**Ph.D Thesis**

# THE ROLE OF THE TRP CHANNELS AND SENSORY NEUROPEPTIDES IN THE INFLAMMATORY DISEASES OF THE NASAL MUCOSA

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# INTRODUCTION

## Pathophysiology of chronic rhinosinusitis

Chronic rhinosinusitis (CRS) is multifactorial and characterized by chronic inflammation of the sinonasal mucosa and its exact ethiology is still unclear. The limitations of earlier assumptions about CRS that it is an incomplete or therapeutically resistant form of acute rhinosinusitis (ARS) has led to the emergence of many alternative hypotheses, but no unified consensus has emerged so far.

According to the classical theory of the 1980s, the development of the disease begins with the occlusion of the osteomeatal complex (OMC) resulting in inflammation due to the blockage of ventilation and drainage. This was followed by the epithel rupture theory according that during inflammation and edema of the nasal mucosa small injuries form that lead to neovascularization and thus polyp formation [1].

A new attempt was made by the fungal theory at the end of the 1990s, which means that all CRS are based on a powerful inflammatory reaction to the Alternaria species. The definition of eosinophilic fungal rhinosinusitis associated with Ponikau [2] and the role of fungus in CRS has fallen for a decade. The fungus, as an influencing factor, changes the character and course of the disease in some CRS variants [3,4].

Studies at the beginning of the 21st century focus on the Staphylococcus superantigen theory. According to Bachert et al. Staphylococcus enterotoxin (SE), which has been implicated through impaired epithelium, initiates multiple immunomodulatory pathways simultaneously leading to Th2 dominant inflammatory process. Due to the strong immune response, polyclonal IgE production, eosinophilic and mast cell inflammation have been observed. The level of serum IgE increases and consequently cause inflammatory symptoms in the upper and lower respiratory tracts, resulting in intrinsic asthma. However, it raises doubts that only about half of patients with nasal polyps show abnormal response to superantigens [5]. For some time attention has shifted towards aspirin intolerance, a disturbance of phospholipid metabolism due to the inhibition of cyclooxygenase enzyme. Through this process, the synthesis of proinflammatory leukotrienes (LTR) increases and the

amount of antiinflammatory prostaglandins (PG) decreases. It is likely to play a role mainly in aspirin exacerbated respiratory disease (AERD), but aspirin and impaired phospholipid metabolism appears to be implicated in aspirin tolerant patient with nasal polyposis, as well [6].

Recently, the damage of the immun barrier has been emphasized. Accordingly, the deficiency of the mechanical barrier and/or the innate immune response of the sinonasal mucosa leads to the development of CRS. The consequence of the negative effect of biofilm and pathogens and the superantigen reaction or fungus exacerbated immun response can contribute to CRS.

Overall, it is assumed that the pathophysiology of the disease involves the impaired response to the adverse effects of the environment, influenced by additional factors as comorbidities, asthma, allergy, or dysbiosis.

## The phenotypes and endotypes of CRS

Two clinically and phenotypically distinct groups are chronic rhinosinusitis with and without nasal polyps are recognised (CRSwNP and CRSsNP, respectively). Based on the severity of CRS, recurrent and "recalcitrant" phenotypes are commonly referred to as recurrent symptoms, the latter being therapeutic-resistant cases. Complicated forms are special entities (mucocele, osteitis, optic neuropathy). Phenotype of CRS can be determined based on serum specific IgE positivity, triggering events and bacterial microenvironment. Recognizing the heterogeneity of CRS has led to the distinction between several biological subtypes based on pathophysiological mechanisms termed as endotype. By understanding the pathophysiology and the corresponding biomarker characteristics, we can understand the course of the disease, predict the likelihood of recurrences, and more individual treatment can be applied. The endotype of Th2 dominant nasal polyposis, predominantly in Europe, can also be characterized by a positive response to anti-IL-5 biological therapy. The presence of biofilm-forming Staphylococcus aureus superantigen enterotoxin (SE), the measurable SE IgE, polyclonal IgE is characteristic of the IgE dominant anti-IgE-therapy responsive endotype [15]. Based on the histopathological differences, two types are predominantly distinguished: remodeling-neutrophils and eosinophilic dominant nasal polyposis.

## TRPV1 receptor

It was firstly investigated and than discovered in Hungary in the 1950-60's that capsaicin, selectively activates the nociceptive nerve terminals. The concept of capsaicin acting on a specific receptor was also demonstrated by Hungarian researchers in the following years. [7]. The receptor was localized to „capsaicin sensitive neurons" in dorsal root (DRG), trigeminal and vagal ganglia. The receptor, named as vanilloid 1 (VR1) was cloned in 1997 refering to the stimuli of vanilloid like structures. Later according to the international nomenclature it has been renamed to transient receptor potential vanilloid 1 (TRPV1). The exact structure of the receptor has been discribed after cloning; the receptor subunit is a 95 kD protein consists of 6 transmembrane domain and a pore part. The 4 subunits form transmembrane, tetrameric and/or heteromeric non-selective ion channel complexes with intracellular N- and C-terminal regions. By specifying the amino acid regions of the protein molecule special functions have been identified. Beside capsaicin TRPV1 can be triggered by noxious heat (> 43 °C), acidic pH (<6), inflammatory and pain inducing molecules, bradykinine peptide, endogenous lipid-like compounds eg. endocannaboids, lipoxynegase products: prostaglandin E2, prostacyline and exogenous vanilloids. TRPV1 activation induces $Na^{+}$ and $Ca^{2+}$-ion influx and depolarisation that generates action potential resulting in sensory activation and nociception. Consequently it transmits different sensory modalities such as thermosensation, pain, itch (afferent function). On the other hand, activation of capsaicin-sensitive sensory nerve fibres leads to the release of various, mainly proinflammatory neuropeptides (neurokinin A, neurokinin B, substance-P, calcitonin gene related peptide: CGRP) mediating inflammation, which is called "efferent function". These molecules cause vasodilatation, edema, and release of plasma proteins from venules, moreover stimulate migration of inflammatory cells [11]. The TRPV1 mediated neurogenic inflammation is the subject of numerous examination of diseases. It plays a role in several inflammatory diseases, such as bronchial asthma, allergic and idiopathic rhinitis, anaphylaxia, migraine [10], inflammatory skin-and bowel diseases. Endomorphins and somatostatin released from the sensory nerve endings have systemic anti-inflammatory "sensocrine" effect [11]. TRPV1 is expressed on primary sensory neurons in nasal mucosa. More recently, TRPV1 receptors have been identified in non-neuronal cells, as well (epidermis, epithelium of various organs, immune system) and reported to have a role in the developement of inflammation and thermosensation. Activated TRPV1 receptors on human

mast cells stimulate the degranulation, acitvate macrophages and phagocytosis, and the defensive mechanisms of dendritic cells and CD4+ T-lymphocytes.

In human nasal mucosa, non-neuronal TRPV1 has been found in seromucosal glands of normal epithelium [12] [13]. It is believed to play a role in the increased mucosa secretion and the pathogenesis of inflammatory diseases [10,14,15].

## TRPA1 receptor

The Transient Receptor Potential Ankyrin 1 (TRPA1) is similar to TRPV1 in terms of molecular structure, function and it is characterized by high repeat of ankyrin regions in the N-terminus that is supposed to have role in gating. As a non-selective $Ca^{2+}$ channel it plays an important role in neurogenic inflammation, pain sensation and many pathophysiological mechanisms. TRPA1 and TRPV1 are extensively coexpressed in a subpopulation of Aδ- and C-fibers cell (afferents of trigeminal and vagal ganglia). It is increasingly clear the functions of the two receptors are interconneted in several respects. TRPA1 was first isolated from lung fibroblast cell line, but for a long time its expression was restricted to certain sensory fibers and accordingly, its primary function as well. The presence of the receptor on non neuronal cells has improved further investigations. It has also been observed that these two receptors are generally co-localized in non neuroanal cells eg. keratinocytes, macrophages, mast cells and monocytes. TRPA1 can be activated by noxious cold temperature (<17 °C), environmental stimuli, endogenous oxidative stress leading to the release of proinflammatoyr mediators, resulting in neurogenic inflammation. Furthermore many irritants found in the human diet and herbs eg. mustard, wasabi, oregano, garlic, cinnamon can excite the receptor [18]. TRPA1 has an important sensory role in respiratory irritation, cough and possibly in cold-induced non-allergic idiopathic rhinitis [19–21]. It has been recently proved that the expression of TRPV1 and TRPA1 is upregulated in oral lichen planus [22,23].

TRPV1, TRPA1 receptors have been described on sensory nerves of the nasal mucosa. They play pivotal roles in mediating neurogenic inflammation, hyperreactivity and histamine-induced itch. However, non-neuronal TRP functions have not been fully elucidated yet.

## Capsaicin desensitization- denervation

The potent TRPV1 agonist capsaicin (the pungent principle in hot pepper) selectively excites and, in repeated high doses, desensitizes or even reversibly damages a subpopulation of sensory nerve fiber terminals. Desensitization induces long-lasting but reversible deficit in function of the sensory nerve endings that is called denervation [24,25]. Repeated high doses of $Ca^{2+}$ ion influx leading to the increase of intracellular $Ca^{2+}$ concentration results in the hypofunction and the depletion of the sensory nerve endings.

In the early 2000s a number of studies have been published trying to demonstrate that locally administered desensitization can be successful in treating chronic inflammation of the nasal mucosa. Capsaicin treatment was used in CRS, allergic and idiopathic rhinitis, and postoperative treatment of CRS. The results are unclear and unconvincing. Treatment in nasal polyps reduced the symptoms and the size of the polyps but its effect were less than the efficacy of evidence-based therapies such as oral steroid treatment or FESS. In a placebo controlled study in patients with postoperative recurrent nasal polyps capsaicin desenzitisation has significantly reduced the size of polyps [27]. In allergic rhinitis the efficacy of intranasal capsaicin therapy was not succesfully proved [28].

# AIMS

The above-mentioned etiopathological uncertainties about CRS and our previous international results in inflammatory diseases have prompted me to examine human patient samples: whether TRP receptors mediating neurogenic inflammation in sensory neurons are present in non neuronal cells, if there is a change in the expression of the receptors that contribute to the pathogenesis of the disease. I summarize my goals as follows:

**1.** To detect changes in gene expression of non neuronal TRPV1 and TRPA1 channels in nasal polyposis compared to healthy nasal mucosa.

**2.** To determine the localization of TRPV1 and TRPA1 receptors in nasal polyposis by immunohistochemistry.

**3.** To analyse of citokine pattern and comparison with the expression of TRP receptors in nasal polyposis.

**4.** Based on the above results, to execute a more detailed study of the subgroup of CRS patients with asthma and allergy.

# EXPERIMENTS (PATIENTS AND METHODS)

## Patients

Healthy control subjects and patients with CRSwNP were recruited in 2010-2012. Nasal polyp (NP) samples were obtained during routine endonasal sinus surgery from 33 patient suffering from CRSwNP. Diagnosis was based on clinical signs, endoscopical findings and CT scans according to the criteria of CRS as defined by the European Position Paper on Rhinosinusitis and Nasal Polyps. All patients used long-term intranasal corticosteroid (INCS) treatment but it was found to be ineffective. Patients ranged from 18-70 years in age, 17 females and 16 males, and divided into subgroups according to comorbidities as appeared in the clinical history: allergic rhinitis and/or asthma. Allergic rhinitis was based on positive prick test and clinical signs; asthma on the evidence of the pulmonolgist. 10 control samples were obtained from healthy subjects without history of any inflammatory upper airway or sinonasal disease, 4 females, 6 males. Samples were taken from the inferior turbinate during routine septal surgery or turbinotomy. In the second part of the study we further examined 9 new polyp samples obtained from NP patients only from the subgroup of asthma and 6 controls.

## Tissue samples

Excised tissue samples were cut into three pieces. One part was put into RNAlater (cat. no. R0901, Sigma-Aldrich, St. Louis, Missouri, USA) and one part was prepared for fresh frozen sample and stored in -80 °C. The third part was fixed in 4% paraformaldehyde and embedded in paraffin for immunohistochemical examination.

## Immunohistochemistry

Tissue samples were evaluated for mast cells, polymorphonuclear leukocytes (PMN), eosinophils (EO), lymphocytes and plasma cells with methylated Giemsa (M&G) staining. Deparaffinized and rehydrated tissue sections were incubated in methylating solution (99 ml

methanol, 1 ml 36% hydrochloric acid) for 20 min at 56 °C followed by Giemsa staining solution (20 ml Giemsa stain, Central Clinical Pharmacy, Medical School, University of Pécs, Pécs, Hungary; 80 ml dH2O) for 20 min at room temperature. Differentiating solution (200 µl cc. acetic acid in 100 ml dH2O) was applied then slides were rinsed in 96% ethanol and washed in xylol for 3 x 5 min. Sections were coverslipped using Pertex mounting medium (Histolab Products AB, Västra Frölunda, Sweden). Slides were scanned using the Pannoramic Desk instrument (3D Histech Ltd., Budapest, Hungary). Cover slips were removed (xylol at 56 °C until detachment followed by 5 min in 96% ethanol). Slides were immunostained using the Leica Bond Max automated stainer (Leica Biosystems, Wetzlar, Germany) in the following main steps. Slides were rinsed in dH2O followed by antigen retrieval (Bond Epitope Retrieval Solution1, Leica Biosystems, Wetzlar, Germany) at pH 6.00 for 20 min. Slides were immunostained by either anti-TRPV1 (1:300; cat. no. GP14100, Neuromics, Edina, MN, USA) or anti-TRPA1 (1:250; cat. no. ARP35205-P050; Aviva Systems Biology, San Diego, CA, USA) rabbit policlonal primary antibody for 15 minutes. Slides were incubated with the Bond Polymer Refine Detection Kit at 37 °C for 20 min with anti-rabbit Poly-HRP-IgG secondary antibody with subsequent chromogenic development using diaminobenzidine tetrahydrochloride hydrate (DAB) for 10 min and hematoxylin nuclear staining for 5 min. Cover slips were mounted again using Pertex medium (Histolab Products AB, Västra Frölunda, Sweden) and re-scanned using the Pannoramic Desk instrument and visualized by Pannoramic Viewer 1.15 software (both from 3D Histech Ltd., Budapest, Hungary). Examination of the tissue sections and identification of immunopositivity and cell morphology were performed by an expert pathologist.

## Quantitative Real-Time RT-PCR (qRT-PCR)

Purification of total RNA was carried out according to the TRI Reagent manufacturer's (Molecular Research Center, Inc., Cincinnati, OH, USA) protocol up to the step of acquiring the aqueous phase. The quantity and purity of the extracted RNA was assessed on Nanodrop ND-1000 Spectrophotometer V3.5 (Nano-Drop Technologies, Inc., Wilmington, DE, USA). 500 ng of total RNA was reverse transcribed using Maxima First Strand cDNA Synthesis Kit (cat. no. K1642, ThermoScientific, Santa Clara, CA, USA) according to the manufacturer's instructions. qRT-PCR was performed on a Stratagene Mx3000P qPCR System (Agilent

Technologies, Santa Clara, USA). PCR amplification was performed using SensiFast Probe/SYBR Lo-ROX Kit (cat. nos BIO-84020/BIO-94020). Transcripts of the reference genes glyceraldehyde 3-phosphate dehydrogenase (GAPDH), hypoxanthine phosphoribosyltransferase 1 (HPRT1), beta-actin and beta-glucuronidase (GUSB) were detected in all samples [29,30]. GAPDH and HPRT1 were eventually chosen as internal controls, the geometric mean of their $C_q$ values were calculated.

## Luminex Multiplex Immunoassay

The excised and frozen tissues were thawed and weighed, and homogenized (TissueLyser II bead mill system, cat. no. 85300, Qiagen, Hilden, Germany) in 450 µl Procarta Cell Lysis Buffer (Affymetrix, Santa Clara, CA, USA). Samples were centrifuged at 2000xg for 20 min. at 4 °C than supernatants were collected and used for further analysis. Procarta Immunoassay (Affymetrix, Santa Clara, CA, USA) using Luminex technology was performed to determine the concentration of interferon gamma (IFNγ) interleukin 4 (IL-4), interleukin 5 (IL-5) and interleukin 8 (IL-8, CXCL8) cytokines in the samples. The experiment was performed according to the manufacturer's instructions.

## Statistical analysis

Unpaired t-test or one-way ANOVA with Tukey's multiple comparisons test on log2 transformed mRNA fold change data, and Mann–Whitney test or Kruskall-Wallis with Dunn's multiple comparisons test on cytokine protein data were performed using GraphPad Prism 5.02 for Windows (GraphPad Software, Inc., La Jolla, CA, USA). Probability values $P < 0.05$ were accepted as significant. Error bars represent standard error of the mean (SEM). Log2 mRNA fold change data were further analyzed by hierarchical cluster analysis then visualized in a heat map using the free web tool Morpheus (Broad Institute, Cambridge, MA, USA). Correlation analysis of log2 gene expression data was performed by biweight mid-correlation algorithm implemented in R 3.2.3 programming language applying *bicor* function of the WGCNA package. Correlation coefficients (r values) were visualized in a heat map using the Morpheus web tool.

# RESULTS

## The change of gene expression of non neuronal TRPV1 and TRPA1 channels in nasal polyps compared to normal healthy mucosa

**a.** Both TRPV1 and TRPA1 transcripts were detected in nasal polyp and normal nasal mucosa.

**b.** In patient group #1, TRPV1, TRPA1 mRNA levels of NP samples decreased significantly by 0.35 and 0.43 fold, respectively, compared to the normal mucosa.

**c.** When comparing TRP mRNA amounts within subgroups of CRSwNP patients, a significant elevation of TRPV1 but not TRPA1 was observed in the allergic (1.9 fold), and allergic + asthmatic (3.9 fold) subgroups compared to NP patients without these comorbidities.

**d.** No significantly change was observed in TRPA1 levels compared to the subgroups.

**e.** In CRSwNP patient group #2 with asthma, TRPV1, TRPA1 mRNA levels were decreased highly similarly to group #1 when compared to controls without inflammatory conditions

## The immunohistochemical localization of TRPV1 and TRPA1 channels in nasal polyposis

**a.** Methylated Giemsa staining revealed the structure and the cellular distribution in each tissue sample In normal nasal mucosa some plasma cells, a few number of mast cells and lymphocytes were observed in the stroma. TRPV1 and TRPA1 immunopositivity were present in a few plasma cells and macrophages.

**b.** In nasal polyposis, intensive TRPV1 immunopositivity was detected in periglandular and interglandular cells around seromucuos glands which were mainly plasma cells and a population of mast cells, and, to lesser extent, other lymphocytes and macrophages. The presence of TRPV1 positive plasma cells was the most prominent, however, only a subpopulation of plasma cells was stained with TRPV1 consistently across samples.

**c.** TRPA1 immunohistochemistry showed positive reaction in a population of macrophages and a population of mast cells, but eosinophils were not stained. We were not able to identify the presence of TRPA1 on plasma cells and lymphocytes.

## Analysis of cytokine pattern and comparison with the expression of TRP receptors in nasal polyposis

**a.** There was no change in the protein levels of interferon gamma (IFNγ), IL-4 in CRSwNP patients, IL-5 protein was not detected in control samples. IL-8 protein amounts increased significantly 2.2 fold to 4.2 pg/mg wet tissue in NP patients compared to the healthy control group.

**b.** When subgroups were compared only IL-4 was significantly altered: it elevated 2.3 fold to 0.070 pg/mg wet tissue in patients with both asthma and allergic rhinitis compared to their NP counterparts without these comorbidities.

**c**. In the study of inflammatory markers and TRPV1/TRPA1 correlation, transcripts of 15 inflammatory mediators or cell markers were further detected in patient group #2. Transcript data were hierarchically clustered and expression patterns were visualized by heat map. Nine genes implicated in Th2-driven inflammation were clustered together and were markedly upregulated in CRSwNP with asthma samples. The expression pattern of TRPV1 mRNA was similar to mast cell markers in the order of: TAC4 **>** CD117 **>** CHM-1 (chymase). TRPA1 demonstrated a highly disparate pattern from all inflammatory markers except IL-8.

**d.** The change in the expression of a gene was determined by the correlation coefficient *r* values. TRPV1 mRNA levels correlated positively at a marked level with TAC4 **>** CD117 **>** CD79a (plasma B cell receptor), while TRPA1 mRNA correlated negatively with the granulocyte cell surface receptor EMR1 (ADGER1) transcripts.

## CONCLUSIONS

**a**. We presented immunohistochemical evidence that non-neuronal TRPV1, TRPA1 receptors are expressed in biopsy samples of NP patients. TRPV1 immunostaining was observed on plasma cells and mast cells, mainly in the interglandular space while TRPA1 was enriched on stromal macrophages in NPs. However, the presence of TRPA1 were not confirmed on plasma cells.

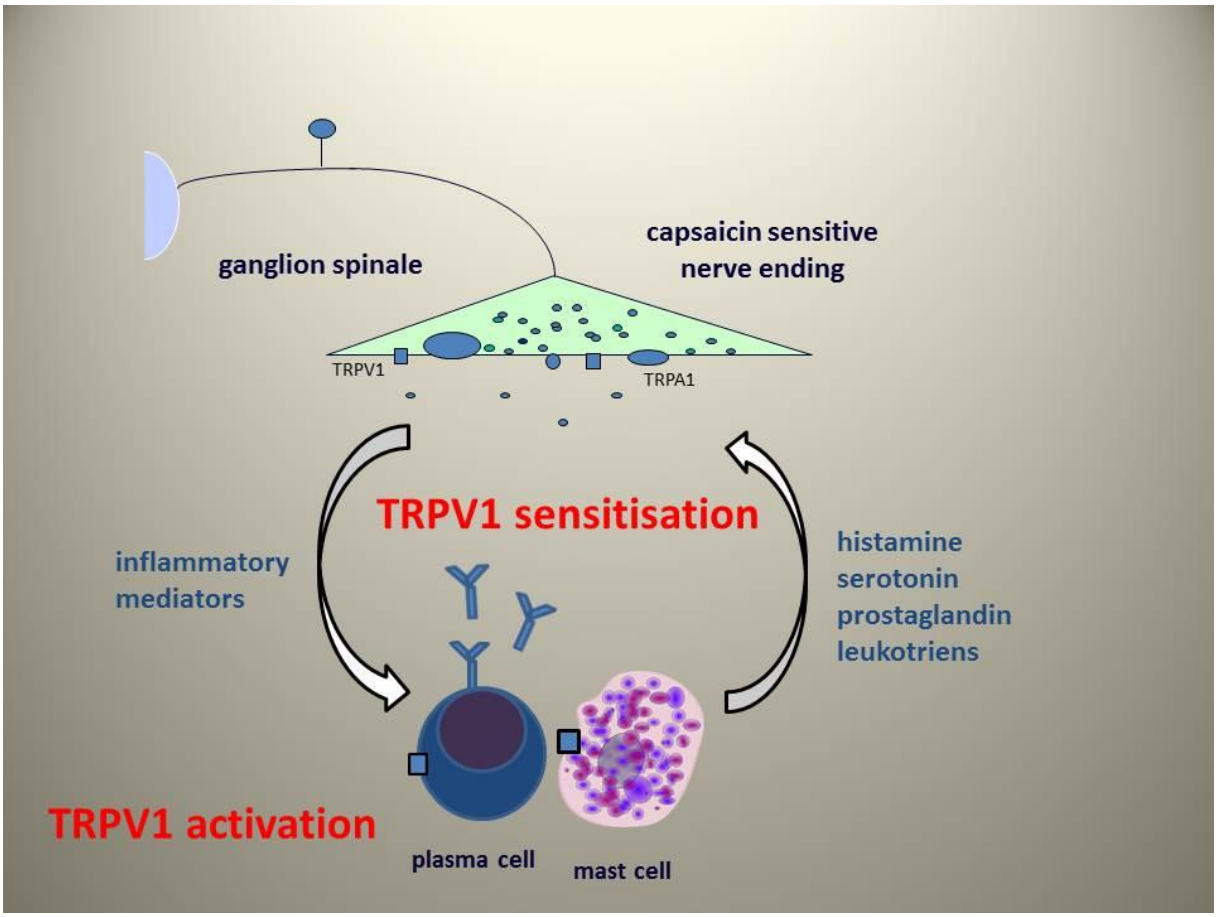
**b.** Mucosal space closely connected to the environment via luminal surface explaining the accumulation of TRPV1 and TRPA1 positive periglandular cells as chemosensors of environmental pollutants, dust particles and chemicals [19,32]. Activation of TRPA1 by environmental factors in mast cells and macrophages can induce proinflammatory changes.

**c.** The presence of TRPV1 on human plasma cells and the non neuronal expression of TRPA1 in nasal polyposis were demonstrated for the first time. Since plasma cells consequently express TRPV1 but not TRPA1 receptors, this could explain the difference between TRP patterns. TRPV1 expression in plasma B cells is an intruiging new result, but its significance is still unclear.

**d.** We observed a marked coexpression of TRPV1 and TAC4 as well as other mast cell markers. The TAC4 gene product hemokinin-1 peptide is known to enhance TRPV1 (but not TRPA1) responses [34], proliferation and antibody production of B cells (55) as well as IgE-mediated mast cell inflammatory responses [35]. We hypothesize that activation of TRPV1 positive plasma cells in CRS patients with asthma contributes to elevated IgE levels and proinflammatory changes leading to more severe symptoms.

## SUMMARY OF THE MAIN RESULTS OF THE THESIS

Elevated TRPV1 levels in comorbid asthma and allergy may have a function in CRSwNP. The presence of subpopulation-specific TRPV1 on plasma and mast cells, furthermore the coexpression of TRPV1 and the immunregulatory TAC4 mRNS may indicate likely roles in regulation of activation and release of inflammatory mediators. TRPV1 positive plasma cells releasing the inflammatory mediators may have a clue to neuronal receptors with a" cross-talk" mechanism, but these processes need more investigation.



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*Upregulation of extraneuronal TRPV1 expression in chronic rhinosinusitis with nasal polyps.*
RHINOLOGY 56:(3) pp. 245-254. (2018)
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Molnár Dávid, **Tóth Eszter**
*Az allergiás rhinitis felismerése és kezelése*
GYÓGYSZERÉSZ TOVÁBBKÉPZÉS 10:(3) pp. 75-78. (2016)

**Tóth Eszte**r
*Az obstruktív alvási apnoe és hypopnoe szindróma mögött meghúzódó fül-orr-gégészeti megbetegedése; az okok feltárásának modern módszere: az alvás alatti endoszkópia*
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