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Macrophage metabolism in N-ERD patients

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Structure

List of abbreviations

AA	arachidonic acid
ACACA	acetyl-CoA carboxylase 1 (gene)
ACC1	acetyl-CoA carboxylase 1
ACSL1	long-chain fatty acid-CoA ligase 1 (gene)
Acyl-CoA	acyl-Coenzyme A
ADIPOR2	adiponectin receptor protein 2 (gene)
AERD	aspirin-exacerbated respiratory disease
ALOX	arachidonate lipoxygenase (gene)
ALOX15	arachidonate 15-lipoxygenase (gene)
ALOX5AP	arachidonate 5-lipoxygenase-activating protein (gene)
ALX	lipoxin A4 receptor
aMDM	alveolar-like monocyte-derived macrophages
AMP	adenosine-monophosphate
AMPK	AMP-dependent protein kinase A
ATP	adenosine-triphosphate
BLT_2	leukotriene B_4 receptor 2
BMI	body mass index
BSA	bovine serum albumin
C/EBP δ	CCAAT/Enhancer binding protein delta
CCAT	calcium channel associated transcriptional regulator
CCL23	C-C motif chemokine 23
CCL5	C-C motif chemokine 5
CD	cluster of differentiation
CD1A	T-cell surface glycoprotein CD1a (gene)
CERS4	ceramide synthase 4 (gene)
CLEC10A	C-type lectin domain family 10 member A (gene)
CLEC18B	C-type lectin domain family 18 member B (gene)
COX	cyclooxygenase
CPT1	carnitine palmitoyl transferase 1
CPT1B	carnitine O-palmitoyltransferase 1 (gene)
CPT2	carnitine O-palmitoyl transferase 2
CRS	chronic rhinosinusitis
CRSsNP	chronic rhinosinusitis without nasal polyps
CRSwNP	chronic rhinosinusitis with nasal polyps
CRTH2	prostaglandin D2 receptor 2

CS	corticosteroids
CXCL2	C-X-C motif chemokine 2
CXCL8	C-X-C motif chemokine 8
CXCR3	C-X-C chemokine receptor type 3
CYP	cytochrome P450
CYSLTR1	cysteinyl leukotriene receptor 1 (gene)
CYSLTR2	cysteinyl leukotriene receptor 2 (gene)
cysLTs	cysteinyl leukotrienes
DCs	dendritic cells
DEGs	database of essential genes
DHA	docosahexaenoic acid
DHET	dihydroxyeicosatrienoic acid
DMSO	dimethyl sulfoxide
DP1	prostaglandin D1 receptor
DP2	prostaglandin D2 receptor
DTT	dithiothreitol
ECP	eosinophilic cationic protein
EDTA	ethylenediaminetetraacetic acid
ENT	ear, nose, throat
EP1	prostaglandin E2 receptor 1
EP2	prostaglandin E2 receptor 2
EP3	prostaglandin E2 receptor 3
EPOS	European Position Paper on Rhinosinusitis and Nasal Polyps
FACS	fluorescens activated cell sorting
FAO	fatty acid oxidation
FBS	fetal bovine serum
FFA	free fatty acids
FFAR2	free fatty acid receptor 2 (gene)
FFAR3	free fatty acid receptor 3 (gene)
FP	prostaglandin F receptor
GA2LEN	Global Allergy and Asthma Network of Excellence
GAC	Genome Analysis Center
GATA3	trans-acting T-cell-specific transcription factor GATA-3
GM-CSF	granulocyte-macrophage colony-stimulating factor
HDHA	hydroxydocosahexaenoic acid
HEPE	hydroxyeicosapentaenoic acid
HETE	hydroxyeicosatetraenoic acid

HLA	human leucocyte antigen
HPETE	hydroperoxyeicosatetraenoic acid
HPGDS	hematopoietic prostaglandin D synthase (gene)
IDO1	indoleamine 2,3-dioxygenase 1 (gene)
IFNγ	interferon γ
Ig	immunoglobulin
IL	interleukin
IL-17RB	interleukin-17 receptor B
IL1RAP	interleukin-1 receptor accessory protein (gene)
IL1RL1	interleukin-1 receptor-like 1 (gene)
IL1β	interleukin-1 *β*
ILC2s	type 2 innate lymphoid cells
IRF4	interferon regulatory factor 4 (gene)
KIT	mast/stem cell growth factor receptor Kit
LC-MS/MS	liquid chromatography–mass spectrometry
LO(X)	lipoxygenase
LOD	level of detail
LRP8	low-density lipoprotein receptor-related protein 8 (gene)
LT	leukotriene
LTA4H	leukotriene A4 hydrolase (gene)
LTB_4	leukotriene B4
LTC_4	leukotriene C4
LTC_4S	leukotriene C4 synthase
LTE4	leukotriene E4
LXA_4	lipoxin A4
lysoPCs	lysophosphatidylcholines
M1 macrophage	classically activated macrophage
M2 macrophage	alternatively activated macrophages
MACS	magnetic cell separation
MCs	mast cells
MDMs	monocyte-derived macrophages
MeOH	methanol
MHC	major histocompatibility complex
N-ERD	NSAID-exacerbated respiratory disease
NaCl	sodium chloride
NLF	nasal lavage fluid
NMU	neuromedin U
NP	nasal polyp

NSAID	non-steroidal anti-inflammatory drug
Ocs	oral corticosteroids
oxo-ETE	oxo-eicosatetraenoic acid
PAMPS	pathogen-associated molecular patterns
PBMC	periphal blood mononuclear cell
PBS	phosphate-buffered saline
PCA	principal component analysis
PGD_2	prostaglandin D2
PGE_2	prostaglandin E2
PGF_{2a}	prostaglandin F2α
PGI_2	prostacyclin
PLS-DA	supervised partial least squares-discriminant analysis
PMN	polymorphonuclear cells
PPBP	platelet basic protein
PTGER3	prostaglandin E2 receptor EP3 subtype (gene)
PTGES	prostaglandin E synthase (gene)
PTGS2	prostaglandin E2 receptor EP2 subtype (gene)
PTGS2	prostaglandin G/H synthase 2 (gene)
PUFA	polyunsaturated fatty acid
RNA	ribonucleic acid
RNA-seq	RNA sequencing
RT	room temperature
SGPP2	sphingosine-1-phosphate phosphatase 2 (gene)
sMac	sputum macrophages
SMPD3	sphingomyelin phosphodiesterase 3 (gene)
sMRM	scheduled multiple reaction monitoring
sMRM	multiple reaction monitoring
SM	sphingomyelin
SN	supernatant
SNOT22	sino-nasal outcome test
ST2	interleukin-1 receptor-like 1
TGF-β	transforming growth factor β
T_H1	type 1 T helper cell
T_H2	type 2 T helper cell
TNF-α	tumor necrosis factor α
TSLP	thymic stromal lymphopoietin
TXB2	thromboxane B2
βME	β-mercaptoethanol

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1 Introduction

Everywhere our organism comes into contact with the environment the immune system has the challenging task to maintain a balance between interaction and protection. The airways form part of this barrier system: The mouth and nose are the entry and exit ports of the airways. Air enters, passes through the pharynx and larynx down the trachea and finally passes through the bronchi. The inhaled air contains a mixture of harmless particles, pathogens, allergens and pollutants challenging the immune system. Airway immunity must be tightly controlled to maintain homeostasis and dysregulation can contribute to serious diseases. Type 2 immune response forms part of the body's defense mechanism and is at the same time known to be involved in the pathogenesis of various diseases whose treatment challenge science until today. Those diseases appear as local inflammation, limited to mainly one (e.g. asthma, CRS, N-ERD, atopic dermatitis, and eosinophilic gastrointestinal disorders[1,2]) or more organs (e.g. allergy[3]).
Despite the introduction of new therapeutic options[4,5] respiratory diseases are widespread and cause an immense worldwide health burden.[6] Thus, a better understanding of airway disease pathomechanism and biomarkers can open the door for more efficient and patient-specific therapies.[7]

1.1 Type 2 immune response

The type 1 and type 2 immune responses describe a paradigm of immune responses that are mainly regulated by subpopulations of Cluster of Differentiation 4^+ T cells ($CD4^+$ T cells). These cells can be distinguished into Type 1 (T_H1) and Type 2 T helper cells (T_H2) and produce characteristic mediators[8]. T_H1 cells secrete interferon-γ (IFNγ), interleukin-2 (IL-2), and lymphotoxin-α. Together they promote an immune reaction with high phagocytic activity[9]. On the contrary T_H2 cells are characterized by the production of the cytokines IL-4, IL-5, IL-9, IL-10 and IL-13 and the stimulation of type 2 immunity[10]. This is reflected in the activation of eosinophils, mast cells (MCs), basophils, and

alternatively activated (M2) macrophages[11]. Different stimuli like helminths[12], cancer cells[13], allergens[14] or endogenous host molecules[15] can trigger type 2 responses (Figure 1)

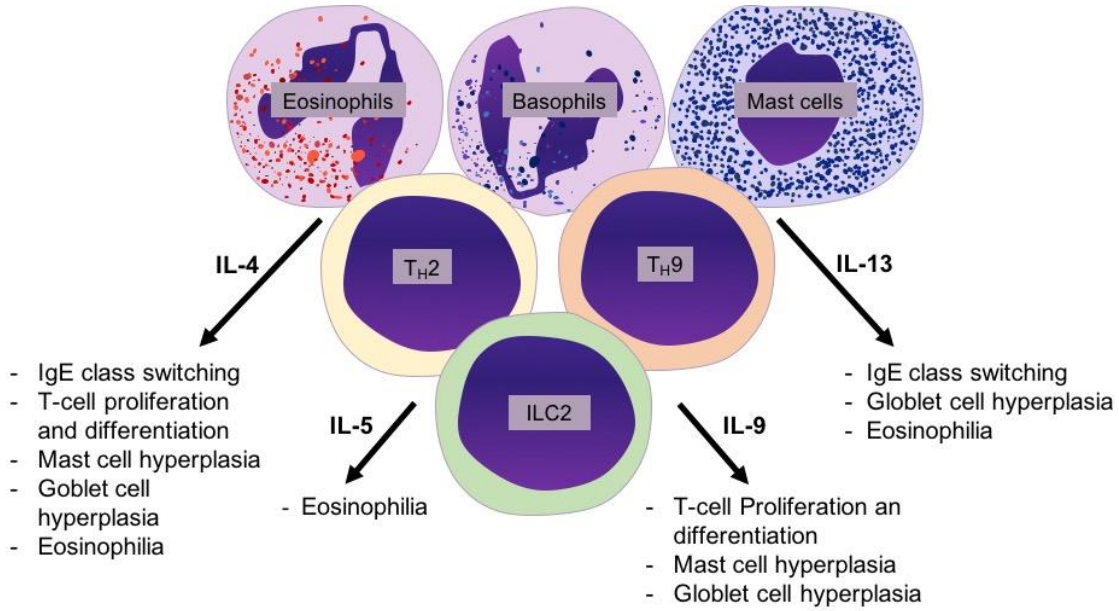


Figure 1 Expression and function of type 2 effector cytokines

Eosinophils, basophils, MCs, T_H2 cells, T_H9 cells and ILC2s produce type 2 immune cytokines (IL-4, IL-5, IL-9, IL-13) leading to various immune response of other cells. Together they drive type 2 inflammation.
This figure was adopted from the paper “Insights into the initiation of type 2 immune responses” (Immunology,2011)[16].

The traditional model of the initiation of type 2 immunity was, that dendritic cells (DCs) sample antigens from the luminal compartment bordering the external environment. DC mobilize to lymph nodes, presenting the processed antigen via major histocompatibility complex (MHC) class II molecules to naïve $CD4^+$ T helper cells. T helper cells differentiate into T_H2 cells, producing characteristic cytokines and stimulating B cells to produce immunoglobulin E (IgE) antibodies. IgE antibodies form immune complexes that bind to high affinity IgE receptors on basophils and mast cells and induce their degranulation and release of pro-inflammatory mediators (histamine, heparin, serotonin).

The discovery of type 2 innate lymphoid cells (ILC2s) changed the DC-T_H2 view to an ILC2-DC-T_H2 cell axis (Figure 2)[1,17]. After an appropriate exogenous stimulus, thymic stromal lymphopoietin (TSLP), IL-25 and IL-33 can be produced

by various cells, above all epithelial cells[18]. But also fibroblasts[19], alveolar macrophages[20] or airway smooth muscle cells[21] can be a source of these metabolites. Those so-called “alarmins” can activate tissue-resident ILC2s and stimulate cytokine production and ILC2s growth itself[18]. ILC2 activity can be modulated by a vast number of other cytokines and lipid mediators like for example eicosanoids (prostaglandins[22,23], cysteinyl leukotrienes[24,25]).

Accordingly, the recruitment and activation of downstream immune cells are caused by type 2 cytokines secreted either by ILC2s, T_H2 cells or both. Beyond that, type 2 cytokines have an effect on another important component of the immune system: macrophages. The following paragraph will focus on this topic.

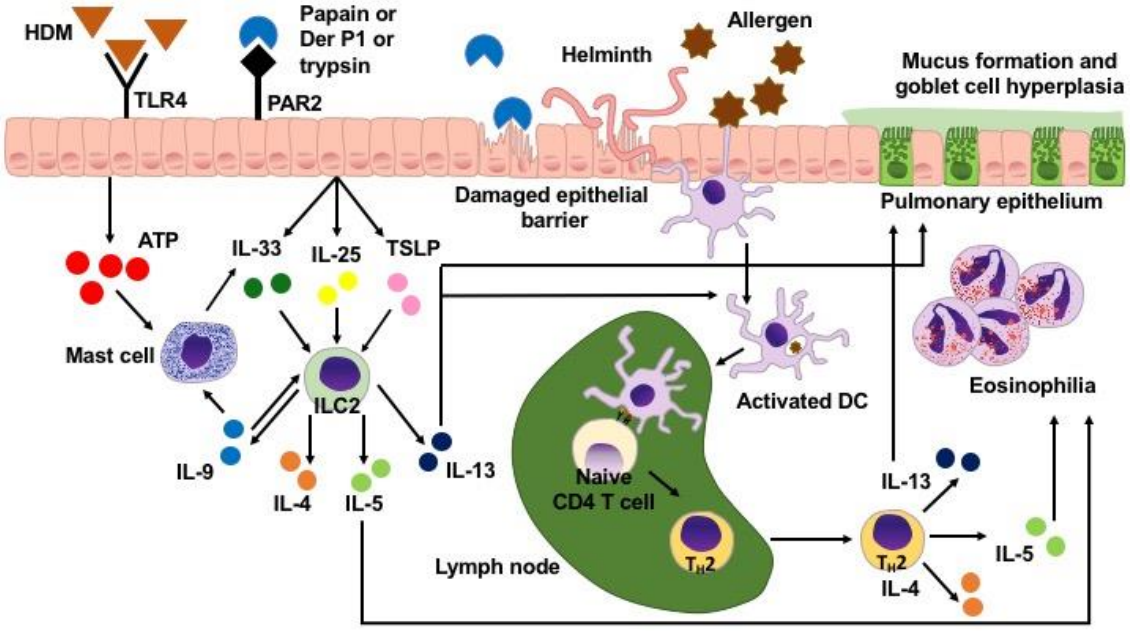


Figure 2 Scheme of the ILC2-DC-TH2 cell-centric axis in type 2 immune response

Epithelial cells are able to produce alarmins like IL-33, IL-25 or TSLP in response to tissue damage (proteases, helminths) or pathogen sensing. Epithelial cells can stimulate mast cell (MC) IL-33 excretion via adenosine-triphosphate (ATP) production. Cysteine proteases, as Der P1 or papain deriving from house dust mite (HDM), can either damage epithelial cells directly or through activation of the protease activated receptor 2 (PAR2). The same effect have receptors recognizing allergens, as toll like receptor 4 (TLR4) for HDM. Other allergens bind to dendritic cells and contribute to the transformation of naïve CD4 T cells into effector T_H2 cells.

The released alarmins in turn activate ILC2s enhancing type 2 cytokine metabolism (IL-4, IL-5, IL-9 and IL-13). TSLP and IL-13 may act together supporting T_H2 cell differentiation. Changes in

respiratory tissue can be induced through IL-13. IL-5 acts on eosinophils and supports their expansion and recruitment from the bone marrow. IL-9 leads to MC recruiting and cell expansion. Taken together, type 2 cytokines drive type 2 immunopathology.
This figure was adopted and modified from "Orchestration between ILC2s and T_H2 cells in shaping type 2 immune responses" (Nature, 2018)[26].

1.2 Monocytes and Macrophages

Monocytes and macrophages represent an important component of the immune system. They serve three main functions: phagocytosis, antigen presentation and mediator production. Macrophages can either be tissue-resident or monocyte-derived macrophages (MDMs). Tissue-resident macrophages establish during embryogenesis and maintain their populations by self-renewal[27]. MDMs derive from progenitor cells in the bone marrow, which circulate after their release between one and three days[28] in the bloodstream[29,30].
During both, homeostasis and inflammation, monocytes immigrate to different body tissues by diapedesis and differentiate into macrophages (and/or DCs). Based on their anatomical location, macrophages differentiate into specialized phenotypes highly adapted to their local environment[31,32]. One example are alveolar macrophages (as extracted for our experiments from sputum samples[33]).
Various stimuli like cell damage, pathogens and cytokine release, attract monocytes towards inflamed tissues. At any point during inflammation, macrophages can functionally adapt to their surrounding by macrophage polarization initiated by distinct antigen and cytokine environment. The term macrophage polarization describes the phenomenon of the differentiation into two major macrophage subtypes characterized by their production of specific factors, cell surface marker and biological activities. Even though macrophage polarization should be viewed as a multidimensional process[22,34], a dualistic model was established to fit complex and incompletely understood macrophage phenotypes and functions into a simplified system. The dichotomous model reflects the T_H1 and T_H2 polarization concept: M1 and M2 macrophages[35].
M1 differentiation can be initiated through infectious microorganism-related molecules (e.g. the gram-negative product Lipopolysaccharide) or through

cytokines like tumor necrosis factor α (TNF-α) or IFN-γ. M1 macrophages produce various pro-inflammatory mediators as IL-1, IL-6 or TNF-α that have an anti-tumoral and anti-microbial effect[36].

M2 polarization is usually induced via T_H2-related cytokines,IL-4, IL-5 or IL-13, and is also described as alternative macrophage activation[1]. M2 macrophages in turn produce cytokines and chemokines leading to the recruitment of immune cells like T_H2 cells and eosinophils and are also an abundant source of lipid mediators[1]. It is assumed that alternative activated macrophages are involved in tumor progression, parasite infestation and tissue remodeling, as they were detected in various diseases driving anti- and pro-inflammatory processes[1,38]. This includes for instance asthma and allergic rhinitis, both pathological conditions closely related to AERD. Asthma is known to provide a type 2-associated cytokine environment driving M2 differentiation[39,40] and patients with allergic rhinitis revealed a M2 predisposition in comparison to healthy controls[41].

1.3 Eicosanoids

Eicosanoids are lipid mediators arising from the 20-carbon polyunsaturated fatty acid (PUFA) arachidonic acid (AA). Figure 3 gives an overview of the AA metabolism. The activation of phospholipase A2 leads to the release of AA from membrane phospholipids. AA is the precursor substance being oxidized via the enzymes cyclooxygenase (COX), lipoxygenase (LOX) and cytochrome P450 (CYP) or by free radical mechanisms. Because of its outstanding importance for inflammatory processes, as for example in the pathophysiology of N-ERD, this thesis focuses on the COX and LOX pathway[42,43,44,45].

In mammals two isoforms of COX are known, referred to as COX-1 and COX-2. The COX1/2 pathway produces thromboxanes, prostacyclin and prostaglandins. Thromboxanes play a role in platelet aggregation[46] and platelet response to injury,[47,48] whereas prostacyclin has an opposite effect and acts antithrombotic[49]. Additionally, prostacyclin (PGI_2) has a vasodilatory function[50].

There exist three types of prostaglandins, Prostaglandin D2 (PGD_2), Prostaglandin E2 (PGE_2) and Prostaglandin F2α (PGF_{2a}), binding on different G-

protein coupled receptors (DP1, DP2, EP1, EP2, EP3, FP). PGs mediate a tremendous amount of versatile biologic activities, of both pathogenic[51] and homeostatic kind, but in the following I will concentrate on their function in inflammation. In peripheral tissue PGD_2 is mainly produced by activated mast cells, but also DCs, macrophages and T_H2 cells.

In the respiratory tract PGD_2 has shown a significant role causing bronchoconstriction and eosinophil infiltration[52,53]. PGE_2, as one of the most abundant PGs in the body, mediates both T_H1[54,55,56]- and T_H2[57,58,59] immune responses, even though its role in type 2 immune response is much better understood. Herein PGE_2 functions via EP2 receptor as an anti-inflammatory[1] mediator inhibiting platelet activation[60], 5-Lipoxygenase (5-LO) function[61] as well as mast-cell[62,63], eosinophil[58] and ILC2 activation. Furthermore, PGE_2 was recognized to transform type 2 macrophages into an anti-inflammatory, IL-10 producing phenotype[66,67]

Leukotrienes (LTs), often described as pro-inflammatory counterpart to PGE_2, derive as well from AA and can be metabolized by four distinct LOs (5-,8-,12-,15-LO). 5-LO is involved in pro-inflammatory LT production, metabolizing AA into 5-HPETE followed by Leukotriene A_4 (LTA_4) production. LTA_4 can be processed by either LTA_4 hydrolase to LTB_4 or by LTC_4 synthase (LTC_4S) to LTC_4. LTC_4 is exported out of the cell and enzymatically converted into LTD_4 and further metabolized into stable LTE_4. 5-LO is primarily found in myeloid cells e.g. macrophages or granulocytes. But LT production does not depend exclusively on 5-LO, since virtually all cells possess LTA_4 hydrolase[68], and activated monocytes can serve as an important source of LTA_4[69].

The metabolites LTC_4, LTD_4 and LTE_4 are referred to as cysteinyl leukotrienes (cysLTs). In addition to the intracellular pathway, transcellular mechanisms for cysLT formation exist. Cells expressing LTC4S, but not the proximal enzyme 5-LO, can generate LTC_4, as it has been observed with leukocyte adherent platelets in N-ERD patients.[47] Through that mechanism, cysLT production can be pathologically increased.

CysLTs bind to the G-protein-coupled $cysLT_1$ or $cysLT_2$ receptor and mediate cardiovascular disease[70], cancer[71], immune response[72,73], and fibrosis[74]. $CysLT_1$

and cysLT$_2$ receptor are expressed by hematopoietic[75] and structural cells[76,77]. CysLT influence the innate immune system for example by recruiting and/or activating macrophages, eosinophils and mast cells[78], as well as adaptive immunity e.g. by the induction of T$_H$2 response[79]. Blood monocytes or MDMs upregulate cysLT$_1$ expression in a T$_H$2 cytokine milieu (IL-4, IL-13) and enhance their chemotactic response to LTD$_4$, thus promoting inflammation.[80] Because of its essential role in the pathophysiology of asthma, cysLT1 receptor is known to mediate airway reactions like bronchoconstriction, hyperresponsiveness, mucus hypersecretion and remodeling.[81]

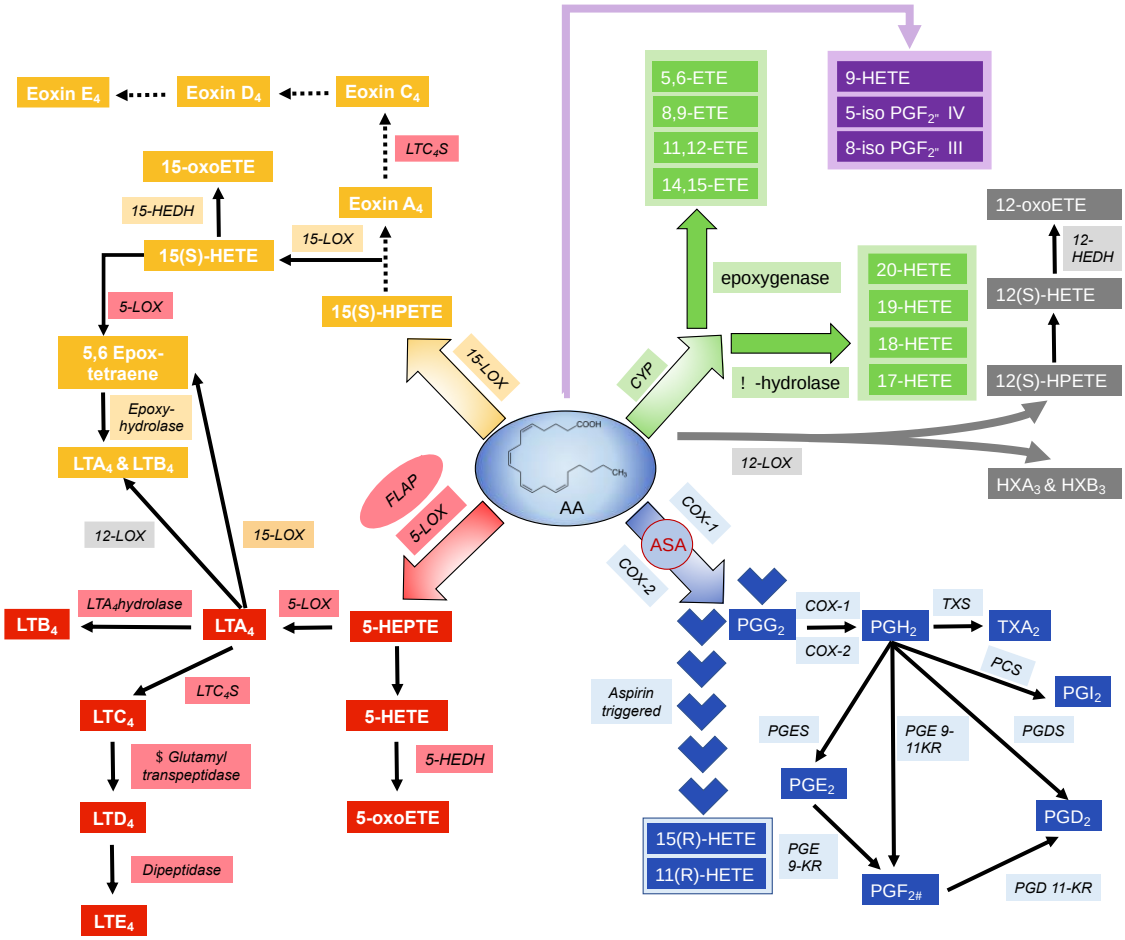


Figure 3 Arachidonic acid metabolism in N-ERD

Metabolites of different pathways are indicated in different colors. The involved enzyme is shown with light shade of the same color as the major metabolites they transform: COX (blue), 5-LOX (red), 15-LOX (yellow), 12-LOX (grey), CYP450 (green), non-enzymatic (violet). Acetyl salicylic

acid (ASA) or other NSAIDs, block COX-1 and partially COX-2 shifting AA metabolism towards the remaining enzymes (5-LOX, 15-LOX, CYP).
This figure was adopted from “Biochemical pathogenesis of aspirin exacerbated respiratory disease (AERD)” (Clinical Biochemistry, 2012).

1.4 Acylcarnitines

Acylcarnitines are free fatty acids (FFAs) that are transported via blood circulation to the cells of the target tissue. FFAs need the fatty acid transport system to pass into the interior of cells. From there FFAs get activated into acyl-Coenzyme A (acyl-CoA) via esterification. Medium-chain acyl-CoAs can diffuse into the mitochondria whereas long-chain acyl-CoAs have to be converted into a transportable metabolite called acylcarnitine. Mitochondrial and peroxisomal enzymes like carnitine palmitoyl transferase 1 (CPT1) produce acylcarnitines, the carnitine acylcarnitine translocase is responsible for the import into the mitochondrion and carnitine O-palmitoyl transferase 2 (CPT2) reconverts them into acyl-CoA. Afterwards they form the basic metabolite for fatty acid oxidation (FAO)[82]. As producer of adenosine-triphosphate (ATP), FAO provides an essential energy source for the cell[83].
Macrophage polarization can be associated to acylcarnitine production as M1 and M2 macrophages show differences in energy metabolism. M2 macrophages depend on FAO for energy production whereas M1 macrophages use the glycolytic metabolism[84,85]. In chronic inflammatory diseases macrophages display an energy metabolism shift towards FAO that contributes to their pathogenic pro-inflammatory function[86,85]. Enhanced FAO was observed in a murine model of allergic asthma[87] and it was shown that acylcarnitines are able to induce pro-inflammatory enzyme and mediator expression (TNF, IL6, COX-2, CXCL2, CCL5, IL1β)[88]. Thus, acylcarnitines may play in general an important role in inflammatory conditions and should be considered as important factor in the pathogenesis of various inflammatory diseases. Further investigation is needed to gain more insight into the complex network of lipid function and regulation during type-2 inflammation.

1.5 Asthma

Asthma is a wide-spread chronic inflammation of the lower respiratory tract characterized by airway obstruction and hyperresponsiveness. Over 235 million people are living with asthma, one of the leading non-communicable diseases worldwide[89,90]. Asthma is defined by recurrent episodes of wheezing, breathlessness, chest tightness and coughing[91]. Depending on the presence or absence of IgE antibodies to common environmental allergens, asthma is classified as allergic or nonallergic. In order to diagnose asthma, symptoms of airway hyperresponsiveness or airflow obstruction have to be present. The latter has to be partially reversible and alternative diagnoses should be excluded[91]. Genetic predisposition[92], possibly alteration in the microbiome[93] and metabolites[94,95] as well as environmental exposures[96,97] are reasons for the disease onset and lead in most instances to a type 2 inflammation of the respiratory tissue.

IL-13 and IL-4 production is triggered by IL-33 from epithelial and endothelial cells via ST2 receptor on M2 macrophages, mast cells and ILC2 cells[98]. IL-13 and IL-4 induce airway hyper-responsiveness, globlet cell hyperplasia and mucus hyper-secretion[99]. Type 2 cytokines like IL-5 and IL-9 activate and recruit eosinophils and mast cells[100]. IgE mediates MC and basophile degranulation supporting type 2 immunity via increased vascular permeability, recruitment of inflammatory cells, in particular eosinophils, and smooth muscle constriction[101]. Like ILC2s and eosinophils, basophils generate IL-4 and promote eosinophilic inflammation, ILC2 and T_H2 activation, as well as mucus production[102]. Stimulated eosinophils in turn produce oxygen radicals, lipid mediators and cytokines among all mainly IL-1b, IL-6, IL-8 and IL-4. Endothelial cell damage, alteration in repair processes, induction of fibrosis and activation of DC chemotactic activity are further reactions caused by activated eosinophils[103,104].

All these immune processes enhance airway hyperactivity and wall remodeling and effect directly the extent of the disease progression. An overview of the pathophysiology of asthma is given in Figure 4.

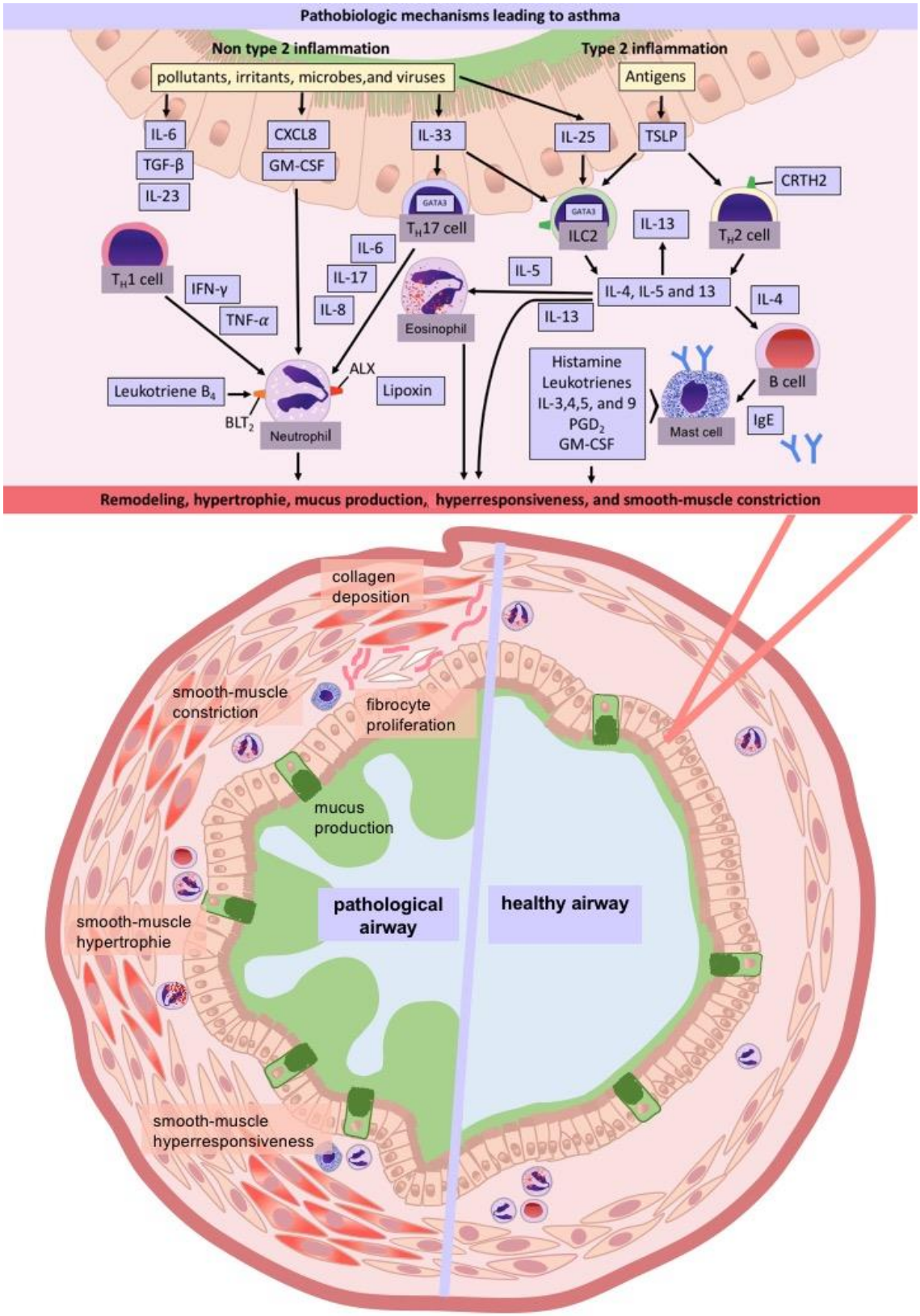


Figure 4 Type 2 and non-type 2 inflammation in asthma

Pathophysiologic models of asthma development are typically distinguished between type 2 and non-type 2 asthma:

1. The adaptive immune system can react to allergens with TSLP, stimulating Type 2 inflammation. GATA3 transcription factor induces type 2 cytokine production (IL-4, IL-5, IL-13) of T_H2 cells and ILC2s. Type 2 cytokines have extensive effects: eosinophil recruitment, smooth muscle remodeling and hyperresponsiveness, mucus production, cytokine production of epithelial cells or IgE production. MCs amplify inflammation via mediator, PGD_2 and cytokine production affecting eosinophil infiltration, remodeling and smooth-muscle contraction as well. MC survival depends on stem cell factor and its receptor KIT. PGD_2acts at the CRTH2 receptor and stimulates eosinophils, basophils, ILC2s and T_H2 cells.
2. Type 2 inflammation can also be initiated by irritants and infectious agents causing innate immune system to produce cytokines like IL-33 and IL-25. ST2 (IL-33 receptor) and IL-17RB (IL-25 receptor) can either stimulate ILC2s and T_H2 cells or cells of the non-type 2 pathway like T_H17 cells or neutrophils. T_H17 cells, additionally activated by cytokines derived from the epithelium, attract and stimulate neutrophils. T_H1 cells are also involved and stimulate neutrophils as well. The production of Lipoxins and other anti-inflammatory mediators, able to diminish neutrophil activation and antagonizing pro-inflammatory leukotrienes, can be reduced in asthmatic patients. Yellow highlighted fields represent substances that were targeted for severe asthma treatment.
ALX (lipoxin A_4 receptor), BLT_2 (leukotriene B_4 receptor 2), CXCL8 (CXC motif chemokine ligand 8), CXCR3 (CXC chemokine receptor 3), GM-CSF (granulocyte-macrophage colony-stimulating factor), TGF-ß (transforming growth factor ß), and TNF-α (tumor necrosis factor α).
This figure was adopted and modified from “Severe and Difficult-to-Treat Asthma in Adults” (The New England Journal of Medicien, 2017)[105].

1.6 Chronic rhinosinusitis with and without nasal polyps

Rhinosinusitis in adults is defined as inflammation of the nose and the paranasal sinuses characterized by two or more symptoms. Nasal blockage, obstruction and congestion can occur together with or without nasal drip. Moreover, these complaints can produce facial pain or pressure and reduce the olfactory sense to the maximal form of total anosmia. Diagnostics can either be confirmed by endoscopy or CT scan. Endoscopic signs can be nasal polyps, mucopurulent discharge and/or mucosal obstruction of the meatus. CT imaging typically shows mucosal changes within the osteomeatal complex and/or sinuses. If the symptoms persist for a period of more than 12 weeks this is classified as chronic rhinosinusitis (CRS). CRS can be classified into two different clinical phenotypes based on the presence or absence of nasal polyps: chronic rhinosinusitis with nasal polyps (CRSwNP) or chronic rhinosinusitis without nasal polyps (CRSsNP)[106]. Figure 5 provides a schematic overview of nasal polyposis in patients with CRSwNP.

Depending on the diagnostic criteria, the prevalence of CRS has been described in multiple studies with a range varying between 1%[107] and 16%[108]. A prevalence of 10.9% in European adult population was stated by the Global Allergy and

Asthma Network of Excellence (GA2LEN)[109] using the anamnestic guidelines mentioned in the European Position Paper on Rhinosinusitis and Nasal Polyps (EPOS)[106]. Besides the frequent occurrence of CRS, the quality of life of affected patients is massively restricted[110]. The quantity of affected people and the health impairment lead to high medical costs[111].

Various approaches towards possible causes of CRSwNP and CRSsNP have been made to improve the current situation. Inter alia immune dysfunction[112,113], allergy[114,115], asthma[116,117], aspirin sensitivity[118,119], ciliary dyskinesia[120,121] or pregnancy[122] seem to be linked to the disease, but a monocausal explanation for the pathogenesis does not exist. Besides all these theories it is suggested that different immune processes are key drivers of CRS. Even though the dichotomous hypothesis of a predominant type 1 immune reaction in CRSsNP and type 2 immune response in CRSwNP[123] is very simplified and not coherent in all ethnical groups[124], it still represents an important finding to understand CRS. The initiation of the type 2 immune response was the objective of numerous studies and was integrated in the 'immune barrier hypothesis'[125] (Figure 6): To this theory a dysfunctional epithelial barrier permits the penetration of pathogen-associated molecular pattern (PAMPs) into the tissue and leads to activation of the innate immune system[126]. TSLP stimulates T_H2 cell differentiation[127,128] and the production of T_H2 cytokines (IL-5, IL-13) by mast cells[129,130]. IL-33 acts synergistically to TSLP and is produced by epithelial cells[131,132]. Together they are able to directly bind dendritic cells and drive the adaptive response into a T_H2 direction[133,134]. IL-33 additionally contributes to this process by regulating the development and function of ILC2s[135]. The production of IL-13 promotes the polarization of M2 macrophages[132] emerging from monocytes attracted by the CCL23 secretion of eosinophils[136]. Type 2 immune response involves also B-cell activation which in turn leads to antibody production. IgE and IgA mediate the activation of airway mast cells and eosinophils in response to antigen exposure[137]. The role of alternative activated macrophages has not been adressed in CRSwNP.

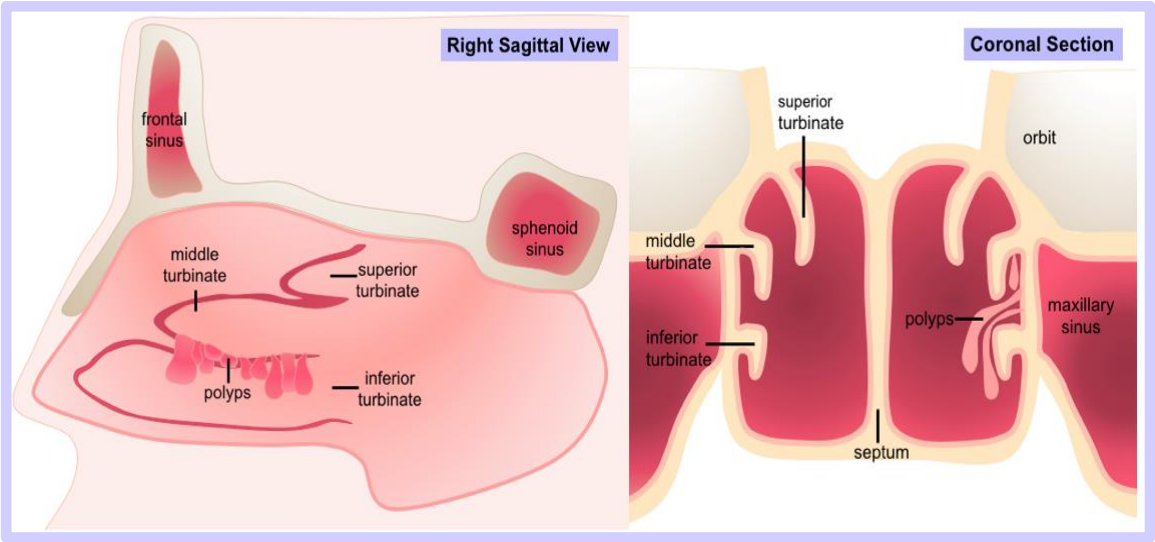


Figure 5 Anterior rhinoscopy in patient with CRSwNP

Polyps fill the nasal cavity between the nasal septum and the middle and inferior turbinates in a patient with CRSwNP.

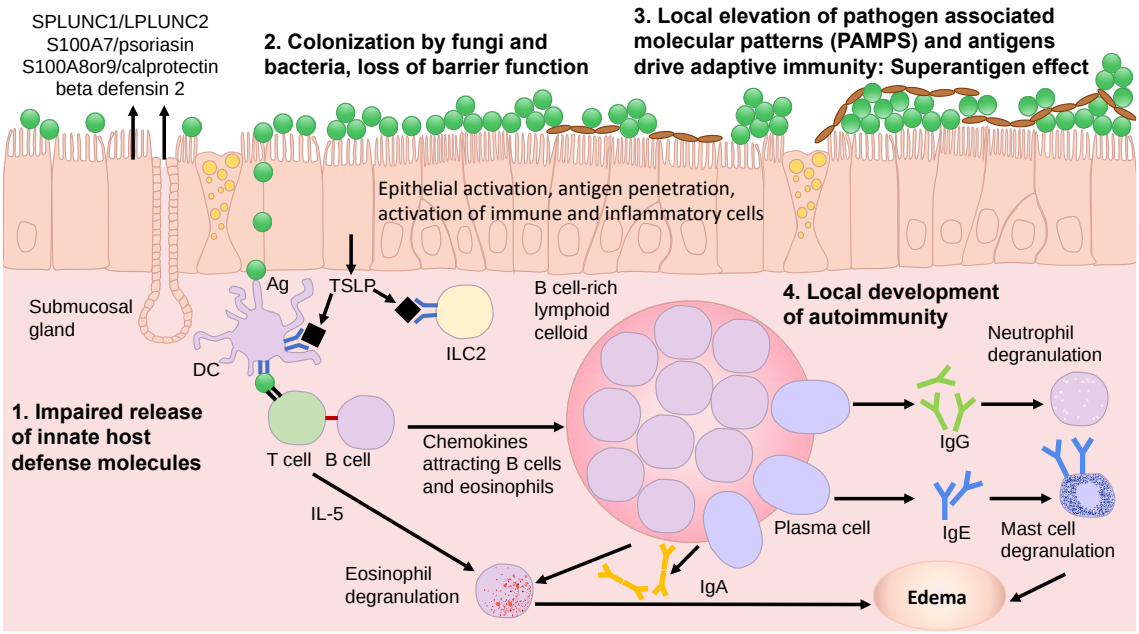


Figure 6 Overview of the 'immune barrier hypothesis' in CRS patients

1. Host deficits of the nasal epithelium lead to a reduced production of innate host defense immune anti-microbial molecules 2. This diminished immune function facilitates the colonization with microbial agents. 3. The integrity of the epithelial barrier is disrupted by the microbial agents. Simultaneously, their pathogen-associated molecular patterns (PAMPS) are detected by cells initiating a series of immune responses e.g. of T and B cells. 4. This pro-inflammatory milieu

provokes the dysregulation of the local inflammatory microenvironment. Pseudo-follicles develop, and immunoglobulins are produced. Antibodies mediate mast cell, neutrophil and eosinophil degranulation releasing cytotoxic and vasoactive mediators into the nasal mucosa.
This figure was adopted and modified from "The Etiology and Pathogenesis of Chronic Rhinosinusitis: a Review of Current Hypotheses" (Current Allergy and Asthma Reports, 2015)[139].

1.7 NSAID-exacerbated respiratory disease

N-ERD, AERD, Samter's triad, Widal triad or aspirin-sensitive asthma are many names for the same clinical picture. The disease pattern is defined by severe and refractory nasal polyposis, bronchial asthma and intolerance to non-steroidal anti-inflammatory drugs[140] and was first described by Widal in 1922[141].
The overall prevalence of N-ERD given in the literature varies widely depending on the diagnostic approach, statistical methods and examined cohort. A literate meta-analysis leads to the assumption that the prevalence of N-ERD is 7.15% in asthmatic patients and doubles in patients with severe asthma[142]. It is presumed that the lifetime prevalence of asthma in Germany constitutes 8,8% of the overall population[143]. Among patients with NP or CRS 9.96% and 8.7% are affected[142]. Taken together, N-ERD occurs much more frequently than clinicians would suspect, and the socioeconomic costs should not be underestimated.
The fact that there does not exist any validated clinically available in vitro diagnostics could be the reason for prevalences varying in such a wide range. The clinical history can suffice to diagnose N-ERD though it is not the most reliable method. Laidlaw proposed that patients with asthma, recurrent nasal polyps and at least 2 previous non-steriodal anti-inflammatory drug (NSAID)-induced respiratory reactions, at least one of which was within the last 5 years, can be diagnosed with N-ERD[144]. The current gold standard are nasal or oral provocation challenges with aspirin or other COX-1 inhibitors[144]. With a sensitivity of 78%[145], intranasal ketorolac challenges show a lower sensitivity than oral aspirin challenges but tend to trigger less severe symptoms. The most important disadvantage of provocation challenges are the unpredictable effects that COX-1 inhibitors can provoke in N-ERD patients. Therefore, they should only be performed under constant observation by qualified medical staff within the facility

and knowledge to fulfill emergency procedures[144,146]. The intake of NSAID can cause a broad range of symptoms starting from nasal discharge and blockage to extreme reactions like anaphylactoid shock with hypotension or loss of consciousness[147]. Additionally, it has to be mentioned that a fraction of the affected patients is totally unaware of their sensibility towards NSAID which makes N-ERD even more dangerous[142].

Besides acute reactions, N-ERD patients typically also present a broad spectrum of chronic symptoms independent from NSAID intake. A typical pattern of the disease's course can be noticed starting in most cases in the third decade of life[148]. First symptoms consist in persistent rhinitis, followed by asthma, aspirin intolerance and nasal polyposis[149]. Nasal polyposis occurs progressively and in severe forms which leads typically to 'white out' of paranasal sinus in cranial CT scans (Figure 7)[150]. This phenomenon expresses the subtotal occlusion of all paranasal sinus. Consequently, the olfactory sense is sharply reduced and can lead to total anosmia[149], facial pain[151] and other symptoms heavily influencing quality of life[152]. Females are affected more than twice as often and show more severe and progressive symptoms than males[149].

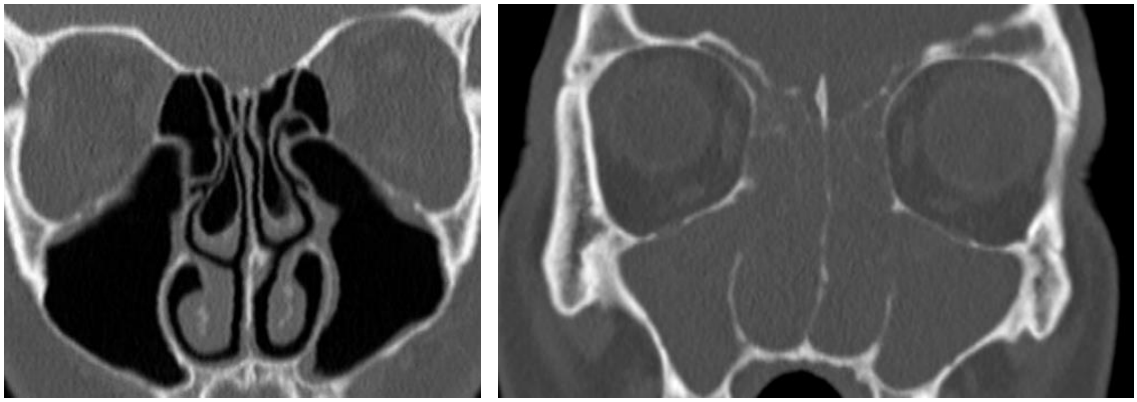


Figure 7 Comparison of cranial CT scans, coronal section plane

Comparison of two cranial CT scans, focused on the orbita, nasal cavity and paranasal sinuses of a N-ERD patient **(right side)** and a healthy control (**left side**). Substances of a low density (e.g. air) present as hypodense areas whereas substances of a high-density (e.g. bones) present as hyperdense regions. The right side shows a subtotal polypous blockage affecting almost the entire nasal cavity, the maxillary sinuses and the cellulae ethmoidales. This leads to the typicial 'white out' of paranasal sinuses in N-ERD patients.

The CT scan on the left was originally published in “Computed tomography findings in patients with Samter’s Triad: an observational study” (Open Medicine, 2019)[153].

Whilst the pathomechanism of the acute symptoms in connection to NSAID-intake seems to be clear, explanations for chronical symptoms are vague. Many studies failed in finding a coherent explanatory model, but an abundance of single aspects possibly being part of the whole pathomechanism are known.

One of the main causes for the acute reactions during NSAID-intake, but also chronic symptoms, is assumed to be a modified AA metabolism. The assumed defect in N-ERD is related to an imbalance between pro-inflammatory Cys-LTs and decreased anti-inflammatory COX1/2 pathway products resulting in a pro-inflammatory milieu[154,155]. After the intake of NSAID, COX1 and COX2 pathways are inhibited leading to a shifted metabolization via the 5-LO pathway. This condition was measured by elevated Cys-LT level in urine[44], sputum[156], blood and exhaled breath[157,158] of N-ERD patients. Simultaneously, chronic symptoms occurring without NSAID-intake can also be attributed to a dysregulated AA metabolism. Key enzymes for Cys-LT production are upregulated in lymphocytes of N-ERD patients[45,159,160] and eosinophils[161,45], MCs[162], macrophages and neutrophils[163] are abundantly present in their respiratory mucosa[,]. It has been suggested that leukocyte-adherent platelets contribute to this overproduction via transcellular LTC_4 formation[47]. Additionally, a higher sensibility due to cysLT receptor upregulation has been shown[164,165,166].

On the other hand, the counterpart to cysLTs presented by anti-inflammatory PGE_2 displays low levels in N-ERD patients and further decreases during COX-1 inhibitor intake[167,95]. Moreover the expression of EP2 receptor is reduced[168] caused by diminished COX-2 expression, the principal enzyme producing PGE2[154].

Laidlaw et al.[169] postulated diminished levels and activity of AMP-dependent protein kinase A (AMPK), an enzyme known for inhibiting LT production, in granulocytes of probands with N-ERD compared to healthy controls. Due to this fact, LT levels of granulocytes were consistently elevated despite of PGE_2 treatment. Laidlaw et al. introduced the assumption of PGE_2-resistance in

granulocytes of N-ERD patients[169]. We had particular interest in these findings and transferred them onto our experiments.

As mentioned before N-ERD lung and sinonasal tissues are characterized by a severe infiltration of eosinophils. Therefore, a T_H2-like profile in N-ERD patients was considered in multiple studies[170,171]. IL-33 and TSLP were found to be strongly expressed in nasal polyp tissue of N-ERD probands[172,173]. Both mediators drive type 2 immune responses via eosinophil activation and support herein the T_H2 thesis[98,174]. Other studies however support the thesis of a T_H1-like milieu based on findings of elevated IFN-γ levels in patients of CRS and N-ERD[175]. Even a mix of T_H1/ T_H2 immune responses is discussed postulating the theory that the type 2 immune response is contained by T_H1 cells and IFN-γ release inhibiting IgE class switch and explaining the often lacking appearance of allergy in N-ERD patients[176].

Neither the role of macrophages, strongly participating in eicosanoid production, nor macrophage polarization in the milieu of N-ERD inflammation were addressed in former investigations.

2 Objectives

Inflammatory airway diseases impose an immense health and financial burden on patients and the society, but numbers of affected subjects keep rising worldwide. NSAID-exacerbated respiratory disease, one of the most serious airway inflammations, plays a special role as the diseases pathomechanism is still incompletely understood, refractory symptoms occur frequently and there exist only inconsistent treatment regimens. All these elements complicate N-ERD treatment and justify its investigation as every new finding could improve the status quo. This study aimed to examine the role of macrophages in the pathogenesis of N-ERD via different approaches as they have been neglected before. Thereby we aimed to reveal unknown treatment possibilities helping to improve CS (corticosteroids) resistance and nasal polyposis.

Primarily a detailed questionnaire should precisely characterize our study participants in order to discover possible disruptive factors (e.g. medication) and to demonstrate causal relations between factors and probands. In the following, we focused on the anti-inflammatory lipid mediator PGE_2 showing reduced effects in granulocytes of N-ERD probands. Based on these previous findings (by Laidlaw et al.) we analyzed the PGE_2-effectiveness on aMDM from N-ERD subjects compared to healthy and CRSwNP probands by liquid chromatography-mass spectrometry (LC-MS/MS) lipid mediator analysis. Furthermore, the chemokine/cytokine (chemokine/cytokine multiplex assay) and transcriptional profile (RNA sequencing) were analyzed and compared among the three groups.

In the subsequent experiment we realized sputum induction for macrophage isolation as well as nasal brushings in N-ERD and healthy subjects from cohort II. Via RNA sequencing (RNA-seq) we compared sputum macrophages (sMac) and aMDM to uncover gene up- and downregulations characterizing N-ERD macrophages and might add to the pathomechanism. At last, sputum and NLF (nasal lavage fluid) served for targeted (lipid) metabolomics as numerous studies implied a relation between obesity, airway inflammation and metabolism alterations.

3 Material and Methods

3.1 Material

Table 1 Chemicals and Reagents

Chemicals and Reagents	**Company**
Acetone for H<PLC >99.8%	Sigma-Aldrich, Merck, Darmstadt, Germany
Aqua ad iniectabilia	Berlin-Chemie, Berlin, Germany
β-mercaptoethanol	Carl Roth, Karlsruhe, Germany
Dimethyl sulfoxide (DMSO) for cell culture	Applichem, Darmstadt, Germany
Disodium phospate (Na_2PO_4)	Merck, Darmstadt, Germany
Methanol for preparative chromatography	Merck, Darmstadt, Germany
Methanol gradient grade for liquid chromatography	Merck, Darmstadt, Germany
Sodium chloride (NaCl)	Roth, Karlsruhe, Germany
UltraPure DEPC-Treated Water	Thermo Fisher Scientific, Waltham, MA, USA
Calcium ionophore A23187	Sigma-Aldrich, Merck, Darmstadt, Germany
CD14 MicroBeads, human	Miltenyi Biotec, Bergisch Gladbach, Germany
human GM-CSF, premium grade	Miltenyi Biotec, Bergisch Gladbach, Germany
human IL4, premium grade	Miltenyi Biotec, Bergisch Gladbach, Germany
human TGFβ1 Recombinant (CHO derived)	Peprotech, Hamburg, germany

human TNF, research grade	Miltenyi Biotec, Bergisch Gladbach, Germany
LymphoPrep	Alere Technologies AS, Oslo, Norway
May-Gruenwald's Solution	Carl Roth, Karlsruhe, Germany
Polymorphprep	Alere Technologies AS, Oslo, Norway
Prostaglandin E2	Cayman Chemical, Ann Arbor, MI, USA
RNAprotect	Qiagen, Hilden, Germany
RosetteSep Human Monocyte Enrichment Cocktail	Stemcell Technologies, Vancouver, Canada
Sodium chloride 5.85% solution (NaCl)	B-Braun, Melsungen, Germany
Sputolysin Reagent (DTT)	Calbiochem, Merck, Darmstadt, Germany
Trypan Blue Solution 0.4%	Thermo Fisher Scientific, Waltham, MA, USA

Table 2 Medium and Medium components

Medium components	**Company**
Bovine Serum Albumin (BSA)	Sigma-Aldrich, Merck, Darmstadt, Germany
DMEM-F12	Thermo Fisher Scientific, Waltham, MA, USA
DPBS, no calcium, no magnesium	Thermo Fisher Scientific, Waltham, MA, USA
Fetal Bovine Serum (FBS), exosome-depleted	Thermo Fisher Scientific, Waltham, MA, USA
Gentamicin (50 mg/mL)	Thermo Fisher Scientific, Waltham, MA, USA
Hyclone Research Grade Fetal Bovine Serum (FBS)	GE Healthcare Life Science, Chicago, IL, USA

L-Glutamine (200 mM)	Thermo Fisher Scientific, Waltham, MA, USA
Penicillin-Streptomycin (10.000 U/mL)	Thermo Fisher Scientific, Waltham, MA, USA
RPMI-1640 Medium	Thermo Fisher Scientific, Waltham, MA, USA
UltraPure 0.5M EDTA, ph 8.0	Thermo Fisher Scientific, Waltham, MA, USA
Medium	**Medium components**
Complete medium	RPMI-1640, 10% FBS, 1% penicillin-streptomycin, 1 µg/mL gentamicin
Freezing medium	50% DMEM-F12, 40% FBS, 10% DMSO
MACS buffer	PBS, 0.5% BSA, 0.5 mM EDTA
PBS_{EDTA}	PBS, 0.5 mM EDTA

Table 3 Commercial Kits

Kit	**Company**
AbsoluteIDQ p180 Kit	Biocrates Life Sciences AG, Innsbruck, Austria
Elute, Prime, Fragment Mix	Illumina, San Diego, CA, USA
Quant-iT PicoGreen dsDNA Assay Kit	Thermo Fisher Scientific, Waltham, MA, USA
Quick-DNA Microprep Plus Kit	Zymo Research, Irvine, CA, USA
Quick-RNA Microprep Kit	Zymo Research, Irvine, CA, USA
RNA 6000 Nano Kit	Agilent, Santa Clara, CA, USA
TruSeq Stranded Total RNA Library Prep Kit with Ribo-Zero	Illumina, San Diego, CA, USA

Table 4 Commercial Assays

Assay type	Analyte(s)	Company
immunoCAP	ECP	Thermo Fisher Scientific, Waltham, MA, USA
immunoCAP	total IgE	Thermo Fisher Scientific, Waltham, MA, USA
17-Multiplex, human	CXCL1, CXCL2, CXCL8, CXCL9, CXCL10, CXCL11, CCL5, CCL11, CCL17, TNF, IL1β, IL6, IL12p70, IL18, IL33, IL10, IL27	R&D Systems, Minneapolis, MN, USA
4-Multiplex, human	CXCL1, CXCL2, PPBP, CCL18	R&D Systems, Minneapolis, MN, USA
2-Multiplex, human	CXCL8, CCL20	R&D Systems, Minneapolis, MN, USA
8-Multiplex, human	CCL2, CCL26, CXCL8, GM-CSF, IL25, IL33, Periostin, TSLP	R&D Systems, Minneapolis, MN, USA

Table 5 Consumables

Item	Company
12 Well Chamber Slide, removable grid	ibidi, Gräfelfing, Germany
96-well plate tissue culture treated, flat/round	Sarstedt, Nümbrecht, Germany
BD Falcon 6-/12-/24-/48-/96-well plate (non-) tissue culture treated, flat	BD Biosciences, San Diego, CA, USA
BD Falcon Cell Strainer 70 µm	BD Biosciences, San Diego, CA, USA

Cell Scraper S/M/L	TPP Techno Plastic Products, Trasadingen, Switzerland
Cellstar Tubes 15/50 mL	Greiner Bio-One, Frickenhausen, Germany
Costar Spin-X centrifuge tube, pore size 0.22 µm	Corning, Corning, NY, USA
CryoPure Tube 1.8 mL, white	Sarstedt, Nümbrecht, Germany
Leukosorb	Pall, New York, NY, USA
MACS LS columns	Miltenyi Biotec, Bergisch Gladbach, Germany
Mediprick	Servoprax, Wesel, Germany
Menzel-Gläser Cover Slips, 20x20/24x50	Thermo Fisher Scientific, Waltham, MA, USA
Micro tube 2mL, with screw lid with rubber ring	Sarstedt, Nümbrecht, Germany
Rhino-Pro Curette	Arlington Scientific, Springville, UT, USA
SafeSeal SurPhob Tips 10/200/1250 µL	Biozym Scientific, Hessisch Oldendorf, Germany
SafeSeal tube 1.5/2 mL	Sarstedt, Nümbrecht, Germany
Safety-Mulftifly-Needle	Sarstedt, Nümbrecht, Germany
Serological Pipettes 5/10/25 mL, graduated	Greiner Bio-One, Frickenhausen, Germany
Shandon Cytoclip	Thermo Fisher Scientific, Waltham, MA, USA
Shandon Filter Cards, white	Thermo Fisher Scientific, Waltham, MA, USA
Shandon TPX Single Chamber	Thermo Fisher Scientific, Waltham, MA, USA
S-Monovette EDTA blood collection tube	Sarstedt, Nümbrecht, Germany

Strata-X 96-well plates	Phenomenex, Aschaffenburg, Germany
SuperFrost Plus Adhesion Slides	Thermo Fisher Scientific, Waltham, MA, USA
SurPhob Low Binding Tips 10/200/1250 µL	Biozym Scientific, Hessisch Oldendorf, Germany
T-25/75 cell culture flasks	Greiner Bio-One, Frickenhausen, Germany

Table 6 Instruments

Device	**Company**
1200/1260 Series HPLC	Agilent, Waldbronn, Germany
Agilent 2100 BioAnalyzer	Agilent, Santa Clara, CA, USA
Analytical Balance ALJ	Kern & Sohn, Bailingen-Frommern, Germany
API 4000 triple quadrupole system	Sciex, Darmstadt, Germany
Axiovert 25/40C Microscope	Zeiss, Oberkochen, Germany
BCV control vacuum pump	Vacuubrand, Wertheim, Germany
Bio Plex 200 System	Bio-Rad, Munich, Germany
Centrifuge 5430R	Eppendorf, Hamburg, Germany
Computer M series	ThinkCentre/ Lenovo, Canada
Cool Cell	BioCision, Larkspur, USA
Cytospin 4 Cytocentrifuge	Thermo Fisher Scientific, Waltham, MA, USA
Hamilton Microlab STARTM robot	Hamilton, Bonaduz, Switzerland
Heracell CO_2 Incubators	Heraeus, Hanau, Germany
HERAFreeze -80 °C Freezer	Thermo Fisher Scientific, Waltham, MA, USA
Herasafe (KS) Biological Safety Cabinets	Heraeus, Hanau, Germany
HTC PAL autosampler	CTC Analytics, Zwingen, Switzerland

Illumina HiSeq4000 platform	Illumina, San Diego, CA, USA
Kinetex C18 reversed phase column	Phenomenex, Aschaffenburg, Germany
MACS Multi Stand	Miltenyi Biotec, Bergisch Gladbach, Germany
Megafuge 1.0R	Heraeus, Hanau, Germany
Megafuge 40R	Thermo Fisher Scientific, Waltham, MA, USA
Microlab STAR robot	Hamilton Bonaduz AG, Bonaduz, Switzerland
Microforcep	Aesculap, Tuttlingen, Germany
NanoDrop ND-1000	NanoDrop Technologies Inc., Wilmington, DE, USA
NanoPhotometer N60	Implen, Munich, Germany
NanoSight LM10	Malvern Instruments, Malvern, UK
Nasal speculum	Aesculap, Tuttlingen, Germany
Pari Boy SX nebulizer	Pari, Starnberg, Germany
Neubauer improved counting chamber	Marienfeld Superior, Lauda Königshofen, Germany
PerfectSpin 24R Refrigerated microfuge	PeqLab Biotechnology, Erlangen, Germany
Sciex QTRAP 5500 mass spectrometer	Sciex, Darmstadt, Germany
Scin Prick Test	Allergopharma, Reinbek, Germany
SecurityGuard Ultra Cartridge C18	Phenomenex, Aschaffenburg, Germany
Sigma 1-15 Microfuge	Sigma Laborzentrifugen, Osterode am Harz, Germany
Sniffin‘ Sticks	Burghart, Wedel, Germany
Stuart Digital Tube Roller SRT9D	Cole-Parmer, Stone, Staffordshire, UK
Transferpipette	Brand, Wertheim, Germany

(2.5-25/5-50/20-200/30-300 µL)	
Ultravap nitrogen evaporator	Porvair Sciences, Leatherhead, UK
Vortex Genie2	Bender + Hobein, Bruchsal, Germany
ZAN 100 spirometer	nSpire Health, Oberthulba, Germany
Ziegra ice machine	Ziegra, Isernhagen, Germany

3.2 Methods

3.2.1 Study cohort recruitment

The study set-up was designed as a three armed case-control study with a clinical study part and an experimental study part. The clinical part was of a descriptive kind, as the experimental part was interventional.

The ethics council of the university hospital “Klinikum rechts der Isar” belonging to the Technical University of Munich (internal reference: 422/16) approved the study and a written informed consent from all study participants was obtained, according to the Declaration of Helsinki. The clinical study part was performed from March 2017 until June 2018. We recruited two cohorts in the ear, nose throat (ENT) ambulance at Klinikum rechts der Isar (Munich, Germany) consisting of thirty-five (cohort I) and nine (cohort II) patients. Depending on their state of health they were divided into three groups: healthy participants, patients with CRSwNP and N-ERD patients. The EPOS-2012 served as a guideline to determine the health condition[106]. Accordingly, healthy participants had never presented symptoms of nasal polyposis, asthma or NSAID intolerance. The occurrence of two or more symptoms like anterior/posterior rhinorrhea, nasal blockage/obstruction/congestion, facial pressure/pain or anosmia/hyposmia for a period of more than twelve weeks were the criteria for chronic rhinosinusitis. The symptoms had to emerge from a state of nasal blockage/obstruction/congestion or anterior/posterior purulent rhinorrhea. The diagnose of N-ERD was made if patients presented CRSwNP, physician-diagnosed chronic asthma and a history of adverse reaction to NSAID intake. As evidence of a severe CRSwNP participants had to fulfill a nasal polyp score of at least 2 and/or a pathologic CT-

scan. Criteria for exclusion were the use of biologicals (Mepolizumab, Reslizumab, Omalizumab, Benralizumab), oral corticosteroids (OCS) and/or 5-lipoxygenase inhibitors (Zileuton) to a minimum of four weeks prior to the study. Also, the presence of certain diseases (systemic immune disorders, malign diseases, acute respiratory infections) and pregnancy were defined as reasons for exclusion. The participants age had to range between 18 and 80 years.
Clinical data of both cohorts are summarized in Table 11.

3.2.2 Sampling process

3.2.3 Collection of the clinical data

All participants were asked for their clinical history and current health status in order to be classified in the corresponding study group. To capture more objectifiable data of the quality of life in patients with possible rinosinonasal symptoms we applied the sinonasal outcome test (SNOT) 22 developed by Picirillo et al[177]. Like in various studies[178,179], the standardized Sniffing test, containing 16 Sniffin' Sticks (Burghart, Wedel, Germany) served as a semi-objective measurement for the smelling capability. We surveyed allergic reactions in the probands clinical history and performed a skin prick test (Allergopharma, Reinbek, Germany) to detect IgE-mediated sensitization towards specific allergens[180]. Using the ZAN 100 spirometer (nSpire Health, Oberthulba, Germany) we performed a pulmonary function test to detect infringements of the pulmonary ventilation. All participants were given an examination of the nasal cavity with a rigid 30° Hopkins endoscope. The occurrence of nasal polyposis was determined and the polyp expansion was measured in relation to the middle meatus via the Malm Score Classification[181].

Table 7 Malm Score Classifiaction

Malm Score	Condition
0	No polyp
1	mild polyposis limited to middle meatus;
2	moderate polyposis reaching below middle concha
3	diffuse polyposis (complete involvement of ipsilateral nasal cavity)

3.2.3.1 Blood sampling and processing

We sampled between ten and fifty milliliter of whole venous blood and collected it in ethylenediaminetetraacetic acid (EDTA) tubes (S-Monovette, Sarstedt). During a time period of maximum two hours we processed the collected blood samples. The blood was carefully layered on Polymorphoprep (Alere Technologies AS) followed by a centrifugation process of 35 minutes at room temperature (RT) at 500 x g without brake. The Plasma was discarded from the tube, distributed into smaller tubes (Nümbrecht, Sarstedt) and stored at -80 °C. The upper cell layer containing the peripheral blood mononuclear cells (PBMC) and the lower layer containing the polymorphonuclear cells (PMN) were distributed into separated 50 mL falcon tubes filled with 30mL sterile phosphate-buffered saline (PBS). For PBMC isolation from blood samples of cohort II LymhoPrep (Alere Technologies AS) was used. PMN were treated separately for further experiments.

3.2.3.2 NLF collection and nasal brushings

By inserting a filter paper strip (Leukosorb, Pall) in the nasal cavity and placing it on the inferior turbinate of healthy probands or polypous tissue of N-ERD or CRSwNP participants, NLF was collected. After 5 minutes the paper stripes were removed and put into a 0.22 µm filter spinning tube (Corning Costar Spin-X, Merck). The tubes were centrifuged at 6.000 x g for 5 minutes at 4°C. While the filter units were discarded, the samples for cohort I were stored at -80°C for metabolomics analysis. Cohort II samples needed a final PBS-Methanol (MeOH; Merck) concentration of 40 to 50% (V/V) so the sample volume was determined beforehand and diluted with -20°C cold MeOH 1:1 (V/V). The NLF samples were

stored at -80°C for lipid mediator analysis. Additionally, we collected nasal brushing samples from the inferior turbinates of healthy participants and from nasal polys of N-ERD patients. The samples were immediately diluted in RNAprotect (Qiagen) and stored at -80°C for RNA isolation for RNA-seq.

3.2.3.3 Sputum Collection

Sputum induction was performed with cohort II. Participants inhaled different concentrations of hypertonic saline solution for 10 minutes. The inhalation was repeated three times with increasing saline concentrations (3,4,5% Na Cl V/V). Between every inhalation step probands were asked to expectorate the accumulated sputum. The collected sputum samples were stored on ice and processed maximum one hour after the induction.

3.2.4 *In vitro*-generation and stimulation of alveolar-like macrophages

For the isolation of CD14-positive monocytes from PBMC we used CD14 MACS beads (Miltenyi Biotec) following the manufacturer's instructions. After the isolation process the CD14-positive monocytes were collected in T-25 and/or T-75 flasks with complete medium (RPMI-1640 supplemented with 10% FBS, 100 units/mL penicillin, 1 µg/mL gentamicin (Thermo Fisher Scientific) and 100 µg/mL streptomycin (Penicillin-Streptomycin, Thermo Fisher Scientific) at a concentration of $0.5x10^6$ monocytes/mL) and differentiated to aMDM. The cell differentiation was driven by 2 ng/mL TGFβ1 (Peprotech) and 10 ng/mL granulocyte-monocyte colony-stimulation factor (GM-CSF) as described previously[182,183]. After seven days the cells were divided into groups of $2x10^6$ or $1x10^5$ aMDM and distributed onto a 6-well or 96-well plate. The cells were stimulated for 24 hours with 100 nM PGE_2 or 10 ng/mL IL4 (Miltenyi Biotec) while PBS stimulation served as control. Besides PGE_2, IL4 and PBS, $2x10^5$ aMDM were additionally stimulated with Calcium ionophore A23187 (5 µM, Sigma-Aldrich). The Calcium ionophore stimulation was performed for 10 minutes at 37°C in order to promote lipid mediator production[184]. aMDM were washed with PBS before being diluted in RLT Buffer (Qiagen) with 1% β-mercaptoethanol (βME, Carl Roth). Afterwards the samples were stored at -80°C for RNA and DNA

isolation. The SN was divided into aliquots, diluted 1:1 (V/V) with -20°C cold MeOH and also transferred to the -80°C refrigerator.

3.2.5 sMac isolation

The sputum samples were weighted and transferred to petri dishes (Greiner, Bio-One) for sputum plug selection. The collected sputum plugs were weighted ($w_{sputum\ plugs}$) and mixed with 4:1 ($V/w_{sputum\ plugs}$) 0.1% dithiothreitol (DTT, Sputolysin Reagent, Calbiochem). The dithiothreitol-sputum solution was incubated for 15 minutes at 4°C at the roller mixer (roller mixer SRT9D, Stuart). An aliquot was taken for metabolomic analysis and stored at -80°C. PBS was added to the solution 2:1 ($V/w_{sputum\ plugs}$) and put on a 70 µm cell filter (BD Biosciences). The filtrated homogenate was centrifuged for 10 minutes at 790 x g, 4 °C without break. The cell pellet was rediluted in cold PBS and the cell number was determined via the Neubauer counting chamber (Marienfeld). 50.000 sputum cells were suspended in 5% BSA (V/V, in PBS) and transferred to SuperFrost Plus Adhesion Slides (Thermo Fisher Scientific). The slides were transferred in a Cytospin 4 cytocentrifuge (Thermo Fisher Scientific) and centrifugated at RT (room termperature) at 300 rpm for 5minutes. Then at least 400 non-squamaous cells were counted by differential cell counts as described previously[185]. The slides were air-dried and fixed with MeOH for 5 minutes. In order to fix the cells, we used the Pappenheim method. The slides were dyed for 8 minutes in May- Gruenwald (Carl Roth), washed with water and put into Giemsa solution (Merck) for another 35 minutes. The treated slides were analyzed at 40x magnification. Cold PBS was added to the sputum cell pellet and as a result we obtained highly purified sputum macrophages (sMac) as previously described[186]. Aliquots of the SN were taken and diluted 1:1 (V/V) with -20 °C MeOH in order to perform lipid mediator analysis. The samples were stored at -80°C. Shortly afterwards the sputum cells were co-incubated with RosetteSep human monocyte enrichment cocktail (Stemcell Technologies) and erythrocytes from the corresponding proband at RT for 20 minutes. The cells were centrifuged at RT without brake on LymphoPreb at 1.500 rpm for 15 minutes. The centrifugation process led to a mononuclear sMac layer. Cell counting with trypan blue (Thermo

Fisher Scientific) was performed to control sMac viability and purity and resulted >90% for all participants. For RNA-seq and RNA isolation sMac were diluted in RLT Buffer with 1% βME and transferred to the -80 °C refrigerator.

3.2.6 Chemokine/cytokine multiplex assays

Multiplex chemokine/cytokine assays (Magnetic Luminex Assay 2-plex: CXCL8, CCL20; 4-plex: CXCL1, CXCL2, PPBP, CCL18; 8-plex: CCL2, CCL26, CXCL8, GM-CSF, TSLP, Periostin, IL25, IL33, 17-plex: CXCL1, CXCL2, CXCL8, CXCL9, CXCL10, CXCL11, CCL5, CCL11, CCL17, TNF, IL-1β, IL-6, IL-12p70, IL-18, IL-33, IL-10, IL-27;; R&D Systems, Minneapolis, MN, USA) of aMDM SN were performed according to the manufacturer's instructions on a Bio Plex 200 System (Bio-Rad, Munich, Germany).

3.2.7 Lipid mediator analysis – LC-MS/MS

Lipid mediator analysis was carried out as previously described[187]. The samples (NLF, SN of cell culture and SN of sputum) were diluted with H_2O until a MeOH content of 15% was reached. Additionally, 10 µl of IS stock solution was added. Hamilton Microlab STAR robot (Hamilton Bonaduz AG, Bonaduz, Switzerland) was used for automated solid phase extraction. Sample extraction was performed with Strata-X 96-well plates (30 mg, Phenomenex, Aschaffenburg, Germany) and eluted with MeOH. The samples were dried through nitrogen stream and diluted in 100 µl MeOH/H_2O (1:1). We used a 1260 Series HPLC (Agilent Technologies Deutschland GmbH, Waldbronn, Germany) with a Kinetex C18 reversed phase column (2.6 µm, 100 x 2.1 mm, Phenomenex, Aschaffenburg, Germany) and a SecurityGuard Ultra Cartridge C18 (Phenomenex, Aschaffenburg, Germany) precolumn for chromatographic eicosanoid separation. Operating in negative ionization mode, a Sciex QTRAP 5500 mass spectrometer (Sciex, Darmstadt, Germany), equipped with a Turbo-VTM ion source was used. Sample injection was performed with HTC PAL autosampler (CTC Analytics, Zwingen, Switzerland) at 4°C. Via scheduled multiple reaction monitoring (sMRM) and retention time the metabolites were determined. Single analyte injections and literature comparison allowed to select a unique Q1/Q3 transition for each

analyte[188]. Whereas the differentiation of analytes with identical sMRM transitions was allowed through their retention time. The LC-MS/MS data was recorded with the Analyst Software 1.6.3 and quantified with MultiQuant Software 3.0.2 (both Sciex, Darmstadt, Germany).

The described method was provided by the Genome Analysis Center (GAC) Metabolomics Core Facility forming part of the Helmholtz Zentrum München, Munich Germany. The data collection was carried out by Dr. Pascal Haimerl and Fiona Henkel.

3.2.8 Metabolomics – Metabolite Quantification

The AbsoluteIDQ p180 Kit (Biocrates Life Sciences AG, Innsbruck, Austria), LC-ESI-MS/MS and FIA-ESI-MS/MS measurements were the methods applied for the targeted metabolomics approach. Using the AbsoluteIDQ p180 Kit agreed with the EMEA-Guideline "Guideline on bioanalytical method validation (July 21st 2011)"[189] and contains proof of reproducibility within a given error range. The use of the AbsoluteIDQ p180 Kit and the metabolite nomenclature have been described previously[190,191]. 188 metabolites could be measured via the assay out of 2.5 µL NLF, 5 µL sputum homogenate or 10 µL plasma. The samples (NLF, sputum homogenate or plasma) did not need any further treatment before being processed. The setting for the LODs was three times the values for the zero samples (PBS for all materials). The 188 identified metabolites encompassed 15 sphingolipids (SMx:y), 21 amino acids (ornithine, citrulline and 19 proteinogenic), 21 biogenic amines, 39 acylcarnitines (Cx:y), 90 glycerophospholipids (76 phoshatidylcholines (PC) and 14 lysophosphatidylcholines (lysoPC) and hexoses (sum of hexoses- around 90-95% glucose). Cx:y is used as an abbreviation indicating the total number of carbons and double bonds of all chains (for more details see [190]).To identify and quantify the compounds of the LC we used sMRM. Additionally to basic laboratory equipment, we utilized a Ultravap nitrogen evaporator (Porvair Sciences, Leatherhead, UK) and a Hamilton Microlab STARTM robot (Hamilton Bonaduz AG, Bonaduz, Switzerland) for sampling process. We used an HTC PAL auto sampler (CTC Analytics, Zwingen, Switzerland) provided with an API 4000 triple quadrupole system (Sciex,

Darmstadt, Germany) and a 1200 Series HPLC (Agilent Technologies Deutschland GmbH, Böblingen, Germany) for mass spectrometric analyses. The process was controlled by the software Analyst (v1.6.2). We used the software MetIDQ, as part of the AbsoluteIDQ Kit, as well as the software MultiQuant (v3.0.1, Sciex, Darmstadt, Germany) to quantify metabolite concentrations and evaluate their quality.

The described method was provided by the Genome Analysis Center (GAC) Metabolomics Core Facility of the Helmholtz Zentrum München, Munich, Germany. Data collection was realised by Dr. Pascal Haimerl.

3.2.9 Whole transcriptome analysis – RNA-seq

Following the manufacturer's instruction, total RNA was extracted via the "Zymo Research Kit" (Zyma Research, Irvine, CA, USA). Using the Nano Photometer N60 (Implen, Munich, Germany) or the NanoDrop ND-1000 (NanoDrop Technologies Inc, Wilmingoton, DE, USA) we controlled the quantity and quality of the extracted RNA. The assessment of the RNA integrity number (RIN) was realized with the Agilent 2100 BioAnalyzer (RNA 6000 Nano Kit, Agilent, Santa Clara, CA, USA).

TruSeq Stranded Total RNA Library Prep Kit with Ribo-Zero (Illumina, San Diego, CA, USA) was used for library preparation. For enrichment of whole transcriptome RNA, cytoplasmatic rRNAs (ribosomal ribonucleic acid) were removed from 1 µg of RNA. Then the depleted RNA was fragmented and transcribed using the Elute, Prime, Fragment Mix. PolyA-enriched RNA-seq was used in sMac and aMDM. Using the Elute, Prime, Fragment Mix (Illumina, San Diego, CA, USA) selection, fragmentation and reverse transcription was performed with 300 ng of RNA, as previously described[192]. The High Throughpu protocol of The TruSeq Stranded mRNA Sample Prep Guide for stranded RNA-seq and the TruSeq RNA Sample Prep guide for total RNA-seq were followed for A-tailing, adaptor ligation and library enrichment. Quality and quantity assessment of the RNA libraries was realized with the Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) and the Agilent 2100 BioAnalyzer. The RNA sequencing was performed on an

Illumina HiSeq4000 platform that was able to produce and output of around 50 to 118 Million single end reads per library. We applied the STAR aligner (v2.4.2a)[193] with modified parameter settings (--twopassMode=Basic) for UCSC knownGene annotation and for split read alignment against the human genome assembly hg19 (GRCh37). For quantification of the number of reads mapping to annotated genes, the HTseq-count (v0.6.0)[194] was utilized. With custom scripts we computed the FPKM (Fragments Per Kilobase of transcript per Million fragments mapped).

The described method was kindly provided by the Institute of Human Genetics Next-Generation Sequencing Core Facility of the Helmholtz Zentrum München, Munich, Germany.

3.3 Bioinformatical analysis and statistics

The Bioinformatical analysis and statistics of Genome-wide transcriptomics, targeted metabolomics and statistical test were realized by Dr. Pascal Haimerl during his PhD project.

3.3.1 Genome-wide transcriptomics – RNA-seq

With GSNAP (version 2018-07-04) [195] and splice-site information from Ensembl release 87 [196] we mapped the raw reads to the human genome (hg38). Gene annotations from Ensembl and mapped reads formed the database for featureCounts (v1.6.2) [197] to create counts per cell and gene. The non-protein coding genes were discarded.

With R (v3.5.0) [198] and the R package DEBrowser (v1.11.9)[199] we carried out various expression analysis. We filtered the data for features with a count of > 10 and uploaded the metadata. Normalization and differential expression analysis were performed utilizing the implemented R package DESeq2[200]. For the nasal brushing and aMDM dataset, significance thresholds were set to adjusted *p*-value (padj) ≤ 0.05 (Benjamini-Hochberg false discovery rate)[201] and log2FoldChange ≥ 1/≤ -1. For the sMac vs. aMDM dataset, the padj threshold was set to ≤ 0.01. To remove epithelial signatures in the sMac dataset, AmiGO 2[202–205] search results of Homo sapiens protein-coding genes associated with the term

“epithelial”, but not “macrophage”, were excluded from the dataset. To further reduce dimensionality, differential expressed genes were matched with the search term results of “carnitine”, “sphingo”, “cytokine”, “chemokine” and “icosanoid” through the biomaRt package (v2.38.0) [206,207]. Enrichment and pathway analysis of database of essential genes (DEGs) was performed with the online platform ToppGene suite (https://toppgene.cchmc.org).[208] Volcano plots were created using the differential expression data with standard R packages and EnhancedVolcano (v1.1.3) [209]. For consistency with metabolomics data, heatmaps were created with MetaboAnalyst 4.0 [210]. RNA-seq data have been deposited in the ArrayExpress database at EMBL-EBI (https://www.ebi.ac.uk/arrayexpress)[211] under accession number E-MTAB-7962 (nasal brushing) and E-MTAB-7965 (sMac and aMDM).

3.3.2 Targeted metabolomics – multi-fluid analyses

Based on the exploratory study design, values below LOD were included in subsequent analysis. Metabolomics data of all measurements were imported in the online platform MetaboAnalyst 4.0 (https://ww.metaboanalyst.ca)[210] for statistical analysis.

Plasma metabolomics data from both metabolomics approaches were pooled and cleansed by excluding features with too many missing values (> 20%) and consisted finally of 204 metabolites. Afterwards the final dataset was normalized, auto-scaled and cube root transformed. Via hierarchical clustering and principal component analysis (PCA) we were able to classify 3 N-ERD and 1 healthy outlier[212] which were left out of further data analysis. For visualization of the maximum variance between the groups we used PCA. The following data analysis was performed with ANOVA and the supervised partial least squares-discriminant analysis. Clustering algorithm (Ward) were used to identify clusters within the metabolites.

Normalization on sample protein concentration was performed with Cohort II NLF data and cohort I sputum data considering patient-specific and matrix alterations. Subsequently the datasets were transmitted to MetaboAnalyst. The number of plasma samples was higher than the collected sputum (N-ERD n=3, Healthy n=5)

and NLF samples (N-ERD n=8, CRSwNP n= 8, Healthy n=11). We did not register any statistical outliers within the data sets.

After normalizing the plasma data set metabolomics pathway analysis was implemented on the MetaboAnalyst 4.0 platform. 211 metabolites of 213 were classified with HMDB IDs. KEGG IDs include the metabolites of a cohesive group without taking into consideration the lipid chain structure. Out of the 211 metabolites we were able to determine 55 specific KEGG IDs. Consequently, we utilized the metabolites with identical KEGG IDs. For topological analysis and pathway enrichment we relied on “Relative Betweenness Centrality” and “Homo sapiens” library with “Global Test”.

3.3.3 Statistical analysis

GraphPad Prism 6 (GraphPad Software, La Jolla, CA, USA) was used for non-parametric statistical analyses. The Kruskal-Wallis test with Dunn’s correction or the Chi-square test, Mann-Whitney-Wilcoxon test served to analyze the differences between two or three groups with single conditions. For the comparison of three groups and multiple conditions 2-way ANOVA with Dunnett’s correction was used. Two tailed Spearman correlation and linear regression was applied for SNOT-22 score, adipokine comparisons and BMI. Whereas sputum eicosanoid data was calibrated to the total number of sputum cells, NLF eicosanoids data were standardized on NLF sample volume. MetaboAnalyst 4.0 was applied for all heatmaps. For all statistical test we set a significance level of (adjusted) p-value <0.05.

Eicosanoids of sputum samples were standardized on total sputum cell numbers, while eicosanoids of NLF were standardized on NLF sample volume. For visualization of the auto-scaled data we did not utilize clustering algorithms but “Euclidean”. MetaboAnalyst 4.0 were used to create all non-metabolomic heatmaps. A p-value of < 0,05 was determined significant for all statistical tests.

4 Results

4.1 Study design

The study was performed with two cohorts. The first one included 15 N-ERD patients, 10 CRSwNP patients and 10 healthy participants and was focused on *in vitro* aMDM experiments from whole blood and metabolite characterization of plasma and NLF. The second cohort included 5 N-ERD patients and 4 healthy controls. aMDM, nasal brushing and sMac were used for RNA-seq. SN from sputum and NLF served for lipid mediator analysis. A detailed overview is given in Table 8Table 8.

Table 8 Study design and experimental overview

cohort	**sample type**	**sample subtype**	**experiment**
I	whole blood	aMDM	PGE_2 resistance
			lipid mediator analysis
			chemokine/cytokine analysis
	NLF		metabolomics
II	whole blood	aMDM	RNA-seq
	nasal brushing		RNA-seq
	induced sputum	sMac	RNA-seq
		SN	lipid mediator analysis
	NLF		lipid mediator analysis

4.2 Questionnaire

Recruited individuals were age- and sex-matched. The treatment with biologics, Zileuton or oral corticosteroids was defined as exclusion criterion or had to be discontinued for 6 weeks prior to the experiment. Inhaled glucocorticoids were commonly used by N-ERD patients and rarely used in CRSwNP. In both groups the intake of nasal corticosteroids reached nearly 100%.

The mean age was overall about 50. Only females were included in cohort II to obviate any sex-bias in RNA-seq data. The percentage of females was generally higher in patients with N-ERD due to higher prevalence in women. Higher Malm scores were observed in CRSwNP and N-ERD patients, which was in line with worse sniffing test scores compared to healthy controls. N-ERD and CRSwNP patients further showed significantly increased symptom (SNOT22) scores with the highest scores observed in N-ERD patients. Total IgE levels were also elevated in N-ERD and CRSwNP. Asthma occurred more frequently in N-ERD patients, forming one characteristic symptom of the triad. No differences were found in the Body Mass Index (BMI), smoking/ever smoked status and atopy.
Taken together, we could characterize respiratory symptoms as expected for N-ERD and CRSwNP and documented further possible influential factors of disease severity. In N-ERD patients, the questionnaire also fulfilled a diagnostical purpose as we documented reaction to NSAIDs. The results of the patient characterization are summarized in Table 9.

Table 9 Patient characterization

	parameter	cohort I		
	group	**Healthy**	**NT CRSwNP**	**N-ERD**
	number of individuals	**10**	**10**	**15**
characteristics	sex (female)	7/10 (70%)	5/10 (50%)	13/15 (87%)
	age (years)	54 (30-76)	52 (28-79)	57 (31-82)
	BMI	23.8 (19.8-28)	26.1 (20.4-32.7)	25.5 (19.3-32.9)
	smoking / ever smoked	1/10 (10%)	0/10 (0%)	2/15 (13.3%)
symptoms and symptom scores	SNOT22 score (0-110)	0.8 (0-8)	24.9 (0-62)*****	33.4 (6-73)*****
	Malm score (0-6)	0	2.8 (1-6)*****	3.1 (1-6)*****
	sniffing test score (0-16)	13.5 (10-16)	10.1 (4-14)	4.9 (0-15)*****
	asthma	0/10 (0%)	4/10 (40%)	15/15 (100%)*****
	atopy	5/10 (50%)	7/10 (70%)	7/15 (47%)
laboratory parameters	total IgE (ng/mL)	91 (32-318)	254 (90-771)*****	193 (50-540)*****
	ECP (ng/mL)	4.4 (1.6-7.7)	6.5 (2.6-12.4)	6.0 (0.2-13.0)
medication	corticosteroids nasal inhaled oral	 0/10 (0%) 0/10 (0%) 0/10 (0%)	 10/10 (100%)***** 4/10 (40%) 0/10 (0%)	 14/15 (93%)***** 11/15 (73%)***** 0/15 (0%)
	LABA	0/10 (0%)	3/10 (30%)	9/15 (60%)*****
	Montelukast	0/10 (0%)	0/10 (0%)	2/15 (13%)
	biologics	0/10 (0%)	0/10 (0%)	0/15 (0%)
	Zileuton	0/10 (0%)	0/10 (0%)	0/15 (0%)

	parameter	cohort II	
	group	**Healthy**	**N-ERD**
	number of individuals	**4**	**5**
characteristics	sex (female)	4/4 (100%)	5/5 (100%)
	age (years)	50 (31-64)	52 (35-69)
	BMI	n.d.	n.d.
	smoking / ever smoked	0/4 (0%)	1/5 (20%)
symptoms and symptom scores	SNOT22 score (0-110)	4.0 (3-7)	25.8 (14-37)*****
	Malm score (0-6)	0	2.6 (1-6)*****
	sniffing test score (0-16)	n.d.	n.d.
	asthma	0/4 (0%)	5/5 (100%)*****
	atopy	1/4 (25%)	1/5 (20%)
laboratory parameters	total IgE (ng/mL)	15 (11-21)	112 (72-172)*****
	ECP (ng/mL)	9.1 (3.0-13.9)	27.3 (3.5-53.7)
medication	corticosteroids		
	nasal	0/4 (0%)	4/5 (80%)*****
	inhaled	0/4 (0%)	5/5 (100%)*****
	oral	0/4 (0%)	0/5 (0%)
	LABA	0/4 (0%)	2/5 (40%)
	Montelukast	0/4 (0%)	0/5 (0%)
	biologics	0/4 (0%)	0/5 (0%)
	Zileuton	0/4 (0%)	0/5 (0%)

NT CRSwNP, non-steroidal anti-inflammatory drug (NSAID)-tolerant chronic rhinosinusitis with nasal polyps; N-ERD, NSAID-exacerbated respiratory disease; BMI, body mass index; Malm polyp scoring, range 0-3 per nostril; SNOT22, sinonasal outcome test with 22 questions; ECP, eosinophilic cationic protein; LABA, long-acting beta-2 agonist; biologics include Omalizumab, Benralizumab, Reslizumab, Mepolizumab, Dupilumab; n.d., not determined. Data are shown as mean (min-max) and count (percentage). Data were analyzed with the Chi-square test, Kruskal-Wallis test with Dunn's correction or Mann-Whitney-Wilcoxon test: *$p < 0.05$.

4.3 Stimulation of aMDM from N-ERD, CRSwNP and healthy probands with IL-4 and PGE2

To investigate potential changes in the responsiveness to PGE_2 as reported by Laidlaw et al.[169] we isolated $CD14^+$ monocytes of cohort I from which aMDM were generated *in vitro*. The aMDM were then stimulated with 10nM IL-4 or 100nM PGE_2 for 24 hours. Lipid mediator analysis and chemokine/cytokine multiplex assay were performed with the SN of the stimulated cells.

All measured lipid mediators did not show consistent significant alterations between CRSwNP, N-ERD probands and healthy controls (Figure 8, Figure 9,Table 13)

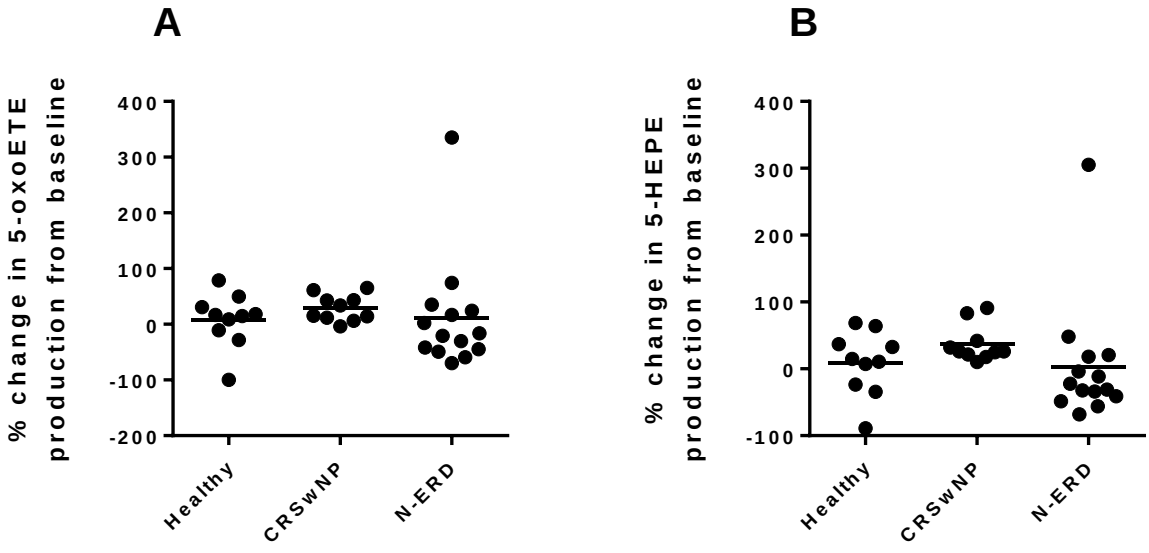


Figure 8 No PGE2 resistance found in N-ERD and CRSwNP aMDM

% change of **A 5-oxo-ETE** and **B 5-HEPE** concentrations in aMDM SN of healthy (n = 8), NT CRSwNP (*n* = 10) and N-ERD (*n* = 14) individuals after 24 hours stimulation with 100 nM PGE_2.

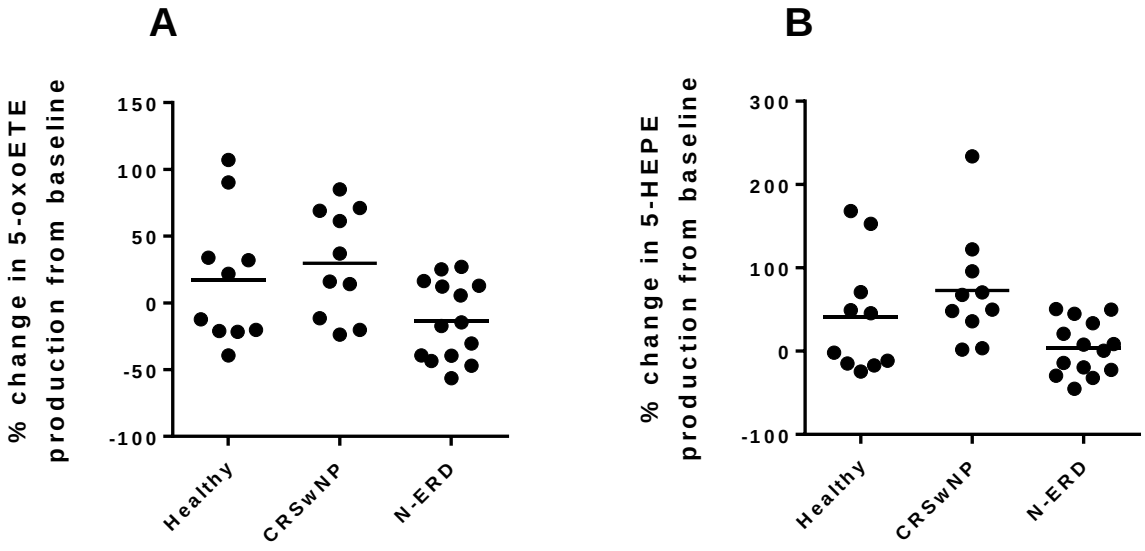
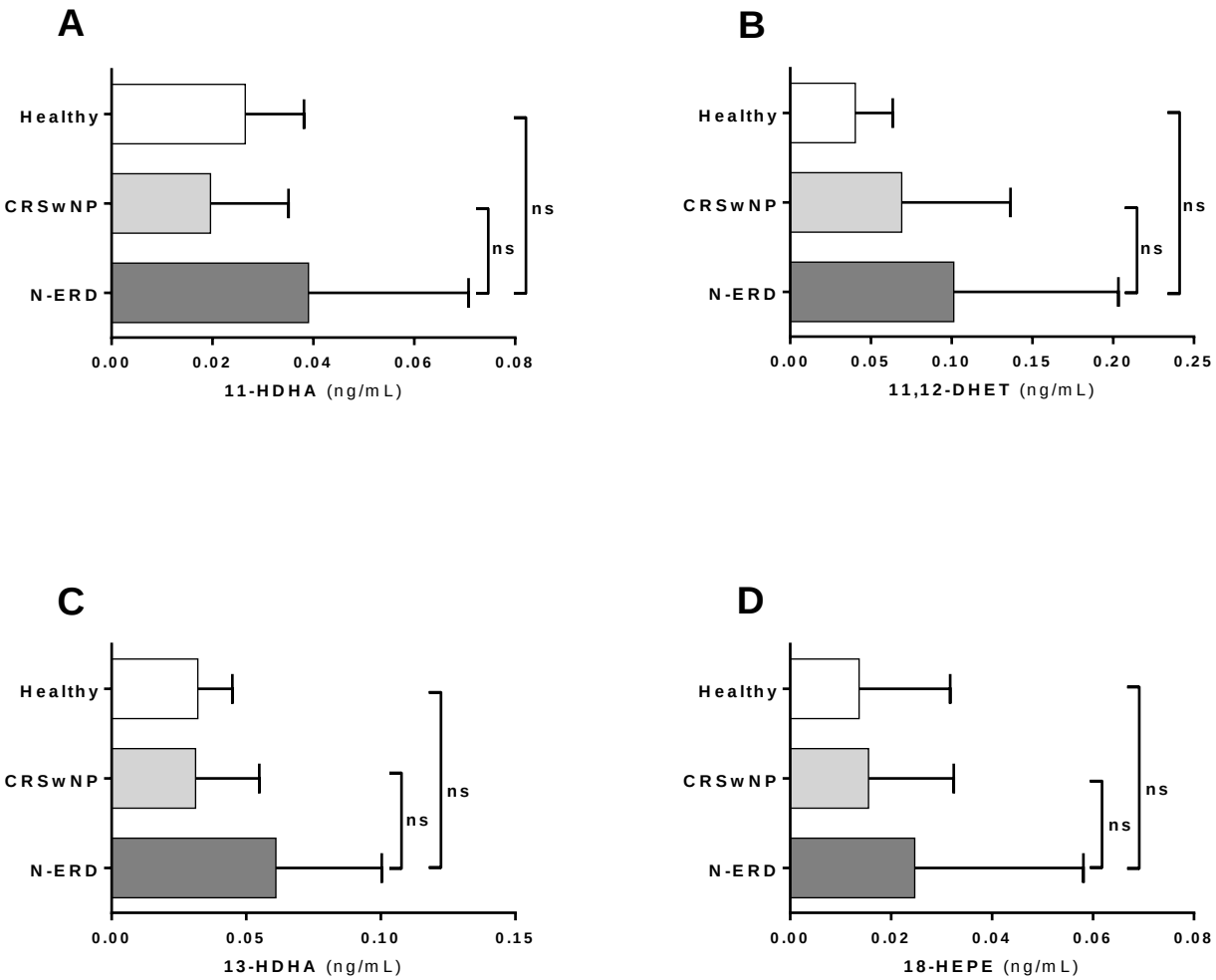


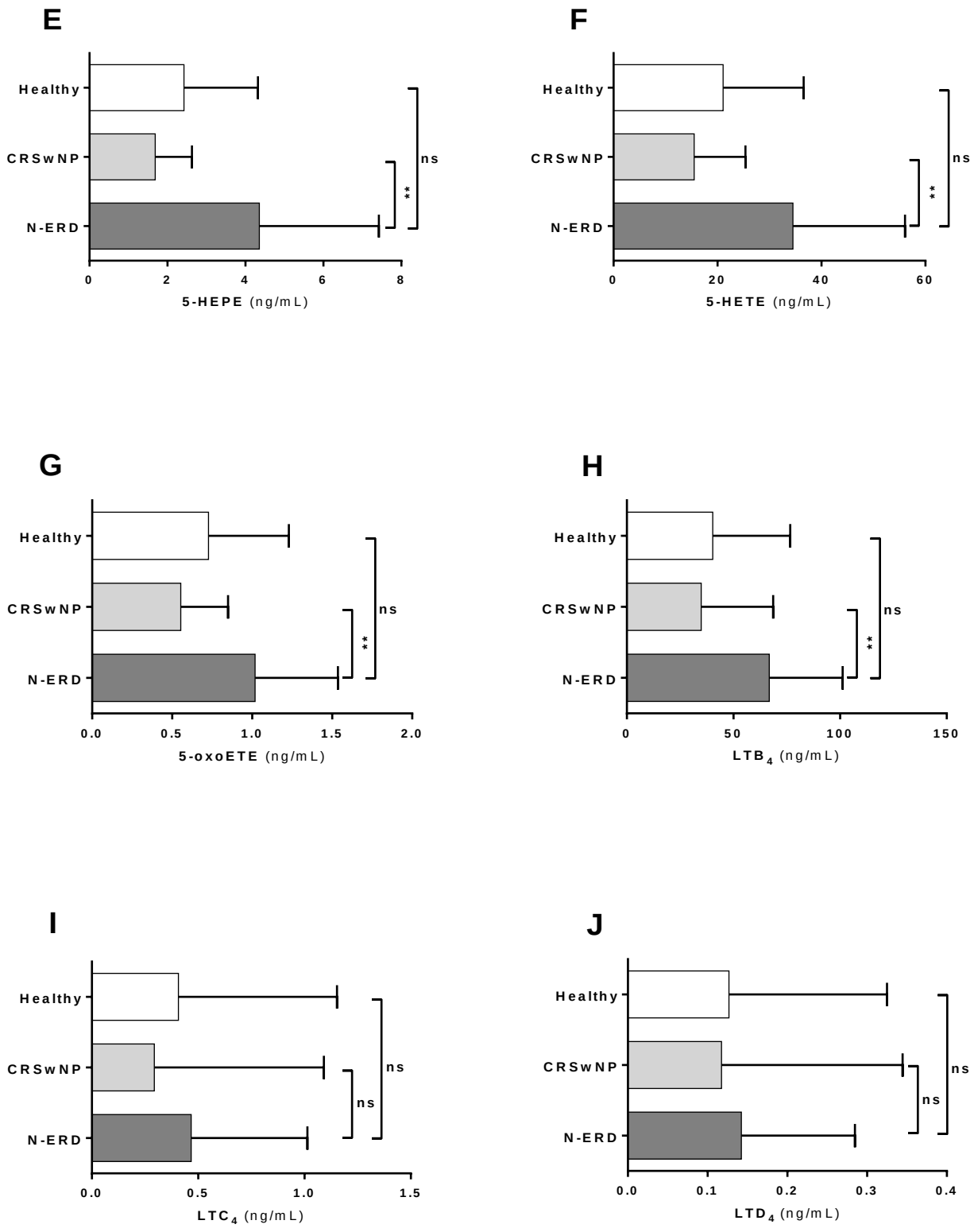
Figure 9 No significant alteration observed via IL-4 stimulation in healthy, N-ERD and CRSwNP aMDM

% change of **A** 5-Oxo-eicosatetraenoic acid (5-oxo-ETE) and **B** 5-HEPE concentrations in aMDM SN of healthy (n = 8), NT CRSwNP (*n* = 10) and N-ERD (*n* = 14) individuals after 24 hours stimulation with 10 ng/mL IL-4.

N-ERD patients showed higher baseline levels of docosahexaenoic acid (DHA) oxidation products like 11-Hydroxydocosahexaenoic acid (11-HDHA), 11,12-Dihydroxyeicosatrienoic acid (11,12-DHET) and 13-HDHA compared to controls (Figure 10) PUFAs and their auto oxidation products are indicative of oxidative stress e.g. under inflammatory conditions[213].

Indeed, 18-Hydroxyeicosapentaenoic acid (18-HEPE), 5-HEPE, 5-Hydroxyeicosatetraenoic acid (5-HETE), 5-oxoETE, which can be produced non-enzymatically (auto-oxidation) or via specific enzymes (5-LOX) were also increased in N-ERD. Similarly, further bioactive 5-LOX products (LTB_4, LTC_4, LTD_4, LTE_4) as well as COX-products PGD_2, PGE_2, $PGF_{2\alpha}$ and TXB_2 tended to be increased at baseline in N-ERD aMDM (Figure 10).


A
Healthy
CRSwNP
N-ERD
ns
ns
0.00
0.02
0.04
0.06
0.08
11-HDHA (ng/mL)
B
Healthy
CRSwNP
N-ERD
ns
ns
0.00
0.05
0.10
0.15
0.20
0.25
11,12-DHET (ng/mL)
C
Healthy
CRSwNP
N-ERD
ns
ns
0.00
0.05
0.10
0.15
13-HDHA (ng/mL)
D
Healthy
CRSwNP
N-ERD
ns
ns
0.00
0.02
0.04
0.06
0.08
18-HEPE (ng/mL)


E
Healthy
CRSwNP
N-ERD
ns
**
0
2
4
6
8
5-HEPE (ng/mL)
F
Healthy
CRSwNP
N-ERD
ns
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20
40
60
5-HETE (ng/mL)
G
Healthy
CRSwNP
N-ERD
ns
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0.0
0.5
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2.0
5-oxoETE (ng/mL)
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CRSwNP
N-ERD
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100
150
LTB4 (ng/mL)
I
Healthy
CRSwNP
N-ERD
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ns
0.0
0.5
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LTC4 (ng/mL)
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ns
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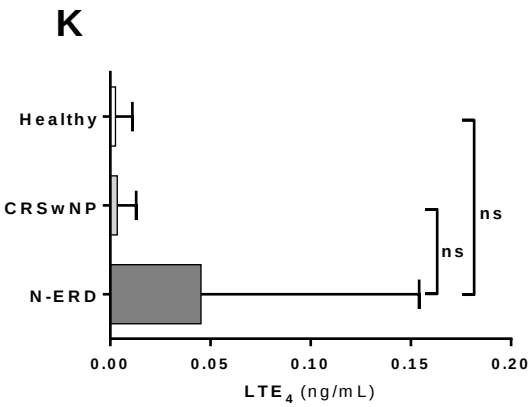
0.1
0.2
0.3
0.4
LTD4 (ng/mL)


K
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CRSwNP
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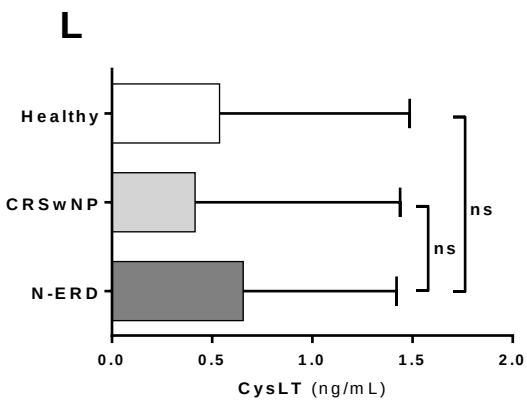
0.10
0.15
0.20
LTE4 (ng/mL)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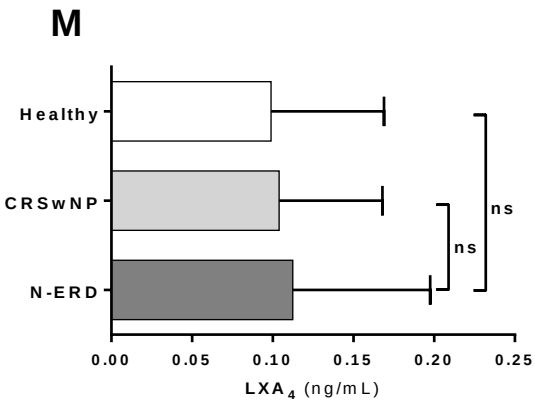
0.5
1.0
1.5
2.0
CysLT (ng/mL)


M
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ns
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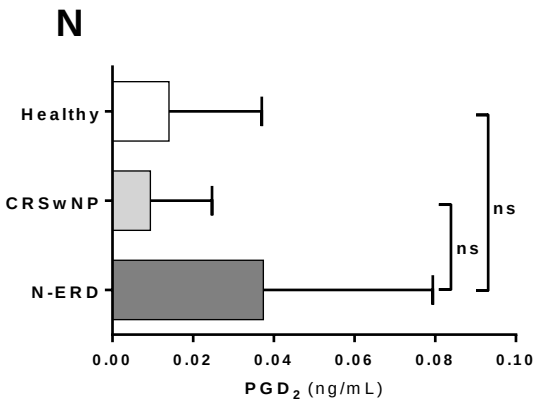
0.05
0.10
0.15
0.20
0.25
LXA4 (ng/mL)


N
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CRSwNP
N-ERD
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ns
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0.02
0.04
0.06
0.08
0.10
PGD2 (ng/mL)

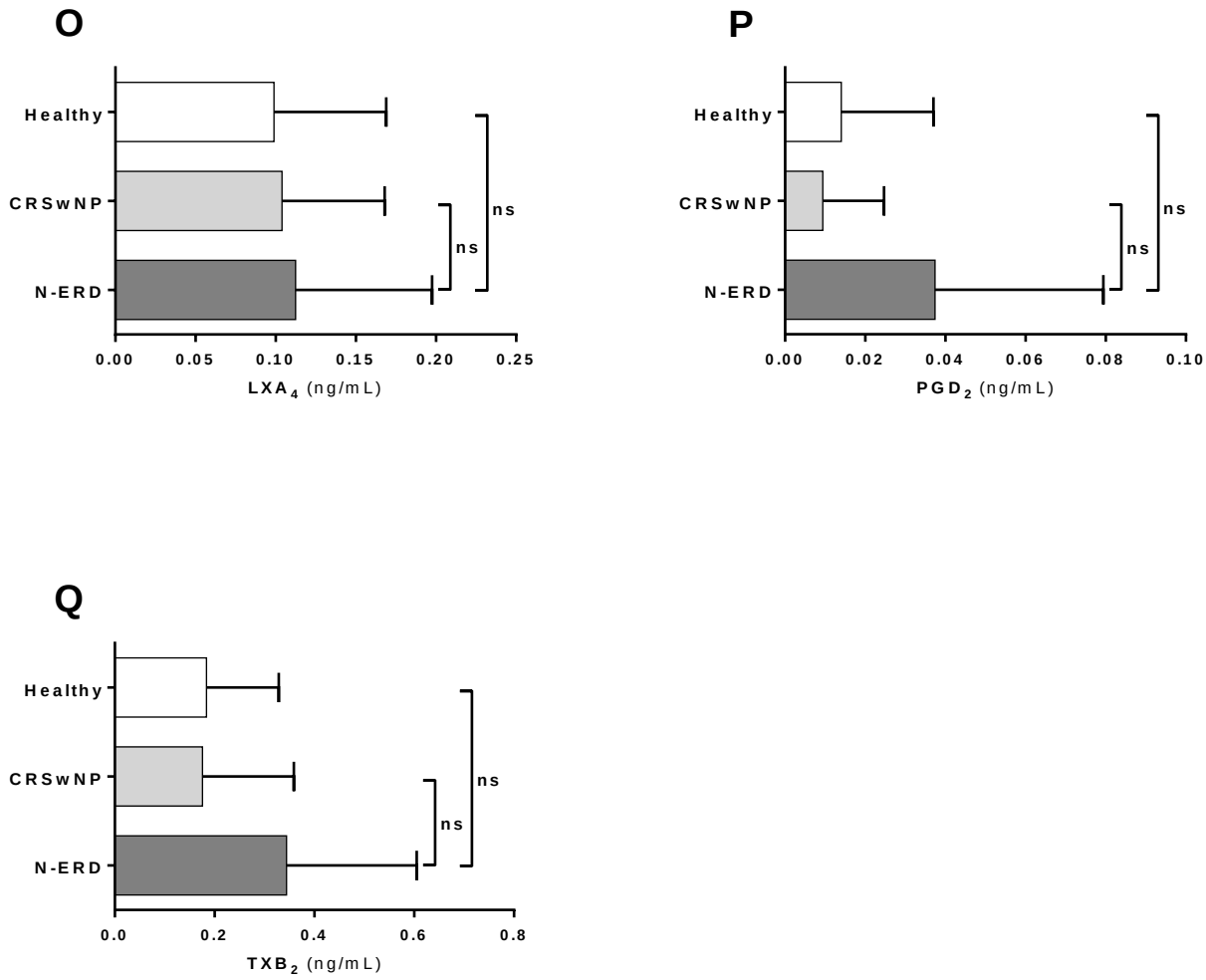

Figure 10 Elevated baseline lipid mediators in N-ERD aMDM

Baseline lipid mediator concentrations of **A** 11-HDHA, **B** 11,12-DHET, **C** 13-HDHA, **D** 18-HEPE, **E** 5-HEPE, **F** 5-HETE, **G** 5-oxoETE, **H** LTB_4, **I** LTC_4, **J** LTD_4, **K** LTE_4, **L** CysLT, **M** LXA_4, **N** PGD_2, **O** PGE_2, **P** $PGF_{2\alpha}$, **Q** TXB_2 in aMDM SN of healthy (*n* = 10), CRSwNP (*n* = 10) and N-ERD (*n* = 14) individuals. Data were analyzed with Kruskal-Wallis test with Dunn's correction: $p < 0.05$. ns= nonsignificant, **=significant.

aMDM of the three N-ERD and CRSwNP subjects with the highest SNOT-22 scores were compared to the SNOT-22 scores from healthy controls. In total, 17 chemokines and cytokines were measured. No consistent alterations were found between healthy, CRSwNP and N-ERD subjects in chemokine and cytokine measurements

It was noticeable that levels of aMDM from N-ERD subjects had the tendency to be elevated for IL1ß and TNF whereas IL-12 trended to be reduced in this cohort. After PGE_2 or IL-4 stimulation of aMDM for 24 hours, baseline levels of pro-inflammatory mediators from N-ERD subjects had the tendency to be increased compared to CRSwNP and healthy subjects.(Figure 11,Table 14)

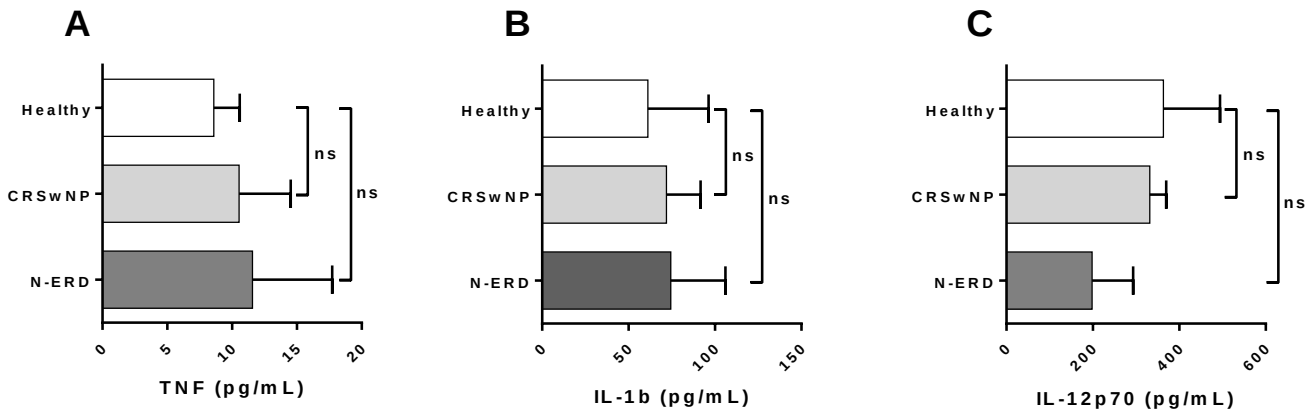

Figure 11 N-ERD aMDM present higher TNF, IL1ß and lower IL-12p70 baseline level

Baseline chemokine/cytokine concentrations of **A** TNF **B** IL1ß **C** IL-12p70 in aMDM SN of healthy (*n* = 10), CRSwNP (*n* = 10) and N-ERD (*n* = 14) individuals. Data were analyzed with Kruskal-Wallis test with Dunn's correction: $p < 0.05$. ns= nonsignificant, **=significant.

To further investigate potential changes in local airway macrophages, sMac from cohort II were isolated and compared to aMDM. Cohort II consisted of female N-ERD subjects from cohort I and healthy controls. Nasal brushing, whole blood sampling, sputum induction and NFL extraction were performed to collect samples for whole transcriptome and mediator analyses.

4.4 Whole transcriptome analysis reveals differences of aMDM in N-ERD subjects compared to healthy controls

We compared aMDM of N-ERD patients and healthy controls via whole transcriptome (RNA-seq) analysis. 86 down- and 19 up- regulated genes were found between the two groups pointing out the differences between 'healthy' macrophages and macrophages of N-ERD patients.

The results suggested an upregulation of chemotactic genes and a downregulation of genes involved in host defense and lipid antigen presentation (Figure 12). Moreover, ToppGene analysis suggested that genes regulated by IL-4 and IL-13 signaling (e.g. PTGS2 and CXCL8) differed between the cohorts. CXCL8, which was upregulated in N-ERD, plays an important role as chemoattractant for leukocytes, especially neutrophils[214]. PTGS2 encodes COX-2, which is involved in the biosynthesis of PGE_2 and other prostanoids[215]. Macrophage-inhibiting transcription factor CCAAT/ enhancer-binding protein delta (C/EBP δ), suspected to regulate M2 macrophage polarization, was downregulated in N-ERD aMDM[216].

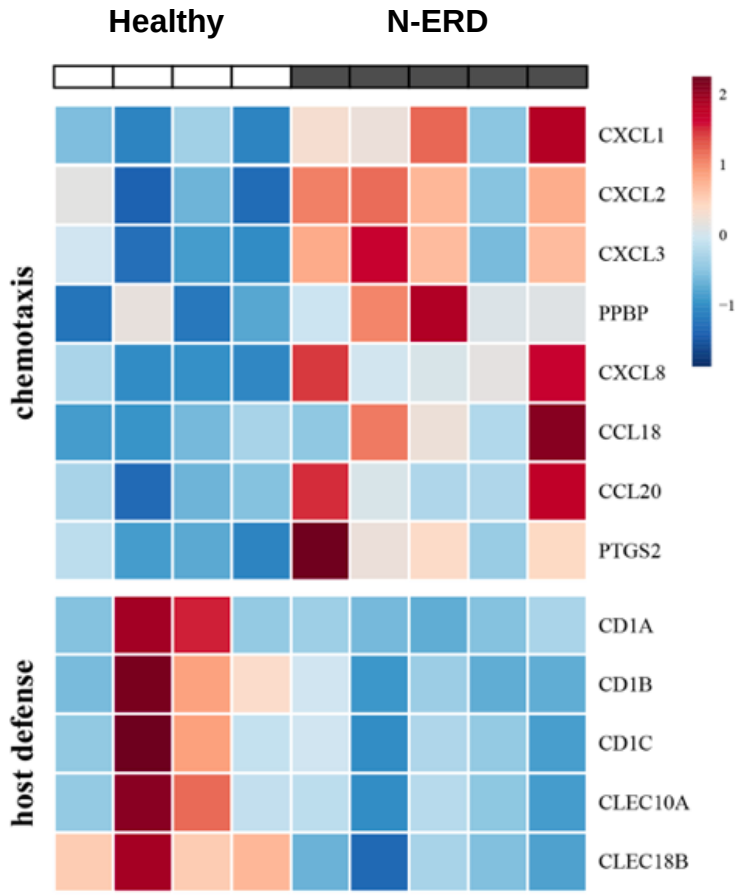

Figure 12 Aberrant aMDM activation in N-ERD patients

Transcriptome analysis of aMDM from N-ERD (*n* = 5) and healthy (*n* = 4) individuals. Heatmap of selected DEGs (z-score) between patients.

Figure made by Dr. Pascal Haimerl

Table 10 Selected pathways dysregulated in N-ERD aMDM

Pathway	*q* value Bonferroni	DEGs in pathway	Total genes in pathway	Pathway DEGs in N-ERD aMDM
Chemokine receptors bind chemokines	**< 0.001**	6	48	↑: PPBP, CXCL1, CXCL2, CXCL3, CCL20, CXCL8
IL-17 signaling pathway	**< 0.001**	7	93	↑: CXCL1, CXCL2, CXCL3, PTGS2, CCL20, CXCL8
				↓: MMP9
Genes encoding secreted soluble factors	**< 0.001**	10	344	↑: PPBP, CXCL1, CXCL2, CXCL3, CCL18, CCL20, CXCL8
				↓: GDF15, LTB, S100B
Interleukin-10 signaling	**< 0.001**	5	49	↑: CXCL1, CXCL2, PTGS2, CCL20, CXCL8
Ensemble of genes encoding extracellular matrix and extracellular matrix-associated proteins	**0.002**	16	1028	↑: PPBP, CXCL1, CXCL2, CXCL3, CCL18, CCL20, CXCL8
				↓: CLEC18B, GDF15, LAMA5, S100B, MMP9, CLEC10A, LTB, AGRN, COL1A1
TNF signaling pathway	**0.003**	6	108	↑: CXCL1, CXCL2, CXCL3, PTGS2, CCL20
				↓: MMP9
Interleukin-4 and 13 signaling	**0.004**	6	114	↑: PTGS2, CXCL8
				↓: CEBPD, POMC, LAMA5, MMP9
Chemokine signaling pathway	**0.006**	7	182	↑: PPBP, CXCL1, CXCL2, CXCL3, CCL18, CCL20, CXCL8

Selected ToppGene pathway analysis results of DEGs between N-ERD and healthy aMDM *a*
revealed by RNA-seq. Significant threshold by Bonferroni: $q < 0.05$
Table made by Dr. Pascal Haimerl

The initial plan to compare sMac of N-ERD probands to healthy controls could not be realized due to the insufficient extraction of sMac from healthy subjects. We decided to compare aMDM to sMac of N-ERD patients instead.

The comparison of sMac to aMDM via RNA-seq resulted in a total of 984 downregulated and 1869 upregulated genes. DEGs were filtered for genes connected to lipid and eicosanoid metabolism and immune response.

SMac presented an upregulation of genes involved in immune regulation, chemotaxis and T-cell priming. In respect to the eicosanoid metabolism sMac showed a higher expression of genes related to immune response regulation. Genes of the prostaglandin metabolism (PTGER2, PTGER3, PTGES, PTGS2, HPGDS), 15-LO (ALOX15) and regulatory leukotriene signaling (CYSLTR2) were upregulated, whereas aMDM demonstrated an upregulation of genes involved in the metabolism and signaling of 5 LO metabolites (ALOX5AP, CYSLTR1, LTA4H).

Differences were also found in the lipid metabolism regulation. sMac showed a simultaneous upregulation of pro-inflammatory (FFAR2[217], FFAR3[218], ACC1[219,220], CPT1B[84,85,221]), anti-inflammatory (SMPD3[222]) and pro/anti-inflammotry genes (SGPP2[223], CERS4[224,225,226]). The same could be observed with different genes in aMDM with ACACA[227,220,219] with a pro-inflammatory effect and ADIPOR2 and LRP8 with anti-inflammatory effect[228,229,230].

The heat map gives a detailed overview of the up- and downregulated genes between aMDM and sMac (Figure 13). It can be concluded that sMac have a more activated phenotype in respect to the immune responses compared to aMDM.

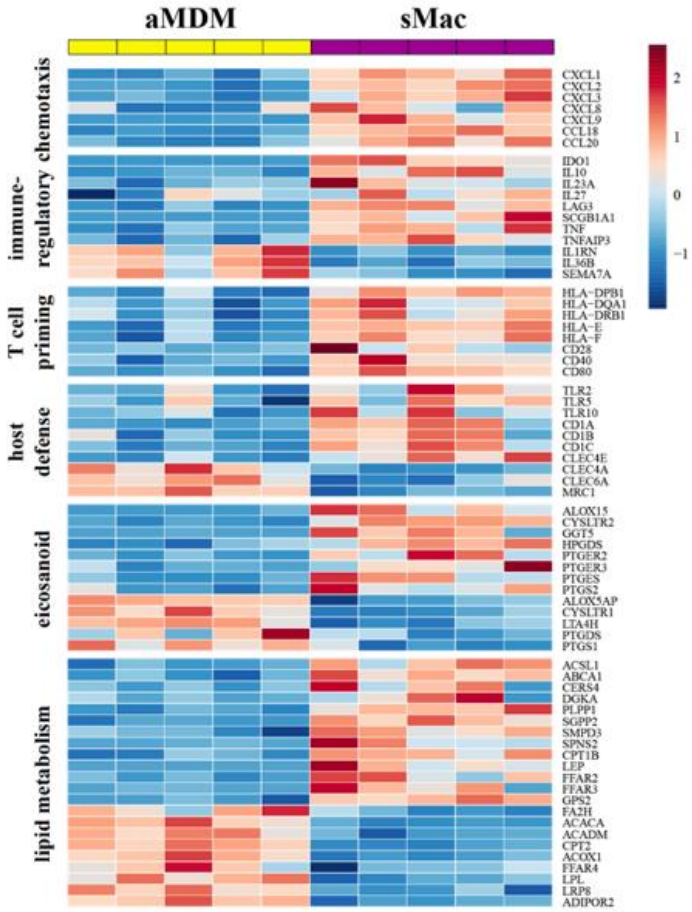

Figure 13 Transcriptome analysis reveals significant differences between N-ERD sMac and aMDM

Transcriptome analysis of sMac *vs.* aMDM from N-ERD (n = 5) patients. Heatmap of selected DEGs (z-score) between cell types.

Figure made by Dr. Pascal Haimerl

4.5 Metabolomic analysis shows higher acylcarnitine levels in NLF and sputum of N-ERD patients compared to control groups

Due to the changes observed in the lipid metabolism of N-ERD macrophages, we further investigated metabolite profiles in N-ERD, CRSwNP and healthy individuals. A targeted metabolomics analysis of 188 metabolites was realized with NLF from cohort I and sputum from cohort II. The measurement included

acylcarnitines, amino acids, biogenic amines, phosphatidylcholines, lysoPCs, SMs, and hexoses.

Hierarchical metabolite clustering led to the identification of four metabolite clusters in sputum. However, there were no significant differences between the three groups, except for the occurrence of different acylcarnitine levels. N-ERD contained higher acylcarnitine concentrations in comparison to the other two groups. Especially in cluster I N-ERD patients clearly presented the highest acylcarnitine levels of all groups. (Figure 14, Table 15).

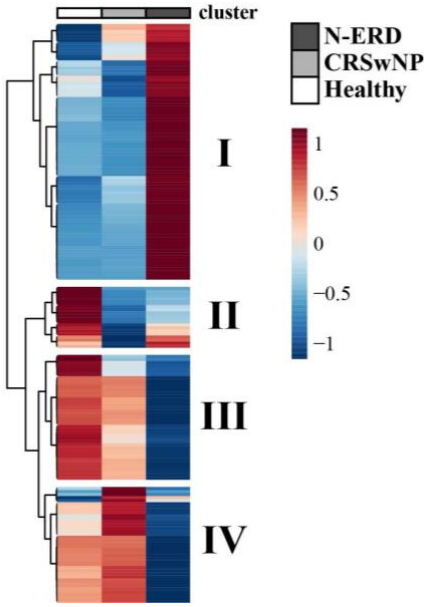

Figure 14 Acylcarnitine levels are elevated in NLF of N-ERD patients

Targeted metabolomics of NLF from N-ERD (n = 11), NT CRSwNP (n = 8) and healthy (n = 8) individuals. Heatmap of metabolites assigned to clusters. Rows represent metabolites, columns show normalized, auto-scaled mean group concentrations. Concentration differences between patient groups are shown as z-score in blue (low) and red (high). See Table 15 for a detailed list of all clusters.

Figure made by Dr. Pascal Haimerl

A similar conclusion was reached when measuring metabolites in sputum of five N-ERD subjects and three healthy controls. Out of the tree metabolite clusters, cluster two included all acylcarnitine metabolites and showed a lower quantity in healthy probands compared to N-ERD patients (Figure 15, Table 15)

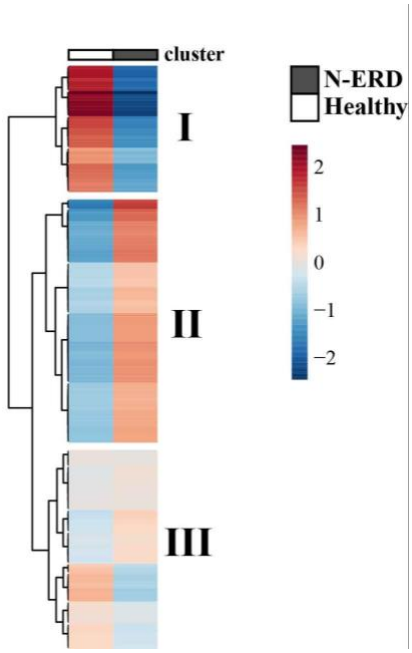

Figure 15 Sputum acylcarnitines are elevated in N-ERD subjects

Targeted metabolomics of sputum from N-ERD (*n* = 5) and healthy (*n* = 3) individuals. Heatmap of metabolites assigned to clusters. Rows represent metabolites, columns show normalized, auto-scaled mean group concentrations. Concentration differences between patient groups are shown as z-score in blue (low) and red (high). See Table 15 for a detailed list of all clusters.

Figure made by Dr. Pascal Haimerl

5 Discussion

N-ERD is a disease characterized by the inflammation of the respiratory tract. Various studies determined a higher presence of leukocytes, especially eosinophils and neutrophils, in the mucosa of N-ERD subjects[162,165,53]. These findings are associated with elevated levels from the 5-LO mediated oxidation products of arachidonic acid e.g. cysLTs and LTB_4[169] . Leukotrienes are potent mediators of inflammation.[231] PGE_2 presents the counterpart of leukotrienes as an anti-inflammatory metabolite in the airways[232]. It is presumed that this anti- and pro-inflammatory balance is dysregulated in N-ERD, at least in part due to an PGE_2-resistance that has been found in polyp fibroblasts[233] and in granulocytes[169] of N-ERD subjects.

We wanted to translate these discoveries onto a new cellular model by using macrophages which are abundant in the airways and potent producers of eicosanoids (LTs and PGE_2)[234]. We stimulated aMDM from 10 healthy, 10 CRSwNP and 15 N-ERD probands with IL-4 or PGE_2 to investigate a potentially different responsiveness to these mediators as observed for other myeloid cell populations by Laidlaw et al.

Results of the RNA-seq with comparison between aMDM from healthy controls and N-ERD did suggest a pro-inflammatory pre-activation of macrophages in N-ERD probands. Multiple lipid metabolism genes associated with pro- and anti-inflammatory functions were significantly altered between sMac and aMDM. Metabolomics analysis from cohort II revealed an association of acylcarnitines with N-ERD.

This study unveils mechanisms that might contribute to a better understanding of N-ERD. Every new finding can open up new treatment possibilities and can help affected individuals.

5.1 PGE_2-resistance could not be shown in aMDM of N-ERD subjects

We could not detect any PGE_2-resistance in aMDM from N-ERD patients compared to CRSwNP and healthy controls as it has been previously postulated by Laidlaw et al. in granulocytes[169] (see **Error! Reference source not found.**). We were also not able to detect any differences between the groups after IL-4 stimulation, a key cytokine in macrophage polarization and type 2 inflammation (see **Error! Reference source not found.**). Thus, the IL-4/PGE_2 stimulation experiment did not show the expected results, as no significant alterations in lipid mediator levels were measured particularly in response to PGE_2 among the groups of N-ERD, CRSwNP and healthy controls. In another experiment, associated to our study, we stimulated PMN with PGE_2 but could not reproduce Laidlaw's results either. Why granulocytes did not show the expected results remains unclear.

Similarities between macrophages and granulocytes making them comparable are for example that both were found to exist in reduced percentages expressing

EP_2 in N-ERD subjects[235]. Moreover, they are important participants in type 2 inflammation and crucial producers of eicosanoids. However, these two cell types represent different myeloid populations and may also respond differently to PGE_2. That could explain why we did not find PGE_2 resistance in macrophages.

Another factor that could have led to a different outcome is our collective of probands. All subjects were recruited in the ambulance of the ENT department at Klinikum Rechts der Isar in Munich. The recruited subjects were of Caucasian origin whereas Laidlaw et al. carried out their study in Boston, US. Divergent results could be caused by the assumingly different ethnical background from patients recruited in North America in addition to a different lifestyle and treatment regimens.

The cohorts compared were also chosen differently because Laidlaw et al. decided on aspirin-tolerant asthmatics as a third cohort whereas we examined aspirin-tolerant CRSwNP patients, which could at least explain different results between these groups.

A disadvantage of our study could be that the determination of N-ERD patients was realized through their medical history standardized in our questionnaire. We justify this decision of a more inaccurate but sufficient categorization because of the dangers the gold standard of an oral provocation implies. Indeed, oral provocation is the most sensitive method to diagnose N-ERD[144] but involves a higher risk of severe reactions for example nasal discharge and blockage, dyspnea or even anaphylactoid shock with hypotension and loss of consciousness[149]. Our research team did not want to put the probands health at unnecessary risk and this approach would have made it very difficult to receive the study's approval from the ethics commission. Moreover there existed a clear history of respiratory reaction after NSAID intake in all N-ERD patients of our study which made further testing redundant following the diagnosis algorithm of Kowalski et al[146].

The number of probands also varied between the two studies. For this project part, we used 10 healthy, 10 CRSwNP and 15 N-ERD patients compared to lower numbers in the study of Laidlaw. Reduced cysLT-production was shown with 7 healthy, 6 ATA and 10 N-ERD patients, the decline of LTB_4 was measured with

only 5 healthy, 3 ATA and 8 N-ERD probands[169]. It can be questioned if the results from cohorts of such a small size lead to significant results[236]. On the other hand it is very difficult to recruit N-ERD subjects due to the unawareness of patients[149], the unpopularity of the disease and the difficulty of diagnosing N-ERD. Most of the studies associated with PGE_2 and lipid metabolism in N-ERD have less than 20 subjects per cohort[169,233, 237].

We decided to use an intermediate concentration of 100 nM PGE_2 for our aMDM stimulation experiments. The best method to determine the reasonable quantity of PGE_2 would have been titration. But due to the low amount of sample material we were not able to use this method. As we could not measure lipid mediator reduction, it is possible that the chosen concentration was too low to provoke cellular reactions. Other studies used a concentration of 1nM[215] to 10µM[238] PGE_2 to stimulate macrophages *in vitro*. Laidlaw et al. used 0.01µM, 0.1µM or 1µM of PGE_2 for granulocyte stimulation and detected a PGE_2 resistance in N-ERD patients. Their leukotriene measurements (LTB_4 and cysLT) in N-ERD subjects showed simultaneously that the leukotriene production does not descent proportional to increasing PGE_2 concentrations[169]. In another experiment realized in our laboratory, aMDM of three healthy and three N-ERD patients were stimulated with 1µM PGE2. Lipid mediator levels showed different tendencies compared to our experiment. Higher PGE2 concentrations may lead to different results but it is questionable to what extend one can defend these concentrations if they do not mirror *in vivo* conditions. Higher PGE_2 concentrations can also cover small but significant metabolite differences. Moreover, N-ERD patients are known to display lower levels of PGE_2 in nasal and sinonasal tissue as well as in peripheral blood cells compared to healthy controls[167,95].

5.2 Mediator profiles are altered in aMDM of N-ERD subjects

Besides a dysregulated PGE_2 metabolism, it has been shown by multiple studies that pro-inflammatory 5-LO metabolites are elevated in N-ERD subjects. Up-regulation of 5-LO and LTC_4 synthase have been observed in nasal polyps, sinuses, lungs and infiltrating leukocytes[45,167,160,159]. In our study, we also

observed elevated baseline levels of 5-LO metabolites (18-HEPE, 5-HEPE, 5-HETE, 5-oxoETE, LTB_4, LTC_4, LTD_4 and LTE_4) in N-ERD patients compared to CRSwNP and healthy controls (see **Error! Reference source not found.**).
Furthermore, we detected higher values of COX metabolites (TXB_2, PGD_2).
In a different study Laidlaw et al. also found baseline (urinary) TXB_2 to be elevated in N-ERD patients compared to healthy controls. They additionally determined a high frequency of platelet-adherent leukocytes in N-ERD probands. Putting their observations into relation, they suggested that adherent platelets are involved in the production of these pro-inflammatory mediators through transcellular exchange[239]. In contrast to Laidlaw et al., we did not find more leukocyte-adherent platelets in N-ERD patients, as we realized FACS (Fluorescens Activated Cell Sorting) with PMN samples from the same patients for a different project. In comparison to Laidlaw et al. we did not use heparin but EDTA anti-coagulation agent for the blood samples. In order to find out if this fact and/or our PMN isolation were the cause for the loss of adhering platelets, we examined whole blood with EDTA or heparin of volunteers at ZAUM and study subjects. Again, we did not find any differences between the patient groups. One aspect we have to take into consideration as a possible influencing factor, is the preparation time of the samples. Laidlaw et al. were able to process the blood samples within one hour whereas we needed two hours for further proceeding. Possibly due to this time lag we were not able to detect more leukocyte-adherent platelets and TXB_2 elevation remains unclear.
Besides TXB_2, Lipoxin A_4 (LXA_4) concentrations were increased in N-ERD subjects. Lipoxins are bioactive metabolites mainly produced by three LOs: 5-LO, 15-LO and 12-LO. In response to e.g. inflammation, immune cells like macrophages secrete lipoxins which accomplish anti-inflammatory function[240]. Elevated LXA_4 and COX-metabolite level have previously been detected in N-ERD patients[241].
In previous studies, lipid metabolites have often been measured after NSAID-intake of N-ERD patients. This set-up could explain why our results did not show lipid metabolite elevation as drastic as in other studies. Indeed, N-ERD is a disease that constantly causes symptoms and not only after NSAID medication,

but it is also known that the extent of complaints and thus pro-inflammatory metabolites, increases tremendously in response to NSAIDs[45,159,157].

Chemokine/cytokine measurements did not show significant differences between the three cohorts. However, we could detect the tendency of diminished IL-12 levels. IL-12 is a cytokine involved in the differentiation of naïve T-cells to T_H1 cells[242]. On the contrary TNF values tended to be elevated. TNF plays an important role as chemoattractant for PMNs and peripheral blood monocytes and as an important negative regulator of M2 polarization[243,244,245]. IL1ß concentration had the tendency to be elevated as well and also represents an important pro-inflammatory factor acting synergistically with TNF[246,247] (**Error! Reference source not found.**).

These findings lead us to the conclusion that chemokine/cytokine and lipid mediator profiles hint to a rather pro-inflammatory phenotype of aMDM in N-ERD compared to controls. Thus, it would be interesting for further studies to perform metabolome, epigenome and transcriptome analyses of macrophages to gain a detailed insight into differences and possible pathways that lead to the pro-inflammatory phenotype of N-ERD macrophages.

5.3 Whole transcriptome analysis reveals differences between aMDM of N-ERD subjects and healthy controls

ToppGene analysis of aMDM showed that genes regulated by the IL-4/IL-13 signaling pathway differ between healthy probands and N-ERD patients. N-ERD subjects presented an upregulation of C/EBP δ, POMC, LAMA5 and MMP9 and a downregulation of PTGS2 and CXCL8 (**Error! Reference source not found.**, Table 10).

IL-4/IL-13 play a central role in type 2 inflammation. The type 2 inflammatory response is characterized by a distinct cell and cytokine milieu[248] which provides host resistance against parasitic helminth infection but can also occur as cause of chronic inflammatory diseases e.g. in response to innocuous allergens in allergic inflammation. These type 2 inflammatory conditions include diseases such as allergic rhinitis[249], CRSwNP[123] or asthma[250] but also N-ERD. N-ERD

holds a special position because it seems to be driven by type 2 inflammatory influence [251,252,253,170] while there exist simultaneously hints for a role of type 1 inflammation [254,255]. Due to previous findings, the hypothesis of a mixed T_H1/ T_H2 milieu in N-ERD has been formulated in an attempt to fit multiple research results into one disease concept [256].

The type 2 inflammation component in N-ERD subjects has previously gained importance, because pharmaceuticals known for their effective treatment of asthma have been successfully used to reduce nasal symptoms in N-ERD patients[257,258,259]. Findings in asthma as an important type 2 inflammatory disease effecting the respiratory tract could possibly be transmitted to N-ERD and vice versa. To date, it is mostly ignored that the nose as well as the nasal sinuses form an immunologically important part of the airways. “The airways” should not be seen as a fragment starting below the glottis and being totally independent of neighboring structures. Taking this into consideration, models have been postulated that treat “the airways” as an anatomical and -even more important- pathophysiological unity starting from the nostrils downwards to the alveoli[260,261]. An argument in favor of this theory, besides the successfully treatment of asthma and N-ERD, is the fact that asthma and nasal pathologies like rhinitis[262] and sinusitis[263] occur frequently in the same patient.

Macrophages are abundant in both the upper and lower airways[264,265]. Nevertheless, we lack N-ERD studies examining the role of macrophages and especially the role of alternative-activated macrophages in N-ERD. Therefore, our results regarding an altered macrophage activation in N-ERD may be potentially relevant in the context of increased M2 polarization in the airways of asthmatic patients [266,267].

C/EBP δ, one of the genes we found to be most downregulated in N-ERD subjects, represents a gene that functions as a transcription factor regulating metabolism[268], immune response[269] and cell differentiation[270]. C/EBP δ especially plays a role in macrophage polarization, phagocytosis and lipid accumulation even though its detailed effects on M2 macrophage activation remain unclear[216,271,272].

We found CXCL8, the gene encoding IL-8, to be upregulated in aMDM of N-ERD subjects compared to healthy controls. CXCL8 (IL-8) is mainly known as neutrophil and mast cell chemoattractant and accelerates inflammation[273,274]. It has also been suggested to attract eosinophils, thus causing eosinophil accumulation in the airways of asthmatic patients.[275] Various studies established a connection between IL-8 and airway disease. Subjects suffering from severe asthma present IL-8 upregulation in airway smooth muscle cells and sputum[276,277]. Pezato et al. found higher median levels of IL-8 in N-ERD patients. In the course of their study they drew the conclusion that AERD patients have a different baseline inflammatory pattern than CRSwNP[278].

We also found PTGS2 to be upregulated, the gene that encodes for prostaglandin synthase 2, a synonym for COX-2. This is unexpected given that COX-2 is generally downregulated in N-ERD tissues [167,154,279]. However, a cell type-specific upregulation of COX-2 may still be present in macrophages and potentially contribute to the synthesis of PGD2 and TXB2, which are pro-inflammatory COX metabolites involved in N-ERD pathogenesis[280,281,282]. PTGS2 upregulation in combination with C/EBP δ downregulation hints to a pro-inflammatory pre-activated state characteristically found in airway macrophages as compared to MDM in healthy individuals[283].

Our results also suggested an upregulation of chemotactic genes (CXCL1-3, PPBP, CXCL8, CCL18, CCL20) and a downregulation of the host defense genes (CD1A-C, CLEC10A, CLEC18B).

Considering all results, we propose that aMDM of N-ERD patients display a pro-inflammatory and pre-activated phenotype compared to aMDM of healthy subjects. Despite the influence of GM-CSF and TGFβ1 for 7 days on *in vitro* aMDM, they presented the aforementioned genetic alterations. This indicates that the altered immunological profile of the aMDM was already set in an earlier stage of the blood cells like monocytes or bone marrow precursors.

5.4 N-ERD sMac present a M2 macrophage phenotype with stronger pro-inflammatory characteristics than aMDM of N-ERD patients

In our second cohort, we performed RNA-seq to compare sMac to aMDM of N-ERD patients. Immunoregulatory enzymes (e.g., PTGS2, PTGES, IDO1), cytokines (e.g. IL1β, IL6, IL10, IL18BP, IL23A, IL27, TNF, TGFβ2) and pro-inflammatory chemokines (e.g. CCL and CXCL family) were increased in sMac confirming the results of previous studies[283,284]. Moreover, sMac expressed an altered profile functionally linked to TNF signaling pathways as well as the chemokines IL17, IL10, IL4, and IL13.

Furthermore, sMac presented an upregulation of genes involved in prostaglandin metabolism (PTGER2, PTGER3, PTGES, PTGS2, HPGDS), LO-15 and leukotriene metabolism (**Error! Reference source not found.**).

PTGS2 and HPGDS upregulation may result in an enhanced production of PGD_2 which has been discovered to be elevated in patients with severe asthma[285]. In N-ERD patients with severe gastrointestinal and cutaneous symptoms during Aspirin desensitization PGD_2 was found to be more elevated than N-ERD patients who could undergo aspirin treatment[286]. Moreover, ILC2s [287] have been found to participate in type 2 immune reactions and also occur in N-ERD subjects, whereby they increase in nasal mucosal samples during the time of COX-1 inhibitor reactions[288]. The important role of leukotrienes in the pathomechanism of N-ERD has been mentioned before and we found CYSLTR2 to be upregulated in sMac of N-ERD subjects. CYSLTR2 encodes for the cysteinyl receptor 2 that binds LTC_4, LTD_4 and LTE_4 and mediates pro-inflammatory reactions like IL-33 production by lung epithelial cells[289] as well as platelet aggregation[290]

We also found ALOX15, the gene encoding the enzyme 15-LOX, to be upregulated in sMac compared to aMDM. 15-LOX converts AA into 12-hydroperoxyeicosatetraenoic acid (12-HPETE) and 15-HPETE. 15-LOX is expressed in healthy airway epithelium but was demonstrated to show higher activity and amounts in asthmatic patients[291,292]. It also induces the release and expression of chemokines in cultured human lung epithelial cells[293]. Alterations have not exclusively been observed in pulmonary disease but also in N-ERD subjects. Song et al. found a higher tendency of 15-HPETE in N-ERD patients compared to healthy controls[294]. Key type 2 cytokine IL-4 enhances 15-LOX expression in MDMs and DCs[295,296, 297]. Simultaneously, IL-4 inhibits 5-LOX and

COX-2 expression in those cells[298,299]. The fact that ALOX15 was upregulated in sMac of N-ERD would additionally support the concept of a type 2-dominated inflammation in this disease. In contrast to sMac, aMDM showed a higher expression of 5-LO pathway genes (ALOX5AP, LTA4H) and an upregulation of other activation markers (e.g. C/EBP δ, TGFβ2, IRF4, IL10, CCL17).

More pro-inflammatory genes were enhanced in sMac, including genes involved in chemotaxis (CXCL1, CXCL2, CXCL3, CXCL8, CXCL9, CXCL17, CCL18, CCL20) as well as immunoregulatory enzymes (e.g. IDO1, PTGS2 (COX-2), PTGES (mPGES-1) and T cell priming- associated genes (e.g. CD1a-c, CD28, CD40, CD80, HLA family).

Taken together, the sMac transcriptome in N-ERD is indicative of an ongoing type 2 airway inflammation that determines a M2 macrophage shifted phenotype in N-ERD patients. However, future studies should compare sMac transcriptome profiles of N-ERD and healthy individuals, possibly by novel single cell RNA-seq approaches to define pro-inflammatory sub-phenotypes with an involvement in N-ERD pathogenesis.

5.5 N-ERD macrophages display an altered expression of genes involved in lipid- and fatty acid- metabolism

Also, lipid metabolism differed between sMac and aMDM. The upregulation of free fatty acid receptor (FFAR2/3) and carnitine palmitoyltransferase 1B (CPT1B) in sMac hint to an increased demand for FFAs for FAO. The enzyme encoded by CPT1B gene is responsible for transporting long-chain fatty acyl-CoAs from cytoplasm into the mitochondria. Additionally, acyl-CoA long-chain family member 1 (ACSL1) was upregulated. ACSL1 metabolizes long-chain fatty acids to acyl-CoA esters and influences inflammatory functions in macrophages[300,84]. CPT2 encodes for carnitine palmitoyltransferase II which oxidizes long-chain fatty acids in the mitochondria. It is possible that reduced CPT2 expression in sMac can lead to an accumulation of acylcarnitines in the airways. As a consequence, pro-inflammatory mediators may be elevated in N-ERD sMac as acylcarnitines can induce TNF, IL1β, CXCL2, COX-2, and IL6[88].

These alterations in lipid metabolism could be explained by the fact that myeloid cells of chronic inflammatory disease models showed in multiple experiments an increased expression of FAO enzymes[301,86,85]. Al Khami et al. discovered the same tendencies in a murine asthma model especially in macrophages of the bronchoalveolar tissue[87]. These macrophages presented a M2 phenotype as macrophage polarization is regulated at transcriptional and metabolic levels[302,80]. M1 and M2 macrophages rely on different energy sources. While M1 macrophages use aerobic glycolysis, M2 macrophages oxidize fatty acids. Our findings of upregulated enzymes linked to fatty acid metabolism in sMac, further support an M2 metabolic phenotype shift in sMac of N-ERD patients.

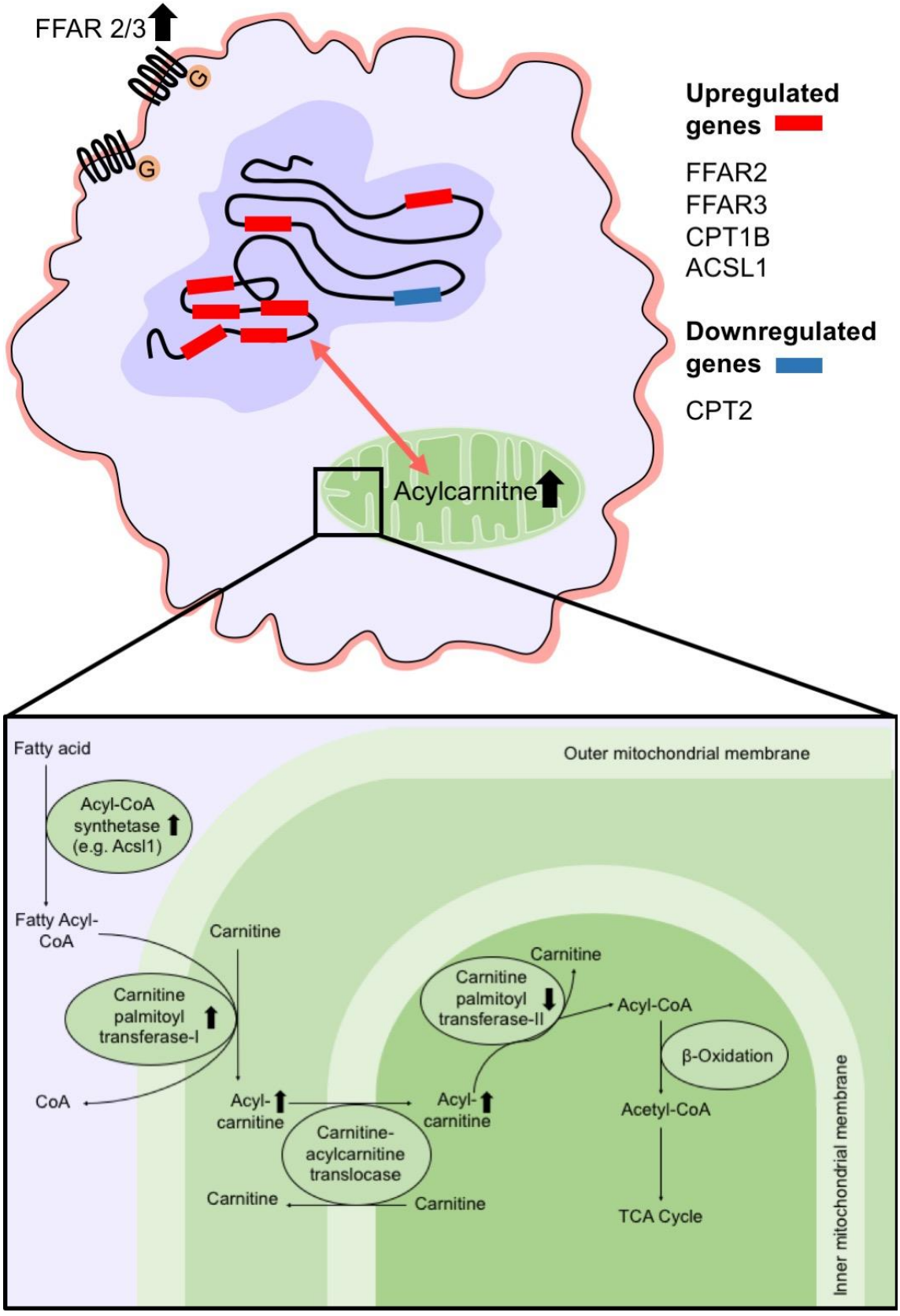

Figure 16 Genetical and metabolic changes in sputum macrophages of N-ERD patients

Genetical up-and downregulation in sputum macrophages of N-ERD patients involve genes of fatty acid metabolism. These alterations lead to acylcarnitine accumulation and enhance β-oxidation. Elevated acylcarnitine levels in turn can induce the upregulation of pro-inflammatory genes like TNF, IL1β, CXCL2, COX-2, and IL6 (symbolized by the pink arrow). Taken together the characteristics found in the sputum macrophages suggest an alternatively activated macrophage type.

5.6 Acylcarnitines are elevated in sputum and NLF of N-ERD patients

After our findings of a pro-inflammatory phenotype of aMDM and sMac of N-ERD patients we hypothesized that this could be associated with changes in lipid / fatty acid metabolism. As we found genes connected to FAO as well as pro-inflammatory genes to be upregulated, acylcarnitines were considered as potential metabolites mediating these alterations.

Acylcarnitines and chronic inflammatory conditions were found to be linked in prior research[303,304]. NLF and sputum served in previous studies as sample material to detect metabolic differences between healthy controls and subjects with upper respiratory disease[305,306,307]. Differences were found e.g. in NLF of CRS patients possibly enabling the differentiation of CRS in contrary to N-ERD and CRSwNP.[306] But no literature has been published so far reporting on acylcarnitine metabolism aberrations in N-ERD. Hence, we performed for further investigation targeted metabolomics analyses with sputum and NLF of N-ERD patients.

N-ERD subjects indeed presented higher levels of long -chain acylcarnitines compared to CRSwNP and healthy controls (**Error! Reference source not found.**, **Error! Reference source not found.**). Acylcarnitines are intermediate metabolites of fatty acids produced to pass the mitochondrial membranes, which acyl-CoA otherwise could not overcome. Carnitine is conjugated with acyl-coenzyme A to form acylcarnitine, is translocated into the mitochondrion and then converted back via carnitine palmitoyltransferase 2 (CPT2) into carnitine and Acyl-CoA, which is used for beta oxidation in order to produce ATP.

The altered lipid/ FA metabolism in N-ERD may be due to metabolic reprogramming of immune cells located in a chronic inflammatory environment.

There is ample evidence that the differentiation, function and longevity of immune cells depend on the use of different metabolic pathways to produce energy[87]. Elevated levels of acylcarnitines have been observed in chronic inflammatory process like obesity[308], insulin resistance[309], non-alcoholic steatohepatitis[310] or in a murine asthma model[87] and are associated with increased FAO and type 2 inflammation. But cells are not only influenced by inflammation but may also contribute to it by upregulating pro-inflammatory genes in response to metabolic cues. Acylcarnitines reportedly act through activation of Toll-like receptor and nuclear factor κB to induce pro-inflammatory effector molecules[88]. Indeed, acylcarnitines were able to induce TNF, IL6, IL1β, PTGS2 and CXCL2 expression in macrophages[88].

Thus, increased acylcarnitines in sputum and NLF could be connected to the pro-inflammatory phenotype of sMac and aMDM of N-ERD subjects. It is possible that acylcarnitines play an essential part in the pathophysiology of N-ERD and should be addressed in further studies. Therefore, it would be interesting to have a closer look on how acylcarnitines influence immune cells involved in the inflammatory process of N-ERD.

Conclusion:

- aMDM of N-ERD probands present a pro-inflammatory imprinting compared to healthy controls
- Upregulation of pro-inflammatory and lipid metabolism genes hint to a type 2 airway inflammation in N-ERD patients yielding macrophages with a type 2 inflammatory pattern
- N-ERD patients exhibit higher acylcarnitine level in NLF and sputum compared to control groups (possibly due to aberrant FAO in macrophages).

6 Summary

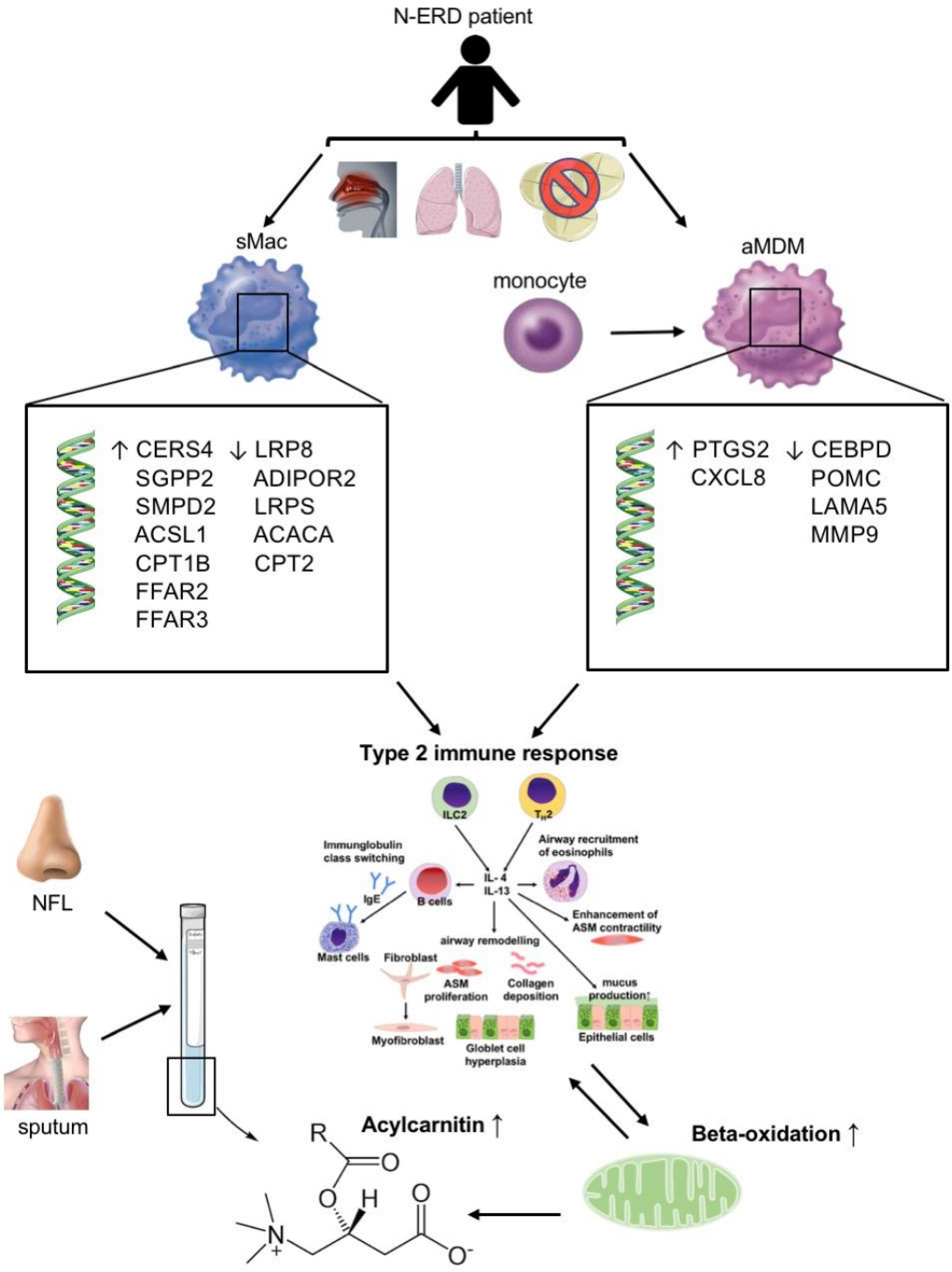

Figure 17 Summary

We gained sputum macrophages (sMac) and monocytes from N-ERD patients. Monocytes were differentiated into alveolar-like monocyte-derived macrophages (aMDM). Genetical alterations were revealed in both macrophage types via RNA-seq. The **left side** represents our findings of altered genes in sMac, regulating lipid metabolism. In sMac these genetical changes lead to a

shift in energy generation towards β-oxidation probably being influenced and influencing ongoing type 2 inflammation. Consequently, acylcarnitines as metabolite of fatty acid oxidation and mediator of inflammatory processes were measured in higher concentrations in sputum and NFL of N-ERD probands. On the **right side** genetic alterations of aMDM in N-ERD subjects are presented. In particular immune regulating genes as for example of the IL-4/IL-13 pathway differed between aMDM of N-ERD and healthy probands. The findings lead to the assumption of a pro-inflammatory phenotype of sMac and aMDM.

6.1 English summary

Non-steroidal anti-inflammatory drug (NSAID)- exacerbated respiratory disease is a severe inflammatory condition affecting particularly the airways and nasal sinuses as the typical symptom triad consist of nasal polyps, asthma and NSAID intolerance. Over 300 million people worldwide suffer from chronic inflammatory airway diseases forming a massive health issue as well as a non-negligible financial burden for society and the affected individuals themselves. So far N-ERD is not curable and its treatment consist of the attempt to alleviate the symptoms. Symptom control is one of the main challenges as treatment regimens are inconsistent and patients often present complex and recurrent complaints. This unsatisfactory condition stems from the incomplete understanding of the pathomechanisms and emphasis the importance of N-ERD research.

It was established before that N-ERD subjects present inter alia alterations in arachidonic acid metabolism and therefore in immune regulation. Macrophages as one of the key players in immune regulation yet have been neglected in N-ERD research. Through our experiments we aimed to reveal possible differences in aMDM and sMac as well as alterations in body fluids (NFL, sputum) of N-ERD, CRSwNP and healthy subjects. We used LC-MS/MS lipid mediator, chemokine/cytokine multiplex and RNA-seq analysis to draw a multidimensional macrophage profile.

We did not detect different effects of anti-inflammatory PGE_2 or IL-4 stimulation of N-ERD aMDM. However, we discovered a (pre-)activated pro-inflammatory phenotype of aMDM and sMac. sMac demonstrated a shift towards beta oxidation possibly being linked to the upregulation of inflammatory type 2 immune

response. Higher concentrations of acylcarnitines, as one of the key metabolites in fatty acid oxidation and participant in inflammatory reactions, were measured via targeted multi-fluid metabolomics (sputum, nasal lining fluid) in N-ERD probands.
Our study demonstrated alterations in activation and lipid metabolism of N-ERD macrophages and revealed potential pathomechanism in N-ERD. These discoveries can be taken as an opportunity for new therapeutic approaches as well as diagnostic methods. As foundation for further studies they offer a new approach in order to improve diagnosis, treatment and understanding of the disease via recovery of homeostatic lipid metabolism and physiological macrophage function.

6.2 Deutsche Zusammenfassung

N-ERD (Non-steroidal anti-inflammatory drug (NSAID)- exacerbated respiratory disease) ist eine schwerwiegende Atemwegserkrankung, die drei charakteristische Symptome umfasst: nasalen Polypen, Asthma und NSAR Intoleranz. Über 300 Millionen Menschen weltweit leiden an chronisch entzündlichen Atemwegserkrankungen, die ein enormes Gesundheitsproblem und eine nicht zu vernachlässigende finanzielle Belastung für die Gesellschaft und die Betroffenen selbst darstellen. Bisher ist N-ERD nicht heilbar und die aktuelle Behandlung zielt lediglich auf Symptomlinderung ab. Dabei stellt die Symptomkontrolle die größte Herausforderung dar, denn Therapieoptionen sind häufig widersprüchlich und die Patienten stellen sich mit komplexen und wiederkehrenden Beschwerden vor. Der Grund für diesen unbefriedigenden Zustand ist, dass der Pathomechanismus von N-ERD immer noch nicht vollständig verstanden ist und unterstreicht wie wichtig seine Erforschung ist.
Es ist seit längerem bekannt, dass N-ERD Patienten unter anderem Veränderungen im Arachidonsäuremetabolismus und damit der Immunregulation aufweisen. Makrophagen, einer der Hauptakteure der Immunregulation, sind bisher jedoch in der N-ERD Forschung vernachlässigt worden. Unsere Experimente sollten daher mögliche Unterschiede zwischen

Knochenmarksmakrophagen und Blutmakrophagen sowie Abweichungen in Köperflüssigkeiten (Nasensekret, Sputum) zwischen N-ERD Patienten, Probanden mit CRSwNP (chronischer Rhinosinusitis mit Polypen) und Gesunden aufdecken. Um ein multidimensionales Makrophagenprofil zu kreieren, wurde LC-MS/MS lipid mediator-, chemokine/cytokine multiplex- und RNA-seq Analyse verwendet.

Unsere Analysen ergaben keine unterschiedliche Wirkung von anti-entzündlichem PGE_2 oder von IL-4 auf N-ERD Makrophagen. Allerdings zeigte sich, dass sowohl aMDM als auch sMac einen proinflammatorischen und (vor)aktivierten Phänotyp aufweisen. Zudem konnten wir einen gesteigerte β-Oxidation in sMac messen, was im Zusammenhang mit einer hochregulierten Typ 2 Immunantwort stehen könnte. Unsere Messungen via targeted multi fluid metabolomics in Sputum und Nasensekret von N-ERD Probanden, ergaben außerdem höhere Acylcarnitin Konzentrationen im Vergleich zu den anderen Probanden. Acylcarnitine sind Schlüsselmetabolite in der Fettsäureoxidation und spielen eine wichtige Rolle in Entzündungsreaktionen.

Zusammenfassend konnte unsere Studie Unterschiede in der Aktivierung und dem Lipidmetabolismus von N-ERD Makrophagen feststellen, und weißt damit einen möglichen Pathomechanismus der Erkrankung auf. Unsere Ergebnisse könnten somit die Grundlage zukünftiger Studien bilden, um neue Erkenntnisse zur Krankheitsentstehung, Diagnoseverfahren und Therapieansätze für N-ERD Patienten zu entwickeln. Dabei könnte ihr Fokus auf einer Normalisierung der Makrophagenfunktion, insbesondere einer Lipidstoffwechsel-Homöostase, liegen.

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8 Supplemental data

Table 11 Lipid mediator profile of PGE_2/IL4-stimulated aMDM

Lipid mediator	aMDM SN - lipid mediator concentrations (ng/mL)								
	Healthy			NT CRSwNP			N-ERD		
	Control	PGE_2	IL4	Control	PGE_2	IL4	Control	PGE_2	IL4
11-HDHA	0.027	0.041	0.043	0.020	0.033	0.027	0.042	0.033	0.045
11,12-DHET	0.040	0.050	0.044	0.069	0.089	0.088	0.101	0.059	0.069
13-HDHA	0.032	0.041	0.043	0.031	0.040	0.049	0.061	0.054	0.053
18-HEPE	0.014	0.035	0.017	0.016	0.025	0.020	0.025	0.031	0.030
5-HEPE	2.426	2.748	2.810	1.689	2.277	2.764	4.359	3.567	4.600
5-HETE	21.067	25.092	26.688	15.509	21.801	24.653	34.500	28.619	37.173
5-oxoETE	0.727	0.830	0.700	0.553	0.700	0.680	1.018	0.854	0.871
LTB_4	40.289	46.076	50.993	34.870	41.050	48.950	66.792	54.218	66.148
LTC_4	0.407	0.586	0.540	0.294	0.565	0.374	0.467	0.456	0.495
LTD_4	0.127	0.197	0.157	0.117	0.170	0.131	0.142	0.138	0.152
LTE_4	0.003	0.003	0.003	0.004	0.004	0.002	0.045	0.037	0.045
cysLTs	0.537	0.785	0.700	0.415	0.739	0.508	0.655	0.632	0.692
LXA_4	0.099	0.160	0.140	0.104	0.122	0.106	0.112	0.114	0.101
PGD_2	0.014	0.013	0.080	0.009	0.014	0.111	0.037	0.020	0.134
PGE_2	0.174	0.000	3.925	0.000	0.253	4.835	0.605	0.000	5.793
$PGF_{2\alpha}$	0.053	0.047	0.062	0.044	0.069	0.079	0.074	0.061	0.081
TXB_2	0.184	0.220	0.236	0.176	0.364	0.257	0.344	0.333	0.423

Mean lipid mediator concentrations of all successfully measured lipid mediators in the SN of aMDM from N-ERD (*n* = 15), NT CRSwNP (*n* = 10) and healthy (*n* = 10) individuals. Measurements were realized 24 hours after *in vitro*-stimulation with PGE_2 or IL4.

Table 12 Baseline aMDM lipid mediator profile

Lipid mediator	Baseline concentration (ng/mL)			*p* value		
	Healthy	CRSwNP	N-ERD	N *vs.* H	N *vs.* C	C *vs.* H
11-HDHA	0.027	0.020	0.042	> 0.999	0.291	0.8733
11,12-DHET	0.040	0.020	0.101	0.319	> 0.999	> 0.999
13-HDHA	0.032	0.069	0.061	0.278	0.136	> 0.999
18-HEPE	0.014	0.031	0.025	> 0.999	> 0.999	> 0.999
5-HEPE	2.426	0.016	4.359	0.100	**0.006**	> 0.999
5-HETE	21.067	1.689	34.500	0.163	**0.012**	> 0.999
5-oxoETE	0.727	15.509	1.018	0.360	**0.044**	> 0.999
LTB_4	40.289	0.553	66.792	0.101	**0.027**	> 0.999
LTC_4	0.407	34.870	0.467	0.589	0.093	> 0.999
LTD_4	0.127	0.294	0.142	0.589	0.200	> 0.999
LTE_4	0.003	0.117	0.045	0.269	0.682	> 0.999
cysLTs	0.537	0.004	0.655	0.504	0.138	> 0.999
LXA_4	0.099	0.415	0.112	> 0.999	> 0.999	> 0.999
PGD_2	0.014	0.104	0.037	0.390	0.168	> 0.999
PGE_2	0.174	0.009	0.605	> 0.999	> 0.999	> 0.999
$PGF_{2\alpha}$	0.053	0.000	0.074	> 0.762	0.250	> 0.999
TXB_2	0.184	0.044	0.344	0.147	0.058	> 0.999

Baseline lipid mediator concentrations in aMDM SN of N-ERD (n = 15), NT CRSwNP (n = 10) and healthy (n = 8) individuals. Data was analyzed with the Kruskal-Wallis test with Dunn's correction: $p < 0.05$. H, healthy; C, NT CRSwNP; N, N-ERD.

Table 13 Statistical analysis of the lipid mediator profile of PGE_2/IL4-stimulated aMDM

Lipid mediator	2-way ANOVA: intergroup stimulations (adjusted *p* value)									2-way ANOVA: intragroup stimulations (adjusted *p* value)					
	Control			PGE_2			IL4			Control *vs.* PGE_2			Control *vs.* IL4		
	N *vs.* H	N *vs.* C	C *vs.* H	N *vs.* H	N *vs.* C	C *vs.* H	N *vs.* H	N *vs.* C	C *vs.* H	Healthy	CRSwNP	N-ERD	Healthy	CRSwNP	N-ERD
11-HDHA	0.516	0.208	0.841	0.653	0.986	0.783	0.994	0.396	0.393	0.463	0.528	0.724	0.373	0.821	0.961
11,12-DHET	**0.044**	0.468	0.479	0.773	0.720	0.316	0.609	0,6	0.192	0.954	0.929	0.224	0.985	0.733	0.329
13-HDHA	0.204	0.184	0.999	0.942	0.486	0.304	0.899	>0.999	0.920	0.587	**0.048**	0.887	0.774	0.500	0.827
18-HEPE	0.678	0.780	0.987	0.996	0.730	0.777	0.612	0.774	0.967	0.374	0.844	0.799	0.949	0.918	0.881
5-HEPE	0.236	0.051	0.777	0.786	0.133	0.510	0.185	0.214	0.997	0.953	0.998	0.755	0.966	0.545	0.965
5-HETE	0.336	0.090	0.790	0.957	0.762	0.921	0.0960	0.063	0.981	0.884	0.740	0.775	0.984	0.929	0.932
5-oxoETE	0.757	0.424	0.870	> 0.999	0.911	0.925	0.453	0.288	0.948	0.9530	0.905	0.908	0.348	0.073	0.8910
LTB_4	0.366	0.213	0.948	0.950	0.812	0.955	0.222	0.188	0.995	0.941	0.933	0.762	0.979	0.998	0.999
LTC_4	0.998	0.945	0.969	0.927	0.944	0.999	0.295	0.311	0.999	0.924	0.834	> 0.999	0.300	0.203	0.998
LTD_4	>0.999	>0.999	>0.999	0.975	0.991	0.996	0.067	**0.032**	0.950	0.977	0.987	> 0.999	0.094	**0.046**	0.999
LTE_4	0.991	0.992	> 0.999	0.994	0.995	> 0.999	0.070	**0.029**	0.928	> 0.999	> 0.999	>0.999	0.082	**0.037**	> 0.999
cysLTs	0.990	0.925	0.974	0.922	0.954	0.996	0.500	0.542	0.997	0.896	0.829	0.999	0.435	0.340	0.997
LXA_4	>0.999	> 0.999	> 0.999	0.987	>0.999	0.993	**0.040**	**0.019**	0.954	0.981	0.998	0.999	**0.0710**	**0.039**	0.999
PGD_2	0.997	0.996	> 0.999	**>0.999**	>0.999	> 0.999	0.080	**0.026**	0.890	> 0.999	>0.999	0.998	0.052	**0.017**	0.938
PGE_2	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
$PGF_{2\alpha}$	0.998	0.996	> 0.999	0.999	>0,999	0.998	0.062	**0.021**	0.907	>0.999	0.997	>0.999	0.079	**0.029**	0.999
TXB_2	0.902	0.891	> 0.999	0.952	0.987	0.907	0.280	0.131	0.910	0.994	0.847	>0.999	0.101	**0.040**	0.963

Statistical analysis of all successfully measured lipid mediators in the SN of aMDM *in vitro*-stimulated with PGE_2 or IL4 for 24 hours. Healthy (n=10), CRSwNP (n=10), Significant threshold by 2-way ANOVA: $p < 0.05$. H, healthy; C, NT CRSwNP; N, N-ERD; N/A, not available (due to not detectable lipid mediators in some conditions).

Table 14 Statistical analysis of the chemokine/cytokine profile of PGE_2/IL4-stimulated aMDM

Analyte	2-way ANOVA: intergroup stimulations (adjusted *p* value)									2-way ANOVA: intragroup stimulations (adjusted *p* value)					
	Control			PGE_2			IL4			Control *vs.* PGE_2			Control *vs.* IL4		
	N *vs.* H	N *vs.* C	C *vs.* H	N *vs.* H	N *vs.* C	C *vs.* H	N *vs.* H	N *vs.* C	C *vs.* H	Healthy	CRSwNP	N-ERD	Healthy	CRSwNP	N-ERD
CXCL1	0.996	> 0.999	0.993	0.998	> 0.999	0.998	0.125	0.197	0.962	0.090	0.989	0.905	> 0.999	0.999	> 0.999
CXCL2	0.991	0.997	0.976	0.967	> 0.999	0.968	0.181	0.486	0.776	**0.035**	0.701	0.287	0.998	0.994	0.975
CXCL8	0.954	0.928	0.997	> 0.999	0.999	> 0.999	0.989	0.789	0.706	0.109	0.147	**0.017**	0.737	0.846	0.297
CXCL9	> 0.999	0.972	0.972	0.892	0.892	> 0.999	> 0.999	**0.035**	**0.035**	0.592	0.457	0.177	0.870	0.738	> 0.999
CXCL10	0.701	0.994	0.636	0.745	0.998	0.712	0.218	0.201	0.999	0.324	0.974	0.842	0.919	0.992	0.970
CXCL11	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
CCL5	0.934	0.718	0.903	0.364	0.739	0.796	0.702	0.953	0.866	0.460	0.793	0.972	0.854	0.979	0.127
CCL11	0.858	0.858	0.550	0.934	0.657	0.447	0.448	0.550	0.082	0.068	**0.043**	> 0.999	0.827	0.953	0.664
CCL17	> 0.999	> 0.999	> 0.999	0.285	0.760	0.101	0.930	0.869	0.986	0.504	0.574	0.659	0.984	0.079	0.207
TNF	0.632	0.945	0.819	0.208	0.313	0.962	0.996	0.991	0.999	0.420	0.123	0.059	0.664	0.903	0.177
IL-1b	0.884	0.996	0.921	0.999	0.799	0.776	0.602	0.892	0.864	**0.001**	0.415	0.766	0.502	**0.009**	0.441
IL-6	0.992	0.999	0.985	0.839	0.913	0.986	0.888	0.998	0.916	0.975	0.603	0.898	> 0.999	> 0.999	0.235
IL-12 p70	0.369	0.514	0.962	0.362	0.822	0.709	0.891	0.908	0.659	0.930	0.891	0.753	0.994	0.766	0.992
IL-18	0.835	0.792	0.996	0.788	0.913	0.965	0.588	0.563	0.999	0.198	0.134	0.987	> 0.999	0.459	0.947
IL-33	0.684	0.985	0.582	0.784	> 0.999	0.783	0.392	0.985	0.482	0.745	0.926	0.350	0.744	0.533	0.637
IL-10	0.759	0.987	0.665	0.849	0.840	0.518	0.214	0.445	0.867	0.256	0.727	0.969	0.987	0.851	0.811
IL-27	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A

Statistical analysis of 17 chemokines/cytokines in the SN of aMDM *in vitro*-stimulated with PGE_2 or IL4 for 24 hours. Significant threshold by 2-way ANOVA: $p < 0.05$. H, healthy; C, NT CRSwNP; N, N-ERD; N/A, not available (due to not detectable chemokines/cytokines in some conditions).

Table 15 Hierarchical clusters of analytes in targeted metabolomics

Analysis	Analyte group	Cluster analysis – number and % of analytes									
		I	%	II	%	III	%	IV	%	V	%
Sputum	Acylcarnitine	0	0	**38**	**95.0**	2	5.0				
	Sphingomyeline	5	33.3	1	6.7	9	**60.0**				
	Amino acid / biogenic amine	0	0.0	13	39.4	**20**	**60.6**				
	Phosphatidylcholine aa/ae	**35**	**38.9**	23	25.6	**32**	**35.6**				
	Sum of carbohydrates	0	0	**1**	**100**	0	0				
NLF	Acylcarnitine	**38**	**95.0**	2	5.0	0	0	0	0		
	Sphingomyeline	2	13.3	1	6.7	**11**	**73.3**	1	6.7		
	Amino acid / biogenic amine	**13**	**35.1**	**13**	**35.1**	5	13.5	6	16.2		
	Phosphatidylcholine aa/ae	**31**	**34.4**	4	4.4	25	27.8	**30**	**33.3**		
	Sum of carbohydrates	0	0	0	0	0	0	**1**	**100.0**		
Plasma	Acylcarnitine	6	15.0	4	10.0	9	22.5	6	15.0	**15**	**37.5**
	Sphingomyeline	0	0	0	0	1	6.7	2	13.3	**12**	**80.0**
	Amino acid / biogenic amine	9	28.1	**11**	**34.4**	8	25.0	1	3.1	3	9.4
	Phosphatidylcholine aa/ae	**24**	**26.7**	12	13.3	15	16.7	14	15.6	**25**	**27.8**
	Sphingolipid metabolomics (II)	0	0	2	8.3	**10**	**41.7**	6	25.0	6	25.0
	Sum of carbohydrates	**1**	**100.0**	0	0	0	0	0	0	0	0

Targeted metabolomics hierarchical clustering analysis of sputum, NLF and plasma in healthy (*n* = 3-9), NT CRSwNP (*n* = 0-10) and N-ERD (*n* = 5-13) individuals. Number and percentage of analytes per analyte group in each cluster are shown. Clusters with highest percentages are marked as bold for each analyte group.

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