redox homeostasis**

The host's innate immunity protects against such stimuli by producing ROS and specific proteins expressed at the first airway barrier. In live cells, reactive oxygen species are continuously generated, for example, by xanthine oxidase to degrade purine nucleotides, by nitric oxide synthase to form nitric oxide, and by other biochemical reactions as a byproduct of the oxidative energy metabolism for the formation of adenosine triphosphate from glucose in mitochondria. Under normal physiological conditions, small amounts of oxygen are constantly converted into superoxide anions, hydrogen peroxide, and hydroxyl radicals. The biological activity of reactive oxygen species at a physiological concentration plays an important role in cell homeostasis and in a wide range of cellular parameters (proliferation, differentiation, cell cycle, and apoptosis) [65]. In the cell, reactive oxygen species arise under the influence of such exogenous prooxidant factors as environmental pollutants, ionizing and ultraviolet radiation, xenobiotics, air pollutants, and heavy metals [66-67]. The main endogenous sites of production of cellular redox-reactive compounds include complexes I and III of the mitochondrial electron transport chain, endoplasmic reticulum, peroxisomes, and such enzymes as membrane-bound nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX) isoforms 1–5 (NOX1–NOX5), complexes of

dual oxidases 1 and 2, xanthine oxidase, polyamine and amine oxidases, enzymes catabolizing lipids, and cytochrome P450 family 1 (CYP1A) [68-69].

Dual oxidase (DUOX1 and DUOX2), hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>)-producing isoforms of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, responsible for the formation of reactive oxygen species (ROS), has recently been found to be up-regulated in CRS patients. These two isoforms have been linked to inflammatory cytokines such as TNF-, MIG (Monokine Induced by Interferon Gamma), IL-8, and eotaxin [70]. NADPH oxidase isoforms such as NOX1 and NOX4, as well as cellular sources of superoxides, are mostly found in the epithelial layer, submucosal glands, vascular endothelium, and inflammatory cells in both healthy and allergic nasal mucosa and nasal polyps. According to a study conducted to investigate the influence of oxidative stress on the development of allergic rhinitis and nasal polyposis [71]. NOX1 and NOX4 play an important role in ROS production, leading to the oxidative stress found in allergic rhinitis and nasal polyposis.

The high reactivity of oxygen and its active species necessitates a multi-level antioxidant defense system that blocks the formation of highly active free radicals [67,72]. Free radicals are usually eliminated by the body's natural antioxidant system. Redox homeostasis in normal cells is maintained by a nonenzymatic system consisting of carotenoids, flavonoids, glutathione, anserine, carnosine, homocarnosine, melatonin, thioredoxin, and vitamins C and E, as well as a network of antioxidant enzymes such as superoxide dismutases, catalases, peroxiredoxins, glutathione peroxidase, glutaredoxins and paraoxonases. In redox homeostasis, a certain role is played by the enzymes of phase II xenobiotic biotransformation, e.g., NADPH:quinone oxidoreductase 1 (NQO1), glutathione-S-transferase P1, GSTA1/2, UDP glucuronosyltransferase 1A6, GPX4, and heme oxygenase. Hydroxyl radicals effectively oxidize unsaturated fatty acids, inducing lipid peroxidation, which is one of the most harmful effects of oxidative stress, causing plasma membrane damage [73].

### **1.9 Oxidative stress in CRS**

An imbalance between formation of oxidative free radicals and antioxidant defense capacity of the body's cells is defined as oxidative stress. Oxidative stress arises from oxidation-reduction (redox) homeostasis. When the balance between oxidants and antioxidants is

maintained in a steady-state redox balance, in which stress from oxidants does not outweigh antioxidants, it is of a reversible and physiological state and thus called “oxidative eustress”. Oxidative eustress is an important concept in redox control, and it acts as physiological redox signalling. Cellular injury as an effect of oxidative stress can be classified into three categories: damage to nucleic acids, proteins, or lipids .

The various effects of long-term uncontrolled oxidative stress are well understood to be linked to the onset of numerous inflammatory cascades that reflect the up-regulation of inflammatory cytokines via various molecular pathways and trigger an intrinsic apoptotic pathway, ultimately leading to caspase activation. Uncontrolled inflammatory responses cause severe cell and tissue damage, leading to tissue loss associated with chronic inflammation .

One of the oxygen-derived free radicals is peroxy radicals; it acts in the peroxidation of fatty acids. Free radicals trigger chain reactions of lipid peroxidation, generated lipid radical reacts with oxygen, and peroxy radicals are produced. Then, peroxy radical transforms polyunsaturated fatty acids into lipid hydroperoxides, which are unstable and disintegrated into unsaturated aldehydes or malondialdehydes (MDAs). MDAs are one of the commonly used oxidative stress markers and are capable of forming cross-linkages of proteins and thus inactivating them [74]. The lipid peroxidation counteracts cell membrane integrity itself, disrupting the membrane lipid bilayer and downregulating membrane receptors and enzymes.

Excessive production of ROS induces lipid peroxidation (LPO), especially of omega-6-polyunsaturated acids, which evolves as the autocatalytic process generates highly reactive aldehydes, notably 4-hydroxy-2-nonenal (HNE), MDA and acrolein [75]. The most intensively studied product of lipid peroxidation is HNE, which is considered to be “the second messenger of free radicals” that effects pathophysiology of cells in various ways. High concentrations of HNE are cytotoxic and mutagenic, while in physiological processes, present at low levels, HNE acts as a signaling molecule involved in growth regulation, interacting with cytokines and regulating the expression of cellular (proto)oncogenes [76-77]. The cytotoxic effects of reactive aldehydes resemble the toxicity of ROS, but because of their higher chemical stability, these aldehydes can spread from the site of origin and react

with major biomolecules, among which proteins appear to be the major target, even at the distant site, because of high reactivity with biomolecules and its multiple biological effects. HNE is one of the most abundant cytotoxic lipid-derived aldehydes, and its biological activity is proportional to intracellular concentration. Lower intracellular concentrations (2 M) have a positive effect on cell survival and proliferation. Higher concentrations (10 to 60 M) are genotoxic because they cause sister chromatid exchange, micronuclei formation, and DNA fragmentation [78]. HNE has been shown to consistently upregulate the expression of transcription factors such as Nuclear Factor Kappa B (NF- $\kappa$ B), which in turn regulates the expression of a variety of genes involved in cell proliferation and differentiation, such as protein kinase C and mitogen activated protein kinase. HNE, on the other hand, has been shown to inhibit the formation of NF- $\kappa$ B at higher concentrations, thereby exerting regulatory effects on inflammatory processes via intracellular concentration. HNE forms relatively stable and little metabolized protein adducts due to its high binding affinity for cysteine, histidine, and lysine [79]. As a result, even after a long period of time, peroxidation products as 4-HNE can be identified. Therefore, slow half-life of 4-HNE allows to using such marker as indicator of oxidative stress in biological materials after several years.

4-HNE is regarded as a major bioactive marker of lipid peroxidation in a variety of diseases. For example, it has been demonstrated that the levels of 4-HNE expression and NADPH oxidase isoform p67phox are significantly higher in nasal polyp tissue than in healthy mucosa [80].

Recently known another lipid peroxidation marker, altering membrane fluidity and yielding the formation of reactive aldehydes, such as acrolein. In the body, acrolein can also be formed during the catabolism of polyamines and amino acids or as a metabolite of some drugs, such as cyclophosphamide. Due to its electrophilic properties, acrolein can effectively bind and form adducts with DNA and proteins, altering their structure and function [81]. The evidence suggests that acrolein induces endothelial cell damage in vitro .

### **1.10 Aryl hydrocarbon receptor and signaling pathway into the cell**

An important function in the the regulation of oxidative stress is performed by AhR signaling pathway via prooxidant and antioxidant mechanisms. AhR is a ligand-activated transcription factor that has recently been identified as a key physiological regulator of

immune response, influencing both innate and adaptive immunity. AhR, its partner protein aryl hydrocarbon receptor nuclear translocator (ARNT) and AhR repressor protein (AhRR) are members of a family of structurally related transcription factors (basic helix–loop–helix (bHLH) motif-containing Per–ARNT–Sim (PAS), whose members carry out critical functions in the gene expression networks that underlie many physiological and developmental processes, especially those participating in responses to signals from the environment [82-83].

By functioning as a transcription factor, AhR takes part in many physiological and pathological processes in cells and tissues. Numerous studies have shown that AhR is activated by many natural and synthetic ligands, which may or may not be planar molecules of the polycyclic aromatic hydrocarbon type [84-85]. In this context, AhR acts as a sensor that connects the external environment and internal environment. AhR participates in processes of development, immune defense, and homeostasis, including cell differentiation and physiological processes in stem cells. AhR exerts this action by regulating fundamental metabolic processes that modulate cell proliferation, cell cycle, cell differentiation and phenotype formation, and cell adhesion and migration . AhR is activated by a wide range of ligands , which can be categorized into endogenous ligands and exogenous ones [86-88]. Among the exogenous AhR ligands, halogenated aromatic hydrocarbons are typical, including dioxins (such as TCDD), polychlorinated biphenyls, and polycyclic aromatic hydrocarbons such as benzo[a]pyrene (BaP) and 3-methylcholanthrene. AhR also binds to a number of drugs such as omeprazole and to compounds present in foods, such as plant polyphenols and flavonoids (e.g., quercetin) .Growing interest in the physiological functions of AhR has led to the identification of many endogenous ligands of AhR . These include heme metabolites bilirubin and biliverdin , tetrapyrroles , arachidonic acid metabolites , tryptophan metabolites such as kynurenic acid and kynurenine , 6-formylindolo[3,2-b]carbazole (FICZ) (which is a photoproduct of the ultraviolet irradiation of L-tryptophan ), indolo[3,2- b]carbazole and estrogen equilenin . Compounds secreted by bacteria can also be AhR ligands . AhR ligands can serve as either agonists or antagonists of the transcription of AhR controlled genes, depending on various conditions in the cell. In different cell types, there are diverse scenarios of gene activation in response to AhR stimulation. Different AhR ligands can induce dissimilar transcriptome profiles within the same cell type, and the same

AhR ligand can give rise to different transcriptome profiles in different cell types [89-90]. The AhR signaling pathway involves both classic (canonical) and non-classic (noncanonical) signal transduction mechanisms [90-91].

The classic (canonical) pathway of xenobiotic metabolism was the first-studied molecular mechanism of AhR action, and adherence to this paradigm has greatly delayed the understanding of the global biological significance of AhR. Under physiological conditions, AhR is localized to the cytosol and forms a complex with specific chaperone proteins, such as hepatitis B virus X-associated protein 2 (XAP2, also known as AIP or ARA9), p23, and hsp90 [92]. Ligand binding results in a conformational change that causes AhR to disassociate from the above complex, and then the ligand–AhR complex is translocated from the cytosol to the nucleus [93]. In the classic mechanism of transcriptional regulation, the complex of AhR with its ligand heterodimerizes with ARNT and binds to xenobiotic-responsive elements in DNA upstream of AhR's inducible target genes. The AhR–ARNT complex initiates the transcription of several genes, including cytochrome P450 family 1 subfamily A member 1 (CYP1A1) and subfamily B member 1 (CYP1B1), and this action has a wide range of physiological and toxic effects understanding of the global biological significance of AhR.

### **1.11 The link between NADPH oxidase and AhR**

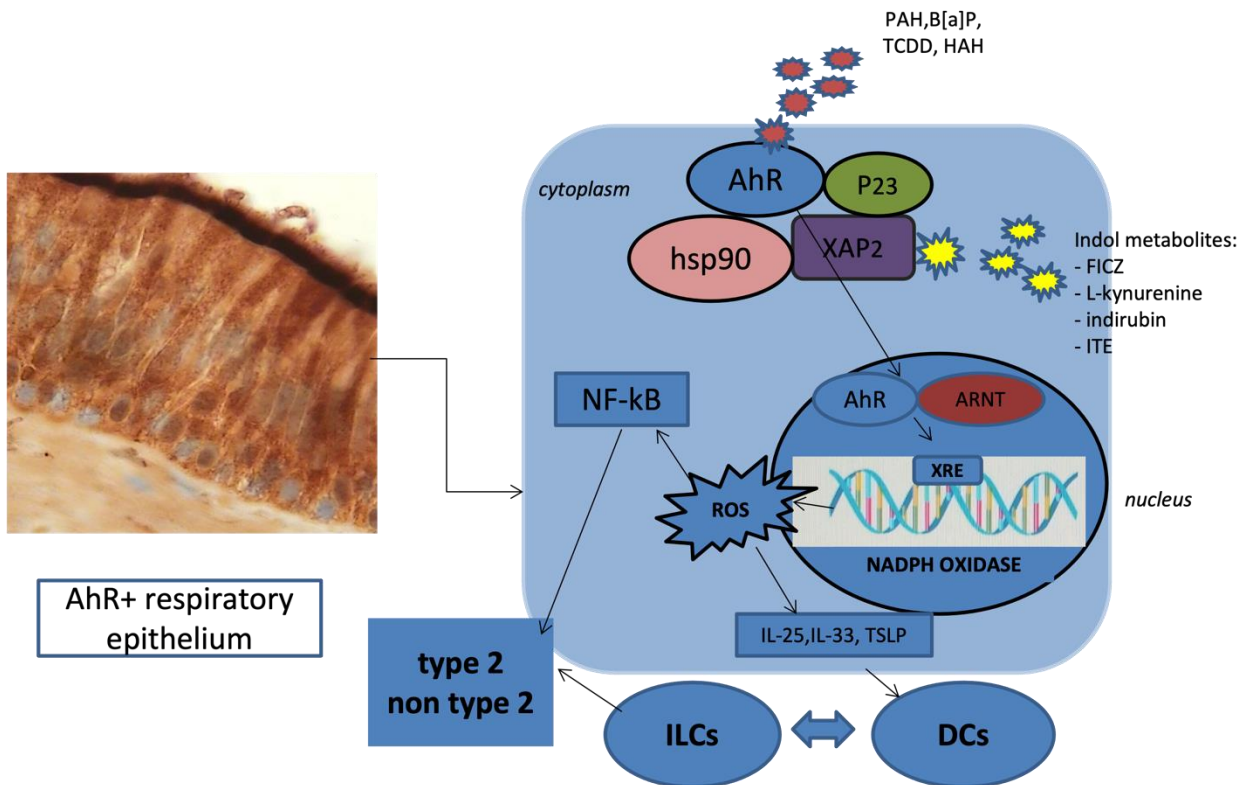
There is evidence that polycyclic aromatic hydrocarbons can stimulate the production of reactive oxygen species via NADPH oxidases, in particular NOX2 and NOX4 [94].

These membrane-bound enzyme complexes are detectable in the plasma membrane of various cell types, such as phagocytes and endothelial and epithelial cells . In the inactive state, NADPH oxidase subunits—three cytoplasmic (Rac1, p47phox, and p67phox) and two intramembrane ones (p22phox and gp91phox)—are not assembled . After activation by cytokines, by opsonized bacteria, by bacterial lipopolysaccharides, or by other stimuli, the complex assembles and the catalytic subunit, i.e., the heterodimeric flavocytochrome composed of gp91phox and p22phox, and transfers one electron from NADPH to molecular oxygen, thus yielding superoxide anions, which are next dismuted

into hydrogen peroxide [95]. Additional proteins, such as p40phox (one of NADPH oxidase subunits), play an important part in the regulation of NADPH oxidase activity and in the subsequent production of reactive oxygen species .

According to the literature, there are several mechanisms of NADPH oxidase activation through the AhR signaling pathway. For example, the NOX2-mediated formation of reactive oxygen species in epidermal keratinocytes under the action of a polycyclic aromatic hydrocarbon is mediated in an AhR-dependent way by the stimulation of the phosphorylation of p47phox (neutrophil cytosolic factor 1), which is necessary for the assembly of the NOX2 complex on the plasma membrane. Another mode of NADPH oxidase activation in human and rat macrophages involves the increased transcription of p47phox because of the direct binding to XRE in the promoter region of this gene after treatment with BaP. In addition, BaP promotes the translocation of the p47phox protein to the macrophage plasma membrane and strengthens the production of superoxide anion under the influence of phorbol myristate acetate [96,97]. We assume that enzyme systems generating ROS could be activated by same AhR pathway in respiratory epithelium. After influence of AhR exogenous or endogenous ligands, AhR translocates into nucleus and binds with ARNT. AhR-ARNT complex binds with promoter XRE of NADPH oxidase gene. Therefore, generated ROS could further activate NF-kB transcription factor responsible for cell proliferation, differentiation and innate immunity's cells. In **Figure 1** illustrated NADPH oxidase activation mechanism on cellular level through AhR signaling pathway. Microscopic image of respiratory epithelium taken with permission from healthy patient participated in the study. Illustration made by the candidate of PhD thesis.





**Figure 1.** NADPH oxidase activation mechanism through AhR signaling pathway in respiratory epithelium. Microscopic image of respiratory epithelium taken from healthy patient participated in the study.

AhR-aryl hydrocarbon receptor, hsp90, p23, XAP<sub>2</sub> -chaperone proteins, ARNT- aryl hydrocarbon nuclear translocator, XRE- xenobiotic response element, NF - k $\beta$  nuclear factor kappa B, ROS- reactive oxygen species, DCs-dendritic cells, ILCs-innate lymphoid cells, PAH-polycyclic aromatic hydrocarbons, TCDD-2,3,7,8 tetrachlorodi benzo -p-dioxin, HAH-halogenated aromatic hydrocarbons, FICZ- 6-formylindolo[3,2-b]carbazole type 2, ITE- 2-(1' H-indole-3'-carbonyl)-thiazole-4-carboxylic acid methyl ester, type 2 immune response and non type 2 immune response, TSLP -thymic stromal lymphopietin.

The activation of AhR by TCDD has been found to induce the expression and activity of COX2 [98]. Unlike COX1 expression, the expression of COX2 can be induced by various stimuli, such as growth factors and cytokines. The upregulation of COX2 has been implicated in chronic inflammation and carcinogenesis. The overexpression of COX2 may enhance the production of reactive oxygen species.

### **1.12 The role of AhR in pathogenesis of CRS**

The AhR signaling pathway has recently received a lot of attention because it is a critical link between environmental stimuli and immune-mediated inflammatory diseases. AhR promotes or facilitates the expression of ILs by T-helper (Th) cells. A recent study in Chinese patients with CRSwNPs discovered that AhR plays an important role in CRS pathogenesis and increases the likelihood of suppressing Th17 cell differentiation, which is otherwise required for Th17 trans-differentiation into regulatory T-cells that help with inflammation resolution [99]. Studies on Th17 cells, human Th22 cells, CD8+ T cells, and NK-22 cells that influence host defense have shown that AhR expression is required for the production of the cytokine IL-22. The synergistic activity of IL-17 and IL-22 plays a protective role in the maintenance of host defense. Low levels of the transmembrane receptor complex IL-22 receptor1 (IL-22R1) and low expression of AhR in nasal polyp tissues may result in low expression of IL-22 in CRSwNP patients particularly in recalcitrant cases [100]. According to a recent study, AhR activation increases mucin production by lower airway epithelial cells, which is induced in part by ROS nascence. Furthermore, an AhR antagonist and an antioxidant reduced mucin formation, implying that antioxidant therapy may be beneficial in AhR-induced hypersecretion diseases of the respiratory tract [101]. Based on a well-established relationship between the AhR, ROS, mucin formation, and protective cytokines, the AhR may be one of the major regulators of local host immunity, shielding the airway mucosa from unfavorable environmental influences. Despite its important role in the host's innate and adaptive immunity, which allows the airway epithelium to fight through Th-cell differentiation, and its role in the regulation of protective cytokines as a detecting sensor of environmental agents, it is still unclear whether AhR regulates ROS production in the sinus mucosa of various CRS subtypes.

## **2. HYPOTHESIS**

We hypothesize that the aryl hydrocarbon receptor signaling pathway regulates reactive oxygen species production in the paranasal sinus mucosa in various subtypes of chronic rhinosinusitis.

### 3. AIMS

#### *General aim*

The purpose of this research is to investigate the role of the aryl hydrocarbon receptor (AhR) in regulating the expression of reactive oxygen species (ROS) and its impact to the inflammatory response in specific subtypes of chronic rhinosinusitis (CRS).

#### *Specific aims*

1. To assess the general oxidative stress level in the paranasal sinus mucosa of the different CRS subtypes;
2. To assess the AhR protein expression in different CRS subtypes;
3. To determine in an experimental *in vitro* model the levels of oxidative stress in human primary sinonasal epithelial cells isolated from tissue samples of the patients with CRS without nasal polyps, CRSsNPs and control study group and ligand which stimulate AhR and NADPH oxidase isoforms expression indicating the inhibition of the receptor;
4. To assess the association between AhR expression, NADPH oxidase activity and its isoforms in the different CRS subtypes, with regard to smoking status and atopy;
5. To assess the impact of AhR expression on inflammatory cytokines in peripheral blood mononuclear cells (PMBC) in patients with CRS, in particular with regard to subgroups of patients classified according to levels of cytokine expression.

## **4. MATERIALS and METHODOLOGY**

Between April 2017 and June 2020, a case-control study was conducted in the Clinical Hospital Zagreb's Department of Ear, Nose and Throat and Head and Neck Surgery, Zagreb, Croatia. The Institutional Review Board approved the study.

### **4.1. Subjects**

The study enrolled a total of 57 patients with CRS who were scheduled for endoscopic sinus surgery. Patients with cystic fibrosis, antrochoanal polyps, and primary ciliary dyskinesia who had taken oral corticosteroids in the previous three months were excluded from the study. Each eligible patient signed an informed consent form to participate in the study and provide tissue, blood samples, and nasal secretion.

Indication for endoscopic rhinosurgical operations of CRS patients participated in study based on assessment of current clinical control. CRS patients participated in the study were uncontrolled to conservative therapy (presented symptoms, presented sleep disturbance, obstruction of osteomeatal complex on nasal endoscopy despite on taken intranasal/oral corticosteroid therapy, extent of opacification on CT scan and recurrence of nasal polyps after surgery).

Intraoperatively, ethmoid and sphenoid sinus mucosa samples were obtained from 12 patients with CRSwNPs, 12 patients with CRSsNP, and 12 patients operated for reasons other than CRS, who served as control cases. The pathomorphological features of chronic inflammation of paranasal sinus mucosa corresponded to CRS phenotype. Macroscopic view of tissue samples obtained from CRSwNPs patients mainly had oedematous type, yellowish color, some of samples was oedematous with signs of exacerbation of chronic inflammation. Tissue samples of CRSsNPs morphologically presented as thickened mucosa without signs of oedema, reddish colour.

The patients were operated in the Clinical Hospital Center Zagreb's Department of Ear, Nose and Throat and Head and Neck Surgery. The 36 patients' tissue and serum samples were analysed at the Ruđer Bošković Institute's Department of Molecular Medicine.

Ex vivo experiment was carried out at the Department for Healthcare Medimurje's Laboratory for Microbiology, on fresh mucosa samples retrieved intraoperatively during

endoscopic rhinosinus surgery from 21 patients: seven from patients with CRSsNPs, seven from patients with CRSwNPs, and seven from CRS-free patients. Nasal secretion was obtained from each patient prior to surgery. The patients were operated in the County Hospital Čakovec's Department of Otorhinolaryngology. The patients were matched in terms of age and gender. Patients were given a questionnaire upon recruitment to assess sociodemographic data, allergy and smoking history, prior sinus surgery, sensitivity to non-steroidal anti-inflammatory drugs (NSAIDs), and co-morbidities.

## 4.2. Molecular analysis

All analyses were carried out at the Ruđer Bošković Institute's Department of Molecular Medicine, Laboratory of Oxidative Stress, in Zagreb, Croatia.

In the Laboratory of Oxidative stress under mentorship of Prof. Neven Žarković we tried to assess on test fresh blood samples the overall levels of oxidative stress in the monocytic cell line from peripheral blood samples of chronic rhinosinusitis patients treated with an AhR agonist/inhibitor analysis accordingly to protocol published by Almeida MC and et al. [102]. Obtained test fresh venous blood samples in volume 20 ml each transferred to Laboratory. First, we used a Ficoll-Hypaque gradient (density = 1.070 g/ml) and afterwards a slight hyperosmolar Percoll gradient (density = 1.064 g/ml). Percoll solutions were done as follows: first an isosmotic Percoll was prepared as usually mixing one volume NaCl 1.5 M with nine volumes of Percoll (Pharmacia, density = 1.130 g/ml). The Percoll gradient was done mixing 1:1 (v/v) isosmotic Percoll with PBS/ Citrate (NaH<sub>2</sub>PO<sub>4</sub> 1.49 mM; Na<sub>2</sub>HPO<sub>4</sub> 9.15 mM; NaCl 139.97 mM; C<sub>6</sub>H<sub>5</sub>Na<sub>3</sub>O<sub>7</sub> 2H<sub>2</sub>O 13mM; pH 7.2). Both gradients were centrifuged at 25-35°C, 400 g for 35 min. Monocyte functional assay after Percoil gradient has done to asses functionality of monocytes: 10<sup>6</sup> monocytes per ml were cultivated in RPMI medium plus 2mM L glutamine with 10% human blood serum. TNF- $\alpha$  production after LPS (10 ng/ml) stimulation was 0, 265pg/ml (mean amount 1,019 pg/ml accordingly protocol) that confirmed weak functionality of isolated monocytes. By this reason we could not perform next step of analysis. Prof. Neven Žarković proposed measuring total oxidative serum capacity and total antioxidative serum capacity across groups as a surrogate measure of general oxidative stress, as well as assessing the expression of stable lipid peroxidation markers (e.g. 4-hydroxynonenal (HNE) and acrolein) in tissue samples. The ELISA assay for

measuring 4-HNE in serum samples provides a reliable estimate of the level of oxidative stress. Because reactive oxygen species are unstable molecules that degrade quickly, determining their levels is difficult. Lipid peroxidation markers, on the other hand, are stable and easily detectable in tissues even after 10 years using standard methods.

#### **4.2.1. Total oxidative serum capacity measurement**

The total oxidative capacity (TOC) assay relied on peroxidase converting 3,5,3',5'-tetramethylbenzidine (TMB) to colored radical cations in the presence of peroxides [103-104].

It was used to calculate total peroxides in mol/L. For the quantitative determination of peroxides in biological fluids, a colorimetric/photometric fast test was used. The reaction between peroxides and peroxidase was followed by a color reaction of the chromogenic substrate tetramethylbenzidine (TMB). After adding the stop solution, the blue solution turns yellow, and a photometric measurement at 450 nm is taken. Serial dilutions of a standard peroxide solution were used to quantify. 10 µl of standards/controls/samples were pipetted into each well, and 200 µl of reaction mixture was added within 1 minute. The optical density (OD) was determined at 450 nm (because the buffer can precipitate plasma/serum proteins). After incubating the plate for 20 minutes, 50 µl of the stop solution was added. Before measuring, the plate was left in place for 2-3 minutes and gently shaken to evenly distribute the color within the well. A second OD measurement at 450 nm was taken.

#### **4.2.2. Determination of total antioxidative serum capacity**

The hydroxyl radical produced by the Fenton reaction using a ferrous ion solution and hydrogen peroxide ( $H_2O_2$ ) was used to calculate total antioxidant capacity (TAC) [103,104]. The produced reacts with the o-dianisidine molecule to produce dianisidyl radicals. Serial dilutions of a standard antioxidant-solution were used to quantify it. Within 1 minute, 25 µl of standards/controls/samples were pipetted into each well, and 200 µl of Reaction mixture A was added. After 3 minutes, the OD at 450 nm was measured (this is done because the buffer can precipitate plasma/serum proteins). Within 1 minute, 50 µl of

Reaction mixture B was added. After incubating the plate for 15 minutes, 50 µl of the stop solution was added. Before measuring, the plate was left in place for 2-3 minutes and gently shaken to evenly distribute the color within the well. A second OD measurement at 450 nm was taken.

#### **4.2.3. The oxidative stress-induced lipid peroxidation marker 4-hydroxynonenal was measured (4-HNE)**

4-HNE levels were determined in formalin fixed tissue samples from patients with CRSwNPs, CRSsNPs, and the control group using immunohistochemistry and a monoclonal antibody according to the manufacturer's instructions (Abcam, USA) [105]. In the procedure, the EnVision-HRP method was used. Fresh tissue sample obtained intraoperatively, fixed in formalin and embedded to paraffin blocks. The tissue samples were deparaffinized in alcohol, then methanol was added over 3 minutes, and the slides were rinsed three times for 5 minutes each with Tris-Buffered Saline + Tween (TBST). Overnight, the Anti-HNE (monoclonal antibody) was diluted 1:10 in 1% bovine serum albumin (BSA) (in TBS solution). The slides were rinsed three times in TBST solution for five minutes each and once in a 3 percent H<sub>2</sub>O<sub>2</sub> solution for twenty minutes in the dark. The slides were rinsed three times in a TBST solution for five minutes each, then incubated for 30 minutes in EN VISION (Dako EnVision+System-HRP, Labelled Polymer Anti Mouse), then rinsed three times in a TBST solution. The slides were rinsed three times with running distilled water and Hemalaun before being dehydrated and blocked. The blocks were cut into 4µm thickness and examined under optical microscope Olympus BX51 at 10x/200x/400x/1000x amplification . The inflammatory cells were identified using microscopical images and morphological features.

#### **4.2.4. Acrolein, an oxidative stress-induced peroxidation marker was measured**

Acrolein levels were measured in formalin fixed tissue samples by immunohistochemistry using a rabbit polyclonal antibody in accordance with the manufacturer's instructions (Abcam, USA) [106]. EnVision-HRP method were used in the procedure. Fresh tissue sample obtained intraoperatively, fixed in formalin and embedded to paraffin blocks The tissue samples were deparaffinized to alcohol, then methanol was added over 3 min, and then



slides were rinsed 3 times with TBST for 5 minutes each. The Anti-ACR (monoclonal antibody) is diluted 1:40 in 1% BSA (in TBS solution) over night. The slides were rinsed 3 times in TBST solution 3 times for 5 minutes each and 3% H<sub>2</sub>O<sub>2</sub> solution for 20 minutes in dark. The Anti-HNE (monoclonal antibody) was diluted 1:10 in 1% bovine serum albumin (BSA) (in TBS solution) over night. The slides were rinsed 3 times for five minutes each in a TBST solution and then incubated for 30 minutes in *EN VISION* (Dako EnVision+System-HRP, Labelled Polymer Anti Mouse), then rinsed once again 3 times for five minutes each in a TBST solution. The DAB (DakoLiquid DAB+Substrate Chromogen System) chromogen kit was used for immunohistochemistry detection. The slides were rinsed three times in running distilled water and Hemalaun and then the samples were dehydrated and blocked. The blocks were cut in 4µm cuts and reviewed under optical microscope Olympus BX51.

#### **4.2.5. Determination of the lipid peroxidation marker 4-hydroxynonenal (4-HNE) in serum samples.**

The serum levels of 4-hydroxynonenal were determined using the ELISA assay [107-108]. To 10 µl of standard/sample, 100 µl of carbonate buffer (0.05M carbonate binding buffer (pH 9.6)) was added first. The samples were rinsed with 200 µl Phosphate Buffer Saline (PBS) and 200 µl blocking solution (5 percent blotto; 5 percent fat-free dry milk in carbonate binding buffer) solution after 4-5 hours at +4°C. Following that, the samples were rinsed in wash buffer (0.1 percent Tween 20 in PBS) and 100 µl 1°Ab on HNE (1:100) in 1 percent BSA in PBS on +4°C monoclonal mouse anti-HNE-His antibody in cell culture supernatant were added (clone HNE1g4). The samples were rinsed five times in 200 µl wash buffer (0.1 percent Tween 20 in PBS and 200 µl peroxidase blocker) within 30 minutes on the first day. Then, in 1 ml of solution, 50 µl H<sub>2</sub>O<sub>2</sub> was added (example: 1 plate = 20 ml + 1 ml H<sub>2</sub>O<sub>2</sub>). During 1 hour at room temperature, plates were rinsed five times with 200 µl wash buffer (0.1 percent Tween 20 in PBS) and 100 µl of 2°Ab anti-mouse (Dako EnVision™ System-HRP Labeled Polymer anti-mouse) 1:100 in 1 percent BSA in PBS. Plates were then rinsed five times in 200 µl wash buffer (0.1 percent Tween 20 in PBS and washed 1x 200 µl citric buffer), followed by 30 minutes in 100 µl TMB solution (0.05 mg/ml) (for 1 plate: 10 ml citric buffer + 100 µl TMB stock + 4 µl H<sub>2</sub>O<sub>2</sub>). Finally, a stop solution (50 µl 2M H<sub>2</sub>SO<sub>4</sub>) was added, and the optical density (OD) was measured at 450/620 nm. The protein

concentration of standards was measured at 10 mg/ml on the second day (HNE-BSA standards range from 0 to 250 pmol of HNE-protein adducts/mg of proteins (0; 3,75; 7,5; 15; 30; 60; 120; 250 pmol/mg). The protein concentration in serum was first determined using the Bradford method and adjusted to 10 mg/ml. All analyses were carried out in triplicate or quadruplicate, and the amounts of HNE-protein adducts determined by ELISA were expressed as pmol HNE/mg of proteins. To remove sample background values, one well of each sample was incubated in PBS with 1 percent BSA (without primary antibody).

### **4.3. Evaluation of the aryl hydrocarbon receptor (AhR) protein expression**

Fresh tissue samples obtained during surgery were fixed in formalin and used to identify AhR protein expression via immunohistochemistry using rabbit polyclonal AhR antibodies as directed by the manufacturer (Antibodies, USA) [109]. Tissue samples fixed in formalin embedded to paraffin blocks. Later samples were deparaffinized in alcohol, then methanol was added over 3 minutes, and the slides were rinsed three times with TBST for 5 minutes each, followed 10% Normal Goat Serum in 1% BSA., then Anti-AhR (polyclonal antibody) diluted 1:500 in 1% BSA, rinsed three times in TBST for five minutes each, then washed in a 3 percent hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) solution for 20 minutes in the dark, and three times in TBST for five minutes each. DakoLiquid DAB+Substrate Chromogen System (DAB) was used until the solution turned brown. After rinsing the plates with distilled water and Hemalaun, the samples were dehydrated and blocked.

#### **4.3.1 4-hydroxynonenal (4-HNE), acrolein (ACR), and aryl hydrocarbon receptor (AhR) expression quantification**

The evaluation was based on the intensity and number of positive cells in 1 mm<sup>3</sup> of tissue under optical microscope Olympus BX51 and was graded as follows: *0 - negative positivity and intensity; I (0-30 percent) - weak positivity and intensity; II (30-50 percent) - intermediate positivity and intensity; and III (> 50 percent) - high positivity and intensity.*

#### **4.4. Ex vivo tissue stimulation model experimental study**

In Upper Airway Research Laboratory (URL), University hospital Ghent, Belgium in 2018 under mentorship of Prof. Claus Bachert we tried on test fresh tissue CRS samples to isolate human sinonasal epithelial cells (SNEC) and further to treat with AhR agonist/antagonist and to assess expression of ROS in cell supernatants using protocol published in study of Soyka MB et al. [110].

Fresh tissue test samples obtained intraoperatively from CRS patients immediately transferred into 0,9%-saline solution and put on ice. Tissue cut into small pieces and trypsinized for 3 h at 37°C (95% O<sub>2</sub>, 5% CO<sub>2</sub>) (Trypsin/ EDTA 0,05%; Lonza, USA), following which trypsin neutralised using the trypsin-neutralising solution (TNS) (Lonza). In all *ex vivo* studies, passage 3–4 cell lines used, seeded into 6 wells and grown up to 90% confluence point prior to the stimulation. The purity of SNECs verified based on the content using confocal microscopy. Under confocal microscope we verified in all test samples contamination of cytokeratin and vimentin. This evidence confirmed presence of other cell types as fibroblasts together with SNECs. We could not perform further analysis due to failure in isolation of SNES line. By this reason an *ex vivo* model of nasal tissue stimulation was proposed by the staff of the collaborative URL instead of cell stimulation model. Experimental study carried out at the Laboratory for Microbiology, Department for Healthcare Medimurje, Čakovec, Croatia, under the mentorship of Prof. Claus Bachert using modified nasal tissue stimulation protocol designed and approved by staff of URL [111].

During endoscopic endonasal sinus surgery, fresh tissue samples were collected. Seven samples of ethmoid and maxillary sinus mucosa from CRSwNP patients, seven samples of maxillary sinus mucosa from CRSsNP patients, and seven samples of ethmoid sinus mucosa from healthy patients were obtained. Tissue culture was performed using *ex vivo* tissue samples. Samples were immediately immersed in a 1:1 mixture of Dulbecco's modified medium and F-12 medium, supplemented with 100 U/mL penicillin, 100 g/mL streptomycin, and 0.25 g/mL amphotericin B (Sigma Aldrich, USA), and processed at room temperature within 2 hours. The specimens were rinsed in PBS and cut into 3-4 mm<sup>3</sup> pieces. The stock solution was made by dissolving the Aryl hydrocarbon receptor agonist 2-(1H-Indol-3-ylcarbonyl)-4-thiazolecarboxylic acid methyl ester (ITE) (MedChemExpress, USA) and antagonist 6,2',4'- trimethoxyflavone (TMF) (Sigma Aldrich, USA) in Dimethyl

Sulfoxide (DMSO) according to the manufacturer's protocol. To make a 1mM ITE concentration, 1 mg of ITE was dissolved in 3.49 ml of DMSO. 1mg of ITE was dissolved in 0.7 ml DMSO solvent to make a 5 mM ITE. 1mg TMF was dissolved in 12.81 ml DMSO to make a 0.25 mM TMF solution, and 1mg TMF was dissolved in 6.4 ml DMSO to make a 0.5 mM TMF solution. Stock solutions were prepared, aliquoted, and stored at -20°C in tightly sealed vials for one month. To prepare the working solution, each 0.40 µl ITE and TMF solution was mixed into a 1 ml bronchial epithelial basal medium (Lonza, Switzerland) in a 24-well plate after warming in a watery bath at 37°C. Tissue cultures were placed in 24-well plates of prepared medium and incubated for 24 hours at 37°C, ( 5% CO<sub>2</sub>, and 95% O<sub>2</sub> ). The working solution did not completely cover the surface of the tissue pieces in each 24 well plate, air-liquid surface preserved. Following incubation, tissue supernatants were collected, centrifuged at 1300 rpm for 5 minutes, and the stimulated tissue specimens was stored at -70°C until analysis.

#### **4.5. Quantitative Real-Time Reverse Transcription-PCR (RT-qPCR) analysis of NADPH oxidase 1 mRNA expression in tissue culture treated with an AhR agonist/inhibitor**

Due to failure in isolation of SNES line from fresh CRS tissue samples , we could not further to assess concentration of NADPH oxidase isoforms by ELISA assay described in proposal of PHD thesis and ROS production in contaminated cell line. Instead of we measured mRNA expression of NADPH oxidase 1 using RT-qPCR analysis in tissue samples treated by AhR ITE ( agonist) and AhR TMF ( antagonist) accordingly to guideline published by Diebold BA et al. [112]. In study of Umekawa T et al. demonstrated comparable results of mRNA expression and protein production of osteopontin and monocyte chemoattractant using RT PCR and ELISA assay [113].

The expression of mRNA NADPH oxidase 1 in treated tissue supernatants was measured as a surrogate marker. Stimulated tissue samples were transferred to the Department of Medical Biology's Laboratory at Zagreb University School of Medicine for real-time qPCR analysis. RT-qPCR was used to examine the expression of NADPH oxidase 1 (NOX1) in mRNA-treated tissue samples. RNA was extracted using a total RNA Extraction Kit (BioTeKe Corporation, China) and reverse transcribed to cDNA using a Prime Script RT Reagent Kit

(TaKaRa, China) as directed by the manufacturer. The RT-qPCR was carried out on an iCycler (Bio-Rad, Hercules, CA, USA) with a SYBR green Real-time PCR Premixture Kit (BioTeKe Corporation, China). The expression of NADPH oxidase 1 mRNA was normalized to that of -actin mRNA. The following primers were used for RT-qPCR: NADPH oxidase 1: 5'-TACTCCACTTCAGCCACCATC-3', reverse: 5'-TATGGGACTCGGCACAATAAA-3'; -actin: 5'-TGACGTGGACATCCGCAAAG-3', reverse: 5'-CTGGAAGGTGGACAGCGAGG-3'. The NOX1 mRNA expression level was determined using the 2 Ct cycle threshold method.

#### **4.6. Cytokine expression in nasal secretion analysis**

Sampling of nasal mucus taken from same patients participated in ex vivo experimental study prior surgery. Nasal secretion obtained from seven CRSwNPs patients, seven CRSSNPs patients and seven from healthy patients.

Using nasal secretion as biological material, we assessed cytokine expression. Tyurin et al. [114] developed a method for obtaining nasal secretion from each patient who participated in the experimental study. Nasal secretion was absorbed for 10 minutes with a cotton swab in the middle nasal concha, transferred to an Eppendorf tube, diluted 1:4 with 0.25 ml of 0.9 percent physiological saline, centrifuged at 1500 rpm for 10 minutes to precipitate cellular elements, and the supernatant was collected and frozen at -70°C. Nasal secretion was tested for IL-4, IL-5, IL-22, and IFN- $\gamma$  using a Bio Cytokine assay or an ELISA in the Molecular Biology Laboratory Labena in Ljubljana, Slovenia.

## 4.7. Statistical analysis

The mean SD of continuous variables was used to summarize them, while the median SD of categorical variables was used to summarize them (range). Pearson's chi-square test of independence was used as a non-parametric statistical procedure, with contingency analysis as an extension for categorical variables. The Mann-Whitney U (Wilcoxon test for independent samples with Wilcoxon each par post-hoc test) was used to compare means between two groups. The nonparametric Kruskal-Wallis test was used to compare means between groups as a generalization of the Wilcoxon (or Mann-Whitney U) test for factors with more than two levels followed by the Steel-Dwass test, which is the nonparametric equivalent of the Tukey HSD, as a post-hoc test. This test is more suitable than proposed Friedman test as we do not have repeated measures. The serum 4-HNE levels were set as the outcome, and the analysis parameters were set as follows: control mean = 9.87.7; experimental group increase 100 percent;  $\alpha=0.05$ ;  $\beta=0.2$ ; power=0.8. The number of subjects required for adequate study power was determined to be ten per group. Alternatively, 4-HNE positivity was chosen as the outcome, with the following analysis parameters: incidence of high and medium positivity 86 percent (12/14); 60 percent decrease in incidence in the control group;  $\alpha=0.05$ ;  $\beta=0.2$ ; power=0.8; and a minimum number of patients per group of 13. JMP 16.0.0 Pro software was used to analysis the data (SAS Institute Inc, Cary, NC).

## 5. RESULTS

### 5.1. Patient characteristics

Endoscopic sinus surgery was performed on 57 patients: 12 with CRS with nasal polyps (CRSwNPs), 12 with CRS without nasal polyps (CRSsNPs), 12 healthy controls, and 21 patients who participated in the ex vivo experimental study.

Between January 2017 and December 2018, patients were recruited in the Cinical Hospital Zagreb's Department of Ear, Nose and Throat and Head and Neck Surgery. Twelve patients with chronic rhinosinusitis with nasal polyps (CRSwNP) were included in the study; their average age was 55 years (range 24-73 years); four were women and eight were men. Following that, 12 patients with chronic rhinosinusitis without polyps (CRSsNP) were included, with a mean age of 37 years (range 14-57 years), eight women and four men. Finally, 12 healthy patients were used as controls, with a mean age of 27 years (range 13-46 years), five women and seven men.

Eight of the CRSwNPs cases had atopic status ; two cases had a positive skin prick test, eight had a history of bronchial asthma, and five had a positive allergy test to non steroidal anti-inflammatory drugs (NSAIDs). In CRSsNPs group were 4 cases of atopic status; four of the CRSsNPs patients had a positive skin prick test, one had atopic dermatitis, four had bronchial asthma and one had NSAID allergy.

In the CRSwNP group only , we found five patients with Samter's triad. Recurrent nasal polyposis despite multiple paranasal sinus surgeries characterizes the recalcitrant subset of CRS. In our study, five patients in the CRSwNPs group underwent more than two (range 2-13) paranasal sinus surgeries, ranging from traditional polypectomy to endoscopic endonasal sinus surgery (FESS) to treat obstructive nasal polyposis. In CRSsNPs group five patients had recalcitrant status.

Other immune pathology, such as disseminated focal encephalitis and urticaria of unknown etiology, was seen only in two patients in the CRSwNPs group.

All patients in CRSwNPs group had diffuse CRS with extent opacification on CT scan. In CRSsNPs group three patients had fungal CRS, and the rest had diffuse CRS. In CRSwNPs group one patient was long-term smoker, in CRSsNPs group four were long-term smokers.

None of the patients in the control group had a history of allergies, bronchial asthma, an NSAID allergy. Five patients in control group were operated on for a unilateral paranasal sinus cyst, two for a mucoencephalocele, and six for a bullous medial nasal concha. Tissue samples in this group were extracted from a healthy paranasal sinus. Three of the patients had been smoking for a long time. **Table 1** summarizes the patient characteristics of the three study groups.

All patients who took part in the study had not used oral corticosteroids for at least three months prior to the surgery.

**Table 1.** *Clinical characteristics of patients with chronic rhinosinusitis with nasal polyps (CRSwNP), chronic rhinosinusitis without nasal polyps (CRSsNP) and SIN-healthy (controls)*

	CRSwNP (n=12)	CRSsNP (n=12)	SIN (n=12)
Mean age (years)	55 (24-73)	37 (14-57)	27 (13-46)
Sex (female:male)	4:8	8:4	5:7
Atopic patients	8	4	0
Samter's triad	5	0	0
Recalcitrant patients	5	5	0
Smokers	1	4	3

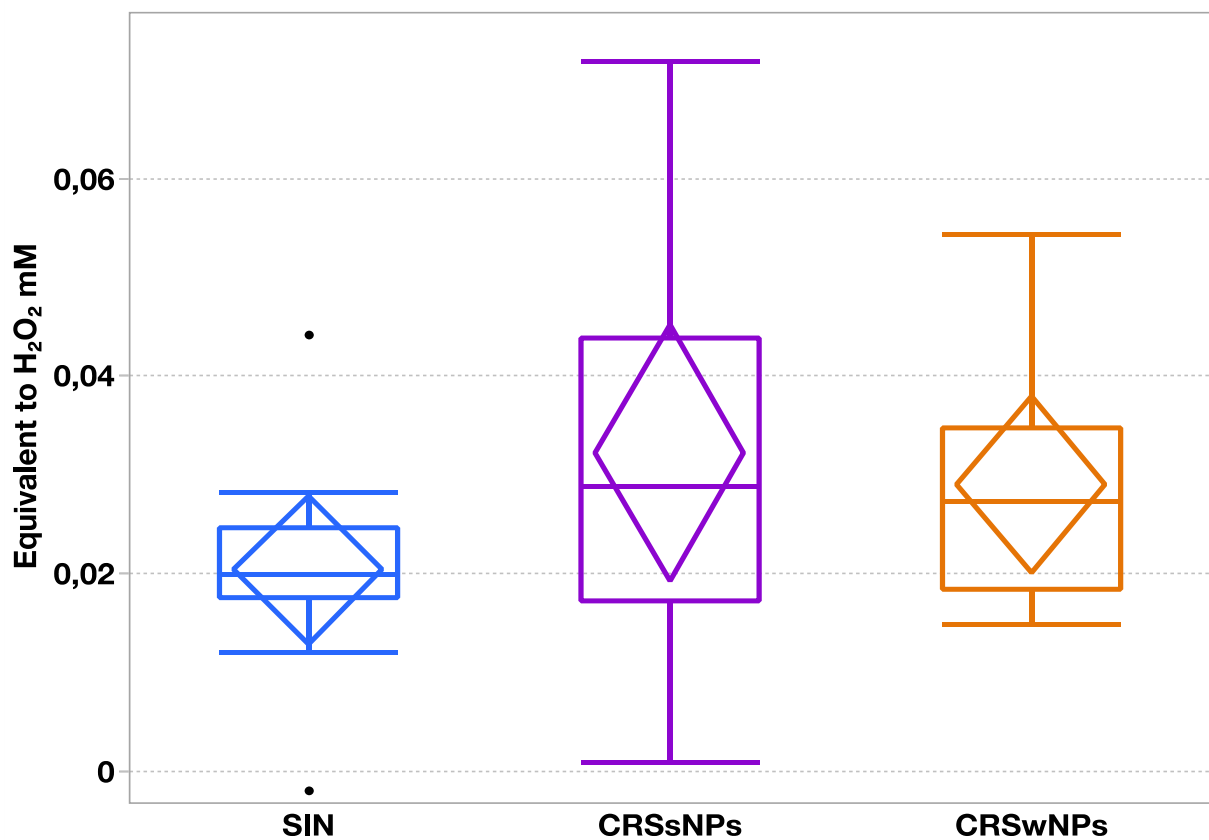
## 5.2 Serum total oxidative capacity

**Table 2** and **Figure 2** show the TOC levels across groups. We excluded patients whose average serum capacity was negative (value before determining H<sub>2</sub>O<sub>2</sub> eq). The CRSsNPs group had the highest mean value (0,032156 mM H<sub>2</sub>O<sub>2</sub> eq. (0,019201), while the SIN (controls) group had the lowest (0,020306 mM H<sub>2</sub>O<sub>2</sub> eq. (0,011162). When the means of the groups were compared, there was no significant difference by Kruskal Wallis ( $\chi^2_2 = 3,4618$ , p=0,1771) with the Steel-Dwass post-hoc test (See **Table 2**).



**Table 2.** Descriptive statistics of the total oxidative serum capacity in groups and results of Kruskal Wallis ( $\chi^2_2 = 3,4618, p=0,1771$ ) with Steel-Dwass post-hoc test.

Group	Number of patients	Mean	Standard deviation	Minimum	Maximum
SIN (controls)	12	0,020306	0,011162	-0,002080	0,044060
CRSsNPs	12	0,032156	0,019201	0,000845	0,071679
CRSwNPs	12	0,028932	0,012408	0,014850	0,054195



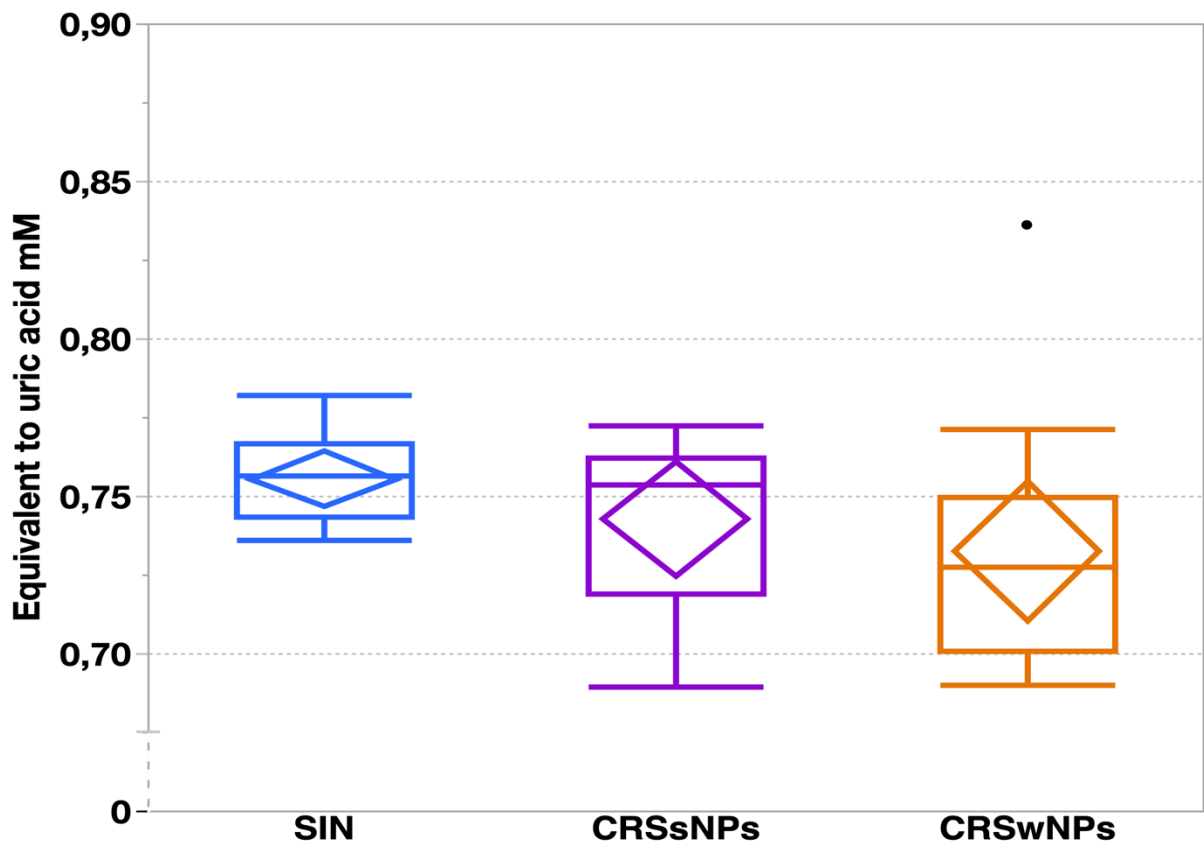
**Figure 2.** Concentration of total oxidative capacity equivalent to hydrogen peroxide ( $H_2O_2$ ) in serum of CRSwNP, CRSsNP, and SIN (controls) patients.

### 5.3 Serum total antioxidative capacity

TAC levels across groups are shown in **Table 3** and **Figure 3**. The SIN (controls) group had the highest mean value (0,755250 mM uric acid eq. (0,013811), while the CRSwNPs group had the lowest (0,732357 mM uric acid eq. (0,038249). When the means of the groups were compared, there was no significant difference by Kruskal Wallis ( $\chi^2_2 = 5,5886$ ,  $p=0,0612$ ) with the Steel-Dwass post-hoc test. (See **Table 3**).

**Table 3.** Descriptive statistics of the total antioxidative serum capacity in groups and results of Kruskal Wallis ( $\chi^2_2 = 5,5886$ ,  $p=0,0612$ ) with the Steel-Dwass post-hoc test.

Group	Number of patients	Mean	Standard deviation	Minimum	Maximum
SIN (controls)	12	0,755250	0,013811	0,736	0,782
CRSsNPs	12	0,742417	0,028532	0,689	0,772
CRSwNPs	12	0,732357	0,038249	0,836	0,690



**Figure 3.** Concentration of total antioxidative capacity equivalent to uric acid in serum of CRSwNP, CRSsNP, and SIN (controls) patients.

#### **5.4. Evaluation of the expression of the oxidative stress-induced lipid peroxidation marker 4-hydroxynonenal (4-HNE) in both phenotype**

In the CRSwNPs group, 12 respiratory epithelium tissue samples (86%) had grade III 4-HNE positivity; 10 samples (71%) had grade III positivity in the nuclei and grade I cytoplasmic positivity. Basal membrane and stromal positivity were grade I in 11 (78 percent) of the tissue samples. Grade I positive cytoplasm was found in the endothelium in 10 tissue samples, while four samples had nuclei grade 0 4-HNE (no staining). 4-HNE expression was grade III in the cytoplasm and grade II in the nucleus of mucosal glands from 8 CRwNPs samples.

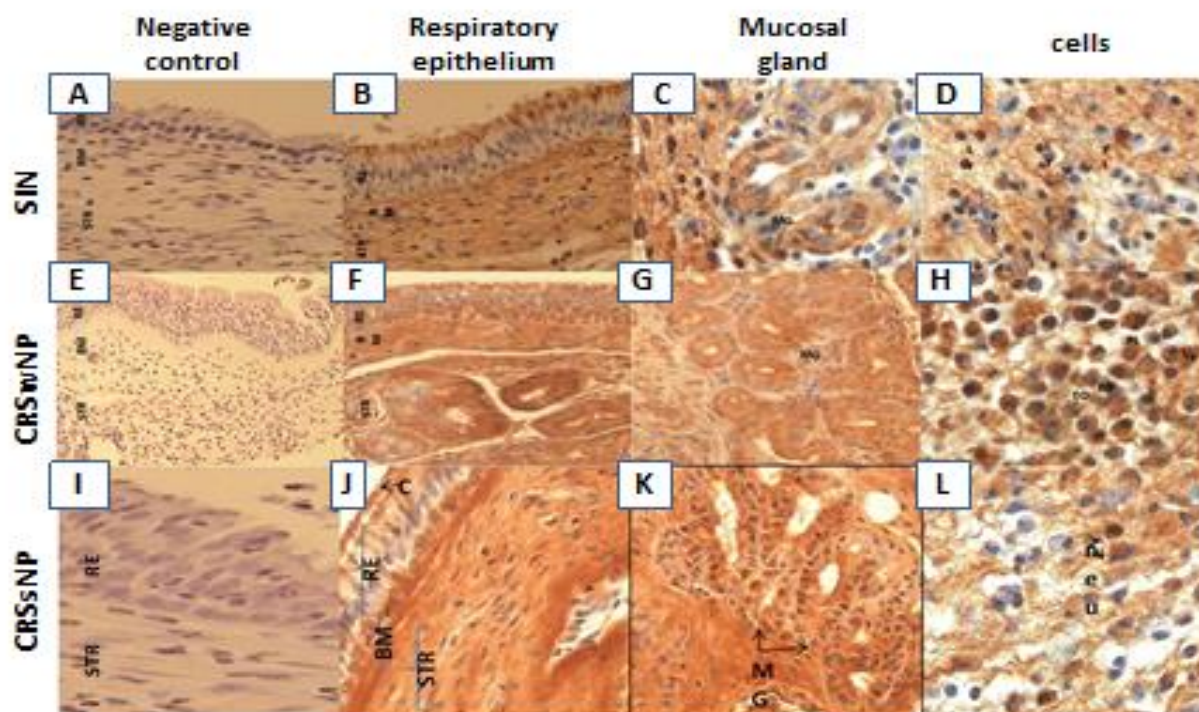
The inflammatory cells were identified by morphological characteristic. Prior immunohistochemical analysis tissue samples of each study group stained in hematoxylin and eosin and examined under optical microscope Olympus BX51 at 1000x amplification. Plasma cells was round-to-ovoid cells contained abundant deep blue cytoplasm with pale perinuclear area. They had a round eccentrically placed nucleus. Eosinophils looked as a nucleus with two lobes, and a thin nuclear bridge connected both lobes. Neutrophils had a characteristic multilobed nucleus, with 3 to 5 lobes joined by slender strands. The cytoplasm of neutrophils contained numerous purplish granules. Lymphocytes looked like an agranular cells with very clear cytoplasm which stained pale blue. Its nucleus was very large for the size of the cell and stained dark purple.

In CRSwNPs tissue samples plasma cells were grade III positive in eight samples, while eosinophils were grade III positive in ten. The nuclei of inflammatory cells were stained with 4-HNE (no staining). At the grade III level, one sample tested positive for intraepithelial neutrophils.

In the CRSsNPs group, nine (69%) samples had grade III 4HNE positivity and intensity in both cytoplasm and nuclei, particularly in respiratory epithelial luminal samples. Nine tissue samples had grade III 4-HNE positivity in nuclei only at the basal membrane. In all samples, Stroma was weakly positive and had grade III 4HNE positivity. Five samples of submucosal glands had weakly positive cells, and the remaining samples had grade 0 4HNE

(no staining). Five (38%) tissue samples had high plasma cell III grade positivity, four (31%) had grade I positivity, and the remaining samples were 4-HNE grade 0. In three samples, lymphocytes were 4-HNE grade 0, eosinophils were grade III positive, and neutrophils were grade III positive. Endothelial cells had grade I cytoplasm in seven samples and grade 0 nuclei in five samples.

Five samples (42 percent) of the respiratory epithelium in the SIN (controls) group (healthy sinus tissue) were grade I positive cells, mostly in the lumen and the cilia. Cytoplasm positivity was grade I at the basal membrane in half of the samples, and stromal cells were grade I positive to 4-HNE. Endothelial cell cytoplasm were grade II positive in six tissue samples, while their nuclei were grade 0 4-HNE positive. In control tissue samples, no inflammatory cells were found. **Figure 4** depicts examples of 4-HNE tissue expression findings. Hematoxylin was used as a negative control stain. Each photograph was taken from a single tissue cut.





**Figure 4.** 4 hydroxynonenal (4-HNE) expression in control (healthy sinus), CRSwNPs, and CRSsNPs tissue samples.


**SIN** - health sinus tissue: **A** - negative control (stained by hematoxylin), **B** - 4HNE<sup>+</sup> respiratory epithelium. **C** - 4HNE<sup>+</sup>, **MG** - mucosal glands. **D**- 4HNE<sup>+</sup> cells.


**CRSwNP** - chronic rhinosinusitis with nasal polyp: **E** - negative control. **F** - 4HNE<sup>+</sup> respiratory epithelium, **G** - 4HNE<sup>+</sup>, **MG** - mucosal glands. **H** - 4HNE<sup>+</sup> inflammatory cells. **CRSsNP** - chronic rhinosinusitis without nasal polyp: **I**- negative control. **J**- 4HNE<sup>+</sup> respiratory epithelium. **K**- 4HNE<sup>+</sup>, **MG**- mucosal glands. **L**- 4HNE<sup>+</sup> inflammatory cells.

**C** - cilia, **RE** - respiratory epithelium, **BM**- basal membrane, **STR**- stroma. **Ly**- lymphocyte, **PL**- plasma cell, **eo**-eosinophil, **neu**-neutrophil

 grade 0 - negative staining ,

 grade I - weak positivity (0-30%) and intensity

 grade II - intermediate positivity (30-50%) and intensity,

 grade III - high positivity (>50%) and intensity.

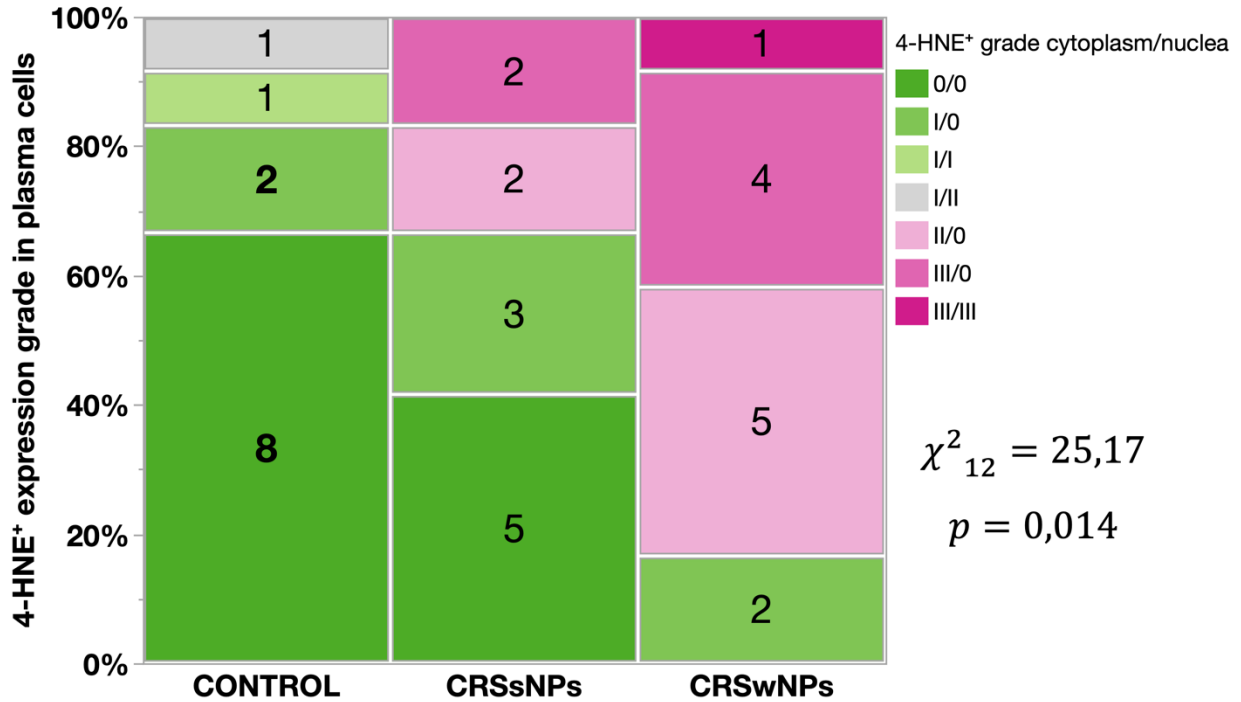
#### **5.4.1. Expression of 4HNE+ in plasma cells from CRSwNP, CRSsNP, and SIN (control) tissue samples**

We discovered a statistically significant difference between study groups using contingency analysis of 4HNE<sup>+</sup> expression in plasma cells ( $\chi^2_{12} = 25,17$ ,  $p = 0,014$ ) (See **Figure 5**). There was a statistically significant difference in 4HNE<sup>+</sup> expression in plasma cell cytoplasm between CRSwNPs, CRSsNPs, and SIN (controls) groups when the overall score was divided into the cytoplasm and the nucleus ( $\chi^2_6 = 20,96$ ,  $p = 0,0019$ ) (See **Table 4**).

CRSwNP patients had a higher prevalence of II and III grade 4HNE<sup>+</sup> expression in their cytoplasm ( II grade  $n = 7$ , 58,33 percent and III grade  $n = 4$ , 33,33 percent) whereas SIN (control) patients had a lower prevalence of 0 and I grade 4HNE expression (0 grade  $n = 8$ , 66,67 percent and I grade  $n = 4$ , 33,33 percent). Five patients with CRSsNPs had 0 grade 4HNE<sup>+</sup> expression in the cytoplasm, five had I grade, and two had II grade.

The nuclei of plasma cells in the SIN (control) group were overwhelmingly negative to 4HNE ( $n = 10$ , 83.33 percent), while two patients had I and II grade 4HNE<sup>+</sup> expression. The nuclei of all plasma cells in all CRSsNPs patients ( $n = 12$ ) tested negative for 4HNE. The nuclei of plasma cells in 11 CRSwNPs patients (92.86 percent) were negative to 4HNE, while one

patient had III grade expression in the nuclea. There was no statistically significant difference in 4HNE+ expression in plasma cell nuclei based on grade between patients with CRSwNPs, CRSsNPs, and SIN (control) ( $\chi^2_6=6,74$ ,  $p=0,372$ ) (See **Table 4**).



**Figure 5.** A contingency analysis with chi square test of the 4HNE+ plasma cells related to grade of expression (cytoplasm/nuclea) in CRSwNPs, CRSsNPs and SIN (controls). Numbers in mosaic plot represents number of patients.

**Table 4.** Distribution and contingency analysis with chi square test of the 4HNE<sup>+</sup> grade in cytoplasm and nuclea of the plasma cells between CRSwNP, CRSsNP, and SIN (controls) patients.

	Group					
	SIN		CRSsNP		CRSwNP	
4HNE <sup>+</sup> cytoplasm $\chi^2_6 = 20,96; p = 0,0019$	N	Column %	N	Column %	N	Column %
0	8	66,67	5	41,66	0	0,00
I	4	33,33	5	41,66	7	58,33
II	0	0,00	2	16,68	4	33,33
III	0	0,00	0	0,00	1	8,34
4HNE <sup>+</sup> nuclea $\chi^2_6 = 6,74; p = 0,372$	N	Column %	N	Column %	N	Column %
0	10	83,33	12	100,00	11	92,86
I	1	8,33	0	0,00	0	0,00
II	1	8,33	0	0,00	0	0,00
III	0	0,00	0	0,00	1	7,14

### 5.5. Acrolein, an oxidative stress-induced peroxidation marker, was examined in tissues from both CRS phenotype.

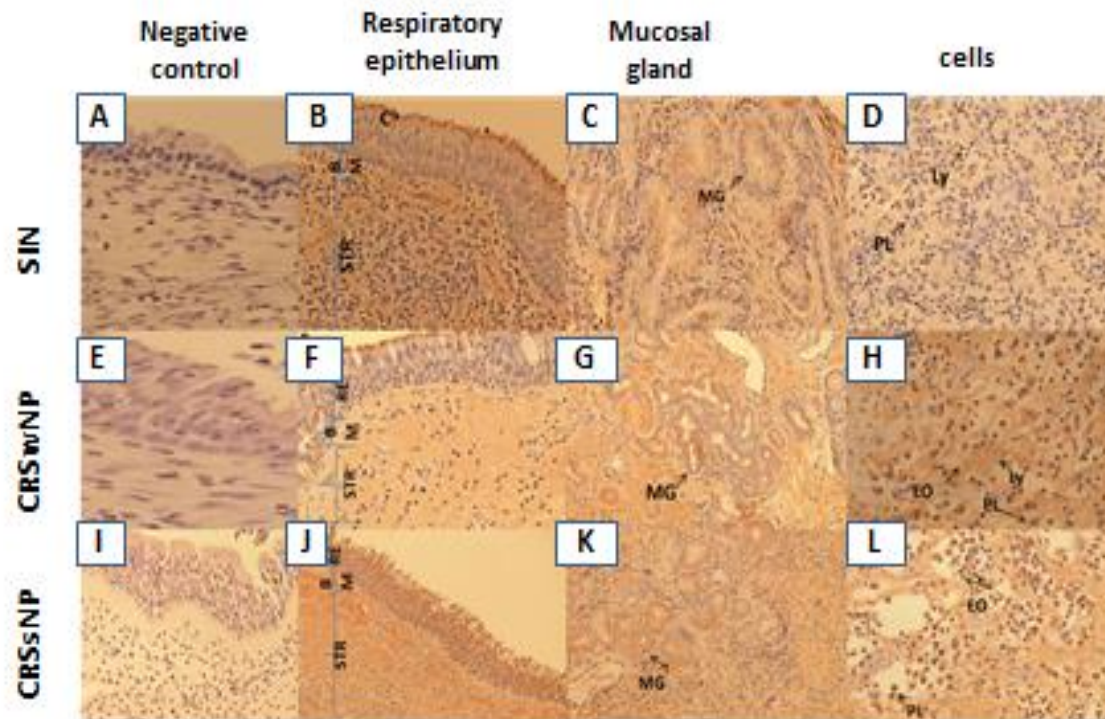
Eight (50 percent) of the CRSwNPs patients' tissue samples tested positive for acrolein at the grade II level, primarily in the lumen. In the cytoplasm of seven (43 percent) of the tissue samples, the intensity was grade II. All basal membranes had grade I positivity and intensity; stroma had grade III positivity in nine (56 percent) of tissue samples. Grade I cytoplasmic positivity was observed in ten tissue samples at the endothelium, and 4-HNE grade 0 positivity was observed in four tissue samples. All samples had grade I acrolein positivity in the submucosal glands. In eight (50 percent) of the tissue samples tested positive for inflammatory cells, with grade III positivity found in the cytoplasm of plasma cells, eosinophils, and lymphocytes. In one sample, intraepithelial neutrophils with grade III

acrolein positivity were found. **Figure 6** depicts the expression of acrolein in tissue samples from different study groups.

Eight (61 percent) of the CRSsNPs tissue samples had grade II acrolein positivity, primarily in the luminal part of the respiratory epithelium; five samples had grade I positivity. At the basal membrane, all tissue samples had grade I acrolein intensities. Stroma positivity was grade II in six samples and grade I in half of the samples. Acrolein positivity was grade III in eight submucosal gland samples and grade I in five. In nine (69%) of the tissue samples, plasma cells were grade I positive, while four (31% of the samples) were acrolein grade 0. Lymphocytes had acrolein grade 0 and eosinophils had grade II; three samples had grade III neutrophils. In seven samples, endothelial cells were grade I cytoplasmic positive, while nuclei were grade 0; five samples were grade 0.

In the control (healthy sinus tissue) group, 10 (77 percent) of respiratory epithelium tissue samples, mostly from the lumen and cilia, were positive for grade II acrolein, while six (23 percent) were positive for grade I acrolein. Cells at the basal membrane had I grade acrolein positivity in four samples, and nine samples had 0 grade acrolein positivity; stroma had I grade positivity in eight samples, and five samples had 0 grade acrolein positivity. Seven samples had grade II acrolein positivity, while six samples had grade 0 acrolein positivity. Acrolein positivity was found in plasma cells and lymphocytes in 11 (85 percent) of the samples. Hematoxylin was used as a negative control stain. Each photograph was taken from a single tissue cut.





**Figure 6.** Acrolein (ACR) expression in control (healthy sinus), CRSsNP, and CRSwNP tissue samples:

**SIN**-health sinus tissue: **A** - negative control (stained by hematoxylin), **B** - ACR<sup>+</sup> respiratory epithelium. **C** - ACR<sup>+</sup>, MG - mucosal glands. **D**- ACR<sup>+</sup> cells.

**CRSwNP** - chronic rhinosinusitis with nasal polyp: **E** - negative control. **F** - ACR<sup>+</sup> respiratory epithelium, **G** - ACR<sup>+</sup>, MG - mucosal glands. **H** - ACR<sup>+</sup> inflammatory cells.

**CRSsNP** - chronic rhinosinusitis without nasal polyp: **I** - negative control. **J** - ACR<sup>+</sup> respiratory epithelium. **K**- ACR<sup>+</sup>, MG- mucosal glands. **L**- ACR<sup>+</sup> inflammatory cells.

**C** - cilia, **RE** - respiratory epithelium, **BM**- basal membrane, **STR**- stroma. **Ly**- lymphocyte, **PL**- plasma cell, **eo**-eosinophil, **neu**-neutrophil

■ grade 0 - negative staining ,

■ grade I - weak positivity (0-30%) and intensity ,

- *grade II - intermediate positivity (30-50%) and intensity,*
- *grade III - high positivity (>50%) and intensity.*

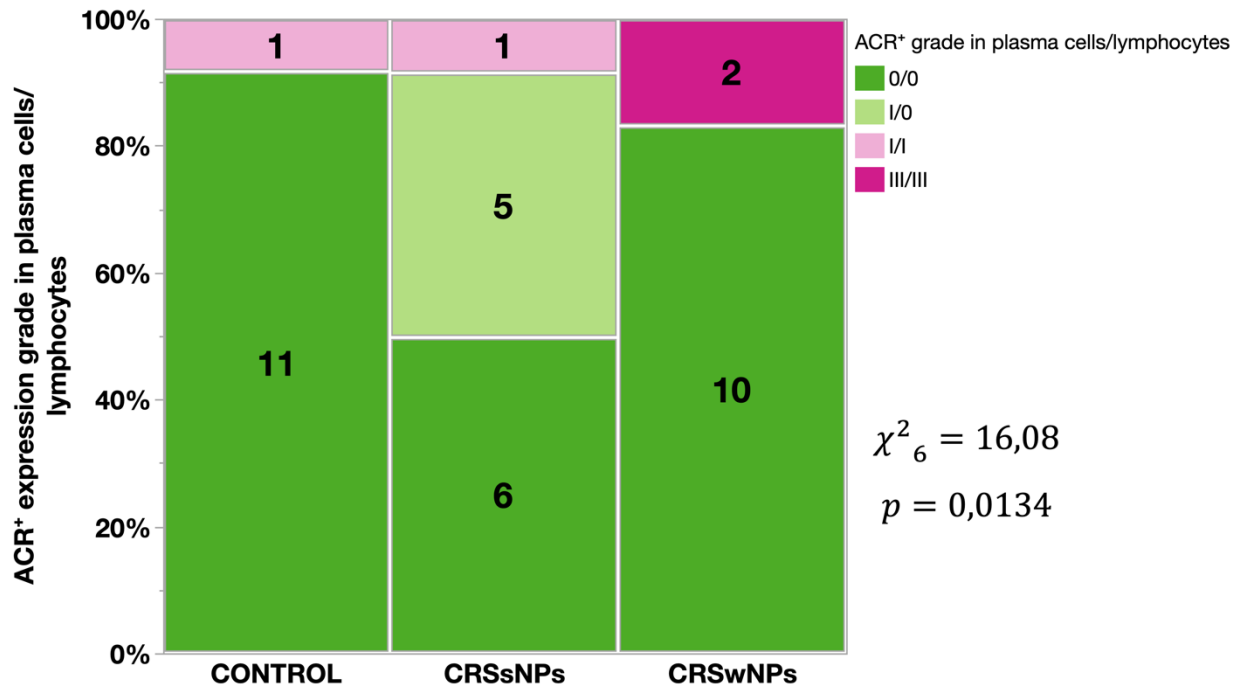
### **5.5.1. Expression of ACR+ in inflammatory cells from CRSwNP, CRSsNP, and SIN (control) tissue samples.**

We discovered a statistically significant difference between study groups using contingency analysis of ACR+ expression in plasma cells and lymphocytes ( $\chi^2_6 = 16,08$ ,  $p=0,0134$ ) (See **Figure 7**). There was also statistically significant difference in ACR+ expression in plasma cells based on grade between the CRSwNPs, CRSsNPs, and SIN (control) groups ( $\chi^2_4=13,84$ ,  $p=0,0078$ ) (See **Table 5**).

Plasma cells in the SIN (control) group were predominantly negative to ACR in 11 patients (91.67 percent), while one patient had I grade of expression. Six patients with CRSsNPs tested negative for ACR in plasma cells, and six patients had I grade expression. There were no patients with ACR expression in plasma cells of the II or III grade. In the CRSwNPs group, 10 patients tested negative for ACR in plasma cells, and two patients tested positive for ACR at the III level.

There was no statistically significant difference in ACR+ expression in lymphocytes by grade between CRSwNPs, CRSsNPs, and SIN (controls) groups ( $\chi^2_4=4,74$ ,  $p=0,3154$ ) (See **Table 5**). Eleven patients in the SIN group were negative for ACR expression in lymphocytes, with one patient having I grade expression. ACR expression in the CRSsNPs group; with eleven patients testing negative for ACR and one patients expressing I grade ACR. Ten CRSwNPs tested negative for ACR in lymphocytes, and two had a III grade.

There was a statistically significant difference in ACR+ expression in eosinophils depending on grade between the CRSwNPs, CRSsNPs, and SIN (control) groups ( $\chi^2_4=28,05$ ,  $p<0,001$ ) (See **Table 5**). Eosinophils were not found in the tissue samples of any of the patients in the control group.



**Figure 7.** A contingency analysis with chi square test of the ACR<sup>+</sup> plasma cells /lymphocytes related to grade of expression in CRSwNPs, CRSsNPs and SIN (controls) . Numbers in mosaic plot represents number of patients.

**Table 5.** Distribution and contingency analysis with chi square test of the ACR<sup>+</sup> grade in plasma cells, lymphocytes and eosinophils between CRSwNP, CRSsNP, and SIN patients.

	Group					
	SIN		CRSsNP		CRSwNP	
ACR <sup>+</sup> plasma cells $\chi^2_4 = 13,84; p = 0,0078$	N	Column %	N	Column %	N	Column %
0	11	91,67	6	50,00	10	85,71
I	1	8,33	6	50,00	0	0,00
II	0	0,00	0	0,00	0	0,00
III	0	0,00	0	0,00	2	14,29
ACR <sup>+</sup> lymphocytes $\chi^2_4 = 4,74; p = 0,3154$	N	Column %	N	Column %	N	Column %
0	11	91,67	11	92,31	10	85,71
I	1	8,33	1	7,69	0	0,00
II	0	0,00	0	0,00	0	0,00
III	0	0,00	0	0,00	2	14,29
ACR <sup>+</sup> eosinophils $\chi^2_6 = 28,05; p < ,0001$	N	Column %	N	Column %	N	Column %
0	12	100,00	6	53,85	0	0,00
I	0	0,00	4	30,77	5	38,46
II	0	0,00	1	7,69	1	7,69
III	0	0,00	1	7,69	6	53,85

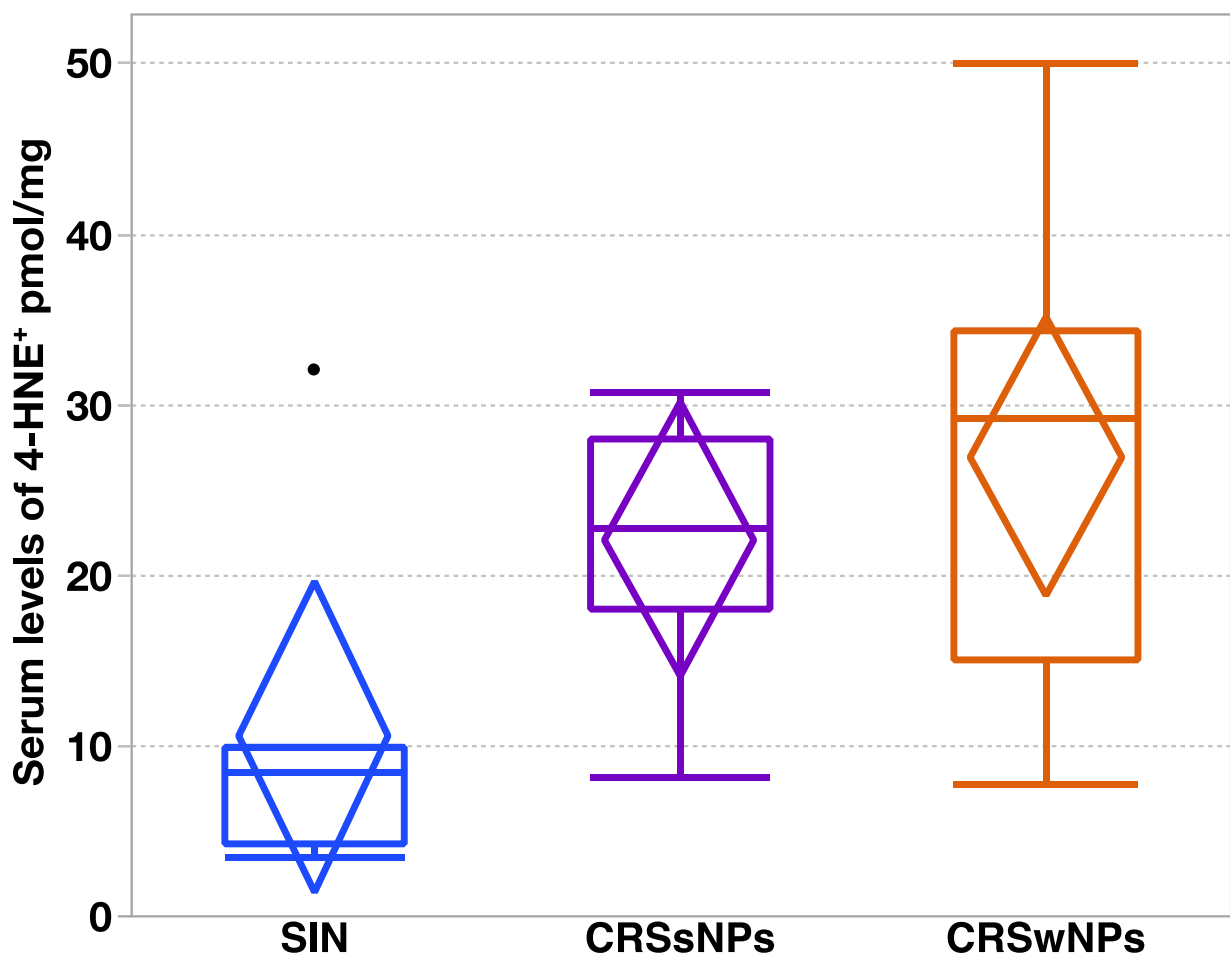
### 5.6. Serum levels of the lipid peroxidation marker 4-hydroxynonenal (4-HNE) differed between CRS phenotype.

The highest overall mean serum concentration of 4-HNE was found in CRSwNPs patients with a serum concentration of 26, 861071 pmol/mg (12,85161). This value differed significantly from that obtained in the SIN (controls) group, which was 10,469184 pmol/mg (9,849568), ( $F_{2,22} = 4,8812$ ,  $p=0,0176$ ). Patients with CRSsNPs had a mean value of 22,047619 (7,662089), which did not differ significantly from SIN(controls) and CRSwNPs.

**Figure 8** and **Table 6** show the distribution of 4-HNE serum concentrations in study groups.

**Table 6.** Descriptive statistics of the serum concentration of 4-HNE in pmol/mg in groups and results of Kruskal Wallis ( $\chi^2_2 = 6,6237, p=0,0364$ ) with the Steel-Dwass post-hoc test.

Group	Number of patients	Mean	Standard deviation	Minimum	Maximum
SIN	12	10,469184	9,849568	3,428571	32,00
CRSsNPs	12	22,047619	7,662089	8,125	30,571429
CRSwNPs	12	26,861071	12,85161	7,714286	49,857143



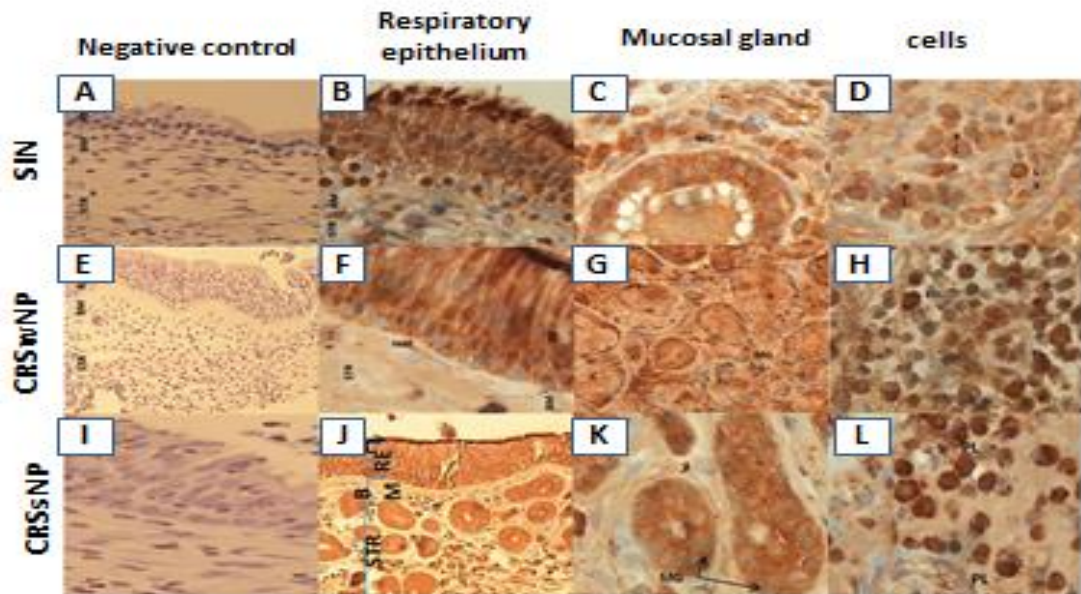
**Figure 8.** Serum levels of lipid peroxidation marker 4-hydroxynonenal (4-HNE) across CRS phenotypes and SIN (controls).

## 5.7. Increased expression of the AhR protein in CRS patients with both phenotype.

In the CRSwNPs group, nine (64 percent) respiratory epithelium samples had grade III AhR cytoplasm positivity and grade 0 AhR (no staining) in the nuclei; five tissue samples had grade III respiratory cilia positivity and the remaining five samples (36%) had grade II positivity. In all tissue samples, the basal membrane was grade 0 AhR (no staining). In eight samples, stromal cells were grade II AhR positive, while seven samples were grade I. Six samples of mucosal gland tissue had grade III cytoplasmic AhR positivity and grade 0 nuclear AhR expression. Plasma cells had grade III AhR positivity and grade 0 nuclear expression in nine (64%) of the CRSwNPs tissue samples; grade II AhR expression was seen in the cytoplasm of eosinophils and lymphocytes in five (36%) of the samples. All inflammatory cell nuclei were grade 0 AhR (no expression).

Ten (83 percent) of the respiratory epithelium tissue samples in the CRSsNPs group had grade III AhR positive cytoplasm and grade I positive nuclei. Grade III AhR positivity was found in all respiratory cilia. Basal membranes were AhR negative, whereas stromal cells were grade I positive and intense. Endothelial cells were grade III positive and intense in six samples and grade II positive in six others. Mucosal glands (seven samples) had cytoplasmic positivity grade III and nuclear positivity grade I. In eight (66 percent) of the samples, cytoplasmic and nuclear grade II positivity was observed in the majority of plasma cells. Grade I AhR expression was found in lymphocytes from four different tissue samples.

Ten (83 percent) of the tissue samples from the respiratory epithelium in the control group had grade III AhR positive cytoplasm and grade I nuclei. Grade III AhR positivity was found in all respiratory cilia. Basal membranes exhibited grade 0 AhR expression, whereas stromal cells exhibited grade I positivity. Endothelial cells were positive in six samples with a grade III positivity and six samples with a grade II positivity. Mucosal glands (seven samples) had cytoplasmic positivity grade III and nuclear positivity grade I. In eight (66 percent) of the samples, the majority of plasma cells had grade II cytoplasmic and nuclear positivity. Grade I AhR expression was found in lymphocytes from four different tissue samples. **Figure 9** depicts examples of AhR tissue expression findings. Hematoxylin was used as a negative control stain. Each photograph was taken from a single tissue cut.



**Figure 9 .** Aryl hydrocarbon receptor (AhR) expression in control (healthy sinus), CRSwNPs and CRSsNPs tissue samples.

**SIN**-health sinus tissue: **A** - negative control (stained by hematoxin), **B** - AhR<sup>+</sup> respiratory epithelium. **C** - AhR<sup>+</sup>, MG - mucosal glands. **D**- AhR<sup>+</sup> cells.

**CRSwNP**-chronic rhinosinusitis with nasal polyp: **E** - negative control. **F** - AhR<sup>+</sup> respiratory epithelium, **G** - AhR<sup>+</sup>, MG - mucosal glands. **H** - AhR<sup>+</sup> inflammatory cells.

**CRSsNP** – chronic rhinosinusitis without nasal polyp: **I**- negative control. **J** - AhR<sup>+</sup> respiratory epithelium. **K**- AhR<sup>+</sup>, MG- mucosal glands. **L**- AhR<sup>+</sup> inflammatory cells.

**C** - cilia, **RE** - respiratory epithelium, **BM**- basal membrane, **STR**- stroma. **Ly**- lymphocyte, **PL**- plasma cell, **eo**-eosinophil.

- grade 0 - negative staining,
- grade I - weak positivity (0-30%) and intensity,
- grade II - intermediate positivity (30-50%) and intensity,
- grade III - high positivity (>50%) and intensity.

### **5.7.1.. Expression of AhR in plasma cells from CRSwNP, CRSsNP and SIN (control) tissue sample.**

Using contingency analysis of AhR+ expression in plasma cells, a statistically significant difference between research groups was discovered ( $\chi^2_{16} = 36,16$ ,  $p=0,0027$ ) (See **Figure 10**). There was also a statistically significant difference in AhR+ expression in plasma cell cytoplasm based on grade between the CRSwNPs, CRSsNPs, and SIN (controls) groups ( $\chi^2_4=17,59$ ,  $p=0,0015$ ) (See **Table 7**).

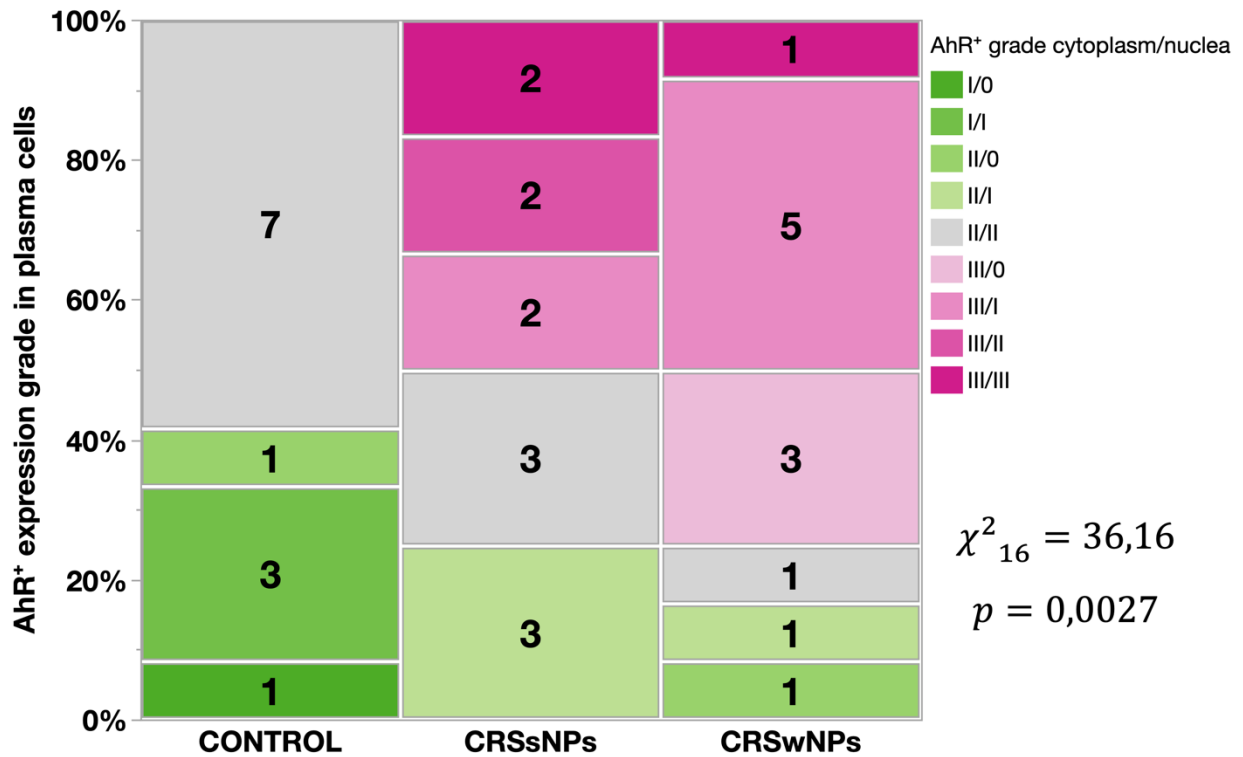
AhR expression in the cytoplasm of plasma cells was distributed as follows in the SIN (control) group: three patients had grade I expression, eight patients had grade II expression, and one had patient had grade III expression. Six patients (50 percent) in the CRSsNPS group received a grade II, and six patients (50 percent) received a grade III of AhR expression . In the CRSsNP group, there were no patients with AhR expression grade of 0 or I. There were no patients in the CRSwNPs group who had AhR expression grades 0 and I in the plasma cell cytoplasm. Three patients had II grade cytoplasm and nine patients had III grade cytoplasm in their plasma cells.

In addition, there was a statistically significant difference in AhR+ expression in plasma cell nuclei based on grade between CRSwNPs, CRSsNPs, and SIN (controls) groups ( $\chi^2_6 = 15,17$ ,  $p=0,0019$ ) (See **Table 7**).

The distribution of AhR expression in nuclei of plasma cells in the SIN (control) group was as follows: two patients had grade 0 expression, three patients had grade I expression, and seven patients had grade I expression. There were no patients who received a grade III. In the CRSsNPS group, there were no patients with 0 grade expression, five patients with I grade expression, and five patients with II grade expression. Three patients of the CRSsNP group had AhR expression grade III.

Three patients in the CRSwNPs group had 0 grade AhR expression and six patients had I grade AhR expression in nuclei of plasma cells. In nuclei of plasma cells, two patients had a grade II and one patient had a grade III.





**Figure 10.** A contingency analysis with chi square test of the AhR<sup>+</sup> grade in plasma cells (cytoplasm/nuclea) and patient group showing statistically significant influence of the type of sinusitis and AhR<sup>+</sup> grade in plasma cells. Numbers in mosaic plot represents number of patients.

**Table 7.** Distribution and contingency analysis with chi square test of the AhR<sup>+</sup> grade in cytoplasm and nuclei of the plasma cells between CRSwNP, CRSsNP, and SIN patients.

	Group					
	SIN		CRSsNP		CRSwNP	
AhR <sup>+</sup> cytoplasm $\chi^2_4 = 17,59; p = 0,0015$	N	Column %	N	Column %	N	Column %
0	0	0,00	0	0,00	0	0,00
I	3	25,00	0	0,00	0	0,00
II	8	66,66	6	50,00	3	25,00
III	1	8,34	6	50,00	9	75,00
AhR <sup>+</sup> nuclei $\chi^2_6 = 15,17; p = 0,0190$	N	Column %	N	Column %	N	Column %
0	2	16,70	0	0,00	3	25,00
I	3	25,00	5	41,66	6	50,00
II	7	58,35	5	41,66	2	16,66
III	0	0,00	2	16,66	1	8,33

When the Kendall rank coefficient was used as a test statistic to determine whether the grades of 4HNE<sup>+</sup> and AhR<sup>+</sup> were statistically dependent, statistical significance was obtained in the case of plasma cell cytoplasm ( $K\tau = 0,41, p=0,004$ ). This suggests that higher levels of 4HNE<sup>+</sup> in the cytoplasm are linked to higher levels of AhR<sup>+</sup> and vice versa. This evidence could not be related to plasma cells nuclei ( $K\tau = -0,19, p=0,193$ ). The grades of the 4HNE<sup>+</sup> and AhR<sup>+</sup> in plasma cells, cytoplasm, and nuclei were then analysed using Kendall rank correlation analysis in CRSwNP, CRSsNP, and SIN patients. There was no statistical significance in this case.

## 5.8. Ex vivo experimental study

### 5.8.1. Patient characteristics

The ex vivo experimental study included 21 patients from a total of 57 patients who had endoscopic sinus surgery. Between January 2020 and June 2020, in the County hospital Čakovec's Department of Otorhinolaryngology were operated seven patients with CRSwNPs (mean age 54 years, range 33-68 years; three males, four females), seven patients with CRSsNPs (mean age 59 years, range 41-80 years; five males, two female), and seven healthy patients (mean age 39 years, range 19-56 years; all male). In CRSwNPs group five cases had atopic status; three patients with CRSwNPs had a positive skin prick test, five patients had a history of bronchial asthma. and one patient with CRSwNPs had a positive allergy test to NSAIDs. In CRSsNPs group one patient had atopic status. One patient suffered from Samter's triad in CRSwNPs group .Recalcitrant form of CRS was three patients only in CRSwNPs group . These three patients had undergone more than two (2-13) paranasal sinus surgeries, i.e. endoscopic endonasal sinus surgery (FESS) for obstructive nasal polyposis. One patient was a smoker in each CRS phenotype .There were no subjects with a history of seasonal allergy, bronchial asthma, NSAID hypersensitivity, or smoking among the control group. Every tissue sample was taken from a healthy paranasal sinus. **Table 8** depicts the patient characteristics of the three study groups.

**Table 8.** *Clinical characteristics of patients with CRSwNP, CRSsNP, and SIN (controls) participated in ex vivo experimental study.*

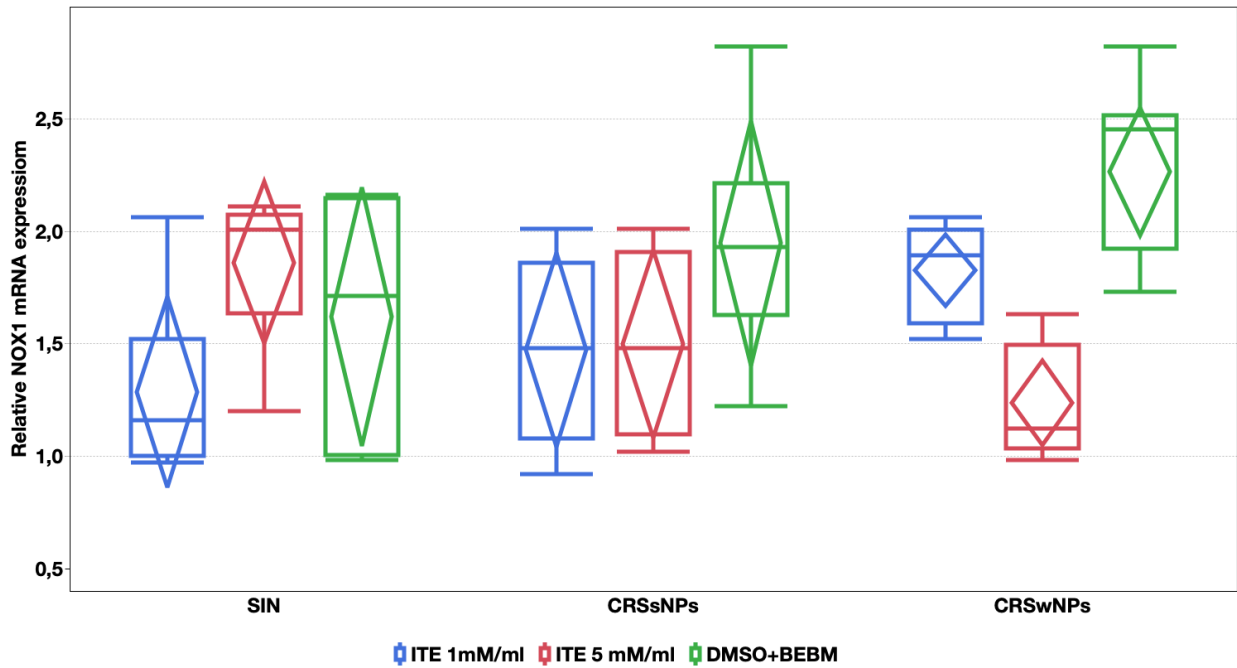
	CRSwNP (n=7)	CRSsNP (n=7)	SIN (n=7)
Mean age (years)	54 (33-68)	59 (41-80)	39 (19-56)
Sex (female:male)	4:3	2:5	0:7
Atopic patients	5	1	0
Samter's triad	1	0	0
Recalcitrant patients	3	0	0
Smokers	1	1	0

### 5.8.2. Effect of 2-(1*H*-Indol-3-ylcarbonyl)-4-thiazolecarboxylic acid methyl ester on NADPH oxidase 1 mRNA expression

Over the course of 24 hours, paranasal sinus tissue samples were treated with a 1mM and 5mM solution containing the Aryl hydrocarbon receptor agonist 2-(1*H*-Indol-3-ylcarbonyl)-4-thiazolecarboxylic acid methyl ester (ITE). Each study group's control sample was Dimethyl-Sulfoxide (DMSO) diluted in bronchial epithelial basal media (BEBM) (DMSO+BEBM).

There was a statistically significant difference in NOX1 mRNA expression between the SIN and CRSwNPs groups when they were treated with ITE 1mM/ml ( $\chi^2_2 = 6,0662$ ,  $p=0,0482$ ) and 5mM/l ( $\chi^2_2 = 8,2496$ ,  $p=0,0162$ ) but not between CRSsNPs and SIN or CRSsNPs and CRSwNPs patients. The same was not true for control sample where we did not find any statistical difference ( $\chi^2_2 = 3,7331$ ,  $p=0,1547$ ) (See **Table 9**). **Figure 11** shows the dose-dependent distribution of relative NOX1 mRNA expression in study groups after ITE stimulation for 24 hours.

There was no statistically significant difference in the mean value of relative NOX1 mRNA in the SIN group (1,28; 1,86; and 1,62, respectively) between ITE 1mM, ITE 5mM, and DMSO +BEBM treatment doses ( $\chi^2_2 = 3,2762$ ,  $p=0,1943$ ). (See **Figure 11, Table 9**). The CRSsNP group obtained the same statistical results. There was no statistically significant difference in the mean value of relative NOX1 mRNA in the CRSsNP group (1,47; 1,50; and 1,95, respectively) between the treatment doses of ITE 1mM, ITE 5mM, and DMSO +BEBM ( $\chi^2_2 = 3,1893$ ,  $p=0,2030$ ). There was a statistically significant difference in the mean value of relative NOX1mRNA (1,83; 1,23; and 2,26, respectively) between the treatment doses of ITE 1mM, ITE 5mM, and DMSO +BEBM in patients from the CRSwNPs group ( $\chi^2_2 = 18,6229$ ,  $p<,0001$ ).



**Figure 11.** Distribution of the relative NOX1 mRNA expression in tissue cultures treated with ITE in a dose-dependent manner over 24 h. ITE reduced NADPH oxidase 1 (NOX1) mRNA expression in CRSwNP tissue cultures, as compared to controls (DMSO+BEBM).

**Table 9.** Descriptive statistics of the relative *NOX1* mRNA expression in tissue cultures treated with ITE in a dose-dependent manner over 24 h, with results of Kruskal Wallis with the Steel-Dwass post-hoc test.

	Group														
	SIN					CRSsNPs					CRSwNPs				
	N	Mean*	Std	Min	Max	N	Mean**	Std	Min	Max	N	Mean***	Std	Min	Max
Significance <sup>+</sup>	<b>A</b>					<b>AB</b>					<b>B</b>				
ITE 1 mM/ml	7	1,28 <sup>A</sup>	0,40	0,97	2,06	A	1,47 <sup>A</sup>	0,41	0,92	2,01	9	<b>1,83<sup>A</sup></b>	0,21	1,52	2,06
Significance <sup>++</sup>	<b>A</b>					<b>AB</b>					<b>B</b>				
ITE 5 mM/ml	7	1,86 <sup>A</sup>	0,34	1,2	2,11	6	1,50 <sup>A</sup>	0,40	1,02	2,01	9	<b>1,23<sup>B</sup></b>	0,24	0,98	1,63
Significance <sup>+++</sup>	<b>A</b>					<b>A</b>					<b>A</b>				
DMSO+BEBM	7	1,62 <sup>A</sup>	0,55	0,98	2,16	6	1,95 <sup>A</sup>	1,95	1,22	2,82	9	<b>2,26<sup>C</sup></b>	0,37	1,73	2,82

\*Levels (vertical) not connected by the same letter are significantly different ( $\chi^2_2 = 3,2762$ ,  $p = 0,1943$ ) within different ITE dose for SIN patients (Steel-Dwass  $p \leq 0,05$ ).

\*\*Levels (vertical) not connected by the same letter are significantly different ( $\chi^2_2 = 3,1893$ ,  $p = 0,2030$ ) within different ITE dose for CRSsNPs patients (Steel-Dwass  $p \leq 0,05$ ).

\*\*\*Levels (vertical) not connected by the same letter are significantly different ( $\chi^2_2 = 18,6229$ ,  $p < 0,0001$ ) within different ITE dose for CRSsNPs patients (Steel-Dwass  $p \leq 0,05$ ).

<sup>+</sup>Levels (horizontal) not connected by the same letter are significantly different ( $\chi^2_2 = 6,0662$ ,  $p = 0,0482$ ) between SIN, CRSsNPs and CRSwNPs patients for ITE 1mM/ml (Steel-Dwass  $p \leq 0,05$ ).

<sup>++</sup>Levels (horizontal) not connected by the same letter are significantly different ( $\chi^2_2 = 8,2496$ ,  $p = 0,0162$ ) between SIN, CRSsNPs and CRSwNPs patients for ITE 5mM/ml (Steel-Dwass  $p \leq 0,05$ ).

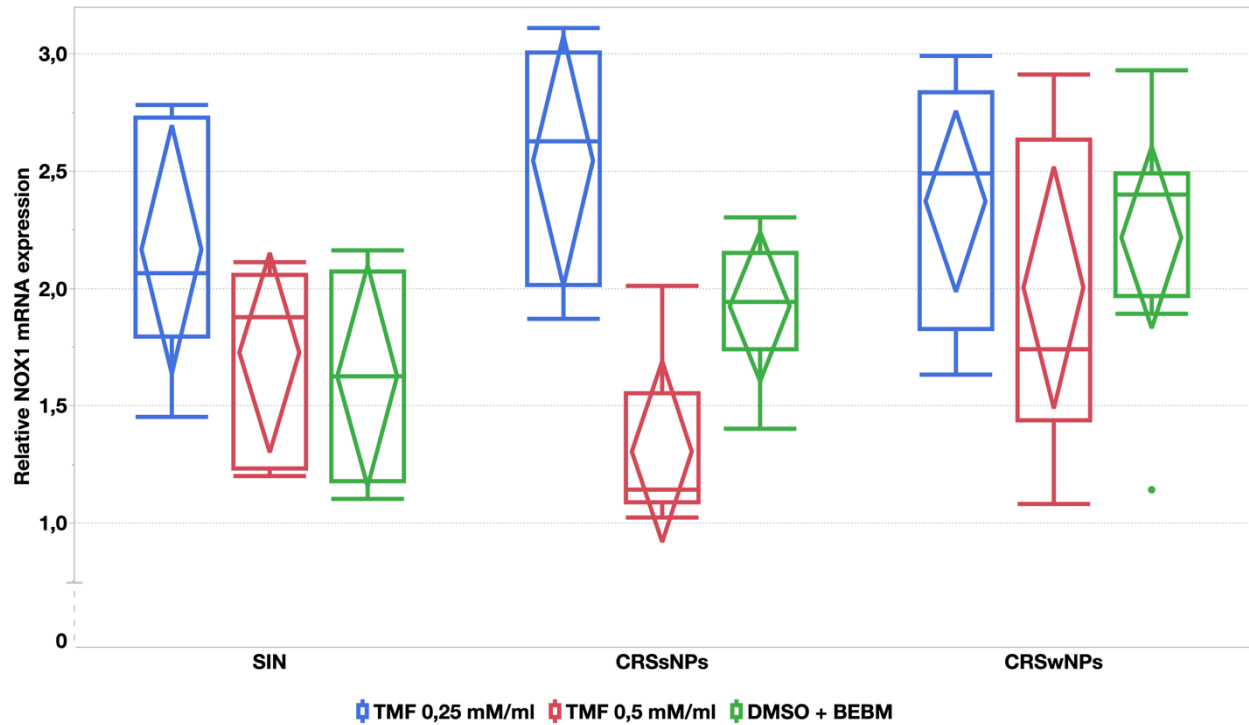
<sup>+++</sup>Levels (horizontal) not connected by the same letter are significantly different between ( $\chi^2_2 = 3,7331$ ,  $p = 0,1547$ ) SIN, CRSsNPs and CRSwNPs patients for DMSO+BEBM (Steel-Dwass  $p \leq 0,05$ ).

### 5.8.3. Effect of 6,2',4'- trimethoxyflavone on NADPH oxidase 1 mRNA expression in both CRS phenotype .

Tissue samples from patients with CRSwNPs and CRSsNPs were treated for 24 hours in a 0.25mM and 0.5mM solution of the aryl hydrocarbon receptor antagonist 6,2',4'-trimethoxyflavone (TMF). Each study group's control samples were DMSO diluted in bronchial epithelial basal medium (DMSO+BEBM).

There was no statistically significant difference in relative NOX1 mRNA expression between the SIN and CRSwNPs groups when TMF 0.25mM/ml, 0.5 percent mM/l, or DMSO + BEBM were used (See **Table 10**). **Figure 12** and **Table 10** show the dose-dependent relative NOX1 mRNA expression in study groups after 24 hours of TMF stimulation.

There was no statistically significant difference in the mean value of relative NOX1 mRNA into the SIN group (2,16, 1,72, and 1,63, respectively) between treatment doses of TMF 0,25mM, TMF 0,5mM, and DMSO +BEBM ( $\chi^2_2 = 3,2465$ ,  $p=0,1973$ ). The mean value of relative NOX1 mRNA in the CRSsNPs group (2,54; 1,30 and 1,92, respectively) was statistically significant ( $\chi^2_2 = 10,7555$ ,  $p=0,0046$ ) between treatment doses of ITE 0,25mM, ITE 0,5mM, and DMSO +BEBM. The mean value of relative NOX1mRNA (2,37; 2,00 and 2,22, respectively) difference between TMF 0,25mM and TMF 0,5mM and DMSO +BEBM was no statistically significant ( $\chi^2_2 = 1,7209$ ,  $p=0,4230$ ) in the CRSwNPs group. **Figure 12** and **Table 10** show the distribution of relative NOX1 mRNA expression after TMF stimulation in both CRS phenotypes and the SIN group (healthy paranasal sinus tissue).



**Figure 12.** Distribution of the relative NOX1 mRNA expression in tissue cultures treated with 6,2'4' trimethoxyflavone (TMF) in a dose- dependent manner over 24 h. TMF reduced NADPH oxidase 1 (NOX1) mRNA expression in both CRS tissue cultures in comparison to controls(DMSO+BEBM).



**Table 10.** Descriptive statistics the relative NOX1 mRNA expression in tissue cultures treated with 6,2'4' trimethoxyflavone (TMF) in a dose-dependent manner over 24 h, with results of Kruskal Wallis with the Steel-Dwass post-hoc test.

	Group														
	SIN					CRSsNPs					CRSwNPs				
	N	Mean*	Std	Min	Max	N	Mean**	Std	Min	Max	N	Mean***	Std	Min	Max
Significance <sup>+</sup>	A					A					A				
TMF 0,25mM/ml	7	2,16 <sup>A</sup>	0,51	1,45	2,78	A	<b>2,54<sup>A</sup></b>	0,51	1,87	3,11	9	2,37 <sup>A</sup>	0,50	1,63	2,99
Significance <sup>++</sup>	A					A					A				
TMF 0,5mM/ml	7	1,72 <sup>A</sup>	0,41	1,20	2,11	6	<b>1,30<sup>B</sup></b>	0,37	1,02	2,01	9	2,00 <sup>A</sup>	0,67	1,08	2,91
Significance <sup>+++</sup>	A					A					A				
DMSO+BEBM	7	1,63 <sup>A</sup>	0,45	1,10	2,16	6	<b>1,92<sup>C</sup></b>	1,30	1,40	2,30	9	2,22 <sup>A</sup>	0,50	1,14	2,93

\*Levels (vertical) not connected by the same letter are significantly different ( $\chi^2_2 = 3,2465$ ,  $p=0,1973$ ) within different TMF dose for SIN patients (Steel-Dwass  $p \leq 0,05$ ).

\*\*Levels (vertical) not connected by the same letter are significantly different ( $\chi^2_2 = 10,7555$ ,  $p=0,0046$ ) within different TMF dose for CRSsNPs patients (Steel-Dwass  $p \leq 0,05$ ).

\*\*\*Levels (vertical) not connected by the same letter are significantly different ( $\chi^2_2 = 1,7209$ ,  $p=0,4230$ ) within different TMF dose for CRSwNPs patients (Steel-Dwass  $p \leq 0,05$ ).

<sup>+</sup>Levels (horizontal) not connected by the same letter are significantly different ( $\chi^2_2 = 1,7003$ ,  $p=0,4274$ ) between SIN, CRSsNPs and CRSwNPs patients for TMF 0,25 mM/ml ((Steel-Dwass  $p \leq 0,05$ ).

<sup>++</sup>Levels (horizontal) not connected by the same letter are significantly different ( $\chi^2_2 = 5,2216$ ,  $p=0,0735$ ) between SIN, CRSsNPs and CRSwNPs patients for TMF 0,5 mM/ml (Steel-Dwass  $p \leq 0,05$ ).

<sup>+++</sup>Levels (horizontal) not connected by the same letter are significantly different ( $\chi^2_2 = 5,9316$ ,  $p=0,0515$ ) between SIN, CRSsNPs and CRSwNPs patients for DMSO+BEBM (Steel-Dwass  $p \leq 0,05$ ).

## 5.8.4 Cytokine concentration in nasal secretion of CRSwNPs, CRSsNPs and SIN (controls) group

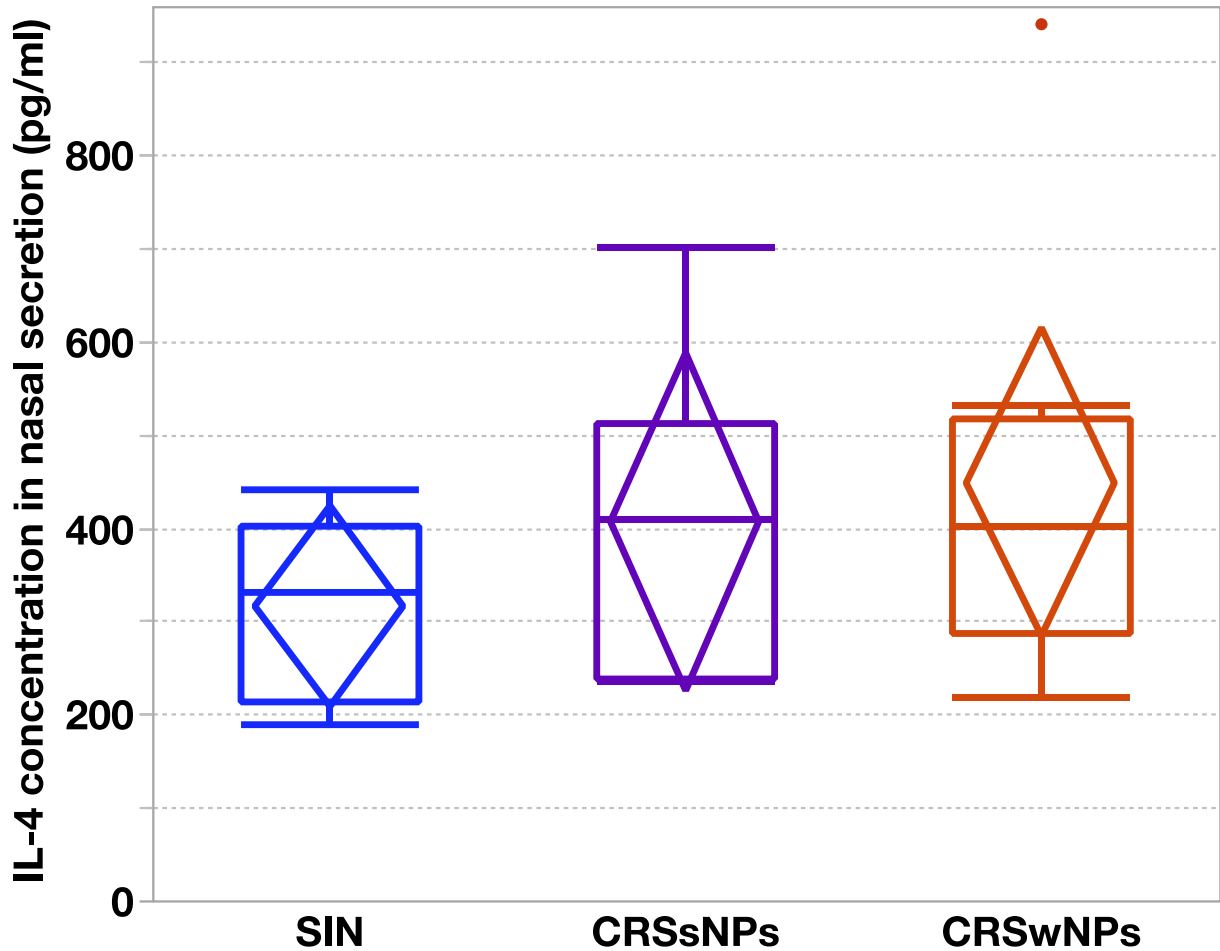
### 5.8.4.1. IL-4 concentration in nasal secretion of CRSwNPs, CRSsNPs and SIN (controls) group

Cytokine concentration in nasal secretions of patients with CRSwNP and CRSsNP, as well as healthy controls, was studied (from the ex vivo experimental study). CRSwNP patients had the highest overall mean IL-4 concentration (mean 448,778 pg/ml, 214,429), followed by CRSsNP patients (mean 406,500 pg/ml, 171,613). SIN (controls) had the lowest value (mean 315,833pg/ml, 102,261). However, there was no statistically significant difference between these groups ( $\chi^2_2 = 2,3626$ ,  $p=0,3069$ ).

**Figure 13** and **Table 11** show the distribution of IL-4 concentration in nasal secretion.

**Table 11.** Descriptive statistics of the IL-4 concentration in pg/ml in nasal secretion in groups and results of Kruskal Wallis ( $\chi^2_2 = 2,3626$ ,  $p=0,3069$ ) with the Steel-Dwass post-hoc test.

Group	Number of patients	Mean	Standard deviation	Minimum	Maximum
SIN	7	315,833	102,261	189,00	440,00
CRSsNPs	7	406,500	171,613	234,00	700,00
CRSwNPs	7	448,778	214,429	217,00	940,00



**Figure 13.** *IL-4 concentration in nasal secretion of study patients from the ex vivo experimental study.*

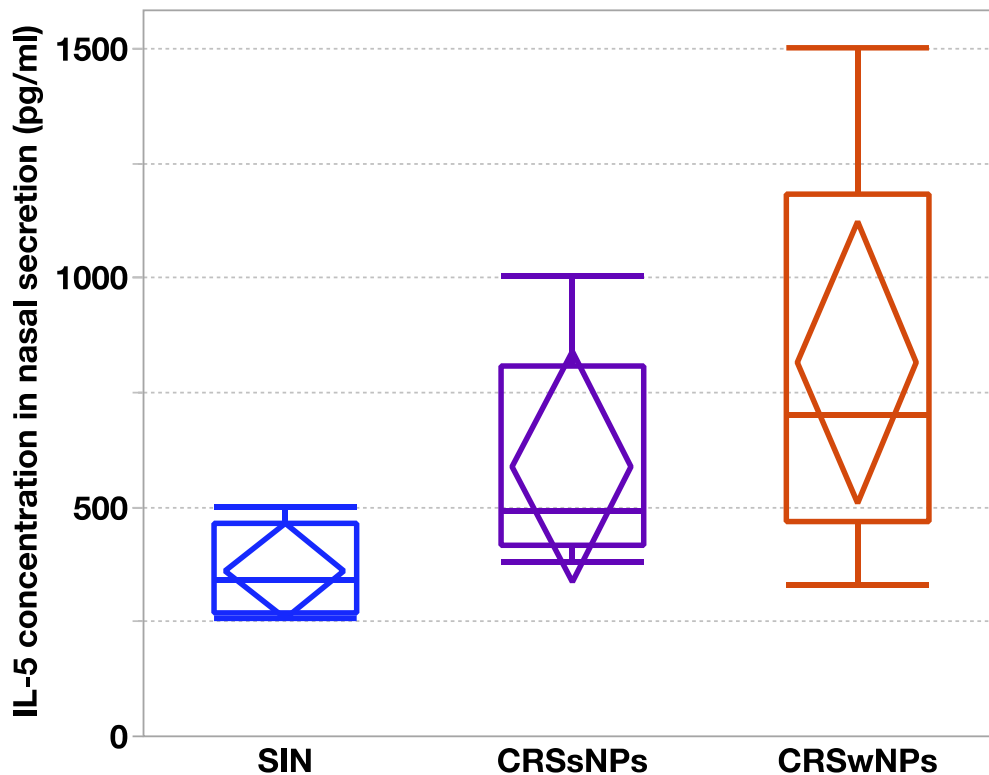
#### **5.8.4.2. IL-5 concentration in nasal secretion of CRSwNPs, CRSsNPs and SIN( controls) group.**

The CRSwNP group had the highest overall mean IL-5 concentration (mean 814,111 pg/ml, 400,653), followed by the CRSsNP group (mean 587,00 pg/ml, 238,185). Patients with SIN (controls) had the lowest overall mean IL-5 concentration (mean 359,50 pg/ml, 100,397). There was a statistically significant difference ( $\chi^2_2 = 7,9104$ ,  $p=0,0192$ ) between SIN and CRSwNPs patients.

**Figure 14** and **Table 12** show the distribution of IL-5 concentration in nasal secretion.

**Table 12.** Descriptive statistics of the IL-5 concentration in pg/ml in nasal secretion in groups and results of Kruskal Wallis ( $\chi^2_2 = 7,9104$ ,  $p=0,0192$ ) with the Steel-Dwass post-hoc test.

Group	Number of patients	Mean	Standard deviation	Minimum	Maximum
SIN	7	359,50	100,397	255,00	500,00
CRSsNPs	7	587,00	238,185	378,00	1000,00
CRSwNPs	7	814,111	400,653	327,00	1500,00



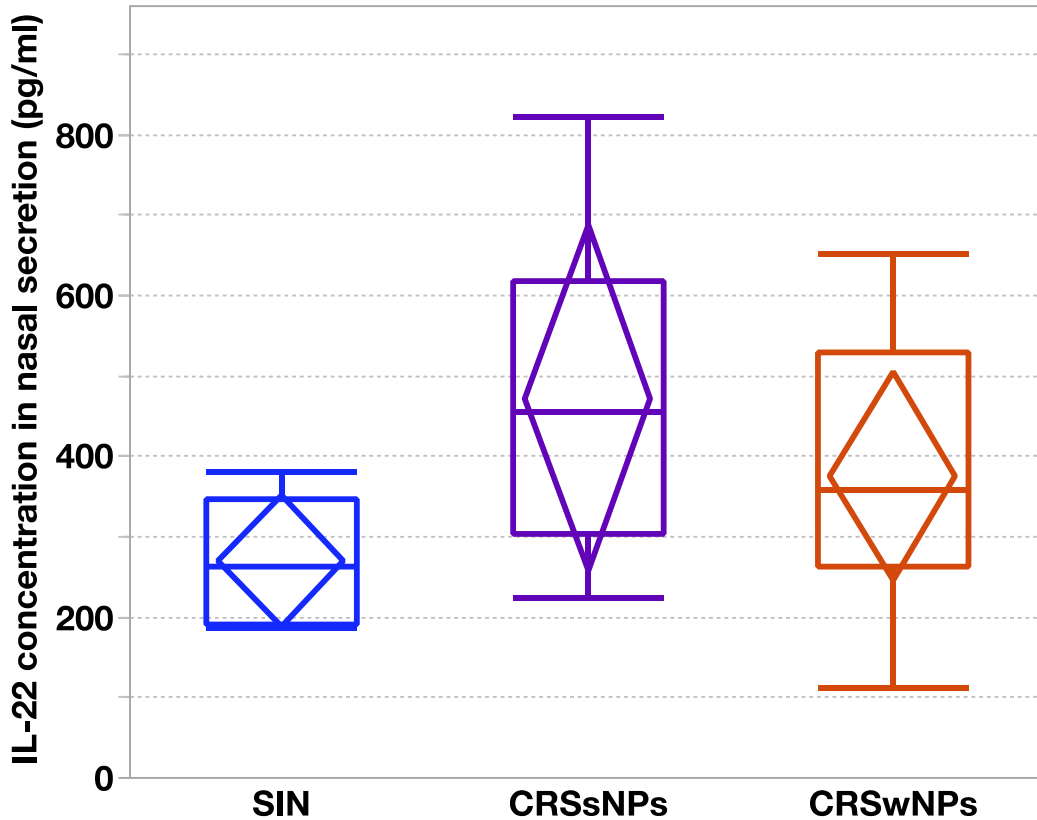
**Figure 14** IL-5 concentration in nasal secretion of study patients from the ex vivo experimental study.

### 5.8.4.3. IL-22 concentration in nasal secretion of CRSwNPs, CRSsNPs and SIN( controls) group.

Patients with CRSsNPs had the highest overall mean IL-22 concentration (mean 471,167 pg/ml, 205,152). The overall mean IL-22 concentration in CRSwNP patients was lower (mean 374,778 pg/ml, 168,587). Patients with SIN (control) had the lowest overall mean IL-22 concentration (mean 268,833 pg/ml, 78,387). These changes, while noticeable, were not statistically significant ( $\chi^2_2 = 3,7648$ ,  $p=0,1522$ ). **Figure 15** and **Table 13** show the distribution of IL-22 concentration in nasal secretion.

**Table 13.** *Descriptive statistics of the IL-22 concentration in nasal secretion in groups and results of Kruskal Wallis ( $\chi^2_2 = 3,7648$ ,  $p=0,1522$ ) with the Steel-Dwass post-hoc test.*

Group	Number of patients	Mean	Standard deviation	Minimum	Maximum
SIN	7	268,833	78,387	185,00	380,00
CRSsNPs	7	471,167	205,152	224,00	820,00
CRSwNPs	7	374,778	168,587	110,00	650,00



**Figure 15.** *IL-22 concentration in nasal secretion of study patients from the ex vivo experimental study.*

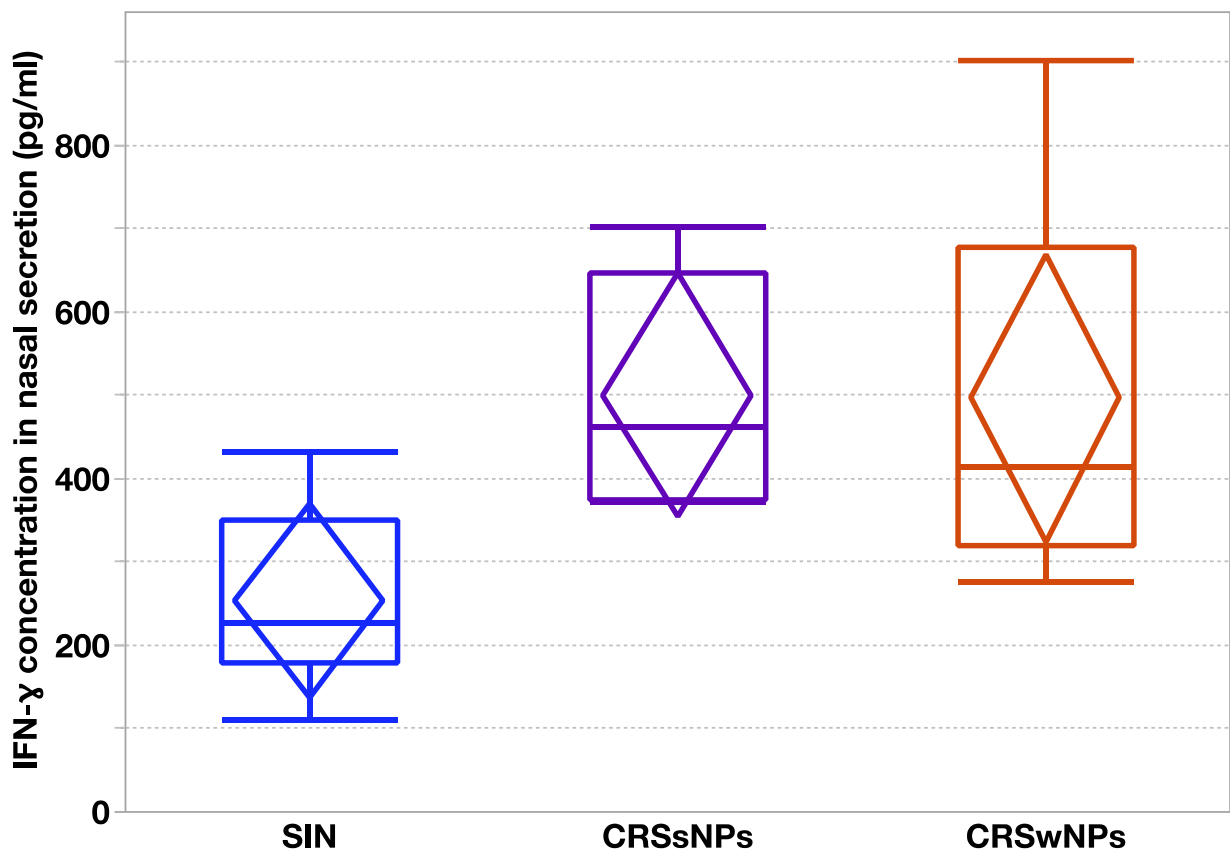
#### **5.8.4.4. IFN- $\gamma$ concentration in nasal secretion of CRSwNPs, CRSsNPs and SIN( controls) group.**

CRSsNPs patients had the highest overall mean IFN-  $\gamma$  concentration (mean 499,333 pg/ml, 139,404), while SIN (control) patients had a much lower value (mean 252,667 pg/ml, 110,919). IFN-  $\gamma$  concentration in nasal secretion was higher in the CRSwNP group (mean 495,222 pg/ml, 223,089) than in the SIN group. There was statistically significant difference between both phenotypes of CRS and controls ( $\chi^2_2 = 7,5397$   $p=0,0231$ ).

**Figure 16** and **Table 14** show the distribution of IFN-  $\gamma$  concentration in nasal secretion.

**Table 14.** Descriptive statistics of the IFN- $\gamma$  concentration in nasal secretion in groups and results of Kruskal Wallis ( $\chi^2_2 = 7,5397$   $p=0,0231$ ) with the Steel-Dwass post-hoc test.

Group	Number of patients	Mean	Standard deviation	Minimum	Maximum
SIN	7	252,667	110,919	110,00	430,00
CRSsNPs	7	499,333	139,404	371,00	700,00
CRSwNPs	7	495,222	223,089	274,00	900,00

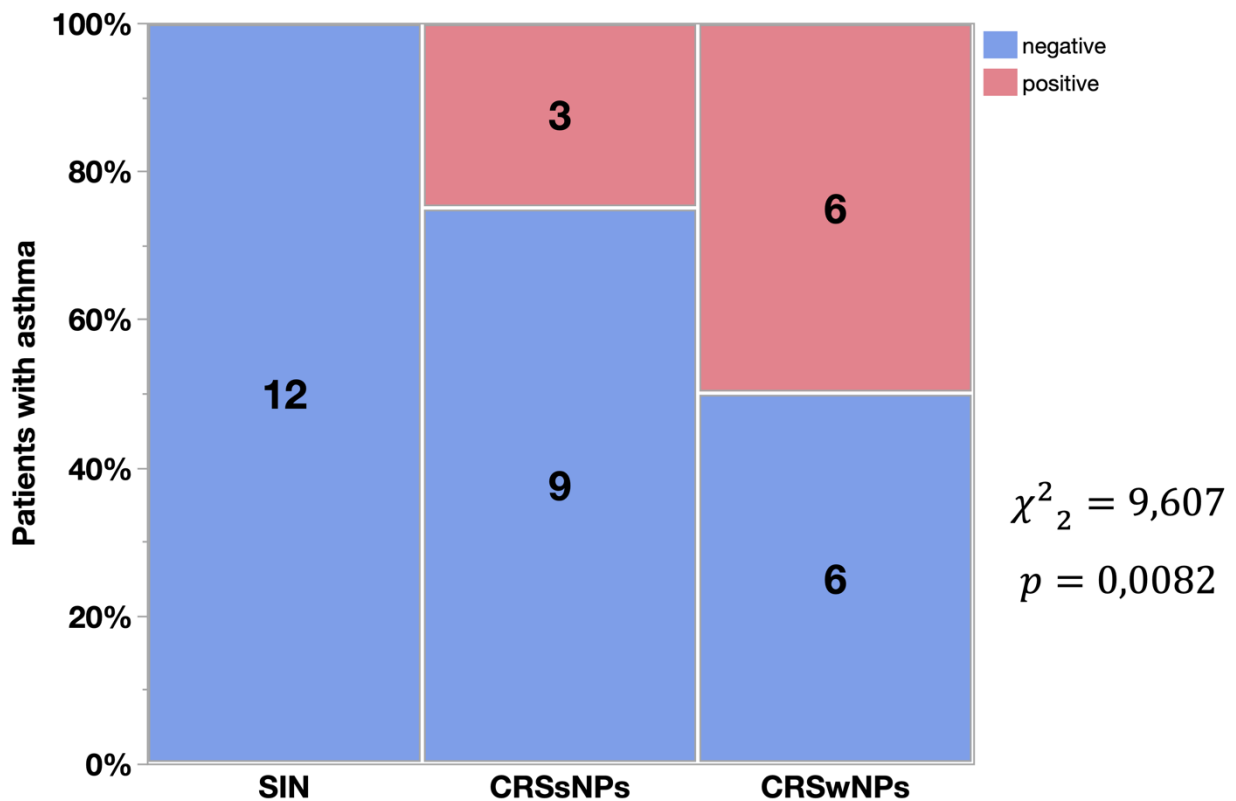


**Figure 16.** IFN- $\gamma$  concentration in nasal secretion of study patients from the ex vivo experimental study.

### 5.9 Propensity for bronchial asthma in CRSsNPs, CRSwNPs, and SIN (healthy) patients.

We did not find any patients with bronchial asthma in a group of healthy patients (n=12). In the CRSsNPs group (n=12), three patients (25 percent ) had a history of bronchial asthma. Bronchial asthma affected the greatest number of patients (50 percent) in the CRSwNPs group, n=6 ( $\chi^2_6 = 9,607$ ,  $p=0,0082$ ) (See **Figure 17**).

We considered the tendency of bronchial asthma as one of the predisposing factor of atopic status in the study groups' patients.



**Figure 17.** A contingency analysis with chi square test of the bronchial asthma status and the phenotype of chronic rhinosinusitis. Numbers in mosaic plot represents number of patients.



## 5.10 Association between AhR expression in plasma cells and predisposing factors

We tried to compare AhR expression into plasma cells of study patients with following factors as smoking, allergy (air pollutants), allergy to NSAIDs and bronchial asthma. There was no statistically significant difference between AhR expression and allergy positive and negative patients, as well as bronchial asthma positive and negative patients. When we compared cytoplasmic AhR expression between smokers and non-smokers we revealed statistically significant difference ( $\chi^2_2 = 7,014$ ,  $p=0,0300$ ). AhR expression into plasma cells of smokers was increased compared to non-smokers. In **Table 15** has shown association between smoking, bronchial asthma, allergy, sensitivity to NSAIDs and AhR expression into cytoplasm and nuclei of plasma cells. Data was not compared between study groups.

**Table 15.** *AhR<sup>+</sup> grade in plasma cells cytoplasm and nuclei showing influence of the smoking, asthma and allergies on AhR<sup>+</sup> grade in plasma cells cytoplasm and nuclei.*

	AhR <sup>+</sup> grade in plasma cells cytoplasm				AhR <sup>+</sup> grade in plasma cells nuclei			
	0 N(%)	I N(%)	II N(%)	III N(%)	0 N(%)	I N(%)	II N(%)	III N(%)
<b>Asthma</b>	$(\chi^2_2 = 2,827, p=0,2433)^*$				$(\chi^2_3 = 0,729, p=0,8664)^*$			
Negative	0 (0,00)	4 (10,26)	13 (33,33)	11 (28,21)	5 (12,82)	9 (23,08)	11 (28,21)	3 (7,69)
Positive	0 (0,00)	0 (0,00)	6 (15,38)	5 (12,82)	2 (5,13)	5 (12,82)	3 (7,69)	1 (2,56)
<b>Allergy (air pollutant)</b>	$(\chi^2_2 = 1,685, p=0,4306)^*$				$(\chi^2_3 = 3,947, p=0,2672)^*$			
Negative	0 (0,00)	3 (8,57)	14 (40,00)	14 (40,00)	6 (17,14)	11 (31,43)	10 (28,57)	4 (11,43)
Positive	0 (0,00)	0 (0,00)	3 (8,57)	1 (2,86)	0 (0,00)	1 (2,86)	3 (8,57)	0 (0,00)
<b>Allergy (NSAID)</b>	$(\chi^2_2 = 2,139, p=0,3432)^*$				$(\chi^2_3 = 3,668, p=0,2996)^*$			
Negative	0 (0,00)	4 (10,26)	16 (41,03)	12 (30,77)	6 (15,38)	11 (28,21)	13 (33,33)	2 (5,13)

Positive	0 (0,00)	0 (0,00)	3 (7,69)	4(10,26)	1 (2,56)	3 (7,69)	1 (2,56)	2 (5,13)
<b>Smoking</b>	<b>(<math>\chi^2_2 = 7,014, p=0,0300</math>)*</b>				<b>(<math>\chi^2_3 = 3,366, p=0,4953</math>)*</b>			
Negative	0 (0,00)	4 (10,53)	10 (26,32)	14 (36,84)	4 (10,53)	11 (28,95)	9 (23,68)	4 (10,53)
Positive	0 (0,00)	0 (0,00)	8 (21,05)	2 (5,26)	2 (5,26)	3 (7,89)	5 (13,16)	0 (0,00)

\* Results of the Kruskal Wallis test.

We also performed **Mann Whitney U test** of the relative NOX1 mRNA expression in tissue cultures treated with 2-(1H-Indol-3-ylcarbonyl)-4-thiazolecarboxylic acid methyl ester (ITE (5 mM/ml) and 6,2'4' trimethoxyflavone (TMF 0,5mM/ml) considering predisposing factors as bronchial asthma, smoking, and allergy .There was no statistically significant difference between relative NOX1 mRNA expression and mentioned factors ( See **Table 16**).

**Table 16.** Descriptive statistics of the relative NOX1 mRNA expression in tissue cultures treated with 2-(1H-Indol-3-ylcarbonyl)-4-thiazolecarboxylic acid methyl ester (ITE (5 mM/ml) and 6,2'4' trimethoxyflavone (TMF 0,5mM/ml) expression in patients with or without asthma, allergy and smoking status.

	ITE 5 mM/ml					TMF 0,5mM/ml				
	N	Mean	Std Dev	Min	Max	N	Mean	Std Dev	Min	Max
<b>Asthma</b>	<b>(z = -1,674, p=0,0941)*</b>					<b>(z = 1,246, p=0,2128)*</b>				
Negative	15	1,585	0,411	1,020	2,110	15	1,599	0,459	1,020	2,450
Positive	6	1,247	0,285	0,980	1,630	6	2,035	0,786	1,160	2,910
<b>Allergy (air pollutant)</b>	<b>(z = -1,187, p=0,2353)*</b>					<b>(z = 1,370, p=0,1707)*</b>				
Negative	18	1,503	0,392	0,980	2,11	18	1,681	0,556	1,020	2,910
Positive	1	1,030	-	1,030	1,030	1	2,780	-	2,780	2,780
<b>Allergy (NSAID)</b>	<b>(z = -0,818, p=0,4135)*</b>					<b>(z = 1,246, p=0,2128)*</b>				

Negative	15	1,530	0,398	1,020	2,110	15	1,599	0,459	1,020	2,450
Positive	6	1,383	0,437	0,980	2,060	6	2,035	0,786	1,160	2,910
<b>Smoking</b>	$(z = -0,476, p = 0,6343)^*$					$(z = -1,625, p = 0,1041)^*$				
Negative	13	1,541	0,430	1,030	2,110	13	1,848	0,614	1,080	2,910
Positive	7	1,463	0,353	1,020	1,972	7	1,383	0,349	1,020	1,970

**\*Results of the Mann Whitney U test.**

Additionally, we performed **Kruskal Wallis test** of the relative NOX1 mRNA expression in tissue cultures treated with ITE (5 mM/ml) and TMF 0,5mM/ml and AhR<sup>+</sup> expression in plasma cells (cytoplasm/nuclea). There was no statistically significant difference between relative NOX1 mRNA expression and mentioned factors. We found sample size too small for appropriate conclusion (See **Table 17**).

**Table 17.** Descriptive statistics of the relative NOX1 mRNA expression in tissue cultures treated with 2-(1H-Indol-3-ylcarbonyl)-4-thiazolecarboxylic acid methyl ester (ITE (5 mM/ml) and 6,2'4' trimethoxyflavone (TMF 0,5mM/ml) considering AhR<sup>+</sup> grade in plasma cells cytoplasm and in nuclea.

	ITE 5 mM/ml					TMF 0,5mM/ml				
	N	Mean	Std Dev	Min	Max	N	Mean	Std Dev	Min	Max
<b>AhR<sup>+</sup> grade in plasma cells cytoplasm</b>	$(\chi^2_2 = 3,7721, p = 0,1517)^*$					$(\chi^2_2 = 1,4342, p = 0,4882)^*$				
0	0	-	-	-	-	0	-	-	-	-
I	2	1,945	0,233	1,780	2,110	2	1,945	0,233	1,780	2,110
II	7	1,574	0,405	0,980	2,040	7	1,624	0,540	1,110	2,490
III	8	1,341	0,352	1,030	2,010	8	1,985	0,675	1,120	2,910
<b>AhR<sup>+</sup></b>	$(\chi^2_3 = 3,3996, p = 0,3340)^*$					$(\chi^2_2 = 0,7462, p = 0,8623)^*$				

<b>grade in plasma cells nuclei</b>										
0	3	1,443	0,592	0,980	2,110	3	1,947	0641	1,240	2,490
I	19	1,384	0,322	1,030	1,870	9	1,828	0,718	1,110	2,910
II	4	1,805	0,404	1,200	2,040	4	1,805	0,404	1,200	2,040
III	1	1630	-	1,630	1,630	1	1,630	-	1,630	1,630

**\* Results of the Kruskal Wallis test.**

## 6. DISCUSSION

Excessive free radical generation, specifically ROS, causes oxidative stress in a biological system and, as a result, mediates the pathogenesis of chronic inflammatory conditions. Reactive oxygen species are biologically active but unstable agents that degrade quickly, making them difficult to identify in tissues. As a result, in order to assess serum and tissue oxidative stress levels, we had to rely on surrogate markers of excessive ROS production – lipid peroxidation markers.

As we mentioned above (Introduction) in study of Zheng et al. level of 4-HNE expression and NADPH oxidase isoform p67 phox are significantly higher in eosinophils and neutrophils of nasal polyp tissue than in healthy mucosa. Our findings revealed a statistically significant difference in 4-HNE expression in plasma cells between the SIN (controls), CRSsNPs, and CRSwNPs groups ( $\chi^2_{12} = 25,17$ ,  $p = 0,014$ ).

When compared to SIN (controls 0%) and CRSsNPs patients, 4-HNE expression in cytoplasm of plasma cells had the highest percent of III grade of 4-HNE (42,86 percent,  $p = 0,0019$ ) in CRSwNPs patients ( $n = 12$ ) (15,38 percent). In 92.86 percent of CRSwNPs patients, the nuclei of plasma cells were negative stained for 4-HNE. Similar findings were found in the nuclei of plasma cells of CRSsNPs (100 percent - 0 grade) and controls (83,33 percent - 0 grade) (**Figure 4, Table 4**).

Furthermore, we measured the levels of expression of Acrolein, a novel lipid peroxidation marker, in the paranasal tissue of CRSwNPs, CRSsNPs, and healthy controls for the first time. We discovered a statistically significant difference in ACR expression between SIN (controls), CRSsNPs, and CRSwNPs ( $\chi^2_6 = 16,08$ ,  $p = 0,0134$ ) ( See **Figure 6**). Thus, in CRSwNPs patients, ACR expression in eosinophils had the highest percentage of III grade (53,85 percent,  $p < 0,001$ ) when compared to SIN (controls 0%) and CRSsNPs patients (7,69%). ( See **Table 5**). We found this as a new evidence which was not yet reported in literature.

According to our findings, oxidative stress exists in the cytoplasm of plasma cells with the highest III grade of intensity in CRSwNPs patients but not in the nuclei of cells. As plasma cells, eosinophils are primarily exposed to oxidative stress in CRSwNPs patients. We believe that the above-mentioned cell types play a critical role in the persistence of chronic inflammation. Furthermore, 4-HNE levels in CRS phenotypes' serum confirm higher

oxidative stress in CRSwNPs (mean value 26,86 pmol/mg, 12,85) compared to CRSNPs and SIN (controls) ( **See Figure 7, Table 6**). The difference between study groups is statistically significant ( $F_{2,22} = 4,8812$ ,  $p=0,0176$ ). The discovery, along with increased protein expression of lipid peroxidation markers in study groups' tissue, confirms the etiopathogenic importance of oxidative stress across CRS phenotypes. We propose that the comorbidities identified in CRSwNPs patients may be an additional factor in exerting oxidative stress status (**See Figure 16, Table 1 and 15**).

NADPH oxidase isoforms, such as NOX1 and NOX4, and the cellular source of superoxides are known to be primarily found in the epithelial layer, submucosal glands, vascular endothelium, and inflammatory cells in both healthy and allergic nasal mucosa, as well as nasal polyps [115]. In our study, we discovered lipid peroxidation markers such as 4-HNE and acrolein in these layers. Similarly, inflammatory cells were discovered in the same layers, which we interpret as an indication of excessive ROS production in CRS patients (Figure 2 and 3). An investigation into the role of oxidative stress in the development of allergic rhinitis and nasal polyposis found that NOX1 and NOX4 play an important role in the production of reactive oxygen species, thus contributing to the oxidative stress seen in allergic rhinitis and nasal polyposis.

In their study, Bozkus et al found that total oxidative status was higher and correlated with age in patients with nasal polyps (NPs) compared to patients without NPs [116]. Our findings are consistent, as we found a higher TOC in CRSsNPs patients compared to controls (mean value 0,027mM, 0,0248 respectively), indicating a role for oxidative stress in the pathogenesis of CRS. There is no statistically significant difference between study groups, owing to the small sample size ( $F_{2,35} = 0,6746$ ,  $p=0,5158$ ).

It has previously been demonstrated that levels of 8-isoprostanes, which are reliable biomarkers of oxidative stress in expired breath condensate, are higher in steroid-naive patients with aspirin-induced asthma (AIA) compared to AIA patients treated with steroids and healthy volunteers. This points to a possible pathological link between AIA and oxidative stress [117]. Among the CRSwNPs (n=12) cases in our study, we found a subgroup of five patients (31%) who had Samter's triad, these patients were resistant to therapy and had to undergo repeated endoscopic endonasal sinus surgery.

Researchers and clinicians have recently become interested in the role of AhR in immune function, particularly in inflammation. Chen et al. discovered that AhR and TGF-1 expression were positively correlated with collagen levels in CRS samples in their study. In CRSsNP patients who do not have allergic rhinitis, elevated AhR expression may be involved in the progression of tissue remodelling. Lower AhR expression, on the other hand, may be involved in allergic reactions in CRSwNP with allergic rhinitis [118].

Our research found a statistically significant difference in AhR expression between SIN (controls), CRSsNPs, and CRSwNPs ( $\chi^2_{16} = 36,16$ ,  $p = 0,0027$ ).

In CRSwNPs patients (n=12), AhR expression in the cytoplasm of plasma cells had the highest percentage of AhR III grade (64,29 percent,  $p = 0,0015$ ) when compared to SIN (controls 0%) and CRSsNPs patients (53,85 percent). In the nuclei of plasma cells, 35.71 percent of CRSwNPs patients had 0 grade AhR expression and 42.86 percent had I grade AhR expression, whereas SIN (controls) had I grade 25 percent and II grade 58.35 percent. In the nuclei of CRSsNP plasma cells (I and II grade equal dominated, 38.46 percent respectively) (See **Figure 9, Table 7**). By looking at AhR expression in plasma cells from different study groups, we discovered that cytoplasmic AhR expression increased in both CRS phenotype as the grade of expression increased. In the nuclei of plasma cells of CRSwNPs patients, AhR expression decreases as the grade of intensity increases (See **Table 7**). We found this as a new evidence which was not yet reported in literature. Taking into account this evidence and increased cytoplasmic 4-HNE into plasma cells of CRSwNPs patients, we propose that this evidence could indicate to weak activity of AhR and therefore weak ability of receptor in regulation of ROS production in CRSwNPs patients compared to CRSsNPs and controls. In addition, there is no difference in nuclear AhR expression between CRSsNPs and SIN (controls), in both group AhR expression in nuclei of plasma cells was weak I grade and moderate II grade of expression. It is known that AhR in the cytoplasm is inactive, and that AhR translocates into cell nuclei and binds with ARNT proteins in response to AhR ligands. When the AhR-ARNT complex becomes active, it can bind to regulatory genes. We could not find reasons for weak activity of AhR in the literature that we observed in CRSwNPs patients in our study due to a predominance of negative and weak positive nuclear AhR compared to SIN (controls). We believe that more research into the AhR activation system in the CRSwNP phenotype is needed.

When the Kendall rank coefficient was used as a test statistic to determine whether the grades of 4HNE+ and AhR+ were statistically dependent, statistical significance was obtained in the case of plasma cell cytoplasm ( $K_{\tau} = 0,41$ ,  $p=0,004$ ). This suggests that higher levels of 4HNE+ in the cytoplasm are linked to higher levels of AhR+ and vice versa. The same could not be said for plasma cell nuclei ( $K_{\tau} = -0,19$ ,  $p=0,193$ ). The grades of the 4HNE+ and AhR+ in plasma cells, cytoplasm, and nuclei were then analyzed using Kendall rank correlation analysis in CRSwNP, CRSsNP, and SIN patients. There was no statistically significance in this case. Thus, the findings support the existence of a link between cytoplasmic AhR and 4-HNE in the cytoplasm of plasma cells in CRSwNPs patients.

This could be interpreted as a link between excessive ROS production and AhR overexpression in patients with CRSwNPs.

AhR is known to play a role in the regulation of Th17 cell differentiation. According to Gu et al [119], polarization of TH17 cells in atopic CRSwNP is higher than in non-atopic individuals. The intervention of an endogenous ligand for the aryl hydrocarbon (Ah) receptor (AhR) – ITE – in peripheral monocyte blood cells (PMBCs) promoted AhR expression and attenuated Th17 responses, demonstrating that AhR was more likely to suppress Th17 cell differentiation in CRSwNP patients. Treatment of NP tissue with ITE in a dose-dependent manner in our study revealed that ITE reduces NOX1 mRNA expression in CRSwNPs patients but not in healthy controls or CRSsNP cases. We found induced NOX1 mRNA expression in healthy paranasal tissue. In allergic rhinitis, there was a significant reduction in Th17 cell differentiation as well as IL-17 and IL-22 production after the ITE intervention [120]. When atopic CRwNPs patients were compared to patients with allergic rhinitis, it was discovered that atopic patients have a distinct pathogenesis from non-atopic CRS cases. It is well known that noxious agents in cigarette smoke and environmental pollutants, such as B (a) P, dioxin, and dioxin-like compounds (AhR agonists), have the potential to cause respiratory tract inflammation by activating growth factors and cytokines [121]. In a study by Souza et al., AhR inhibition was associated with significantly increased neutrophilia in the airways in response to cigarette smoke in an animal model [122]. These findings support the previously proposed link between AhR activity and cigarette smoke.

In our study we were unable to find a link between bronchial asthma and allergy and NOX1 mRNA expression in either of the CRS phenotypes studied in ex vivo (**See Table 15,16 and**



17). As a result, we believe that AhR signaling is regulated by endogenous pathways that are unaffected by exogenous triggers. However, we revealed statistically significant difference in cytoplasmic AhR expression of plasma cells between smokers and non-smokers ( $\chi^2_2 = 7,014, p=0,0300$ ) (See **Table 15**). Smokers had predominantly moderate grade II and high grade III of cytoplasmic AhR expression compared to non-smokers. It is well known that neutrophils can be recruited and are abundant in the nasal mucosa of patients suffering from chronic rhinosinusitis (CRS) in both Asians and Caucasians. Increased neutrophil infiltration in CRS patients has been linked to poor corticosteroid response and disease prognosis. Meanwhile, tissue neutrophils may have distinct phenotypic characteristics that distinguish them from resting blood counterparts and are endowed with specific functions such as cytokine and chemokine production, suggesting that they may contribute to the pathogenesis of CRS [123]. In our study, we found intraepithelial neutrophils in respiratory epithelium tissue samples from CRSwNPs patients, which could indicate a role in the maintenance of chronic inflammation in paranasal sinus tissues.

Recently, it was discovered that 6,2',4'-trimethoxyflavone (TMF) acts as an AhR ligand, that is, as a strong antagonist with agonist-competing capacity. Furthermore, TMF antagonism is not species or promoter dependent. Because of these characteristics, we chose TMF as an AhR ligand strong antagonist in evaluating the response to NOX1 mRNA expression.

In our study, we found that TMF intervention significantly reduced relative NOX1 mRNA expression in CRSsNP in a dose-dependent manner. TMF concentration (mean value 2,54 to 0,25 mM and 1,30 to 0.5 mM). There is a statistically significant difference (Kruskal Wallis with the Steel-Dwass post-hoc test,  $p < 0,05$ ) mentioned. (See **Figure 11** and **Table 10**). TMF intervention suppresses NOX1 mRNA expression in the CRSsNPs phenotype, indicating that the strong AhR antagonist TMF is capable of suppressing NOX1 mRNA. We found this as a new evidence which was not yet reported in literature.

In our ex vivo study, we used a tissue stimulation model to treat SIN-healthy sinus tissue, CRSsNP, and CRSwNPs tissue with AhR agonist by ITE in a dose-dependent manner over 24 hours. There is a statistically significant difference between SIN-healthy and CRSwNPs patients. Patients in the CRSwNPs group reported a statistically significant difference in the mean value of relative NOX1 mRNA (1,83; 1,23; and 2,26, respectively) between the treated doses of ITE 1mM, ITE 5mM, and DMSO +BEBM (Kruskal Wallis with the Steel-Dwass post-

hoc test  $p < 0,05$ ). We propose that higher doses of AhR ligand stimulation can reduce ROS production in the CRSwNP phenotype.

According to the new EPOS 2020 classification mentioned above (Introduction) , three endotypes are defined based on immunological response. Based on emerging evidence linking effector T cells in adaptive immunity and ILC lineages, it has been hypothesized that innate and adaptive immune systems will converge into type 2 and non type 2 ( type 1 and type 3) immune responses.

We measured cytokine concentration in nasal secretion of the patients in ex vivo experimental study. IL-4 concentration in nasal secretion was higher ( mean 448,778 pg/ml ,214,429) in CRSwNPs patients than in controls , However, there was no statistically significant difference between study groups ( $\chi^2_2 = 2,3626$ ,  $p=0,3069$ ). IL-5 concentration in nasal secretion of CRSwNPs patients was significantly higher (mean 814,111 pg/ml, 400,653) than in healthy controls (mean 359,50 pg/ml, 100,397) ( $\chi^2_2 = 7,9104$ ,  $p=0,0192$ ) . CRSsNPs patients had the highest overall mean IFN-  $\gamma$  concentration (mean 499,333 pg/ml, 139,404) than in controls , however was no statistically significant compared to controls. In addition , IFN-  $\gamma$  concentration in both phenotypes of CRS patients' nasal secretion was significantly higher (mean 495,222 pg/ml, 223,089 and 499, 333 pg/ml , 139,404) than in controls (mean 252,667 pg/ml, 110,919), ( $\chi^2_2 = 7,5397$   $p=0,0231$ ) . IL-22 concentration was increased in CRSsNPs patients (mean 471,167 pg/ml, 205,152) however was no significantly different compared to controls (mean 268,833 pg/ml, 78,387), ( $\chi^2_2 = 3,7648$ ,  $p=0,1522$ ).

Thus , we suggest that more research in regulatory mechanism of ROS production through AhR pathway is required in both phenotypes of chronic rhinosinusitis.

## 7. CONCLUSION

1. General oxidative stress level, based on acrolein and 4HNE measurement on cellular level (eosinophils in acrolein and plasma cells in 4-HNE) is significantly higher in the paranasal sinus mucosa of CRSwNP compared to CRSsNP and control samples.
2. AhR protein expression is significantly higher in cytoplasm of plasma cells CRSwNP and CRSsNP, but not in nuclei, while there is no difference between AhR expression in both CRSsNP and controls.
3. The levels of oxidative stress in plasma cells isolated from tissue samples and in serum were significantly higher in samples of the patients with CRS with nasal polyps, than CRSsNPs and control group.
4. AhR ligands regulate NOX1 mRNA expression in both CRS phenotypes and ligand which stimulate AhR and NADPH oxidase isoform expression indicating the inhibition of the receptor.
5. Increased concentration of IL-4 and IL-5 in nasal secretion of CRSwNPs patients suggests predominantly type 2 immune response in patients with CRSwNPs, but only IL-5 concentration is significantly elevated compared to CRSsNP. In CRSsNPs patients increased concentration of IL-22 and IFN- $\gamma$  confirms non type 2 immune response.
6. AhR is significantly more expressed in plasma cells of smokers than in non smokers in persistence of oxidative stress and chronic inflammation.
7. There is no an association between predisposing factors as bronchial asthma, allergy, sensitivity to NSAIDs and AhR expression in plasma cells.

## 8. SAŽETAK

Kronični rinosinitis je heterogena upalna bolest nosne i sinusne sluznice koja se pojavljuje u više patogenetski različitih podtipova u čemu ulogu ima i odgovor na stalnu izloženost prekomjernoj proizvodnji reaktivnih kisikovih radikala kao reakcija na endogene ili egzogene podražaje.

**Ciljevi:** Istražiti ulogu arilnog ugljikovodičnog receptora u regulaciji ekspresije reaktivnih kisikovih radikala i njegov utjecaj na upalni odgovor u određenim podvrstama kroničnog rinosinitisa.

**Metode:** U istraživanju su sudjelovali bolesnici koji su bili podvrgnuti endoskopskoj operaciji sinusa: 12 bolesnika imalo je od kronični rinosinitis s nosnim polipima, 12 bolesnika kronični rinosinitis bez nazalnih polipa i 12 kontrola bez simptoma rinosinitisa. Dvadeset jedan pacijent sudjelovao je u eksperimentalnoj *ex vivo* studiji. Koristeći imunohistokemijske metode, analizirali smo ekspresiju markera peroksidacije lipida 4-hidroksinonenal i akroleina u uzorcima tkiva fenotipova kroničnog rinosinitisa sa i bez nosnih polipa i sinusne sluznice uzoraka kontrolne skupine. Da bismo identificirali ekspresiju AhR, koristili smo imunohistokemijsku metodu. Razina oksidativnog stresa određena je pomoću ELISA testa i razina ukupnog oksidacijskog kapaciteta i antioksidacijskog kapaciteta u serumu svake ispitivane skupine i kontrola. U eksperimentalnoj studiji *ex vivo* izložili smo tkivne kulture oba fenotipa kroničnog rinosinitisa i uzoraka kontrola AhR ligandu agonistu receptora 2-(1H-Indol-3-ylcarbonyl)-4-thiazolecarboxylic acid methyl ester i antagonistu receptora 6,2',4'- trimethoxyflavone . Analizirali smo ekspresiju citokina IL-4, IL-5, IFN- $\gamma$  i IL-22 u nosnom sekretu ispitanika u studiji.

**Rezultati:** Marker peroksidacije lipida izazvan oksidativnim stresom 4- hidroksinonenal prekomjerno je izražen u citoplazmi plazma stanice fenotipa kroničnog rinosinitisa s nosnim polipima ( $p = 0,0019$ ) u usporedbi s kroničnim rinosinitisom bez polipa i zdravim kontrolama. Vrijednosti akroleina u tkivima oba fenotipa kroničnog rinosinitisa ukazuju prisutnost oksidacijskog stresa, u odnosu na izostanak istog kod zdravih kontrola. Koncentracija 4-HNE značajno je viša u uzorcima seruma bolesnika s kroničnim rinosinitisom s nosnim polipima ( $p=0,0364$ ), u usporedbi s kontrolnim uzorcima.

Ekspresija AhR i 4-HNE povećana je u citoplazmi plazma stanica kod bolesnika fenotipa s nosnim polipima. ITE smanjuje ekspresiju NADPH oksidaze 1 mRNA kod bolesnika fenotipa s nosnim polipima ovisno o dozi (1,83; 1,23; i 2,26, Kruskal Wallis s Steel-Dwass post-hoc test,  $p < 0,05$ ), ali ne i kod bolesnika sa kroničnim rinosinitisom bez polipa i zdravih kontrola. 6'2'4' TMF inhibira izraženost NADPH oksidaze 1 mRNA u oba fenotipa kroničnog rinosinitisa, ali ne i kod zdravih kontrola.

U nosnom sekretu koncentracija IL-4 ( $p = 0,3069$ ) i IL-5 ( $p = 0,0192$ ) značajno je jače izražena kod kroničnog rinosinitisa s nosnim polipima nego u kontrolama. Prema endotipizaciji međunarodnim smjernicama kronični rinosinitis s nosnim polipima smo kategorizirali kao imunološki odgovor tipa 2, a kronični rinosinitis bez polipa kao imunološki odgovor ne-tip 2. Kod obje skupine KRS-a, koncentracija IFN- $\gamma$  bila značajno više nego kod kontrola te je statistički značajna razlika zapažena između oba fenotipa KRS-a te kontrole ( $p = 0,0231$ ). Koncentracija IL-22 bila je viša u skupini bolesnika s kroničnim rinosinitisom bez nosnih polipa nego kod kontrola.

**Zaključak:** Opća razina oksidativnog stresa, mjerena razinama akroleina i 4HNE na staničnoj razini (akrolein u eozinofilima i 4-HNE u plazma stanicama) značajno je viša u paranazalnoj sluznici sinusa bolesnika s nosnim polipima u usporedbi s uzorcima kroničnog rinosinitisa bez nosnih polipa i kontrolnim uzorcima. Ekspresija AhR proteina značajno je viša u citoplazmi plazma stanica oba fenotipa kroničnog rinosinitisa, ali ne i u jezgrama, dok nema razlike između ekspresije AhR-a i u kroničnom rinosinitisu bez nosnih polipa i u uzorcima kontrola. AhR ligandi mogu smanjivati ekspresiju NOX1 mRNA, kod oba fenotipa kroničnog rinosinitisa, što potvrđuje regulacijsku ulogu receptora u proizvodnji reaktivnih kisikovih radikala kod pacijenata s kroničnim rinosinitisom.

## 9. SUMMARY

Association between reactive oxygen species and aryl hydrocarbon receptor in chronic rhinosinusitis, Aigerim Kvarantan, 2023

**Objectives:** To investigate the role of the aryl hydrocarbon receptor in the regulation of the expression of reactive oxygen radicals in subtypes of chronic rhinosinusitis .

**Methods:**Using immunohistochemical methods, we analyzed the expression of 4-hydroxynonenal , acrolein and aryl hydrocarbon receptor in tissue samples of both chronic rhinosinusitis phenotypes and controls. In an *ex vivo* experimental study , we exposed tissue cultures of both phenotypes and healthy paranasal sinuses to AhR ligand agonist and antagonist receptor .

**Results:**4-hydroxynonenal was overexpressed in plasma cell cytoplasm of chronic rhinosinusitis phenotype with nasal polyps( $p = 0,0019$ ) compared to chronic rhinosinusitis without polyps and healthy one. AhR increased in the cytoplasm of plasma cells in patients with nasal polyp phenotype. ITE decreases NADPH oxidase 1 mRNA expression in nasal polyp patients, but not in patients with CRSsNPs and healthy controls.TMF inhibits NADPH oxidase 1 mRNA expression in both CRS phenotypes, but not in healthy controls.

**Conclusion:** AhR ligands can reduce the expression of NOX1 mRNA in both phenotypes , which confirms the regulatory role of the receptor in the production of ROS in patients with chronic rhinosinusitis .

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## 11. CURRICULUM VITAE

The PhD candidate Aigerim Kvarantan MD, univ. mag. med. was born in Feb 19<sup>th</sup> 1982 in Kazakhstan. She is a mother of 2 children . She graduated from State Medical Academy as Doctor of Medicine in 2005 in Kazakhstan. In 2005-2006 graduated Internship in Otorhinolryngology in Kazakhstan. In 2013, she entered to specialization on Otorhinolaryngology in Zagreb, Croatia and graduated its in 2018. After graduation ,she defended and got the title univ. magister in the field of Otorhinolaryngology in 2019. Then , she was worked in County hospital of Cakovec as ENT specialist between 2019-2021 and nowadays she works as ENT specialist in General hospital “Ivo Pedisic” in Sisak . During the last 10 years , she regularly participated in Domestic and International congresses, symposium and professional courses to improve professional skills in the field of Otorhinolaryngology especially in Rhinology. In 2014 , she entered to PhD programme in the filed of Biomedicine and Healthcare , School of Medicine, University of Zagreb. During her doctoral study she several times visited Research Upper airways Laboratorium in University of Ghent, Belgium to get skills in *ex vivo* experimental study on tissue stimulation model under mentorship Prof. dr.sc. Claus Bachert.