



Faculty of Medicine and Health Sciences
Department of Respiratory Medicine

**Role of Transient Receptor Potential Channel A1, serotonin
receptor 5-HT₄R and soluble guanylyl cyclase in the pathogenesis
of Chronic Obstructive Pulmonary Disease**

Lisa Dupont

Promotor: Prof. Dr. Guy Brusselle

Co-promotor: Prof. Dr. Guy Joos

Thesis submitted to fulfil the requirements for the degree of 'Doctor in Medical Sciences'

2014

ABBREVIATIONS	4
SUMMARY	6
SAMENVATTING	8
PART I: INTRODUCTION	10
CHAPTER 1. CHRONIC OBSTRUCTIVE PULMONARY DISEASE (COPD).....	10
1.1 Introduction	10
1.2 Symptoms and diagnosis	11
1.3 Comorbidities.....	12
1.4 Therapy.....	13
1.5 Genetics.....	13
1.6 Pathogenesis and pathophysiology.....	15
CHAPTER 2. TRANSIENT RECEPTOR POTENTIAL (TRP) CHANNELS AND TRPA1	17
2.1 Transient Receptor Potential (TRP) channels.....	17
2.2 Transient Receptor Potential (TRP) channels in inflammation and disease	18
2.2.1 TRPA1 in inflammation and disease	19
2.3 TRP channels in airway diseases.....	20
2.3.1 TRPA1 in airway diseases.....	23
CHAPTER 3. SEROTONIN (5-HT) AND SEROTONIN RECEPTORS.....	24
3.1 Serotonin (5-HT)	24
3.2 Serotonin receptors in inflammation and disease	25
3.2.1 5-HT ₄ receptors in inflammation and disease	26
3.3 Serotonin receptors in airway diseases.....	27
3.3.1 5-HT ₄ receptors in airway diseases	28
CHAPTER 4. SOLUBLE GUANYLYL CYCLASE	31
4.1 Nitric oxide/soluble guanylyl cyclase(sGC)/cGMP pathway	31
4.2 Soluble guanylyl cyclase in inflammation and disease.....	33
4.3 Soluble guanylyl cyclase in pulmonary hypertension	35
4.4 Soluble guanylyl cyclase in airway diseases	35
REFERENCES	37
PART II: RESEARCH WORK	45
CHAPTER 5. RATIONALE AND AIMS OF THE THESIS	45
CHAPTER 6. TRANSLATIONAL RESEARCH IN COPD – METHODS.....	47
6.1 Ex vivo: Human lung tissue	47
6.2 In vivo: Murine model of cigarette smoke exposure and COPD	47
6.2.1 Protocol	48
6.2.2 Airway inflammation	49
6.2.3 mRNA expression	52
6.2.4 Protein expression.....	52
6.2.5 Emphysema	54
6.2.6 Pulmonary function: bronchoconstriction.....	56

REFERENCES	57
CHAPTER 7. ROLE OF TRANSIENT RECEPTOR POTENTIAL CHANNEL A1 IN CHRONIC OBSTRUCTIVE PULMONARY DISEASE.....	58
CHAPTER 8. INVESTIGATION OF 5-HT ₄ RECEPTORS IN BRONCHIAL HYPERRESPONSIVENESS IN CIGARETTE SMOKE-EXPOSED MICE	60
CHAPTER 9. THE ROLE OF SOLUBLE GUANYLYL CYCLASE IN CHRONIC OBSTRUCTIVE PULMONARY DISEASE	88
CHAPTER 10. REVIEW: 'ROLE OF THE NITRIC OXIDE - SOLUBLE GUANYLYL CYCLASE PATHWAY IN OBSTRUCTIVE AIRWAY DISEASES'	125
CHAPTER 11. DISCUSSION AND FUTURE PERSPECTIVES	145
11.1 Transient Receptor Potential channels.....	145
11.2 Serotonin receptor 4 (5-HT ₄ R).....	145
11.3 Soluble guanylyl cyclase (sGC).....	147
REFERENCES	151
CURRICULUM VITAE	157

Abbreviations

4-HNE	4-hydroxynonenal
5-HT	5-hydroxytryptamine
5-HT4R	5-hydroxytryptamine receptor 4 (protein)
AC	Adenylate cyclase
Ach	Acetylcholine
ADAM	A disintegrin and metalloproteinase
AP	Alkaline phosphatase
BAL	Bronchoalveolar lavage
CGRP	Calcitonin gene-related peptide
CNS	Central nervous system
CO	Carbon monoxide
COPD	Chronic Obstructive Pulmonary Disease
CS(E)	Cigarette smoke (extract)
DAMP	Damage-Associated Molecular Pattern
DC	Dendritic cell
DI	Destructive index
EFS	Electrical field stimulation
ELISA	Enzyme-linked immunosorbent assay
FEV₁	Forced Expiratory Volume in 1 second
FVC	Forced Vital Capacity
GDP	Guanosine monophosphate
GOLD	Global Initiative for Chronic Obstructive Lung Disease
GPCR	G protein-coupled receptor
GTP	Guanosine triphosphate
GWAS	Genome Wide Association Study
HHIP	Hedgehog Interacting Protein
HRP	Horseradish peroxidase
HTR4	5-hydroxytryptamine receptor 4 (gene)
KC	Keratinocyte-derived chemokine
Lm	Mean linear intercept
LPS	Lipopolysaccharide
MMP	Matrix metalloproteinase
NKA	Neurokinin A
NO	Nitric oxide

Pbm	Length of the basement membrane
PDE	Phosphodiesterase
PLC	Phospholipase C
PRR	Pathogen recognition receptor
qRT-PCR	Quantitative real-time polymerase chain reaction
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
sGC	Soluble guanylyl cyclase
SNP	Single Nucleotide Polymorphism
SP	Substance P
TRP	Transient Receptor Potential

Summary

Chronic obstructive pulmonary disease (COPD) is mainly caused by cigarette smoking and is characterized by a chronic inflammatory response of the lung leading to a persistent airflow limitation. Cigarette smoke (CS) contains more than 4500 components, including reactive oxygen species, nicotine, acrolein and endotoxin (lipopolysaccharide), which can bind to inflammatory cells and epithelial cells, further increasing the pulmonary inflammation.

(part on Transient Receptor Potential Channels has been removed from the electronic version of the dissertation)

To investigate the altered pulmonary function present in patients with COPD, we measured the bronchial hyperresponsiveness (BHR) to serotonin (5-HT) in CS-exposed mice, using pharmacological and genetic approaches.

First, the potential role of **5-hydroxytryptamine 4 receptors (5-HT₄R)** was analyzed. Genome Wide Association studies have demonstrated that genetic variants in the gene for 5-HT₄R are associated with pulmonary function, airway obstruction and COPD. In our study in mice, CS exposure increased the levels of 5-HT₄R mRNA in the lungs and induced BHR to 5-HT. Antagonism of 5-HT₄R did not alter the response to 5-HT in CS-exposed mice. The BHR was also not different between wild-type and 5-HT₄R KO mice. These data suggest that the 5-HT₄R is not involved in the BHR to 5-HT in CS-exposed mice.

In patients with COPD, irreversible airflow limitation is progressive, despite ample amounts of the smooth muscle relaxation factor nitric oxide (NO). Therefore, we analyzed the role of the principal NO receptor, **soluble guanylyl cyclase (sGC)** in BHR to 5-HT. In mice, CS exposure decreased sGC at the mRNA and protein level. Mice deficient in the sGC α 1 subunit had a higher responsiveness to serotonin after CS exposure compared to wild-type

mice. Reactivation of sGC in wild-type mice, using a pharmacological activator of sGC, restored the sGC signaling and attenuated BHR in CS-exposed mice. In addition, the levels of sGC were decreased in smokers without airflow limitation and in patients with COPD, and correlated with disease severity. These new translational findings show that the sGC-cGMP pathway is a promising drug target in the treatment of COPD.

Samenvatting

Chronisch obstructief longlijden (COPD) wordt voornamelijk veroorzaakt door het roken van sigaretten en wordt gekenmerkt door een chronische inflammatoire reactie van de longen, wat leidt tot een aanhoudende luchtstroombeperking. Sigarettenrook (SR) bevat meer dan 4500 componenten, waaronder reactieve zuurstofradicalen, nicotine, acroleïne en endotoxine (lipopolysaccharide), die kunnen binden aan inflammatoire cellen en epitheelcellen. Dit proces zorgt voor een verdere toename van de pulmonale inflammatie.

(deel over Transient Receptor Potential Channels werd verwijderd uit de elektronische versie van het proefschrift)

Om de wijzigingen in pulmonale functie bij patiënten met COPD te onderzoeken, hebben we de bronchiale hyperreactiviteit (BHR) ten opzichte van serotonine (5-HT) gemeten in SR-blootgestelde muizen. Voor dit onderzoek werden farmacologische en genetische benaderingen gebruikt.

Eerst werd de mogelijke rol van **5-hydroxytryptamine 4 receptoren (5-HT₄R)** onderzocht. Genome Wide Association studies hebben aangetoond dat genetische varianten in het gen voor 5-HT₄R geassocieerd zijn met wijzigingen in pulmonale functie, luchtwegobstructie en COPD. Blootstelling aan SR verhoogde 5-HT₄R mRNA in de longen en induceerde BHR ten opzichte van 5-HT in onze studie in muizen. Het neutraliseren van 5-HT₄R in SR-blootgestelde muizen veranderde de respons ten opzichte van 5-HT niet. De BHR ten opzichte van 5-HT was ook niet verschillend tussen wild-type en 5-HT₄R KO muizen. Deze resultaten suggereren dat de 5-HT₄R niet betrokken is in de BHR ten opzichte van 5-HT in SR-blootgestelde muizen.

In patiënten met COPD is de onomkeerbare luchtstroombeperking progressief, ondanks de aanwezigheid van de gladde spier relaxerende factor zuurstofmonoxyde (NO). Daarom hebben we de rol van de voornaamste NO receptor, **soluble guanylyl cyclase (sGC)**, in BHR ten opzichte van 5-HT geanalyseerd. SR-blootstelling in muizen verlaagde de hoeveelheid sGC, zowel op mRNA als op eiwitniveau. Muizen deficiënt voor de sGC α 1 subunit vertoonden een hogere reactiviteit ten opzichte van 5-HT na SR-blootstelling vergeleken met wild-type muizen. Reactivering van sGC in wild-type muizen, aan de hand van een farmacologische activator van sGC, herstelde de sGC signalisatie en verminderde de BHR in SR-blootgestelde muizen. In longweefsel van rokers zonder luchtstroombeperking en patiënten met COPD was de hoeveelheid sGC afgenomen; en gecorreleerd met de ernst van de ziekte. Deze nieuwe translationele bevindingen tonen aan dat de sGC-cGMP pathway een veelbelovend doelwit voor geneesmiddelen is voor de behandeling van COPD.

PART I: INTRODUCTION

Chapter 1. Chronic Obstructive Pulmonary Disease (COPD)

1.1 Introduction

Chronic obstructive pulmonary disease (**COPD**) is a chronic respiratory disease, affecting more than 200 million people worldwide ¹. It is currently the fourth leading cause of death, following ischemic heart disease, stroke and lower respiratory infections (**Figure 1**) ². The main risk factor for developing COPD is tobacco smoke (including passive smoking). Other important risk factors are exposure to indoor and outdoor air pollution, occupational dusts and chemicals ³. Moreover, tobacco smoke is responsible for the death of about 1 in 10 adults worldwide, as it also causes cardiovascular disease and lung cancer ².

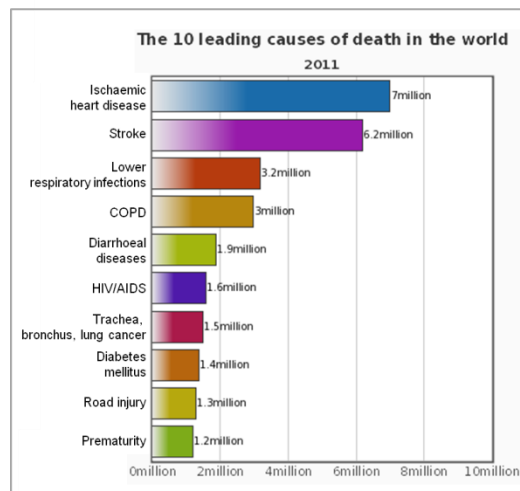


Figure 1. The top 10 causes of death in 2011, according to the World Health Organization (WHO) ².

COPD is defined by the Global Initiative for Chronic Obstructive Lung Disease (GOLD) as “a common preventable and treatable disease, characterized by persistent airflow limitation that is usually progressive and associated with an enhanced chronic inflammatory response in the airways and the lung to noxious particles or gases. Exacerbations and comorbidities contribute to the overall severity in individual patients.” ⁴.

The chronic airflow limitation in patients with COPD is caused by a combination of small airway disease (obstructive bronchiolitis) and parenchymal destruction (emphysema). **Chronic bronchiolitis** is caused by infiltration of inflammatory cells in the small airways. This process induces thickening of the airway wall and increased deposition of connective tissue (fibrosis). Due to goblet cell metaplasia, mucus production is increased, leading to obstruction of the lumen. **Emphysema** is the abnormal enlargement of the airspaces, resulting from disruption of the alveolar attachments. This diminishes the ability of the airways to remain open during expiration. Chronic obstructive bronchiolitis and emphysema can occur separately or together, both causing a progressive decline of lung function ⁴⁻⁷. Patients with COPD often suffer from other extrapulmonary or systemic manifestations, such as osteoporosis, skeletal muscle dysfunction and cardiovascular diseases ^{6,8}.

1.2 Symptoms and diagnosis

Patients with COPD have symptoms like dyspnea, chronic cough or sputum production. Although COPD is a very heterogeneous disease, airway limitation is considered as the main diagnostic indicator for COPD. The golden standard to measure airflow limitation, is spirometry. A post-bronchodilator FEV₁/FVC (also known as Tiffeneau index) < 0.70 confirms the presence of persistent airflow limitation and of COPD. However, to assess the severity of COPD, a classification is made based on FEV₁, expressed as a percentage of the normal FEV₁ of age-, sex- and height-matched individuals (**Table 1**) ⁴.

Table 1. Classification of severity of airflow limitation in COPD according to GOLD (in patients with FEV₁/FVC < 0.70)

GOLD 1	Mild	FEV ₁ ≥ 80% predicted
GOLD 2	Moderate	50% ≤ FEV ₁ < 80% predicted
GOLD 3	Severe	30% ≤ FEV ₁ < 50% predicted
GOLD 4	Very severe	FEV ₁ < 30% predicted

FEV₁: forced expiratory volume in 1 second; FVC: forced vital capacity

An acute worsening of the respiratory symptoms of the patient, or exacerbation, can be triggered by an infection with bacteria or viruses, by environmental pollutants or by other unknown factors ^{6,9}. The frequency of these short periods of increased cough, dyspnea and production of sputum is different between COPD patients. To take the exacerbations into account, the GOLD classification was updated in 2012, now combining the symptoms, spirometric classification and/or the risk of exacerbations (**Figure 2**). This new approach reflects the complex pathophysiology of COPD better than the previous unidimensional analysis of airflow limitation ⁴.

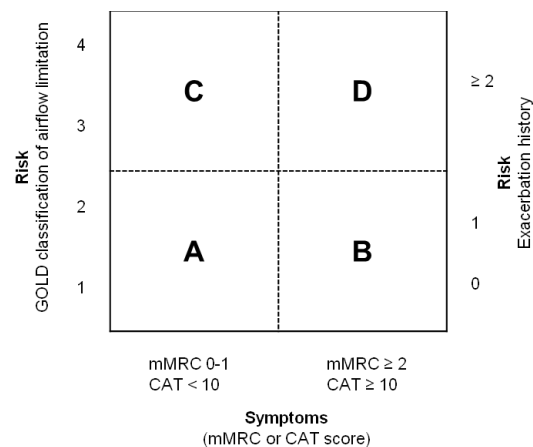


Figure 2. Combined COPD assessment. An mMRC grade ≥ 2 or a CAT score ≥ 10 indicates a high level of symptoms. The exacerbation risk is based on the amount of exacerbations in the preceding year. A patient with a low level of symptoms, 0 to 1 exacerbations per year and/or in GOLD category 1 or 2, belongs to group A (low risk and less symptoms). Figure reproduced from ⁴.
mMRC: Modified Medical Research Council questionnaire; CAT: COPD Assessment Test

1.3 Comorbidities

COPD is not only a disease of the lungs, but is also associated with systemic manifestations and comorbidities such as diabetes, osteoporosis, lung cancer and cardiovascular diseases ^{6,8,10}. These extrapulmonary manifestations of COPD increase the risks of admission to hospital and death. The mechanisms underlying the comorbidities in COPD are not yet fully elucidated.

1.4 Therapy

The most important therapy for patients who smoke, is smoking cessation. Several effective treatments exist that can help the patient to stop smoking, such as nicotine replacement products and smoking cessation counseling ¹¹.

None of the currently existing medications for COPD can modify the long-term decline in lung function. However, pharmacologic therapy can reduce symptoms of COPD, reduce the frequency and severity of exacerbations and improve the health status and exercise tolerance of the patient. Since COPD is a heterogeneous disease, with differences in the severity of symptoms, airflow limitation and frequency of exacerbations between patients, the pharmacological treatment should be patient-specific. Inhaled bronchodilators are the main treatment for COPD. Patients with very severe disease (GOLD stage 4) may need surgery such as lung transplantation or lung-volume reduction ⁶.

Several anti-inflammatory therapies have been tested in patients with COPD (e.g. IL-1-specific antibodies, TNF-specific antibody, leukotriene B4 receptor antagonists), but none of them have shown clinical benefits ¹².

1.5 Genetics

There is a high variation in susceptibility to COPD between individuals, due to differences in genetic predisposition to the disease ¹³. The best known genetic risk factor is a severe hereditary deficiency of α -1 antitrypsin, a major circulating inhibitor of serine proteases. However, this genetic deficiency is rare. In the past, mainly candidate gene studies were performed. In these studies, genetic variants in genes thought to have a function in causing or preventing diseases such as COPD are analyzed ¹⁴. An important drawback of the candidate gene approach is that many genes with unknown functions are missed, whereas the more recent **Genome Wide Association (GWA) studies** are hypothesis-free. GWA studies include a panel of hundreds of thousands of single nucleotide polymorphisms (**SNP**) across the entire genome and relate them to diseases or health-related traits ^{15,16}.

A SNP is a variation at a single position in a DNA sequence among individuals. The DNA sequence is composed of four nucleotide bases: adenine, cytosine, guanine, and thymine. If more than 1% of a population does not carry the same nucleotide at a specific position in the DNA sequence, then this variation can be classified as a SNP. If a SNP occurs within a gene, then the gene is described as having more than one allele. A SNP that occurs in a coding region or exon is called coding SNP. A coding SNP may be nonsynonymous, meaning that it results in a change in the amino acid sequence of a protein. This may affect the function of the protein, in contrast to a synonymous SNP which codes for the same amino acid¹⁶. Several SNPs within genes are known and have been associated with lung function and COPD, such as SNPs in Hedgehog Interacting Protein (*HHIP*), A disintegrase and metalloproteinase 19 (*ADAM19*) and 5-hydroxytryptamine receptor 4 (*HTR4*) (**Figure 3**)¹⁷⁻²⁰. To identify the role of these newly discovered genes in the pathogenesis of COPD, experimental studies of the biological function of these genes are needed.

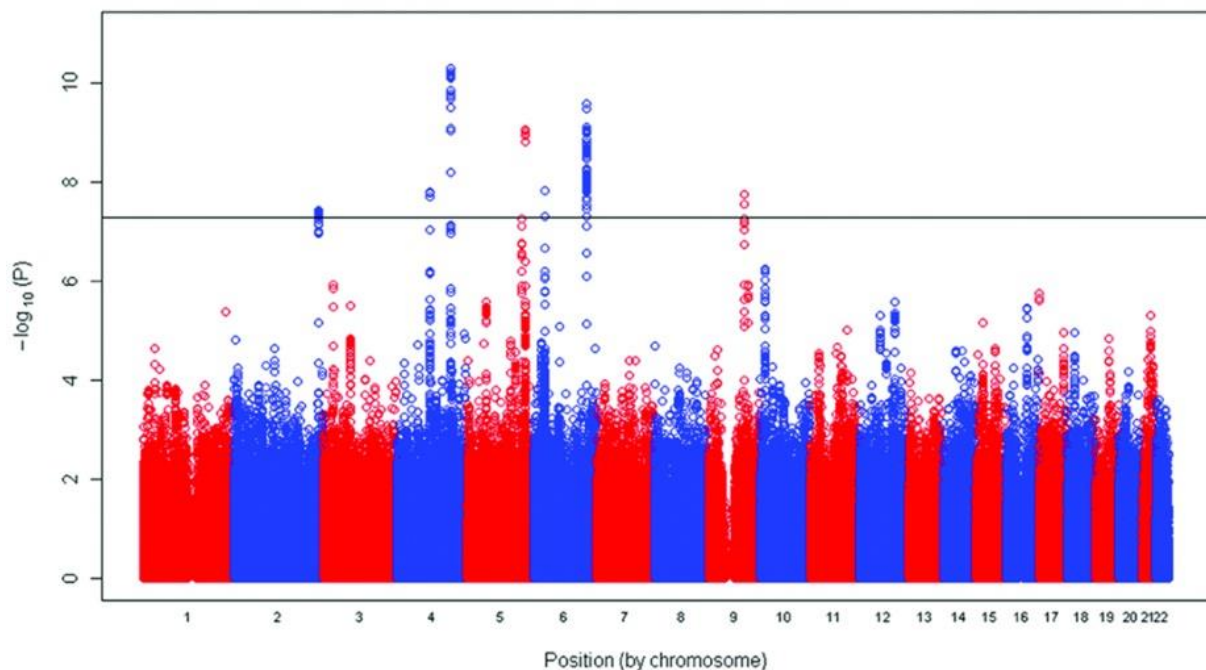


Figure 3: Manhattan plot of the Genome Wide Association study of lung function (FEV₁/FVC). The chromosomal position of SNPs exceeding the threshold (black horizontal line) of genome-wide significance ($P < 5 \times 10^{-8}$) is shown. Figure reprinted with permission from Hancock DB *et al. Nature Genetics* 2010; 42(1):45-52¹⁸.

1.6 Pathogenesis and pathophysiology

COPD is characterized by an influx of **inflammatory cells** of both the innate and the adaptive immune system ²¹⁻²³ in the lungs in response to the inhalation of noxious gases, such as cigarette smoke (**Figure 4**).

Cigarette smoke contains more than 4500 components, including nicotine, acrolein and endotoxin, in its gaseous and particulate phases. Compounds such as endotoxin (lipopolysaccharide (LPS)) can directly activate pattern recognition receptors (PRR) expressed on alveolar macrophages, dendritic cells and epithelial cells ^{22,24}. Cigarette smoke also causes injury to epithelial cells, which release damage-associated molecular patterns (DAMPs), such as uric acid, ATP and HMGB-1. DAMPs can also activate PRRs, leading to increased IL-1 β . The cigarette smoke-induced release of proinflammatory cytokines and chemokines by airway epithelial cells and alveolar macrophages, attracts neutrophils and inflammatory monocytes to the lungs ^{22,25,26}. Upon activation, neutrophils and macrophages cause lung destruction (**emphysema**) by releasing oxygen radicals and proteolytic enzymes such as neutrophil elastase and matrix metalloproteinases (MMPs) (**Figure 4**).

Dendritic cells are localized in the lumen and directly beneath the epithelium of the airways, where they can easily pick up antigens and present them to naïve T lymphocytes in the draining lymph nodes ^{27,28}. The exact nature of these antigens is not clear yet, but components of CS, microbial antigens or auto-antigens are plausible candidates ²⁹⁻³¹. The activated antigen-specific CD4⁺ and CD8⁺ T lymphocytes and antibody-producing B cells migrate back to the lungs. After progression of the disease, **lymphoid follicles** containing segregated B- and T-cell zones, develop around the small airways and in the lung parenchyma ^{22,32,33} (**Figure 4**).

Moreover, structural changes in the airways appear, including peribronchial fibrosis, airway smooth muscle hyperplasia and hypertrophy and goblet cell hyperplasia. This thickening of the airway wall and narrowing of the lumen is called **airway wall remodeling** (**Figure 4**).

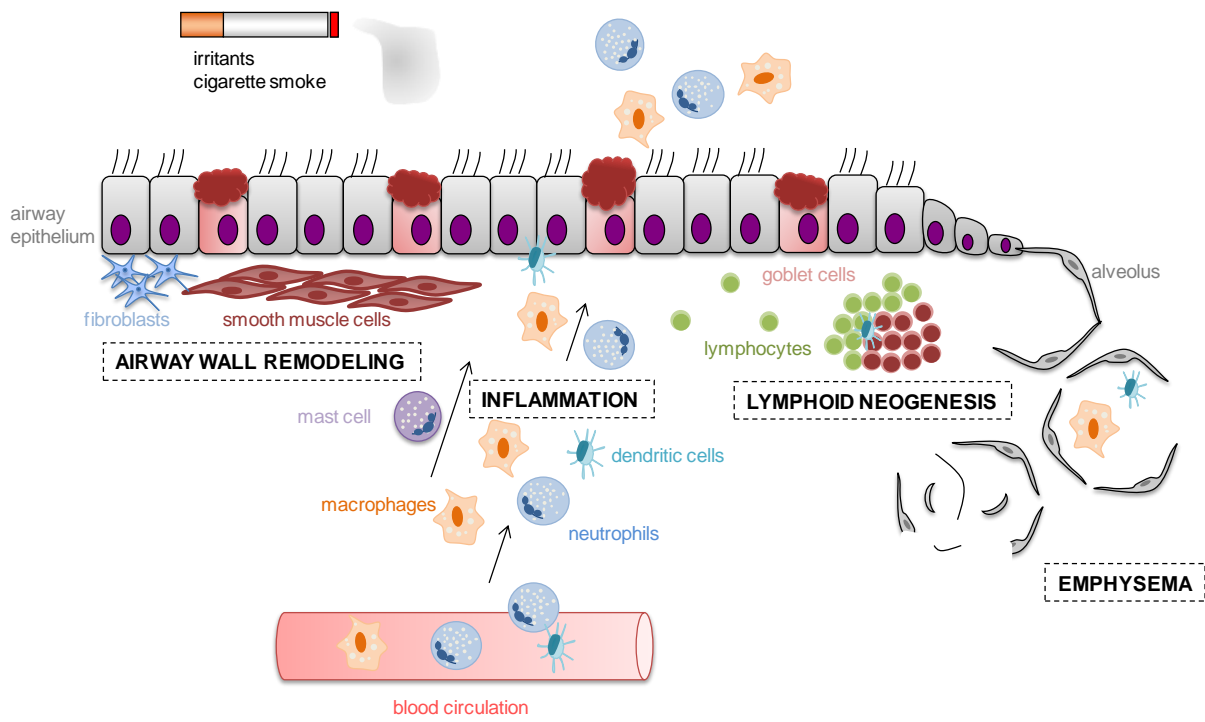


Figure 4. Schematic overview of the pathophysiology of COPD.

Chapter 2. Transient Receptor Potential (TRP) channels and TRPA1

2.1 Transient Receptor Potential (TRP) channels

Transient Receptor Potential (TRP) channels are cation channels that are able to respond to a large variety of physical and chemical stimulants. They play a critical role in sensing both the outside world (thermal sensation, touch, chemosensation) and the local environment (inflammation, tissue injury). The TRP superfamily is subdivided in six subfamilies: TRPC (canonical), TRPV (vanilloid), TRPM (melastatin), TRPA (ankyrin), TRPP (polycystin) and TRPML (mucolipin)^{34,35} (**Figure 5**).

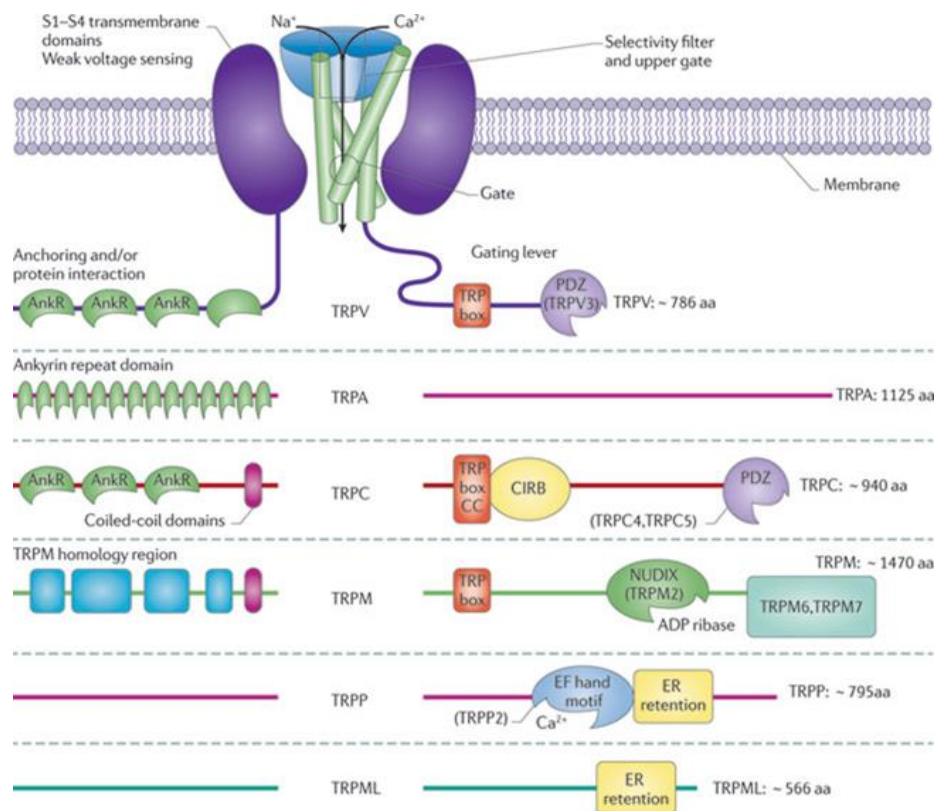


Figure 5. Structure of the 6 subfamilies of the Transient Receptor Potential (TRP) superfamily. TRPV: vanilloid, TRPA: ankyrin, TRPC: canonical, TRPM: melastatin, TRPP: polycystin, TRPML: mucolipin. Figure reprinted with permission from Moran M *et al. Nature Reviews Drug Discovery* 10:601-620³⁵.

TRP channels are expressed and functional in various multicellular organisms (fruit flies, worms, zebrafish, mice, humans). Apart from their 6 transmembrane domains with a pore loop between the fifth and sixth segment and their selectivity for cations, the majority of TRP

channels share a low level of structural similarity (**Figure 5**). Consistent with their diverse structure and expression on both neuronal and non-neuronal cells, TRP channels have very diverse functions throughout the body ³⁵.

2.2 Transient Receptor Potential (TRP) channels in inflammation and disease

TRP channels have been mainly studied in the area of pain research. Several TRP channels are expressed on sensory nerves and are activated by heat, cold, pH and reactive chemicals. This activation can trigger an action potential in the sensory nerve, which is transmitted to the central nervous system where the sensation of pain is experienced ³⁵ (**Figure 6**).

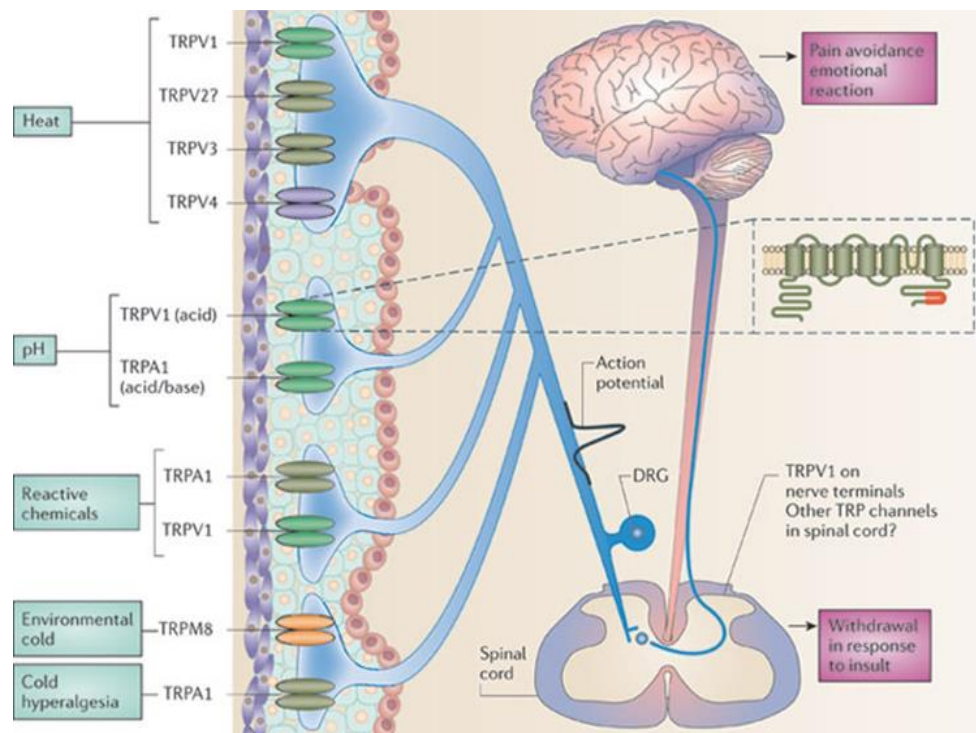


Figure 6. Transient Receptor Potential (TRP) channels on sensory neurons.

TRPV: vanilloid, TRPA: ankyrin, TRPM: melastatin, DRG: dorsal root ganglion. Figure reprinted with permission from Moran M *et al. Nature Reviews Drug Discovery* 10:601-620 ³⁵.

TRPV1, localized on sensory nerves, is activated by capsaicin, a component of chili peppers. Repeated administration of capsaicin desensitizes the sensory nerve, making it unresponsive to further attacks ³⁶. Therefore, TRPV1 could have therapeutic potential in the treatment of pain.

TRP channels are also implicated in other physiological processes such as bladder function, formation of the skin epidermal barrier and glucose tolerance. In the bladder, TRPV1-positive nerves mediate the micturition reflex³⁷. TRPV4 channels are expressed in the urothelium and in the detrusor muscle, making TRPV4 an interesting candidate in the management of an overactive bladder³⁷. In the skin, TRPV1 is highly present on various cell types (keratinocytes, sensory neurons,...)³⁸. TRPV1, TRPV3, TRPV4 and TRPV6 are involved in regulating the formation of the epidermal barrier of the skin^{38,39}. Inactivation of TRPV1 protects against the development of type 1 diabetes and improves the glucose tolerance in type 2 diabetes⁴⁰.

Mutations in TRP channels are the cause of certain diseases, such as autosomal dominant polycystic kidney disease (TRPP)⁴¹, mucopolysaccharidosis type IV (TRPML)⁴², autosomal dominant segmental glomerulosclerosis (TRPC6)⁴³, hypomagnesemia and hypocalcemia (TRPM6)⁴⁴.

2.2.1 TRPA1 in inflammation and disease

Transient Receptor Potential channel ankyrin 1 (TRPA1) has an unusually high number of ankyrin repeats at the amino terminus. TRPA1 can be activated directly by covalent modification of specific cysteine residues located in the ankyrin repeat domains⁴⁵ (**Figure 7**). TRPA1 can also be activated in an indirect way, by modulation by G protein-coupled receptors (GPCRs) through second messenger signaling pathways. Bradykinin activates the GPCR-coupled receptor BK2R, leading to activation of phospholipase C (PLC). Generally, PLC cleaves phosphatidylinositol 4,5-bisphosphate (PIP₂) to diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP₃). After diffusion through the cytosol, IP₃ interacts with calcium (Ca²⁺) channels in the membrane of the endoplasmic reticulum, leading to opening of these channels and subsequent release of Ca²⁺ in the cytosol. This increase in Ca²⁺ could activate TRPA1. DAG, present on the membrane, can lead to activation of protein kinase C, together with Ca²⁺. Phosphorylation by PKC can also activate TRPA1. However, the specific

molecules downstream of PLC, that lead to activation of TRPA1 are not known yet ⁴⁶
(Figure 7).

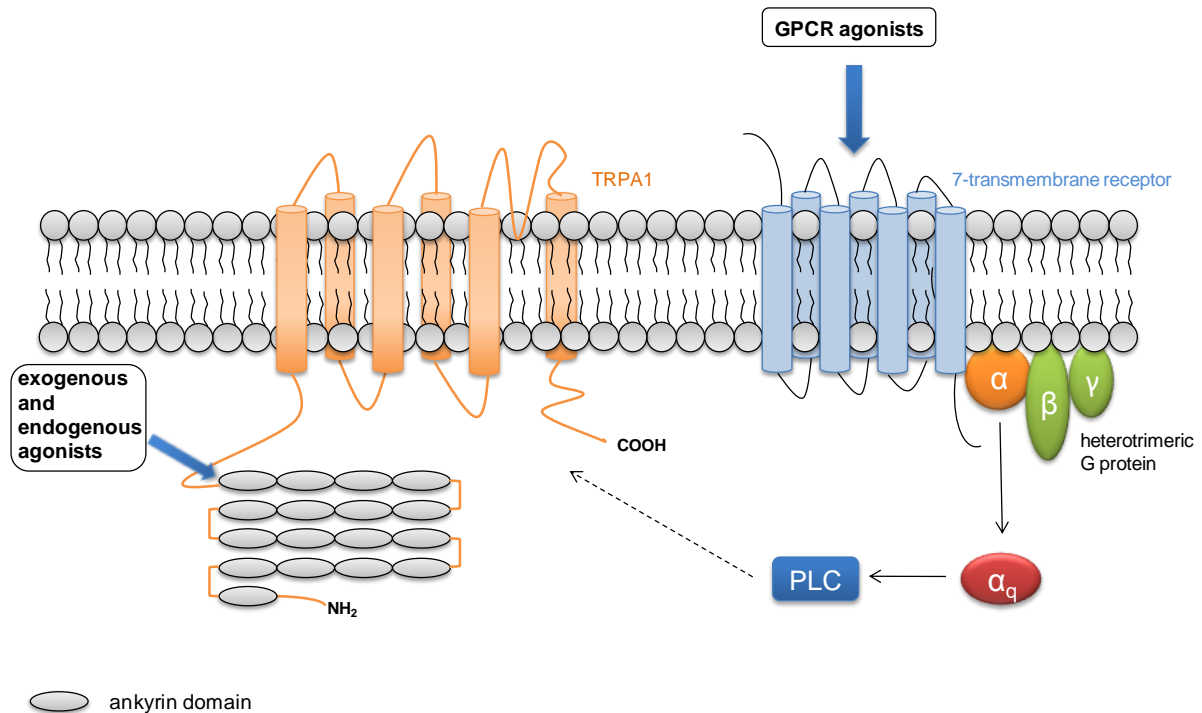


Figure 7. Activation mechanisms of TRPA1. TRPA1 can be activated directly by covalent modification of specific cysteine residues located in the ankyrin repeat domains; and indirectly by modulation by G protein-coupled receptors (GPCRs) through second messenger signaling pathways. GPCR: G protein-coupled receptor, PLC: phospholipase C.

Many of the activators of TRPA1 cause pain in humans and mice. TRPA1 knockout mice show a reduced sensitivity to environmental irritants and reduced pain behavior ⁴⁷. Therefore, TRPA1 antagonists are being investigated in pain research.

TRPA1 is a sensor of inflammation throughout the body, including the gastrointestinal tract. TRPA1 is expressed on sensory nerves that innervate the colon and mediates the release of neuropeptides upon activation ⁴⁸.

2.3 TRP channels in airway diseases

The airways are innervated by a dense network of nerve fibers. The majority of the fibers that innervate the respiratory tract are carried in the vagus nerve, and their neuronal cell bodies are localized in the **nodose and jugular ganglia** ⁴⁹. Also nerves originating from the spinal cord innervate the airways with sensory neurons originating from the **dorsal root ganglia**.

The **trigeminal ganglia** contain the cell bodies of the trigeminal nerve fibers, with nerve endings in the nasal mucosa. The terminals of the sensory fibers, expressing TRP channels, are localized close to the epithelium, which allows them to detect noxious compounds, tissue injury and inflammation in the lungs ^{49,50}. After activation of the TRP channels, the ganglia project the information from the airways through afferent fibers to the brain (nodose, jugular and trigeminal ganglia) or to the spinal cord (dorsal root ganglia) and initiate a **nocifensive reflex** such as bronchoconstriction, mucus secretion or cough ⁴⁹ (**Figure 8**). The majority of sensory nerves in the lower airways are C-fibers that sense injury and mainly respond to chemical stimulation such as capsaicin and bradykinin ^{51,52}. C-fibers, also often referred to as 'nociceptors', contain neuropeptides such as substance P (SP), neurokinin A (NKA) and calcitonin gene-related peptide (CGRP) ⁵³. Neuropeptides are **locally** released upon stimulation and induce a neurogenic inflammatory response (including plasma protein extravasation, bronchoconstriction, mucus secretion and chemotaxis of inflammatory cells to the site of injury) ⁵³⁻⁵⁵ (**Figure 8**).

TRP channels are not only present on nerves, but also on non-neuronal cells. TRPA1 is expressed on airway epithelial cells and airway smooth muscle cells, TRPV1 on airway epithelial cells, TRPV4 on airway epithelial cells, airway smooth muscle cells, endothelium and macrophages, and TRPM8 on airway epithelial cells. The expression of TRP channels on inflammatory cells types has also been suggested ⁵⁶⁻⁶¹.

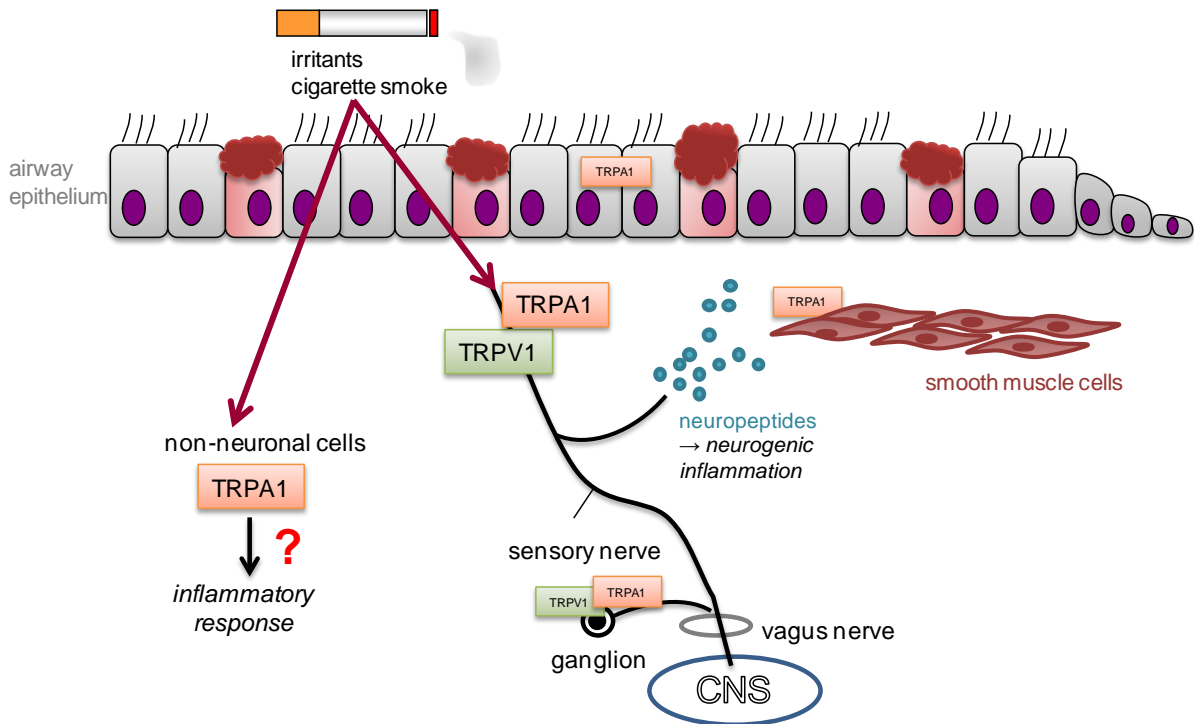


Figure 8. Innervation of the airways. Activation of TRP channels on airway sensory nerve endings can lead to a nocifensive reflex, such as coughing; and to the local release of neuropeptides, inducing neurogenic inflammation. TRPA1 is also present on non-neuronal cells, and its activation could lead to an additional inflammatory response. CNS: central nervous system, TRP: Transient Receptor Potential.

Capsaicin, a **TRPV1** agonist, causes coughing, sneezing and fluid secretion after application to human respiratory mucosa. However, in patients with idiopathic rhinitis, capsaicin induces symptomatic relief by desensitizing TRPV1-expressing sensory neurons⁶². Multiple SNPs in the gene encoding **TRPV4** are associated with COPD⁶³. TRPV4 is expressed on airway epithelium, endothelium and smooth muscle and could play a role in airflow obstruction by inducing contraction of the smooth muscle cells⁵⁹. Moreover, TRPV4 is important in regulating the airway epithelial barrier function and mucociliary transport^{64,65}. Activation of TRPV4 on alveolar macrophages triggers the production of reactive oxygen species and reactive nitrogen species⁶⁶. These effects of TRPV4 are implicated in the pathogenesis of COPD, so TRPV4 may be crucial in the development of COPD.

TRPM8 is activated by cold and menthol. Activation of TRPM8 on lung epithelial cells induces production of inflammatory cytokines⁶⁷. TRPM8 may also be involved in cold-induced exacerbations of asthma⁶⁸.

2.3.1 TRPA1 in airway diseases

A large number of the TRPV1⁺ sensory neurons express TRPA1, which responds to endogenous mediators such as lipid peroxidation byproducts (4-hydroxynonenal: 4-HNE) and to exogenous irritants such as ozone, components of cigarette smoke (acrolein, nicotine, α,β -unsaturated aldehydes, LPS) and chlorine^{69-71,72}. Apart from its localization on nerve fibers, TRPA1 is also present on non-neuronal cells (epithelial cells, smooth muscle cells)^{57,60}.

The activation of TRPA1 on airway sensory nerves can lead to reflex coughing, mucus production, bronchoconstriction; and to a local release of neuropeptides, inducing neurogenic inflammation (**Figure 8**). This indicates that TRPA1 may be a good target in the treatment of various airway diseases. The importance of TRPA1 in the airways has already been demonstrated in a murine model of allergic airway inflammation⁷³. The OVA-induced infiltration of leucocytes in the airways, production of cytokines and mucus, and airway hyperreactivity is significantly reduced in TRPA1 KO mice. Treating OVA-challenged wild-type mice with the TRPA1 antagonist HC-030031 inhibits the increased inflammation in bronchoalveolar lavage (BAL) fluid and prevents airway hyperreactivity. Both genetic deletion and pharmacologic inhibition of TRPA1 decrease the levels of neuropeptides in BAL fluid⁷³.

TRPA1 KO mice, exposed to acute cigarette smoke for 3 days, have significantly lower levels of the neutrophil attracting chemokine KC (keratinocyte-derived chemokine; CXCL1). After intratracheal instillation with cigarette smoke extract (CSE) and treatment with HC-030031, wild-type mice also show lower levels of KC compared to vehicle-treated mice. Moreover, antagonism of the NK1 receptor, which binds substance P, has no influence on KC levels, while it does affect plasma protein extravasation⁵⁷, suggesting a role for TRPA1 in both neuronal and non-neuronal cells of the lung.

Since TRPA1 is activated by several components of CS and is present on both airway sensory nerves and non-neuronal cells in the airways, we analyzed the role of TRPA1 in a murine model of CS-induced inflammation (see **Chapter 7**).

Chapter 3. Serotonin (5-HT) and serotonin receptors

3.1 Serotonin (5-HT)

Serotonin (5-hydroxytryptamine, 5-HT) is a monoamine neurotransmitter (**Figure 9**) and regulates the function of several human organ systems and is involved in multiple disease processes. 5-HT signaling in the central nervous system (CNS) has been thoroughly described, where it is implicated in many psychiatric and neurological conditions such as depression, anxiety disorders, eating disorders and obsessive-compulsive disorder ^{74,75}. However, the majority of total body 5-HT is found peripherally, where it is mainly synthesized (**Figure 9**), stored and released by enterochromaffin cells of the gut ⁷⁶. Production of 5-HT has also been described in pulmonary neuroendocrine cells ⁷⁷ and in rodent mast cells ⁷⁸. After release in the blood circulation, 5-HT is rapidly taken up by platelets where it is stored and released during platelet aggregation.

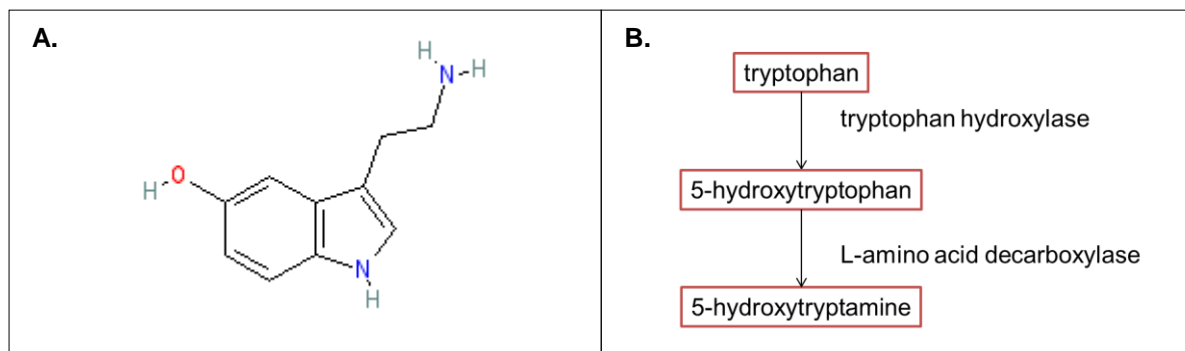


Figure 9. A. Structure of 5-hydroxytryptamine (5-HT) (adapted from IUPHAR database ⁷⁹) **B.** Synthesis of 5-HT. 5-HT is synthesized from the naturally occurring essential amino acid tryptophan. Tryptophan hydroxylase 1 is present in enterochromaffin cells, tryptophan hydroxylase 2 in neurons.

3.2 Serotonin receptors in inflammation and disease

Serotonin receptors are ubiquitously expressed in the human body. Seven major families of 5-HT receptors exist: 5-HT₁, 5-HT₂, 5-HT₃, 5-HT₄, 5-HT₅, 5-HT₆ and 5-HT₇ receptor⁸⁰. The 5-HT₅ receptor has not yet been fully characterized and is therefore not identified as an official receptor. Except for 5-HT₃ receptors, which are ligand-gated ion channels, all 5-HT receptors are G protein-coupled receptors with the typical structure of 7 hydrophobic transmembrane domains, 3 intracellular and 3 extracellular loops. 5-HT₁R are mostly coupled to inhibitory Gα_i proteins, leading to inhibition of adenylyl cyclase (AC) and a subsequent decrease of cAMP levels. 5-HT₄R, 5-HT₆R and 5-HT₇R are linked to Gα_s proteins, resulting in activation of AC and increased cAMP levels. 5-HT₂R are coupled to Gα_q proteins, which are linked to the phospholipase C signaling pathway^{81,82} (**Figure 10**).

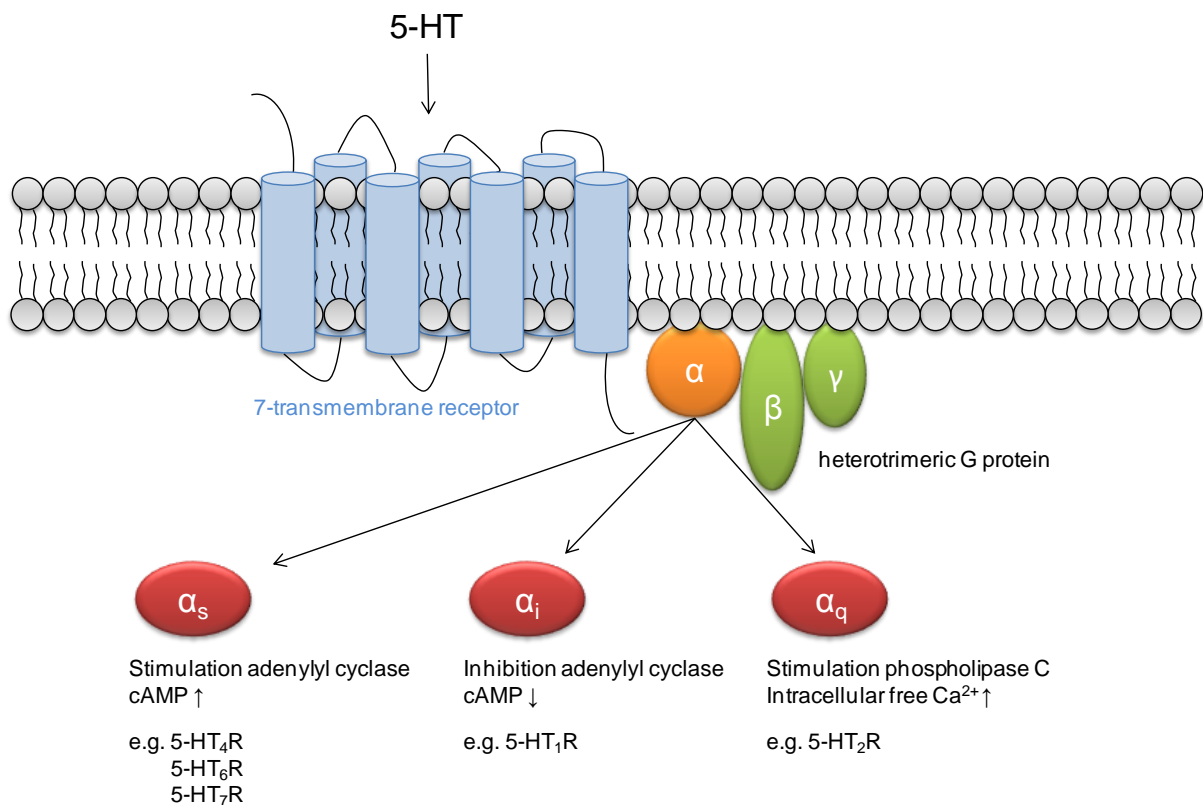


Figure 10. Main signaling pathways of 5-HT receptors. The receptor is coupled to a heterotrimeric G protein, composed of α, β and γ subunits. In the inactive state, the Gα subunit is bound to guanosine diphosphate (GDP). Binding of 5-HT triggers a conformational change in the receptor, which catalyzes the replacement of GDP by GTP and the dissociation of Gα from the Gβγ subunits. A single 5-HTR can couple to one or more Gα subfamilies resulting in different outcomes. The main pathways of 5-HT receptors are depicted in the figure. Figure based on Dorsam RT and Gutkind JS. *Nature reviews Cancer* 2007; 7(2):79-94⁸².

Similar to their ligand 5-HT, the 5-HT receptors are found centrally, as well as peripherally, in the gastrointestinal tract, cardiovascular system, platelets, adrenal gland, bladder and lung. Consequently, 5-HT receptors have divergent functions.

5-HT₁R are clinically relevant in anxiety- and depression-related mood states. Mice deficient for 5-HT₁ receptors show increased anxiety behavior and stress responses⁸³, while partial agonists of 5-HT_{1A} receptor are effective anxiolytic agents^{84,85}. Moreover, 5-HT_{1A}R is extensively expressed in the central respiratory network and pharmacological treatment with 5-HT_{1A}R agonists has anti-apneustic effects^{86,87}.

5-HT_{2A}R and 5-HT_{2B}R mediate the contraction answer of the smooth muscle in peripheral tissues⁸⁸. The activation of 5-HT_{2B}R in the heart may induce fibrotic cardiac valvulopathy (thickening of the heart valves)⁸⁹. In the central nervous system, 5-HT_{2A}R antagonists are used for the treatment of schizophrenia⁹⁰.

5-HT₃R antagonists are currently used as anti-emetic drugs^{91,92} and are beneficial as symptomatic drugs for patients with irritable bowel syndrome⁹³.

5-HT₄R will be described more thoroughly in section **3.2.1** and **3.3.1**.

5-HT₆R are selectively expressed in the central nervous system, but their clinical significance remains unclear⁹⁴.

5-HT₇R are located in the thalamus, hypothalamus, hippocampus and cerebral cortex. In the periphery, 5-HT₇R are present in smooth muscle cells of blood vessels and in the gastrointestinal tract⁹⁵. The pharmacological inhibition of 5-HT₇R (present on dendritic cells) in mice reduces experimentally induced colitis, associated with lower proinflammatory cytokine levels⁹⁶. Similarly, 5-HT₇R KO mice have significantly reduced intestinal inflammation⁹⁶.

3.2.1 5-HT₄ receptors in inflammation and disease

5-HT₄ receptors have been studied in learning and memory processes. 5-HT₄R agonists have pro-cognitive effects, both on short term and long term memory⁹⁷. Moreover, 5-HT₄R

are involved in the processing of the amyloid precursor protein⁹⁸, and a decreased density of 5-HT₄R binding sites has been demonstrated in the brain of patients with Alzheimer's disease⁹⁹, suggesting that 5-HT₄R agonists could be beneficial in the treatment of memory disorders¹⁰⁰. Polymorphisms of the *Htr4* gene, encoding the 5-HT₄ receptors, have been correlated with bipolar disorders and depression¹⁰¹. The brain of depressed suicide victims shows an altered expression of 5-HT₄ receptors¹⁰². Recently, evidence has been found that 5-HT₄ receptors may also be a direct target for treating depression¹⁰³. A study has shown that 5-HT₄R KO mice have reduced hypophagia following stress¹⁰⁴, suggesting that 5-HT₄R antagonists may be useful drugs to treat anorexia-related disorders¹⁰⁵.

The use of 5-HT₄R agonists has been most extensively studied in the gastrointestinal tract, for the treatment of conditions such as irritable bowel syndrome, constipation and gastro-esophageal reflux^{106,107}. However, the 5-HT₄R agonist cisapride has been withdrawn from the market due to cardiovascular adverse effects¹⁰⁸. The production of more selective 5-HT₄R agonists is therefore emerging.

3.3 Serotonin receptors in airway diseases

Several publications suggest a role for the serotonergic system in airway diseases. Symptomatic **asthma** patients have elevated levels of 5-HT in plasma compared with non-asthmatics¹⁰⁹. Moreover, the 5-HT plasma levels are significantly correlated with FEV₁¹⁰⁹. In line with these findings, ketanserin, a 5-HT₂R antagonist, has a beneficial effect on FEV₁ in patients with chronic airflow limitation¹¹⁰. Tianeptine, a drug that reduces the concentration of plasma 5-HT by enhancing the re-uptake of 5-HT, improves pulmonary function in children with asthma¹¹¹. Also patients with **COPD** have elevated plasma 5-HT levels¹¹².

Dietary supplementation of 5-hydroxytryptophan, the precursor of 5-HT, significantly reduces allergen-induced lung inflammation in a murine model, mainly by inhibiting the endothelial cell function during leukocyte recruitment¹¹³. The expression of cytokines and chemokines is not different between mice on a diet with or without 5-hydroxytryptophan, and the systemic 5-HT levels are also not affected¹¹³.

Pulmonary hypertension is a complication of COPD, associated with increased risks of exacerbation and decreased survival ¹¹⁴. There is currently no pharmacological treatment for pulmonary hypertension in COPD ¹¹⁴. A commonly used murine model of PH is the chronic hypoxia model. 5-HT_{1B}R KO mice develop less severe PH than wild-type mice, although they still respond to hypoxia ¹¹⁵. In mice deficient for 5HT_{2B}R, PH is completely absent, indicating that activation of 5-HT_{2B}R is an important step in the development of PH ¹¹⁶.

3.3.1 5-HT₄ receptors in airway diseases

5-HT receptors, including 5-HT₄R, are expressed by human airway epithelial cells ¹¹⁷ and by a broad range of inflammatory cell types, such as dendritic cells ¹¹⁸ and monocytes ¹¹⁹. In dendritic cells, the expression of 5-HT₄R mRNA increases after maturation by LPS ¹²⁰. The activation of 5-HT₄R or 5-HT₇R, using specific agonists, significantly increased the secretion of IL-8 and IL-1 β in mature dendritic cells, while reducing IL-12 and TNF α levels ¹²⁰.

A meta-analysis of Genome Wide Association Study results (GWAS, see **1.5 Genetics**) from 4 CHARGE consortium studies (Atherosclerosis Risk in Communities (ARIC), Cardiovascular Health Study (CHS), Framingham Heart Study (FHS) and Rotterdam Study (RS-I and RS-II)) analyzed the pulmonary function in 20890 participants of European ancestry (11963 ever-smokers and 8927 never-smokers). The meta-analyses were performed with adjustment for smoking status and quantity (pack-years). Polymorphisms in 8 regions were associated with FEV₁/FVC, including *HTR4*, the gene encoding 5-HT₄R in humans ¹⁸ (**Figure 11**).

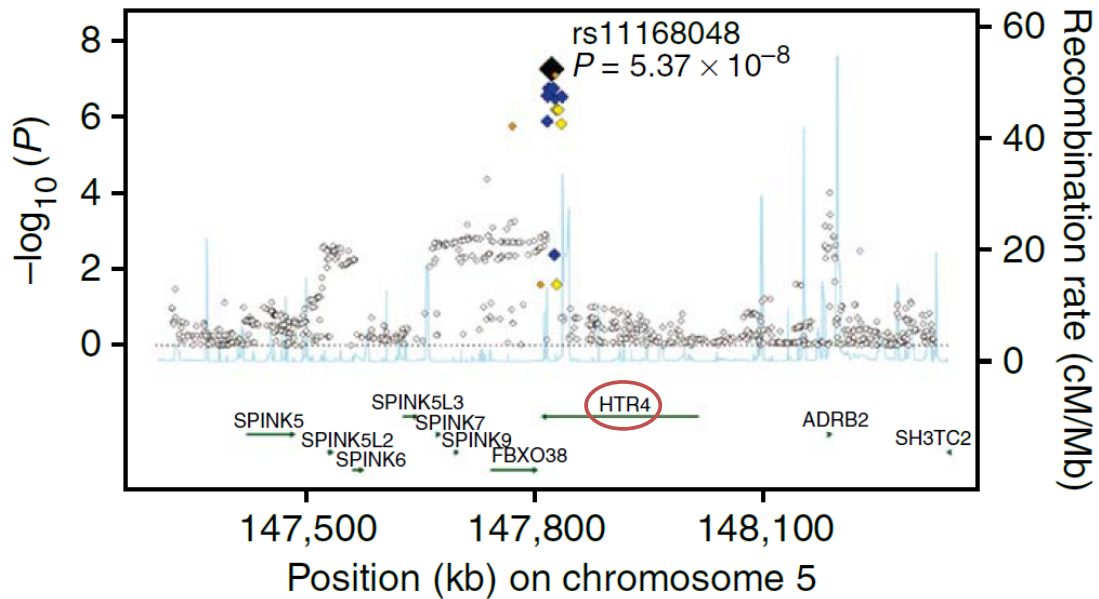


Figure 11. Regional association plot for the locus including *HTR4*, associated with FEV₁/FVC. Figure reprinted with permission from Hancock DB *et al. Nature Genetics* 2010; 42(1):45-52¹⁸.

These loci, and other high-signal hits were replicated in the independent SpiroMeta consortium, counting 20288 participants¹²¹. The association of *HTR4* and FEV₁/FVC was confirmed, and polymorphisms in *HTR4* were also associated with FEV₁¹²¹. In another GWAS, the *HTR4* gene was implicated in the pathogenesis of airflow obstruction¹²².

The original CHARGE meta-analysis was repeated in individuals without asthma and COPD, remaining 17855 of the 20890 participants. Several SNPs in *HTR4* again reached genome-wide significance in individuals with normal pulmonary function¹⁸. Soler Artigas *et al.* analyzed the clinical relevance of five loci reported by the SpiroMeta Consortium. Interestingly, genetic variants in *HTR4* were associated with COPD¹²³.

The functional role of 5-HT₄R in airway diseases has already been investigated in some studies. The protein expression of 5-HT₄R in human lung tissue is very low¹²⁴. In an *in vitro* study of human airways, electrical field stimulation (EFS) induces cholinergic contractions, which are further enhanced in the presence of 5-HT. This response is significantly antagonized by tropisetron, an antagonist of both 5-HT₃R and 5-HT₄R. Selective antagonists of 5-HT₃R and 5-HT₄R are also able to attenuate the facilitatory effect of 5-HT on cholinergic contraction, although the effect is less pronounced than tropisetron. The authors suggest that

both 5-HT₃R and 5-HT₄R are present on postganglionic cholinergic nerves, which enhance the EFS-induced cholinergic contraction of human airways *in vitro*¹²⁵ (**Figure 12**). A study in guinea pigs has shown that 5-HT directly activates 5-HT_{2A} receptors on airway smooth muscle cells, leading to bronchoconstriction. 5-HT₄ receptors, present on cholinergic nerves, can be stimulated indirectly by 5-HT, causing the release of acetylcholine. The binding of acetylcholine to muscarinic receptors on airway smooth muscle cells eventually results in bronchoconstriction¹²⁶ (**Figure 12**). Recently, researchers have shown that rhesus monkeys, sensitized with house dust mite allergen (HDMA) and challenged with ozone + HDMA, have increased airway hyperresponsiveness compared to filtered air-exposed monkeys, even after prolonged recovery¹²⁷. This response is exacerbated by 5-HT, and significantly decreased in the presence of 5-HT_{2A}R, 5-HT₃R or 5-HT₄R antagonists.

The 5-HT-induced contraction in murine airways has been described in several publications^{128,129}, but 5-HT₄R have not been studied in mice before.

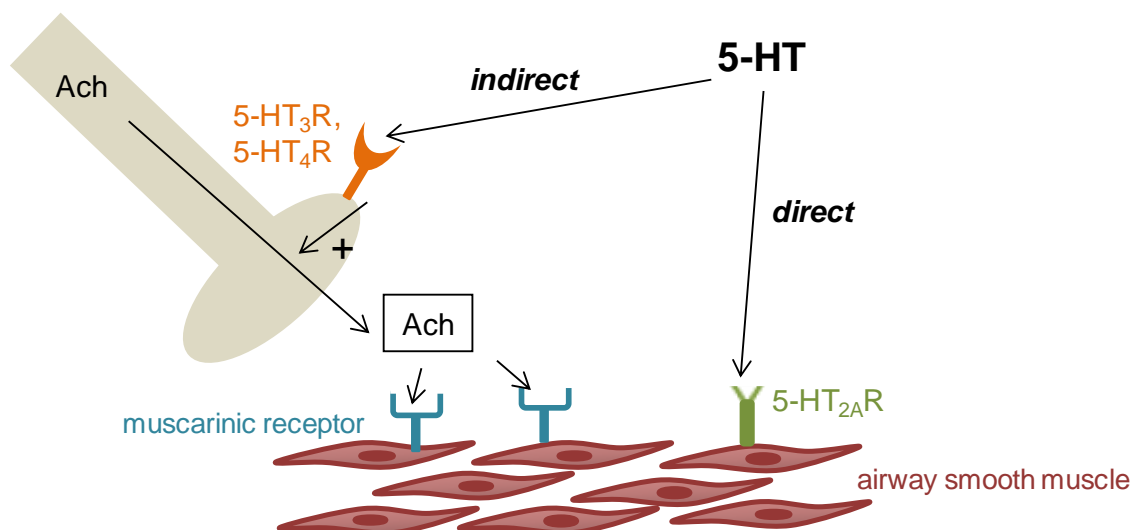


Figure 12. Potential pathways of 5-HT-induced contraction, as suggested in previous studies^{125,126}. 5-HT can directly stimulate 5-HT_{2A}R on airway smooth muscle cells. However, in humans, 5-HT₃R and 5-HT₄R¹²⁵ on parasympathetic cholinergic nerves can also be stimulated by 5-HT, leading to release of acetylcholine (Ach) and the indirect induction of bronchoconstriction.

Chapter 4. Soluble guanylyl cyclase §

4.1 Nitric oxide/soluble guanylyl cyclase(sGC)/cGMP pathway

Nitric oxide (NO) is synthesized from the semi-essential amino acid L-arginine. This reaction is catalyzed by nitric oxide synthases (NOS) (**Figure 13**). The endothelial NOS (eNOS) and neuronal NOS (nNOS) are constitutive isoforms, present in respectively endothelial cells and neuronal cells of the brain and peripheral nerves ¹³⁰. eNOS and nNOS are calcium dependent and induce the transient production of NO in response to various physiological stimuli. A third isoform, inducible NOS (iNOS), is upregulated by endotoxins or cytokines such as TNF- α , IFN- γ and IL-1 β ¹³¹. In the lungs, the main sources of NO under basal conditions are vascular endothelial cells, macrophages, airway nerves and airway epithelial cells; the main effector cells for NO are the vascular smooth muscle and the airway smooth muscle ^{132,133}.

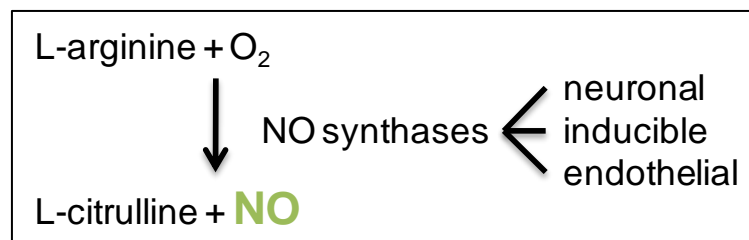


Figure 13: Synthesis of nitric oxide (NO)

Guanylyl cyclases (GCs), members of the family of nucleotide cyclizing enzymes, are widely distributed signal-transduction enzymes that catalyze the conversion of GTP to cGMP (**Figure 13**). Soluble GC (sGC) is a receptor for gaseous ligands (NO and CO) and is able to associate with the plasma membrane through protein-protein interactions in a Ca²⁺-dependent manner ¹³⁴. The downstream effects of the second messenger cGMP are mediated by 3 major types of intracellular effectors: cGMP-dependent protein kinases I and II, cGMP-gated ion channels and cGMP-regulated phosphodiesterases (PDEs) ^{135,136}. The degradation of cGMP is catalyzed by PDE families (including PDE5).

§ Based on 'Role of the nitric oxide - soluble guanylyl cyclase pathway in obstructive airway diseases' Dupont LL, Glynos C, Bracke KR, Brouckaert P, Brusselle GG. Pulm Pharmac Ther 2014.

sGC is a heterodimer, consisting of a larger α -subunit and a smaller β -subunit (**Figure 14**). There are 2 forms of the α -subunit (α_1 and α_2) and of the β -subunit (β_1 and β_2). $\alpha_1\beta_1$ and $\alpha_2\beta_1$ are equally present in the brain, while $\alpha_1\beta_1$ is the most prevalent form in other tissues such as the lung¹³⁷. Both forms have a similar catalytic rate and sensitivity towards NO. The β -subunit has an amino-terminal haem-binding domain and a prosthetic haem moiety, for sensing of NO (**Figure 14**). The haem moiety is a large heterocyclic organic ring with a central metal ion (Fe). sGC is activated by nanomolar concentrations of NO in the presence of the reduced Fe^{2+} (ferrous) haem moiety. Oxidized, Fe^{3+} (ferric) haem is insensitive to NO. Oxidation is induced by exogenous molecules, such as ODQ (1H-[1,2,4]oxadiazolo-[4, 3-a]quinoxalin-1-one), and by endogenous molecules, including reactive oxygen species (ROS) and reactive nitrogen species (RNS)¹³⁴. The highly proinflammatory molecule peroxynitrite (ONOO^-) is an example of RNS and can cause tissue injury in various organs. ROS are free radicals, such as superoxide ($\text{O}_2^{\cdot-}$) and hydrogen radical (HO^{\cdot}), which can cause cell dysfunction and cell death. Normally, they are counterbalanced by antioxidants and rapidly removed from the body. An imbalance between ROS/RNS and antioxidants leads to oxidative/nitrative stress¹³⁸⁻¹⁴⁰.

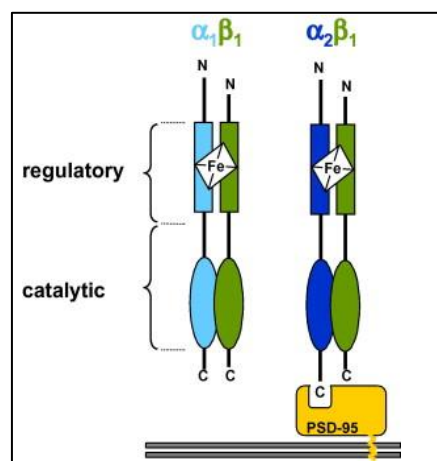


Figure 14: Structure of soluble guanylyl cyclase (sGC). Both sGC $\alpha_1\beta_1$ and sGC $\alpha_2\beta_1$ heterodimers contain an N-terminal regulatory heme group, and a catalytic domain in the C-terminal part. The α_2 subunit can be bound to the plasma membrane by interaction with proteins such as post-synaptic density protein 95 (PSD-95). Figure reprinted with permission from Friebe A and Koesling D. *Nitric Oxide* 2009¹⁴¹.

Both transmembrane and soluble forms of guanylyl cyclases exist. The transmembrane, particulate GC (pGC) acts as a receptor for atrial, brain (B-type) and C-type natriuretic peptides.

4.2 Soluble guanylyl cyclase in inflammation and disease

The NO/sGC/cGMP pathway is impaired in various diseases, and several steps in the pathway are potential therapeutic targets ¹³⁴. The reduced bioavailability and/or responsiveness to endogenously produced NO contributes to the development of pathologies such as cardiovascular, pulmonary, endothelial, renal and hepatic diseases and erectile dysfunction. NO-donors, including organic nitrates, release NO by spontaneous decomposition or bioconversion, thereby activating sGC. Although NO-donors can be beneficial in certain groups of patients, their use is limited because of the potential lack of response, the development of tolerance and the non-specific interactions of NO with biomolecules ¹⁴².

Two types of NO-independent drugs, that target sGC, may be more beneficial than NO-donors. **sGC stimulators** stimulate sGC directly and enhance the sensitivity of the reduced enzyme to low levels of bioavailable NO (**Figure 15**) ¹⁴³. While sGC stimulators are haem-dependent, **sGC activators** activate the NO-unresponsive, haem-oxidized or haem-free enzyme (**Figure 15**) ¹⁴⁴. BAY 58-2667 is the most potent sGC activator. It replaces the sGC oxidized, weakly bound prosthetic haem, leading to activation of the enzyme. Reduced haem is unresponsive to BAY 58-2667 ¹³⁴.

Under physiological conditions, sGC is thought to exist as a pool of reduced NO-sensitive sGC and oxidized or haem-free NO-insensitive sGC. In pathophysiological conditions associated with oxidative stress, such as heart failure, the pool of NO-insensitive sGC is increased ¹⁴⁵.

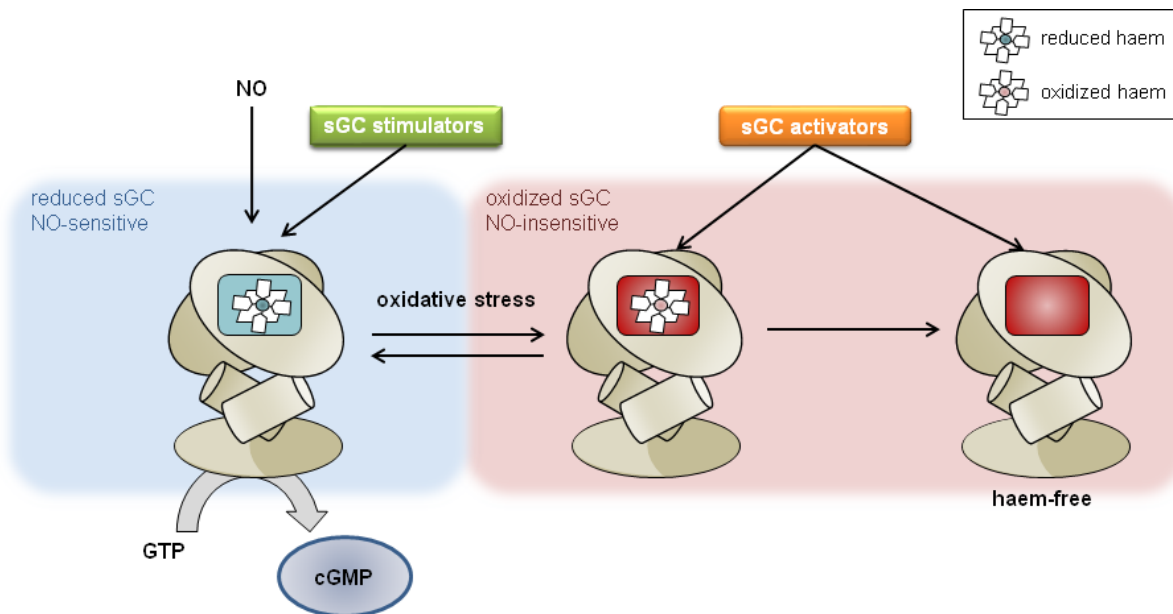


Figure 15: Soluble guanylyl cyclase (sGC). Under physiological conditions, there is a balance between the reduced, NO-sensitive sGC and the oxidized, NO-insensitive sGC. Oxidative stress and reactive oxygen species shift the balance to the oxidized form, resulting in an impaired sGC/cGMP pathway. The enzyme can even lose the haem group (haem-free sGC). sGC stimulators enhance the sensitivity of the reduced enzyme to low levels of bioavailable NO, while sGC activators activate the NO-unresponsive, haem-oxidized or haem-free enzyme.

In patients with **arterial hypertension**, the mRNA and protein levels of $\alpha 1$ and $\beta 1$ subunits and the activity of sGC are reduced. The efficacy of the sGC activator BAY 58-2667 is further enhanced by oxidative stress, and therefore it selectively targets diseased blood vessels ¹³⁴.

The selectivity of BAY 58-2667 was confirmed in a mouse model, where treatment with the sGC activator protects against lethal **endotoxic shock**, while the sGC stimulator BAY 41-2272 and the PDE-5 inhibitor sildenafil have no beneficial effect ¹⁴⁶.

To investigate the functional role of sGC, several genetic knockout mice were generated, which lack one of the three subunits ($\alpha 1$, $\alpha 2$ or $\beta 1$). Because sGC is a heterodimer and the α subunits are less stable in the absence of the β subunit, sGC $\beta 1$ KO mice have reduced or undetectable levels of the α subunits. Also the levels of the $\beta 1$ subunit are reduced in sGC $\alpha 1$ and sGC $\alpha 2$ KO mice. Male sGC $\alpha 1$ KO mice on a 129S6 background develop hypertension, while female mice showed no change in blood pressure. However, on a C57Bl/6 background, male sGC $\alpha 1$ KO mice do not develop hypertension, due to strain-dependent differences in renin genes ¹⁴⁷.

4.3 Soluble guanylyl cyclase in pulmonary hypertension

An impaired NO/sGC/cGMP pathway is involved in the pathogenesis of **pulmonary hypertension**. Pulmonary hypertension is classified in 5 main groups: 1) Pulmonary arterial hypertension, 2) pulmonary hypertension due to left heart disease, 3) pulmonary hypertension due to lung diseases (such as COPD) and/or hypoxia, 4) chronic thromboembolic pulmonary hypertension and 5) pulmonary hypertension with unclear multifactorial mechanisms. One of the main treatments of pulmonary arterial hypertension (group 1), are PDE-5 inhibitors. By inhibiting the degradation of cGMP, PDE-5 inhibitors cause pulmonary arterial vasodilation. However, treating patients with COPD-associated pulmonary hypertension (group 3) with the short-acting PDE-5 inhibitor sildenafil or the long-acting PDE-5 inhibitor tadalafil did not improve exercise capacity or quality of life ^{148,149}. Other studies have analyzed the effect of the sGC stimulator riociguat as a therapy for chronic thromboembolic pulmonary hypertension (group 4) and pulmonary arterial hypertension (group 1). In both patient groups, the exercise capacity was significantly improved after treatment with riociguat ¹⁵⁰⁻¹⁵². sGC stimulators and activators are also effective when endogenous NO is depleted, have limited off-target effects and remain efficacious with prolonged use ¹³⁴. In a murine model of chronic hypoxia, both the sGC activator BAY58-2667 and the sGC stimulator BAY41-2272 significantly decreased pulmonary hypertension. However, a 10 times higher dose of the sGC stimulator was needed to obtain similar effects as the sGC activator ¹⁵³.

4.4 Soluble guanylyl cyclase in airway diseases

The highest content of sGC is found in the lungs ¹³⁷ and is therefore implicated in respiratory diseases, such as asthma.

Patients with **asthma** have an elevated airway tone, despite the presence of large amounts of NO in the airways that could activate sGC and cause relaxation of the smooth muscle. In a murine model of allergic asthma, the levels of sGC α 1, α 2 and β 1 are reduced in the lungs, both on mRNA and protein level. Mice treated with the selective sGC inhibitor ODQ have an

increased airway reactivity to methacholine compared to naïve mice ¹⁵⁴. This finding suggests that sGC could be inhibited in patients with asthma, leading to the observed airway hyperresponsiveness.

Also in patients with **COPD**, the lungs contain ample amounts of NO, but the airway tone remains elevated. We have investigated the role of sGC in airway hyperresponsiveness in COPD (see **Chapter 8**) (**Figure 16**).

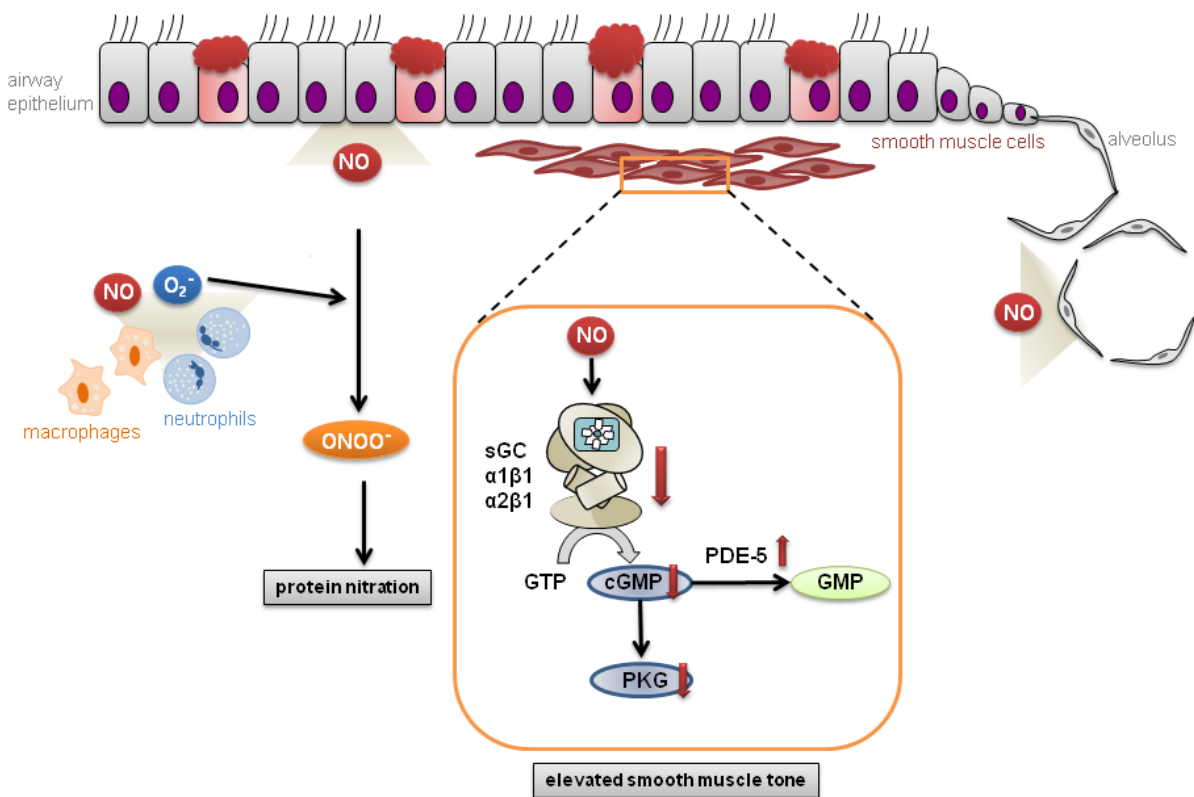


Figure 16: The NO/sGC/cGMP signaling pathway in obstructive airway diseases. Bronchial and alveolar epithelial cells produce NO, which activates sGC under normal conditions. However, decreased sGC levels in COPD and asthma lead to an impaired downstream pathway; while upregulated PDE₅ levels further decrease cGMP levels. Activated inflammatory cells such as macrophages and neutrophils also release NO and reactive oxygen species such as O₂⁻. NO and O₂⁻ form ONOO⁻, leading to protein nitration.

NO: nitric oxide, sGC: soluble guanylyl cyclase, GTP: guanosine triphosphate, cGMP: cyclic guanosine monophosphate, PDE₅: phosphodiesterase 5, PKG: protein kinase G, O₂⁻: superoxide, ONOO⁻: peroxynitrite.

REFERENCES

1. Bousquet J, Kiley J, Bateman ED, Viegi G, Cruz AA, Khaltayev N, Ait Khaled N, Baena-Cagnani CE, Barreto ML, Billo N, Canonica GW, Carlsen KH, Chavannes N, Chuchalin A, Drazen J, Fabbri LM, Gerbase MW, Humbert M, Joos G, Masjedi MR, Makino S, Rabe K, To T and Zhi L. Prioritised research agenda for prevention and control of chronic respiratory diseases. *Eur Respir J* 2010; 36(5):995-1001.
2. The top 10 causes of death. <http://who.int/mediacentre/factsheets>, 2013.
3. Salvi SS and Barnes PJ. Chronic obstructive pulmonary disease in non-smokers. *Lancet* 2009; 374(9691):733-743.
4. Global Initiative for Chronic Obstructive Lung Disease. Global Strategy for the Diagnosis, Management, and Prevention of Chronic Obstructive Pulmonary Disease. <http://www.goldcopd.com>, 2013.
5. Hogg JC, Chu F, Utokaparch S, Woods R, Elliott WM, Buzatu L, Cherniack RM, Rogers RM, Sciurba FC, Coxson HO and Pare PD. The nature of small-airway obstruction in chronic obstructive pulmonary disease. *N Engl J Med* 2004; 350:2645 - 2653.
6. Decramer M, Janssens W and Miravittles M. Chronic obstructive pulmonary disease. *Lancet* 2012; 379(9823):1341-1351.
7. Pauwels RA and Rabe KF. Burden and clinical features of chronic obstructive pulmonary disease (COPD). *Lancet* 2004; 364(9434):613-620.
8. Barnes PJ and Celli BR. Systemic manifestations and comorbidities of COPD. *Eur Respir J* 2009; 33(5):1165-1185.
9. Hurst JR, Vestbo J, Anzueto A, Locantore N, Mullerova H, Tal-Singer R, Miller B, Lomas DA, Agusti A, Macnee W, Calverley P, Rennard S, Wouters EF and Wedzicha JA. Susceptibility to exacerbation in chronic obstructive pulmonary disease. *N Engl J Med* 2010; 363(12):1128-1138.
10. Agusti AG, Noguera A, Sauleda J, Sala E, Pons J and Busquets X. Systemic effects of chronic obstructive pulmonary disease. *Eur Respir J* 2003; 21:347 - 360.
11. Schermuly RT, Pullamsetti SS, Kwapiszewska G, Dumitrescu R, Tian X, Weissmann N, Ghofrani HA, Kaulen C, Dunkern T, Schudt C, Voswinckel R, Zhou J, Samidurai A, Klepetko W, Paddenberg R, Kummer W, Seeger W and Grimminger F. Phosphodiesterase 1 upregulation in pulmonary arterial hypertension: target for reverse-remodeling therapy. *Circulation* 2007; 115(17):2331-2339.
12. Barnes PJ. New anti-inflammatory targets for chronic obstructive pulmonary disease. *Nat Rev Drug Discov* 2013; 12(7):543-559.
13. Siafakas NM and Tzortzaki EG. Few smokers develop COPD. Why? *Respir Med* 2002; 96(8):615-624.
14. Silverman EK, Spira A and Pare PD. Genetics and genomics of chronic obstructive pulmonary disease. *Proc Am Thorac Soc* 2009; 6(6):539-542.
15. Hirschhorn JN and Daly MJ. Genome-wide association studies for common diseases and complex traits. *Nat Rev Genet* 2005; 6(2):95-108.
16. Pearson TA and Manolio TA. How to interpret a genome-wide association study. *JAMA* 2008; 299(11):1335-1344.
17. Castaldi PJ, Cho MH, Litonjua AA, Bakke P, Gulsvik A, Lomas DA, Anderson W, Beaty TH, Hokanson JE, Crapo JD, Laird N, Silverman EK, Copdgene and Eclipse I. The association of genome-wide significant spirometric loci with chronic obstructive pulmonary disease susceptibility. *Am J Respir Cell Mol Biol* 2011; 45(6):1147-1153.
18. Hancock DB, Eijgelsheim M, Wilk JB, Gharib SA, Loehr LR, Marciante KD, Franceschini N, van Durme YM, Chen TH, Barr RG, Schabath MB, Couper DJ, Brusselle GG, Psaty BM, van Duijn CM, Rotter JI, Uitterlinden AG, Hofman A, Punjabi NM, Rivadeneira F, Morrison AC, Enright PL, North KE, Heckbert SR, Lumley T, Stricker BH, O'Connor GT and London SJ. Meta-analyses of genome-wide association studies identify multiple loci associated with pulmonary function. *Nat Genet* 2010; 42(1):45-52.
19. Repapi E, Sayers I, Wain LV, Burton PR, Johnson T, Obeidat M, Zhao JH, Ramasamy A, Zhai G, Vitart V, Huffman JE, Igl W, Albrecht E, Deloukas P, Henderson J, Granel R, McArdle WL, Rudnicka AR, Barroso I, Loos RJ, Wareham NJ, Mustelin L, Rantanen T, Surakka I, Imboden M, Wichmann HE, Grkovic I, Jankovic S, Zgaga L, Hartikainen AL, Peltonen L, Gyllenstein U, Johansson A, Zaboli G, Campbell H, Wild SH, Wilson JF, Glaser S, Homuth G, Volzke H, Mangino M, Soranzo N, Spector TD, Polasek O, Rudan I, Wright AF, Heliovaara M, Ripatti S, Pouta A, Naluai AT, Olin AC, Toren K, Cooper MN, James AL, Palmer LJ, Hingorani AD, Wannamethee SG, Whincup PH, Smith GD, Ebrahim S, McKeever TM, Pavord ID, Macleod

- AK, Morris AD, Porteous DJ, Cooper C, Dennison E, Shaheen S, Karrasch S, Schnabel E, Schulz H, Grallert H, Bouatia-Naji N, Delplanque J, Froguel P, Blakey JD, Britton JR, Morris RW, Holloway JW, Lawlor DA, Hui J, Nyberg F, Jarvelin MR, Jackson C, Kahonen M, Kaprio J, Probst-Hensch NM, Koch B, Hayward C, Evans DM, Elliott P, Strachan DP, Hall IP and Tobin MD. Genome-wide association study identifies five loci associated with lung function. *Nat Genet* 2009.
20. Wilk JB, Chen TH, Gottlieb DJ, Walter RE, Nagle MW, Brandler BJ, Myers RH, Borecki IB, Silverman EK, Weiss ST and O'Connor GT. A genome-wide association study of pulmonary function measures in the Framingham Heart Study. *PLoS Genet* 2009; 5(3):e1000429.
 21. Barnes PJ. Immunology of asthma and chronic obstructive pulmonary disease. *Nat Rev Immunol* 2008; 8(3):183-192.
 22. Brusselle GG, Joos GF and Bracke KR. New insights into the immunology of chronic obstructive pulmonary disease. *Lancet* 2011; 378(9795):1015-1026.
 23. Cosio MG, Saetta M and Agusti A. Immunologic aspects of chronic obstructive pulmonary disease. *N Engl J Med* 2009; 360(23):2445-2454.
 24. Stampfli MR and Anderson GP. How cigarette smoke skews immune responses to promote infection, lung disease and cancer. *Nat Rev Immunol* 2009; 9(5):377-384.
 25. Chung KF. Cytokines in chronic obstructive pulmonary disease. *Eur Respir J Suppl* 2001; 34:50s - 59s.
 26. Hansel TT and Barnes PJ. New drugs for exacerbations of chronic obstructive pulmonary disease. *Lancet* 2009; 374(9691):744-755.
 27. Van Pottelberge GR, Bracke KR, Joos GF and Brusselle GG. The role of dendritic cells in the pathogenesis of COPD: liaison officers in the front line. *COPD* 2009; 6(4):284-290.
 28. Vermaelen KY, Carro-Muino I, Lambrecht BN and Pauwels RA. Specific migratory dendritic cells rapidly transport antigen from the airways to the thoracic lymph nodes. *J Exp Med* 2001; 193(1):51-60.
 29. Lee SH, Goswami S, Grudo A, Song LZ, Bandi V, Goodnight-White S, Green L, Hacken-Bitar J, Huh J, Bakaeen F, Coxson HO, Cogswell S, Storness-Bliss C, Corry DB and Kheradmand F. Antiasthma autoimmunity in tobacco smoking-induced emphysema. *Nat Med* 2007; 13(5):567-569.
 30. Feghali-Bostwick CA, Gadgil AS, Otterbein LE, Pilewski JM, Stoner MW, Csizmadia E, Zhang Y, Scirba FC and Duncan SR. Autoantibodies in patients with chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 2008; 177(2):156-163.
 31. Brusselle GG, Demoor T, Bracke KR, Brandsma CA and Timens W. Lymphoid follicles in (very) severe COPD: beneficial or harmful? *Eur Respir J* 2009; 34(1):219-230.
 32. Demoor T, Bracke KR, Vermaelen KY, Dupont L, Joos GF and Brusselle GG. CCR7 modulates pulmonary and lymph node inflammatory responses in cigarette smoke-exposed mice. *J Immunol* 2009; 183(12):8186-8194.
 33. Bracke KR, Verhamme FM, Seys LJ, Bantsimba-Malanda C, Cunoosamy DM, Herbst R, Hammad H, Lambrecht BN, Joos GF and Brusselle GG. Role of CXCL13 in cigarette smoke-induced lymphoid follicle formation and chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 2013; 188(3):343-355.
 34. Venkatachalam K and Montell C (2007). TRP channels. *Annual Review of Biochemistry*:387-417.
 35. Moran MM, McAlexander MA, Bíró T and Szallasi A. Transient receptor potential channels as therapeutic targets. *Nat Rev Drug Discov* 2011; 10(8):601-620.
 36. Bevan S and Szolcsanyi J. Sensory Neuron-Specific Actions of Capsaicin - Mechanisms and Applications. *Trends Pharmacol Sci* 1990; 11(8):330-333.
 37. Andersson KE, Gratzke C and Hedlund P. The role of the transient receptor potential (TRP) superfamily of cation-selective channels in the management of the overactive bladder. *BJU international* 2010; 106(8):1114-1127.
 38. Bodo E, Kovacs I, Telek A, Varga A, Paus R, Kovacs L and Biro T. Vanilloid receptor-1 (VR1) is widely expressed on various epithelial and mesenchymal cell types of human skin. *J Invest Dermatol* 2004; 123(2):410-413.
 39. Kida N, Sokabe T, Kashio M, Haruna K, Mizuno Y, Suga Y, Nishikawa K, Kanamaru A, Hongo M, Oba A and Tominaga M. Importance of transient receptor potential vanilloid 4 (TRPV4) in epidermal barrier function in human skin keratinocytes. *Pflugers Arch - Eur J Physiol* 2012; 463(5):715-725.
 40. Suri A and Szallasi A. The emerging role of TRPV1 in diabetes and obesity. *Trends Pharmacol Sci* 2008; 29(1):29-36.

41. Mochizuki T, Wu GQ, Hayashi T, Xenophontos SL, Veldhuisen B, Saris JJ, Reynolds DM, Cai YQ, Gabow PA, Pierides A, Kimberling WJ, Breuning MH, Deltas CC, Peters DJM and Somlo S. PKD2, a gene for polycystic kidney disease that encodes an integral membrane protein. *Science* 1996; 272(5266):1339-1342.
42. Dong XP, Cheng XP, Mills E, Delling M, Wang FD, Kurz T and Xu HX. The type IV mucopolipidosis-associated protein TRPML1 is an endolysosomal iron release channel. *Nature* 2008; 455(7215):992-U978.
43. Reiser J, Polu KR, Moller CC, Kenlan P, Altintas MM, Wei C, Faul C, Herbert S, Villegas I, Avila-Casado C, McGee M, Sugimoto H, Brown D, Kalluri R, Mundel P, Smith PL, Clapham DE and Pollak MR. TRPC6 is a glomerular slit diaphragm-associated channel required for normal renal function. *Nat Genet* 2005; 37(7):739-744.
44. Walder RY, Landau D, Meyer P, Shalev H, Tsolia M, Borochowitz Z, Boettger MB, Beck GE, Englehardt RK, Carmi R and Sheffield VC. Mutation of TRPM6 causes familial hypomagnesemia with secondary hypocalcemia. *Nat Genet* 2002; 31(2):171-174.
45. Macpherson LJ, Dubin AE, Evans MJ, Marr F, Schultz PG, Cravatt BF and Patapoutian A. Noxious compounds activate TRPA1 ion channels through covalent modification of cysteines. *Nature* 2007; 445(7127):541-545.
46. Bandell M, Story GM, Hwang SW, Viswanath V, Eid SR, Petrus MJ, Earley TJ and Patapoutian A. Noxious Cold Ion Channel TRPA1 Is Activated by Pungent Compounds and Bradykinin. *Neuron* 2004; 41(6):849-857.
47. Bautista DM, Jordt SE, Nikai T, Tsuruda PR, Read AJ, Poblete J, Yamoah EN, Basbaum AI and Julius D. TRPA1 mediates the inflammatory actions of environmental irritants and proalgesic agents. *Cell* 2006; 124(6):1269-1282.
48. Engel MA, Leffler A, Niedermirtl F, Babes A, Zimmermann K, Filipović MR, Izydorczyk I, Eberhardt M, Kichko TI, Mueller-Tribbensee SM, Khalil M, Siklosi N, Nau C, Ivanović-Burmazović I, Neuhuber WL, Becker C, Neurath MF and Reeh PW. TRPA1 and Substance P Mediate Colitis in Mice. *Gastroenterology* 2011; 141(4):1346-1358.
49. Grace MS, Dubuis E, Birrell MA and Belvisi MG. Pre-clinical studies in cough research: role of Transient Receptor Potential (TRP) channels. *Pulm Pharmacol Ther* 2013; 26(5):498-507.
50. Simon SA and Liedtke W. How irritating: the role of TRPA1 in sensing cigarette smoke and aerogenic oxidants in the airways. *J Clin Invest* 2008; 118(7):2383-2386.
51. Carr MJ and Undem BJ. Bronchopulmonary afferent nerves. *Respirology* 2003; 8(3):291-301.
52. (2002). In: Barnes PJ, Drazen JM, Rennard S, Thomson NC, editors. *Asthma and COPD Basic Mechanisms and Clinical Management*.
53. Joos GF, De Swert KO, Schelfhout V and Pauwels RA. The role of neural inflammation in asthma and chronic obstructive pulmonary disease. *Ann N Y Acad Sci* 2003; 992:218-230.
54. Geppetti P, Nassini R, Materazzi S and Benemei S. The concept of neurogenic inflammation. *BJU international* 2008; 101:2-6.
55. Chiu IM, von Hehn CA and Woolf CJ. Neurogenic inflammation and the peripheral nervous system in host defense and immunopathology. *Nat Neurosci* 2012; 15(8):1063-1067.
56. Banner KH, Igney F and Poll C. TRP channels: emerging targets for respiratory disease. *Pharmacol Ther* 2011; 130(3):371-384.
57. Nassini R, Pedretti P, Moretto N, Fusi C, Carnini C, Facchinetti F, Viscomi AR, Pisano AR, Stokesberry S, Brunmark C, Svitacheva N, McGarvey L, Patacchini R, Damholt AB, Geppetti P and Materazzi S. Transient Receptor Potential Ankyrin 1 Channel Localized to Non-Neuronal Airway Cells Promotes Non-Neurogenic Inflammation. *PLoS One* 2012; 7(8):e42454.
58. McGarvey LP, Butler CA, Stokesberry S, Polley L, McQuaid S, Abdullah H, Ashraf S, McGahon MK, Curtis TM, Arron J, Choy D, Warke TJ, Bradding P, Ennis M, Zholos A, Costello RW and Heaney LG. Increased expression of bronchial epithelial transient receptor potential vanilloid 1 channels in patients with severe asthma. *J Allergy Clin Immunol* 2014; 133(3):704-712 e704.
59. Jia Y, Wang X, Varty L, Rizzo CA, Yang R, Correll CC, Phelps PT, Egan RW and Hey JA. Functional TRPV4 channels are expressed in human airway smooth muscle cells. *Am J Physiol Lung Cell Mol Physiol* 2004; 287(2):L272-278.
60. Buch TRH, Schafer EAM, Demmel MT, Boekhoff I, Thiermann H, Gudermann T, Steinritz D and Schmidt A. Functional expression of the transient receptor potential channel TRPA1, a sensor for toxic lung inhalants, in pulmonary epithelial cells. *Chem-Biol Interact* 2013; 206(3):462-471.

61. Delany NS, Hurlle M, Facer P, Alnadaf T, Plumpton C, Kinghorn I, See CG, Costigan M, Anand P, Woolf CJ, Crowther D, Sanseau P and Tate SN. Identification and characterization of a novel human vanilloid receptor-like protein, VRL-2. *Physiol Genomics* 2001; 4(3):165-174.
62. Van Gerven L, Alpizar YA, Wouters MM, Hox V, Hauben E, Jorissen M, Boeckstaens G, Talavera K and Hellings PW. Capsaicin treatment reduces nasal hyperreactivity and transient receptor potential cation channel subfamily V, receptor 1 (TRPV1) overexpression in patients with idiopathic rhinitis. *J Allergy Clin Immunol* 2014; 133(5):1332-1339, 1339 e1331-1333.
63. Zhu G, Gulsvik A, Bakke P, Ghatta S, Anderson W, Lomas DA, Silverman EK and Pillai SG. Association of TRPV4 gene polymorphisms with chronic obstructive pulmonary disease. *Hum Mol Genet* 2009; 18(11):2053-2062.
64. Alenmyr L, Uller L, Greiff L, Högestätt ED and Zygmunt PM. TRPV4-Mediated Calcium Influx and Ciliary Activity in Human Native Airway Epithelial Cells. *Basic Clin Pharmacol Toxicol* 2014; 114(2):210-216.
65. Li JJ, Kanju P, Patterson M, Chew WL, Cho SH, Gilmour I, Oliver T, Yasuda R, Ghio A, Simon SA and Liedtke W. TRPV4-Mediated Calcium Influx into Human Bronchial Epithelia upon Exposure to Diesel Exhaust Particles. *Environ Health Persp* 2011; 119(6):784-793.
66. Hamanaka K, Jian M-Y, Townsley MI, King JA, Liedtke W, Weber DS, Eyal FG, Clapp MM and Parker JC. TRPV4 channels augment macrophage activation and ventilator-induced lung injury. Hamanaka K, Jian M-Y, Townsley MI, King JA, Liedtke W, Weber DS, et al., editors 2010.
67. Sabnis AS, Reilly CA, Veranth JM and Yost GS. Increased transcription of cytokine genes in human lung epithelial cells through activation of a TRPM8 variant by cold temperatures. *Am J Physiol Lung Cell Mol Physiol* 2008; 295(1):L194-L200.
68. Xing H, Ling JX, Chen M, Johnson RD, Tominaga M, Wang CY and Gu JG. TRPM8 mechanism of autonomic nerve response to cold in respiratory airway. *Molecular Pain* 2008; 4.
69. Andre E, Campi B, Materazzi S, Trevisani M, Amadesi S, Massi D, Creminon C, Vaksman N, Nassini R, Civelli M, Baraldi PG, Poole DP, Bunnett NW, Geppetti P and Patacchini R. Cigarette smoke-induced neurogenic inflammation is mediated by alpha,beta-unsaturated aldehydes and the TRPA1 receptor in rodents. *J Clin Invest* 2008; 118(7):2574-2582.
70. Bessac BF, Sivula M, von Hehn CA, Escalera J, Cohn L and Jordt SE. TRPA1 is a major oxidant sensor in murine airway sensory neurons. *J Clin Invest* 2008; 118(5):1899-1910.
71. Talavera K, Gees M, Karashima Y, Meseguer VM, Vanoirbeek JA, Damann N, Everaerts W, Benoit M, Janssens A, Vennekens R, Viana F, Nemery B, Nilius B and Voets T. Nicotine activates the chemosensory cation channel TRPA1. *Nat Neurosci* 2009; 12(10):1293-1299.
72. Meseguer V, Alpizar YA, Luis E, Tajada S, Denlinger B, Fajardo O, Maneschijn JA, Hernandez-Pena C, Talavera A, Kichko T, Navia B, Sanchez A, Senaris R, Reeh P, Perez-Garcia MT, Lopez-Lopez JR, Voets T, Belmonte C, Talavera K and Viana F. TRPA1 channels mediate acute neurogenic inflammation and pain produced by bacterial endotoxins. *Nat Commun* 2014; 5:3125.
73. Caceres AI, Brackmann M, Elia MD, Bessac BF, del Camino D, D'Amours M, Witek JS, Fanger CM, Chong JA, Hayward NJ, Homer RJ, Cohn L, Huang X, Moran MM and Jordt SE. A sensory neuronal ion channel essential for airway inflammation and hyperreactivity in asthma. *Proc Natl Acad Sci U S A* 2009; 106(22):9099-9104.
74. Lucki I. The spectrum of behaviors influenced by serotonin. *Biol Psychiat* 1998; 44(3):151-162.
75. Bellivier F, Leboyer M, Courtet P and et al. Association between the tryptophan hydroxylase gene and manic-depressive illness. *Archives of General Psychiatry* 1998; 55(1):33-37.
76. Berger M, Gray JA and Roth BL. The expanded biology of serotonin. *Annu Rev Med* 2009; 60:355-366.
77. Lauweryns JM, Van Ranst L and Verhofstad AA. Ultrastructural localization of serotonin in the intrapulmonary neuroepithelial bodies of neonatal rabbits by use of immunoelectron microscopy. *Cell Tissue Res* 1986; 243(3):455-459.
78. Weigand LA, Myers AC, Meeker S and Udem BJ. Mast cell-cholinergic nerve interaction in mouse airways. *J Physiol* 2009; 587(Pt 13):3355-3362.
79. Sharman JL, Benson HE, Pawson AJ, Lukito V, Mpamhanga CP, Bombail V, Davenport AP, Peters JA, Spedding M, Harmar AJ and NC-IUPHAR. IUPHAR-DB: updated database content and new features. *Nucleic Acids Res* 2013; 41(Database Issue):D1083-1088.
80. Hoyer D, Hannon JP and Martin GR. Molecular, pharmacological and functional diversity of 5-HT receptors. *Pharmacology, biochemistry, and behavior* 2002; 71(4):533-554.
81. Richter DW, Manzke T, Wilken B and Ponimaskin E. Serotonin receptors: guardians of stable breathing. *Trends Mol Med* 2003; 9(12):542-548.

82. Dorsam RT and Gutkind JS. G-protein-coupled receptors and cancer. *Nat Rev Cancer* 2007; 7(2):79-94.
83. Parks CL, Robinson PS, Sibille E, Shenk T and Toth M. Increased anxiety of mice lacking the serotonin_{1A} receptor. *Proc Natl Acad Sci U S A* 1998; 95(18):10734-10739.
84. Rickels K, Mathew S, Banov MD, Zimbroff DL, Oshana S, Parsons EC, Donahue SR, Kauffman M, Iyer GR and Reinhard JF. Effects of PRX-00023, a novel, selective serotonin 1A receptor agonist on measures of anxiety and depression in generalized anxiety disorder - Results of a double-blind, placebo-controlled trial. *J Clin Psychopharmacol* 2008; 28(2):235-239.
85. Beneytez MaE, López Rodríguez MaL, Rosado MaL, Morcillo MaJ, Orensanz L, Fuentes JA and Manzanares J. Preclinical pharmacology of B-20991, a 5-HT_{1A} receptor agonist with anxiolytic activity. *Eur J Pharmacol* 1998; 344(2-3):127-135.
86. Wilken B, Lalley P, Bischoff AM, Christen HJ, Behnke J, Hanefeld F and Richter DW. Treatment of apneustic respiratory disturbance with a serotonin-receptor agonist. *J Pediatr-US* 1997; 130(1):89-94.
87. Lalley PM, Bischoff A-M and Richter DW. Serotonin 1A-receptor activation suppresses respiratory apneusis in the cat. *Neurosci Lett* 1994; 172(1-2):59-62.
88. Cohen ML, Fuller RW and Wiley KS. Evidence for 5-HT₂ receptors mediating contraction in vascular smooth muscle. *J Pharmacol Exp Ther* 1981; 218(2):421-425.
89. Rothman RB, Baumann MH, Savage JE, Rauser L, McBride A, Hufeisen SJ and Roth BL. Evidence for Possible Involvement of 5-HT_{2B} Receptors in the Cardiac Valvulopathy Associated With Fenfluramine and Other Serotonergic Medications. *Circulation* 2000; 102(23):2836-2841.
90. Kim SW, Shin IS, Kim JM, Youn T, Yang SJ, Hwang MY and Yoon JS. The 5-HT₂ receptor profiles of antipsychotics in the pathogenesis of obsessive-compulsive symptoms in schizophrenia. *Clin Neuropharmacol* 2009; 32(4):224-226.
91. Naylor RJ and Rudd JA. Emesis and anti-emesis. *Cancer surveys* 1994; 21:117-135.
92. Rudd JA and Naylor RJ. Effects of 5-HT₃ receptor antagonists on models of acute and delayed emesis induced by cisplatin in the ferret. *Neuropharmacology* 1994; 33(12):1607-1608.
93. Spiller RC. Targeting the 5-HT₃ receptor in the treatment of irritable bowel syndrome. *Curr Opin Pharmacol* 2011; 11(1):68-74.
94. Pytliak M, Vargova V, Mechirova V and Felsoci M. Serotonin receptors - from molecular biology to clinical applications. *Physiological research / Academia Scientiarum Bohemoslovaca* 2011; 60(1):15-25.
95. Vanhoenacker P, Haegeman G and Leysen JE. 5-HT₇ receptors: current knowledge and future prospects. *Trends Pharmacol Sci* 2000; 21(2):70-77.
96. Kim JJ, Bridle BW, Ghia J-E, Wang H, Syed SN, Manocha MM, Rengasamy P, Shajib MS, Wan Y, Hedlund PB and Khan WI. Targeted Inhibition of Serotonin Type 7 (5-HT₇) Receptor Function Modulates Immune Responses and Reduces the Severity of Intestinal Inflammation. *J Immunol* 2013; 190(9):4795-4804.
97. Bockaert J, Claeysen S, Compan V and Dumuis A. 5-HT₄ receptors. *Curr Drug Targets CNS Neurol Disord* 2004; 3(1):39-51.
98. Lezoualc'h F and Robert SJ. The serotonin 5-HT₄ receptor and the amyloid precursor protein processing. *Exp Gerontol* 2003; 38(1-2):159-166.
99. Reynolds GP, Mason SL, Meldrum A, De Keczer S, Parnes H, Eglen RM and Wong EH. 5-Hydroxytryptamine (5-HT)₄ receptors in post mortem human brain tissue: distribution, pharmacology and effects of neurodegenerative diseases. *Br J Pharmacol* 1995; 114(5):993-998.
100. Brodney MA, Johnson DE, Sawant-Basak A, Coffman KJ, Drummond EM, Hudson EL, Fisher KE, Noguchi H, Waizumi N, McDowell LL, Papanikolaou A, Pettersen BA, Schmidt AW, Tseng E, Stutzman-Engwall K, Rubitski DM, Vanase-Frawley MA and Grimwood S. Identification of Multiple 5-HT₄ Partial Agonist Clinical Candidates for the Treatment of Alzheimer's Disease. *J Med Chem* 2012; 55(21):9240-9254.
101. Ohtsuki T, Ishiguro H, Detera-Wadleigh SD, Toyota T, Shimizu H, Yamada K, Yoshitsugu K, Hattori E, Yoshikawa T and Arinami T. Association between serotonin 4 receptor gene polymorphisms and bipolar disorder in Japanese case-control samples and the NIMH Genetics Initiative Bipolar Pedigrees. *Mol Psychiatry* 2002; 7(9):954-961.
102. Rosel P, Arranz B, Urretavizcaya M, Oros M, San L and Navarro MA. Altered 5-HT_{2A} and 5-HT₄ Postsynaptic Receptors and Their Intracellular Signalling Systems IP₃ and

- cAMP in Brains from Depressed Violent Suicide Victims. *Neuropsychobiology* 2004; 49(4):189-195.
103. Lucas G. Serotonin receptors, type 4: a new hope? *Current drug targets* 2009; 10(11):1085-1095.
104. Compan V, Zhou M, Grailhe R, Gazzara RA, Martin R, Gingrich J, Dumuis A, Brunner D, Bockaert J and Hen R. Attenuated response to stress and novelty and hypersensitivity to seizures in 5-HT₄ receptor knock-out mice. *J Neurosci* 2004; 24(2):412-419.
105. Bockaert J, Claeysen S, Compan V and Dumuis A. 5-HT₄ receptors, a place in the sun: act two. *Curr Opin Pharmacol* 2011; 11(1):87-93.
106. De Ponti F and Tonini M. Irritable bowel syndrome: new agents targeting serotonin receptor subtypes. *Drugs* 2001; 61(3):317-332.
107. De Maeyer JH, Lefebvre RA and Schuurkes JA. 5-HT₄ receptor agonists: similar but not the same. *Neurogastroenterol Motil* 2008; 20(2):99-112.
108. Wysowski DK, Corken A, Gallo-Torres H, Talarico L and Rodriguez EM. Postmarketing reports of QT prolongation and ventricular arrhythmia in association with cisapride and food and drug administration regulatory actions. *Am J Gastroenterol* 2001; 96(6):1698-1703.
109. Lechin F, van der Dijns B, Orozco B, Lechin M and Lechin AE. Increased levels of free serotonin in plasma of symptomatic asthmatic patients. *Ann Allergy Asthma Immunol* 1996; 77(3):245-253.
110. Cazzola M, D'Amato G, Lobefalo G, Guillaro B, Sepe J, Assogna G, Pietroletti R and Lauria D. Ketanserin, a new blocking agent of serotonin S₂-receptors. Respiratory functional effects in chronic obstruction of the airways. *Chest* 1987; 92(5):863-866.
111. Lechin F, van Der Dijns B, Orozco B, Jara H, Rada I, Lechin ME and Lechin AE. The serotonin uptake-enhancing drug tianeptine suppresses asthmatic symptoms in children: A double-blind, crossover, placebo-controlled study. *J Clin Pharmacol* 1998; 38(10):918-925.
112. Lau WKW, Chan-Yeung MMW, Yip BHK, Cheung AHK, Ip MSM, Mak JCW and the CSGotHKTS. The Role of Circulating Serotonin in the Development of Chronic Obstructive Pulmonary Disease. *PLoS One* 2012; 7(2):e31617.
113. Abdala-Valencia H, Berdnikovs S, McCary CA, Urick D, Mahadevia R, Marchese ME, Swartz K, Wright L, Mutlu GM and Cook-Mills JM. Inhibition of allergic inflammation by supplementation with 5-hydroxytryptophan. *Am J Physiol Lung Cell Mol Physiol* 2012; 303(8):L642-L660.
114. Chaouat A, Naeije R and Weitzenblum E. Pulmonary hypertension in COPD. *Eur Resp J* 2008; 32(5):1371-1385.
115. Keegan A, Morecroft I, Smillie D, Hicks MN and MacLean MR. Contribution of the 5-HT_{1B} Receptor to Hypoxia-Induced Pulmonary Hypertension: Converging Evidence Using 5-HT_{1B}-Receptor Knockout Mice and the 5-HT_{1B/1D}-Receptor Antagonist GR127935. *Circ Res* 2001; 89(12):1231-1239.
116. Launay JM, Herve P, Peoc'h K, Tournois C, Callebert J, Nebigil CG, Etienne N, Drouet L, Humbert M, Simonneau G and Maroteaux L. Function of the serotonin 5-hydroxytryptamine 2B receptor in pulmonary hypertension. *Nat Med* 2002; 8(10):1129-1135.
117. Bayer H, Muller T, Myrtek D, Sorichter S, Ziegenhagen M, Norgauer J, Zissel G and Idzko M. Serotonergic receptors on human airway epithelial cells. *Am J Respir Cell Mol Biol* 2007; 36(1):85-93.
118. Muller T, Durk T, Blumenthal B, Grimm M, Cicko S, Panther E, Sorichter S, Herouy Y, Di Virgilio F, Ferrari D, Norgauer J and Idzko M. 5-hydroxytryptamine modulates migration, cytokine and chemokine release and T-cell priming capacity of dendritic cells in vitro and in vivo. *PLoS One* 2009; 4(7):e6453.
119. Durk T, Panther E, Muller T, Sorichter S, Ferrari D, Pizzirani C, Di Virgilio F, Myrtek D, Norgauer J and Idzko M. 5-Hydroxytryptamine modulates cytokine and chemokine production in LPS-primed human monocytes via stimulation of different 5-HTR subtypes. *Int Immunol* 2005; 17(5):599-606.
120. Idzko M, Panther E, Stratz C, Muller T, Bayer H, Zissel G, Durk T, Sorichter S, Di Virgilio F, Geissler M, Fiebich B, Herouy Y, Elsner P, Norgauer J and Ferrari D. The serotonergic receptors of human dendritic cells: identification and coupling to cytokine release. *J Immunol* 2004; 172(10):6011-6019.
121. Repapi E, Sayers I, Wain LV, Burton PR, Johnson T, Obeidat Me, Zhao JH, Ramasamy A, Zhai G, Vitart V, Huffman JE, Igl W, Albrecht E, Deloukas P, Henderson J, Granell R, McArdle WL, Rudnicka AR, Barroso I, Loos RJF, Wareham NJ, Mustelin L, Rantanen T, Surakka I, Imboden M, Wichmann HE, Grkovic I, Jankovic S, Zgaga L, Hartikainen A-L, Peltonen L,

- Gyllensten U, Johansson A, Zaboli G, Campbell H, Wild SH, Wilson JF, Glaser S, Homuth G, Volzke H, Mangino M, Soranzo N, Spector TD, Polasek O, Rudan I, Wright AF, Heliövaara M, Ripatti S, Pouta A, Naluai AT, Olin A-C, Toren K, Cooper MN, James AL, Palmer LJ, Hingorani AD, Wannamethee SG, Whincup PH, Smith GD, Ebrahim S, McKeever TM, Pavord ID, MacLeod AK, Morris AD, Porteous DJ, Cooper C, Dennison E, Shaheen S, Karrasch S, Schnabel E, Schulz H, Grallert H, Bouatia-Naji N, Delplanque J, Froguel P, Blakey JD, Britton JR, Morris RW, Holloway JW, Lawlor DA, Hui J, Nyberg F, Jarvelin M-R, Jackson C, Kahonen M, Kaprio J, Probst-Hensch NM, Koch B, Hayward C, Evans DM, Elliott P, Strachan DP, Hall IP and Tobin MD. Genome-wide association study identifies five loci associated with lung function. *Nat Genet* 2010; 42(1):36-44.
122. Wilk JB, Shrine NR, Loehr LR, Zhao JH, Manichaikul A, Lopez LM, Smith AV, Heckbert SR, Smolonska J, Tang W, Loth DW, Curjuric I, Hui J, Cho MH, Latourelle JC, Henry AP, Aldrich M, Bakke P, Beaty TH, Bentley AR, Borecki IB, Brusselle GG, Burkart KM, Chen TH, Couper D, Crapo JD, Davies G, Dupuis J, Franceschini N, Gulsvik A, Hancock DB, Harris TB, Hofman A, Imboden M, James AL, Khaw KT, Lahousse L, Launer LJ, Litonjua A, Liu Y, Lohman KK, Lomas DA, Lumley T, Marciante KD, McArdle WL, Meibohm B, Morrison AC, Musk AW, Myers RH, North KE, Postma DS, Psaty BM, Rich SS, Rivadeneira F, RoCHAT T, Rotter JI, Artigas MS, Starr JM, Uitterlinden AG, Wareham NJ, Wijmenga C, Zanen P, Province MA, Silverman EK, Deary IJ, Palmer LJ, Cassano PA, Gudnason V, Barr RG, Loos RJ, Strachan DP, London SJ, Boezen HM, Probst-Hensch N, Gharib SA, Hall IP, O'Connor GT, Tobin MD and Stricker BH. Genome-wide association studies identify *CHRNA5/3* and *HTR4* in the development of airflow obstruction. *Am J Respir Crit Care Med* 2012; 186(7):622-632.
123. Soler Artigas M, Wain LV, Repapi E, Obeidat M, Sayers I, Burton PR, Johnson T, Zhao JH, Albrecht E, Dominiczak AF, Kerr SM, Smith BH, Cadby G, Hui J, Palmer LJ, Hingorani AD, Wannamethee SG, Whincup PH, Ebrahim S, Smith GD, Barroso I, Loos RJ, Wareham NJ, Cooper C, Dennison E, Shaheen SO, Liu JZ, Marchini J, Medical Research Council National Survey of H, Development Respiratory Study T, Dahgam S, Naluai AT, Olin AC, Karrasch S, Heinrich J, Schulz H, McKeever TM, Pavord ID, Heliövaara M, Ripatti S, Surakka I, Blakey JD, Kahonen M, Britton JR, Nyberg F, Holloway JW, Lawlor DA, Morris RW, James AL, Jackson CM, Hall IP, Tobin MD and SpiroMeta C. Effect of five genetic variants associated with lung function on the risk of chronic obstructive lung disease, and their joint effects on lung function. *Am J Respir Crit Care Med* 2011; 184(7):786-795.
124. Hodge E, Nelson C, Miller S, Billington C, Stewart C, Swan C, Malarstig A, Henry A, Gowland C, Melen E, Hall I and Sayers I. *HTR4* gene structure and altered expression in the developing lung. *Respir Res* 2013; 14(1):77.
125. Dupont LJ, Pype JL, Demedts MG, De Leyn P, Deneffe G and Verleden GM. The effects of 5-HT on cholinergic contraction in human airways in vitro. *Eur Respir J* 1999; 14(3):642-649.
126. Segura P, Vargas MH, Cordoba-Rodriguez G, Chavez J, Arreola JL, Campos-Bedolla P, Ruiz V, Garcia-Hernandez LM, Mendez C and Montano LM. Role of 5-HT(2A), 5-HT(4) and 5-HT(7) receptors in the antigen-induced airway hyperresponsiveness in guinea-pigs. *Clin Exp Allergy* 2009; 40:327-338.
127. Moore BD, Hyde DM, Miller LA, Wong EM and Schelegle ES. Persistence of Serotonergic Enhancement of Airway Response in a Model of Childhood Asthma. *Am J Resp Cell Mol* 2014.
128. Martin TR, Cohen ML and Drazen JM. Serotonin-induced pulmonary responses are mediated by the 5-HT₂ receptor in the mouse. *J Pharmacol Exp Ther* 1994; 268(1):104-109.
129. Cyphert JM, Kovarova M, Allen IC, Hartney JM, Murphy DL, Wess J and Koller BH. Cooperation between mast cells and neurons is essential for antigen-mediated bronchoconstriction. *J Immunol* 2009; 182(12):7430-7439.
130. Redington AE. Modulation of nitric oxide pathways: therapeutic potential in asthma and chronic obstructive pulmonary disease. *Eur J Pharmacol* 2006; 533(1-3):263-276.
131. Asano K, Chee CB, Gaston B, Lilly CM, Gerard C, Drazen JM and Stamler JS. Constitutive and inducible nitric oxide synthase gene expression, regulation, and activity in human lung epithelial cells. *Proc Natl Acad Sci U S A* 1994; 91(21):10089-10093.
132. Ricciardolo FLM. Multiple roles of nitric oxide in the airways. *Thorax* 2003; 58(2):175-182.
133. Barnes PJ and Belvisi MG. Nitric oxide and lung disease. *Thorax* 1993; 48(10):1034-1043.
134. Evgenov OV, Pacher P, Schmidt PM, Hasko G, Schmidt HH and Stasch JP. NO-independent stimulators and activators of soluble guanylate cyclase: discovery and therapeutic potential. *Nat Rev Drug Discov* 2006; 5(9):755-768.

135. Friebe A and Koesling D. Regulation of nitric oxide-sensitive guanylyl cyclase. *Circ Res* 2003; 93(2):96-105.
136. Francis SH, Busch JL, Corbin JD and Sibley D. cGMP-dependent protein kinases and cGMP phosphodiesterases in nitric oxide and cGMP action. *Pharmacol Rev* 2010; 62(3):525-563.
137. Mergia E, Russwurm M, Zoidl G and Koesling D. Major occurrence of the new alpha2beta1 isoform of NO-sensitive guanylyl cyclase in brain. *Cell Signal* 2003; 15(2):189-195.
138. Bargagli E, Olivieri C, Bennett D, Prasse A, Muller-Quernheim J and Rottoli P. Oxidative stress in the pathogenesis of diffuse lung diseases: a review. *Respir Med* 2009; 103(9):1245-1256.
139. Nathan C and Cunningham-Bussel A. Beyond oxidative stress: an immunologist's guide to reactive oxygen species. *Nat Rev Immunol* 2013; 13(5):349-361.
140. Kirkham PA and Barnes PJ. Oxidative stress in COPD. *Chest* 2013; 144(1):266-273.
141. Friebe A and Koesling D. The function of NO-sensitive guanylyl cyclase: what we can learn from genetic mouse models. *Nitric Oxide* 2009; 21(3-4):149-156.
142. Munzel T, Daiber A and Mulsch A. Explaining the phenomenon of nitrate tolerance. *Circ Res* 2005; 97(7):618-628.
143. Stasch JP and Hobbs AJ. NO-independent, haem-dependent soluble guanylate cyclase stimulators. *Handbook of experimental pharmacology* 2009; (191):277-308.
144. Schmidt HH, Schmidt PM and Stasch JP. NO- and haem-independent soluble guanylate cyclase activators. *Handbook of experimental pharmacology* 2009; (191):309-339.
145. Frey R, Mück W, Unger S, Artmeier-Brandt U, Weimann G and Wensing G. Pharmacokinetics, Pharmacodynamics, Tolerability, and Safety of the Soluble Guanylate Cyclase Activator Cinaciguat (BAY 58-2667) in Healthy Male Volunteers. *J Clin Pharmacol* 2008; 48(12):1400-1410.
146. Vandendriessche B, Rogge E, Goossens V, Vandenaabeele P, Stasch J-P, Brouckaert P and Cauwels A. The Soluble Guanylate Cyclase Activator BAY 58-2667 Protects against Morbidity and Mortality in Endotoxic Shock by Recoupling Organ Systems. *PLoS ONE* 2013; 8(8):e72155.
147. Buys ES, Raheer MJ, Kirby A, Shahid M, Baron DM, Hayton SR, Tainsh LT, Sips PY, Rauwerdink KM, Yan Q, Tainsh RE, Shakartzi HR, Stevens C, Decaluwe K, Rodrigues-Machado Mda G, Malhotra R, Van de Voorde J, Wang T, Brouckaert P, Daly MJ and Bloch KD. Genetic modifiers of hypertension in soluble guanylate cyclase alpha1-deficient mice. *J Clin Invest* 2012; 122(6):2316-2325.
148. Goudie AR, Lipworth BJ, Hopkinson PJ, Wei L and Struthers AD. Tadalafil in patients with chronic obstructive pulmonary disease: a randomised, double-blind, parallel-group, placebo-controlled trial. *Lancet Respir Med* 2014; 2(4):293-300.
149. Blanco I, Gimeno E, Munoz PA, Pizarro S, Gistau C, Rodriguez-Roisin R, Roca J and Barberà JA. Hemodynamic and Gas Exchange Effects of Sildenafil in Patients with Chronic Obstructive Pulmonary Disease and Pulmonary Hypertension. *Am J Resp Crit Care Med* 2010; 181(3):270-278.
150. Archer SL. Riociguat for pulmonary hypertension--a glass half full. *N Engl J Med* 2013; 369(4):386-388.
151. Ghofrani HA, D'Armini AM, Grimminger F, Hoepfer MM, Jansa P, Kim NH, Mayer E, Simonneau G, Wilkins MR, Fritsch A, Neuser D, Weimann G, Wang C and Group C-S. Riociguat for the treatment of chronic thromboembolic pulmonary hypertension. *N Engl J Med* 2013; 369(4):319-329.
152. Ghofrani HA, Galie N, Grimminger F, Grunig E, Humbert M, Jing ZC, Keogh AM, Langleben D, Kilama MO, Fritsch A, Neuser D, Rubin LJ and Group P-S. Riociguat for the treatment of pulmonary arterial hypertension. *N Engl J Med* 2013; 369(4):330-340.
153. Dumitrascu R, Weissmann N, Ghofrani HA, Dony E, Beuerlein K, Schmidt H, Stasch J-P, Gnoth MJ, Seeger W, Grimminger F and Schermuly RT. Activation of Soluble Guanylate Cyclase Reverses Experimental Pulmonary Hypertension and Vascular Remodeling. *Circulation* 2006; 113(2):286-295.
154. Papapetropoulos A, Simoes DC, Xanthou G, Roussos C and Gratziau C. Soluble guanylyl cyclase expression is reduced in allergic asthma. *Am J Physiol Lung Cell Mol Physiol* 2006; 290(1):L179-184.

PART II: RESEARCH WORK

Chapter 5. Rationale and aims of the thesis

Current therapy for COPD can only reduce the symptoms, but not the disease progression. Investigating the processes that are involved in the pathogenesis of COPD is important to identify new therapeutic targets.

Transient Receptor Potential (TRP) A1 can be activated by components of cigarette smoke (including nicotine and acrolein). Activation of TRPA1 on sensory nerves innervating the airways can lead to reflex coughing, mucus production, bronchoconstriction; and to a local release of neuropeptides, inducing neurogenic inflammation. **We hypothesized that TRPA1 plays a crucial role in the induction of the pulmonary inflammation after CS exposure.** We analyzed the expression of TRPA1 in murine ganglia containing the cell bodies of airway nerves and in human and murine lung tissue. To analyze the potential role of TRPA1 in CS-induced pulmonary inflammation, wild-type mice were treated with a specific TRPA1 inhibitor.

COPD arises from an interplay between environmental exposures (e.g. cigarette smoke) and genetic predisposition. Therefore, the study of gene-environment interactions is essential to elucidate the pathogenesis of COPD. Genome Wide Association studies have found associations between genetic variants in the gene encoding the human 5-hydroxytryptamine 4 receptor (5-HT₄R) and lung function, airflow obstruction and COPD. These associations suggest that 5-HT₄R could be implicated in the pathogenesis of COPD. 5-HT₄ receptors have already been implicated in the 5-HT-induced bronchoconstriction *in vitro* in human airways and *in vivo* in guinea pigs. **We hypothesized that the 5-HT₄R facilitates the cholinergic contraction of the airways, thereby contributing to the pathogenesis of COPD.** We have tested our hypothesis using genetic and pharmacologic approaches. Using specific antagonists, we analyzed the role of 5-HT₄R, 5-HT_{2A}R and muscarinic receptors in the bronchial responsiveness to 5-HT in CS-exposed mice (pharmacologic approach). The

bronchial responsiveness to 5-HT was also investigated in CS-exposed 5-HT₄R KO mice and their wild-type littermates (genetic approach).

In physiological conditions, the NO/soluble guanylyl cyclase (sGC)/cyclic guanosine monophosphate (cGMP) pathway has a relaxing effect on smooth muscle, leading to vasodilation and bronchodilation. However, although NO is present in the lungs of patients with COPD, the airway smooth muscle tone is elevated, implicating suboptimal activity of NO. Inflammatory stimuli, such as interleukin 1 β and reactive oxygen species (ROS), down-regulate the expression of sGC in animals models of lung injury and asthma. Therefore, **we hypothesized that the expression of sGC is downregulated in COPD.** We analyzed the expression level of sGC in smokers and patients with COPD; and in a murine model of CS exposure. The functional role of sGC was investigated in CS-exposed mice, using an activator of sGC (pharmacologic approach) and sGC α 1 KO mice (genetic approach).

Chapter 6. Translational research in COPD – Methods

In this dissertation, we have performed translational research combining functional *in vivo* studies in a murine model of COPD and *ex vivo* expression studies in humans. This chapter addresses all techniques used for the research work.

6.1 *Ex vivo*: Human lung tissue

The department of Respiratory Medicine of Ghent University Hospital has set up a tissue bank, consisting of lung tissue and induced sputum of never smokers, smokers without airflow obstruction and patients with COPD^{1,2}. The categories are made based on the GOLD classification strategy (see **Chapter 1**). Specimens of peripheral lung tissue are obtained from human subjects with solitary pulmonary tumors undergoing lung resection surgery in Ghent University Hospital. Lung tissue is taken at a maximal distance of the pulmonary lesion by a pathologist. All subjects provided written informed consent and were interviewed about their smoking habits and medication use. The studies are approved by the Medical Ethical Committee of the Ghent University Hospital.

A part of the lung tissue is snap-frozen in liquid nitrogen, and stored at -80°C for use in Western Blot. A second part is stored in an RNA Stabilization Reagent (Qiagen, Hilden, Germany), and used for RNA extraction and qRT-PCR. Thirdly, the lung is fixed in 4% paraformaldehyde, embedded in paraffin and cut into 3 µm transverse sections followed by immunohistochemical staining. Another part, used for cryosections, is frozen in an Optimum cutting temperature (OCT) medium (Fisher Scientific, Erembodegem, Belgium) and stored at -80°C.

The procedures for qRT-PCR, Western Blot and immunohistochemical staining are explained more thoroughly in paragraph **6.2**.

6.2 *In vivo*: Murine model of cigarette smoke exposure and COPD

Extensive studies in humans are often technically not feasible or morally inconceivable; and *in vitro* studies only study an isolated part of the body. Although these studies are

indispensable for research, additional *in vivo* animal models are needed to elucidate the cellular and molecular mechanisms of a disease and to test potential new therapies ^{3,4}. To study the pathogenesis of COPD, our research group has developed a murine model of cigarette smoke exposure ⁵ (described in detail in **6.2.1 Protocol**). Mice are relatively inexpensive and easy to breed. Moreover, their genome has been sequenced and transgenic mice, which overexpress or lack the gene of interest, are available ^{4,6,7}. Although the physiology, anatomy and inflammatory pattern are different between mice and humans, mice remain the model of interest for COPD research ^{6,8}. Several experimental models of COPD exist ⁶. The tracheal instillation of elastase or other proteases induces enlargement of the airspaces and serves as a model to study emphysema ^{9,10}. A major drawback of this model is that the lung injury is caused by a single insult, instead of a continuous exposure as seen in humans ⁶. Another murine model is the LPS (lipopolysaccharide) model ¹¹. Mice chronically exposed to LPS develop similar pathological changes as observed in patients with COPD (emphysema, lymphoid aggregates, pulmonary inflammation, thickening of the airway walls) ¹¹. However, LPS is only one single component of tobacco, which can not mimic the complex mechanisms caused by cigarette smoke. Therefore, cigarette smoke exposure appears to be the best choice when examining inflammation, emphysema, airway remodeling and lymphoid neogenesis ¹².

6.2.1 Protocol

Mice are put in a plexiglass chamber and exposed whole body to the smoke of 5 cigarettes (Kentucky Reference Cigarette 3R4F, without filters). A smoke:air ratio of 1:6 is obtained during the exposure. This procedure is repeated 4 times a day, 5 days a week. There is a 30 minute smoke-free interval in between smoke exposures.

Mice are exposed to cigarette smoke for 3 days (acute exposure), 4 weeks (subacute exposure) or 24 weeks (chronic exposure). After 3 days smoke exposure, mainly **innate immune cells** (macrophages, neutrophils) are present in the BAL fluid. After 4 weeks smoke exposure, also cells of the **adaptive immune system** appear in BAL fluid and lungs (CD4⁺

and CD8⁺ T-lymphocytes). **Dendritic cells** act as a link between the innate and adaptive immune system. Only after chronic smoke exposure (24 weeks), also other hallmarks of COPD are present: **airway wall remodeling, emphysema** and **lymphoid follicles**.

6.2.2 Airway inflammation

6.2.2.1 Bronchoalveolar lavage

Bronchoalveolar lavage is performed to obtain inflammatory cells from the airways and alveolar spaces. Through a tracheal cannula, a salt solution (HBSS, free of Ca²⁺ and Mg²⁺ and supplemented with 1% BSA) is gently injected and subsequently withdrawn from the lungs. This is repeated a second time with another salt solution (HBSS supplemented with 0.6 mM EDTA). The resulting 2 fractions are centrifuged and the cell-free supernatant of the first fraction is collected for analysis of proteins (e.g. by Enzyme-linked immunosorbent assay (ELISA)). The remaining cell pellets of the 2 fractions are used for total cell counting by using a Bürker chamber, for differential cell counting by means of cytopsin, and for flow cytometry. Cytospins are stained with May-Grünwald (Sigma-Aldrich, Bornem, Belgium) and Giemsa (VWR, Leuven, Belgium). Cells are counted based on standard morphological criteria of the cells. Macrophages are large mononuclear cells with abundant cytoplasm and contain numerous cytoplasmic granules. Neutrophils have a multi-lobed nucleus, with a very faint stained cytoplasm. Lymphocytes are the smallest leucocytes with a big round-shaped nucleus and little cytoplasm (**Figure 17**).

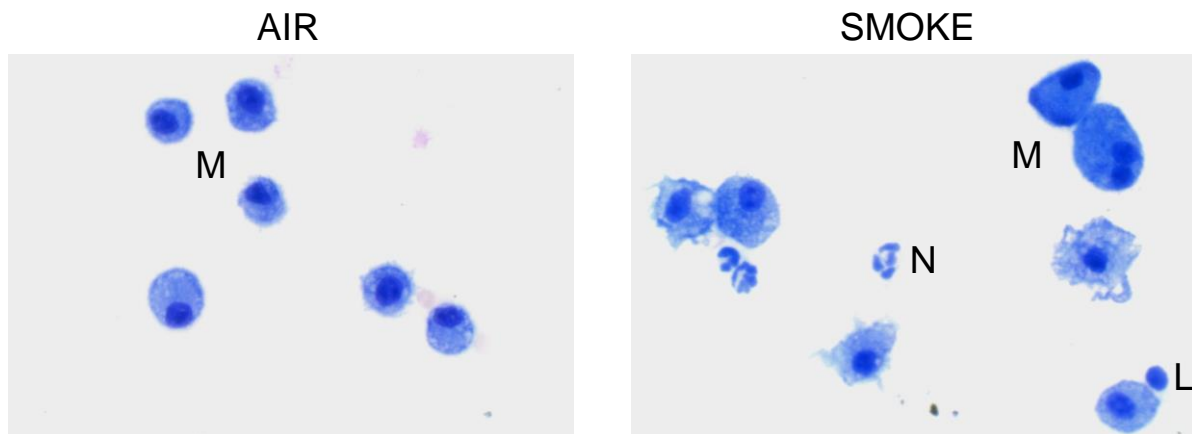


Figure 17: Cytospins of BAL fluid stained with May-Grünwald-Giemsa. BAL fluid of air-exposed mice mainly contains resident macrophages (M); BAL fluid of smoke-exposed mice contains macrophages (M), neutrophils (N) and lymphocytes (L).

6.2.2.2 Lung single cell suspension

After rinsing of pulmonary and systemic circulation, the right lung is clamped and removed for RNA extraction (small lobe) (6.2.3), Western Blot (middle lobe) (6.2.4.1) and preparation of single-cell suspensions for flow cytometry (largest lobe) (6.2.2.4). Briefly, the largest lobe of the right lung is thoroughly minced, digested with collagenase and DNase, subjected to red blood cell lysis, passed through a 50- μ m cell strainer and kept on ice until labelling. Cell counting occurs with a Z2 particle counter (Beckman-Coulter, Fullerton, CA, USA).

6.2.2.3 Isolation of ganglia

After removal of the skull and the brain, the trigeminal ganglia are dissected. By pulling the ear bone and the first vertebra apart, the nodose and jugular ganglia become visible and can be isolated. Next, the spine is isolated and cut transversally to isolate the dorsal root ganglia. The ganglia are stored in paraformaldehyde for histology, in RNA Stabilization Reagent for RNA extraction or in medium for functional analysis.

6.2.2.4 Flow cytometry

Flow cytometry is used to quantify the percentage of cells that is positive for certain antigens. Antibodies against these antigens are labeled with fluorochromes. The labeled BAL and lung

cells pass along an excitation source, where positive cells absorb the light and transmit a fluorescent signal, measured by the detectors.

To reduce aspecific binding of the antibodies, the cell suspensions are pre-incubated with a FcR-blocking antibody. The main cell types that are studied in this dissertation are macrophages, neutrophils, dendritic cells and CD4⁺ and CD8⁺ T-lymphocytes. Macrophages are CD11c⁺ and high-autofluorescent cells, while dendritic cells are CD11c⁺, low-autofluorescent and MHCII⁺. Inflammatory neutrophils are CD11c⁻, CD11b⁺, Ly6G⁺ and Ly6C⁺. CD3⁺ T-lymphocytes are further characterized as CD4⁺ (T-helper cells) or CD8⁺ (cytotoxic T cells) (**Figure 18**). To exclude dead cells, 7-aminoactinomycin D is incorporated in the analysis.

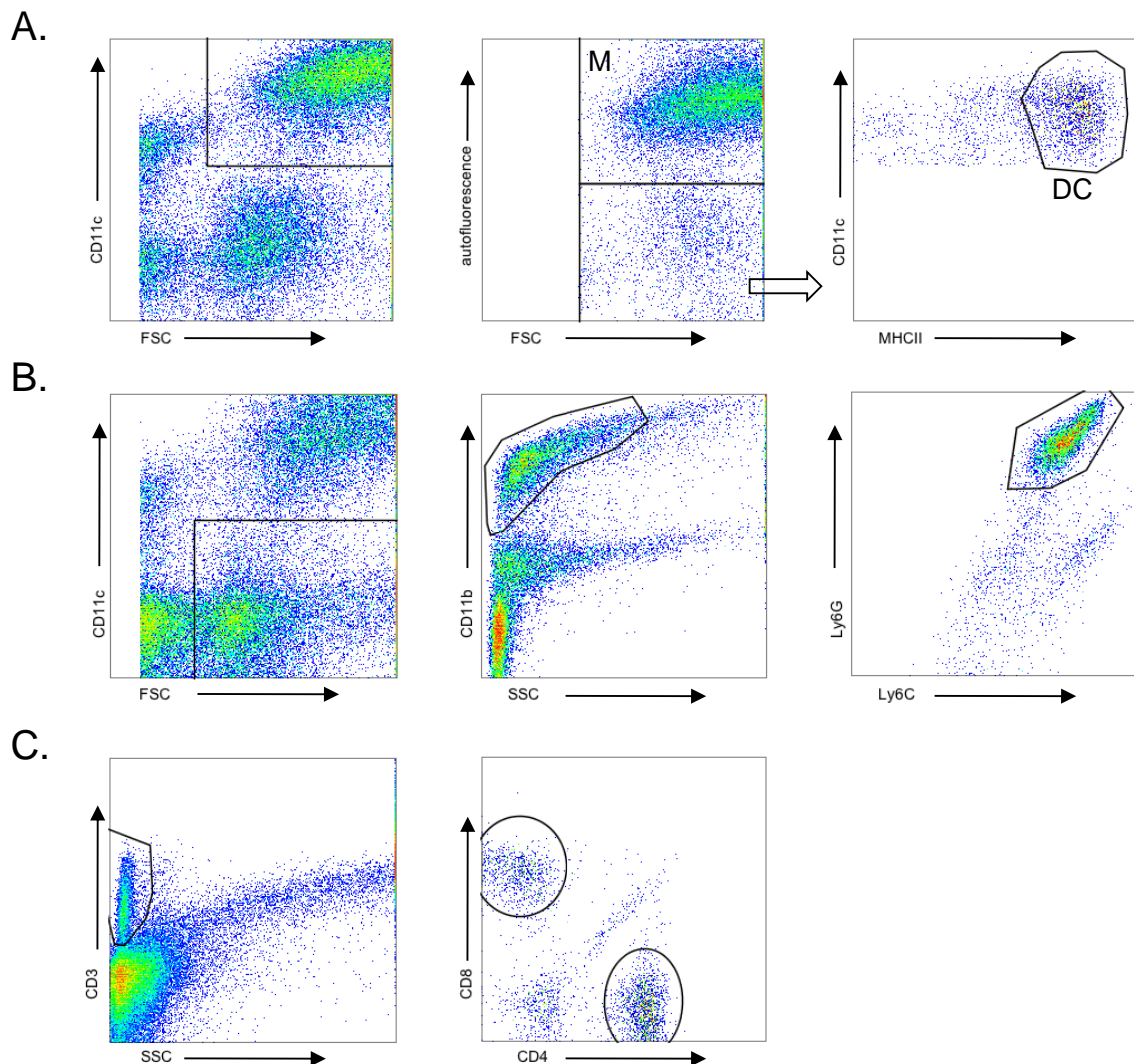


Figure 18. Gating strategy for **A.** macrophages (M), dendritic cells (DC), **B.** inflammatory neutrophils and **C.** CD4⁺ and CD8⁺ T-lymphocytes in bronchoalveolar lavage fluid.

6.2.3 mRNA expression

Human or murine lung tissue, and murine trigeminal, nodose/jugular and dorsal root ganglia are isolated to perform RNA extraction and subsequent determination of mRNA levels of genes of interest by quantitative real-time PCR (qRT-PCR). Total lung mRNA from human or murine lung tissue or murine TG, NG and DRG is extracted using the miRNeasy Mini kit (Qiagen). The expression of target genes, relative to reference genes is measured by qRT-PCR using custom designed primers (Sigma) or Taqman Gene Expression assays (Applied Biosystems), in a LightCycler[®] 480 (Roche).

6.2.4 Protein expression

6.2.4.1 Western Blot

Western Blot is an analytical technique to detect and quantify specific proteins of interest in a sample of tissue homogenate. In this dissertation, lung tissue homogenates are used. One ml of T-Per tissue protein extraction reagent and 10 µl Protease Inhibitor Cocktail Kit (Thermo Fisher Scientific, Waltham, MA) is added to the middle lobe of the murine right lung, or to the isolated part of the human lung. The lung tissue is minced mechanically (TissueRuptor, Qiagen). After centrifugation, the middle layer is transferred to a tube and the total protein concentration is defined using the Bradford protein assay (Bio-Rad Laboratories, Hercules, CA).

The proteins are denatured and protein disulfide bonds are reduced using NuPAGE[®] LDS Sample Buffer and Reducing Agent (Life Technologies) and by heating the samples for 10 minutes at 70°C. Next, the samples are loaded onto a pre-cast gel. The running buffer contains an antioxidant to maintain the proteins in a reduced state. After electrophoresis, a PVDF membrane is placed on the gel to transfer the proteins from the gel to the membrane. Next, a blocking solution (Life Technologies) is applied to the membrane before incubating the membrane with the primary antibody. After some washing steps, the alkaline phosphatase (AP)-conjugated secondary antibody is added. Visualization of the proteins occurs by using a Chemiluminescent Substrate (Life Technologies) and a film developer

(**Figure 19**). The Western blot images are analyzed with the program Image J (Image J 1.44P, National Institutes of Health, USA). The blots are scanned from the developed films and displayed as a 32-bit image in jpeg format. The bands of the gels are analyzed by generating lane profile plots, drawing lines to enclose peaks of interest, and finally measuring peak areas. Values obtained by densitometry for the proteins are normalized for actin (1 μ g/mL; Sigma).

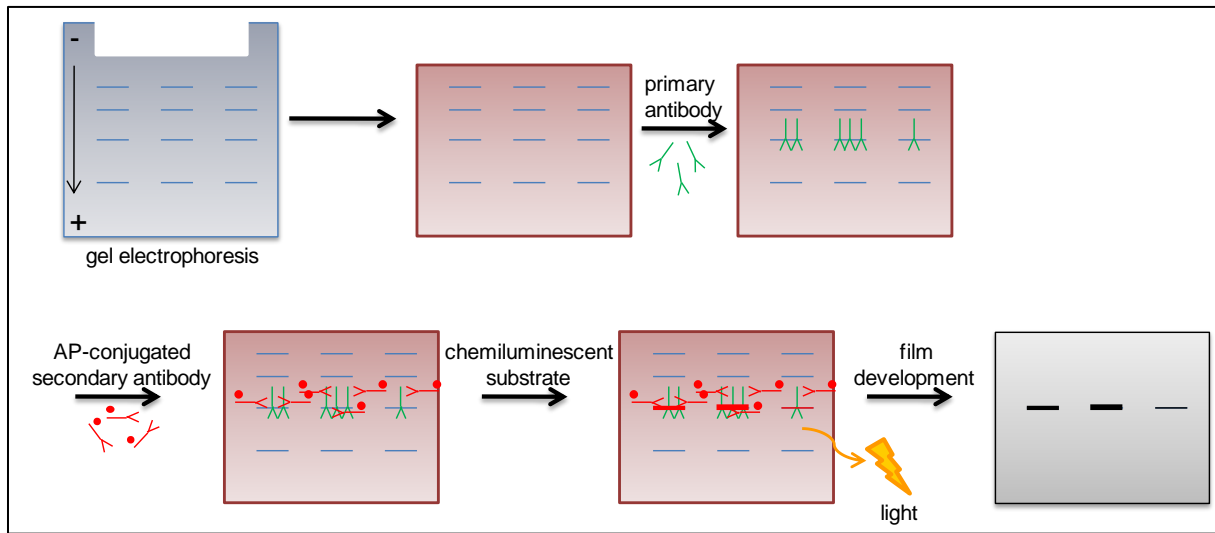


Figure 19: Overview of Western Blot analysis
AP: alkaline phosphatase

6.2.4.2 Immunohistochemical staining

After fixation in 4% paraformaldehyde, the murine or human lung or murine ganglia are embedded in paraffin and cut into 3 μ m transversal sections. Subsequently, immunohistochemical staining or histological staining (with haematoxylin/eosin for measurement of emphysema, see **6.2.5 Emphysema**) is performed. First, tissue sections are incubated with Boehringer blocking agent with 0.3% triton and primary antibody or isotype control. Next, the slides are incubated with an enzyme-conjugated (AP or HRP) secondary antibody (Immunovision Technologies, Burlingame, CA, USA) and stained with the according substrate. Sections are counterstained with haematoxylin and mounted using mounting medium (Thermo Fisher Scientific).

The staining of the protein of interest is quantified in the murine airway epithelium, in a marked area between the airway lumen and the epithelial basement membrane using AxioVision software (Zeiss) (**Figure 20**). The area with positive protein staining is normalized to the length of the basement membrane (Pbm).

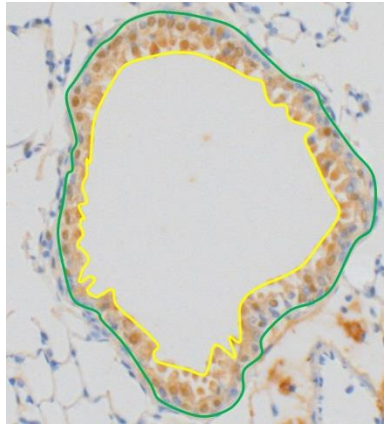


Figure 20: Quantification of immunohistochemical staining. The airway lumen is marked in yellow, the epithelial basement membrane in green. The staining is quantified in the airway epithelium, between the 2 lines.

6.2.5 Emphysema

Chronic CS exposure causes a destruction of the alveolar walls and enlargement of the airspaces. To determine the degree of emphysema, photomicrographs of haematoxylin/eosin-stained parenchymal lung sections are used. To measure the destruction of the alveolar walls, the **destructive index (DI)** is determined¹³, while the enlargement of the airspaces is quantified by the **mean linear intercept (Lm)**^{5,14}.

For determination of the Lm, a 100x100 µm grid is placed over each image. The total length of each line of the grid is divided by the number of alveolar intercepts, giving the average distance between alveolated surfaces, or the Lm (**Figure 21**).

The DI is measured by placing a grid on the image with 42 points that are at the center of hairline crosses. Alveolar and/or duct spaces lying under these points are classified as normal (N) or destroyed (D) (**Figure 22**). The DI is calculated from the formula: $DI = D/(D+N) \times 100$.

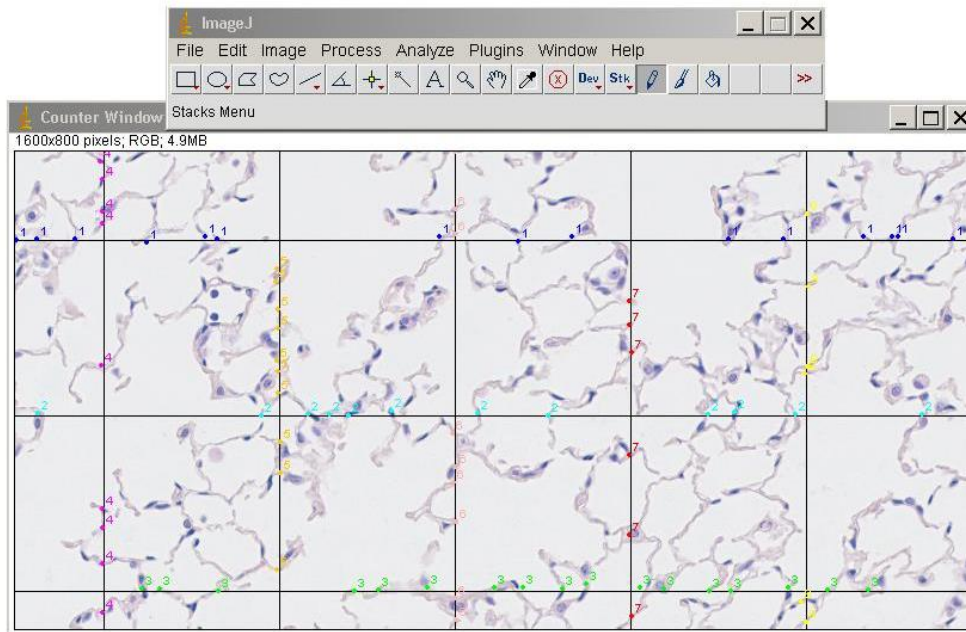


Figure 21 : Quantification of emphysema in mice by mean linear intercept (Lm). The figure shows the lung parenchyma of a mouse after chronic (24 weeks) smoke exposure. The destruction of the alveolar walls induces an enlargement of the airspaces, resulting in a larger mean linear intercept.

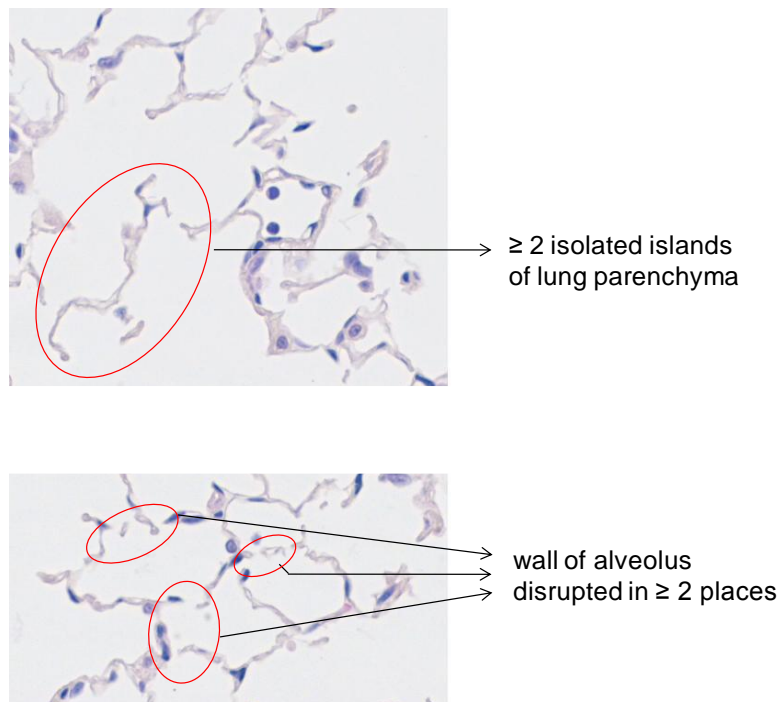


Figure 22: Quantification of emphysema in mice by destructive index (DI). The figure shows the lung parenchyma of a mouse after chronic (24 weeks) smoke exposure, with clear destruction of the alveolar walls and ducts.

6.2.6 Pulmonary function: bronchoconstriction

The airway resistance is measured invasively in tracheostomized, anaesthetized mice using the FlexiVent System (SCIREQ, Montreal, QC, Canada). The mice are placed on a 37°C heated blanket and are ventilated by a computer-controlled small animal ventilator with a breathing frequency of 150 breaths/minute. A muscle relaxant (pancuronium bromide (1mg/kg i.v., Organon, Oss, The Netherlands)) is administered to the mice, to prevent autonomous breathing. During the entire lung function experiment, the *arteria carotis* is cannulated to monitor the blood pressure.

To measure bronchial hyperresponsiveness, increasing doses of carbachol or serotonin (5-HT) are administered to the mice (2 – 4 – 8 – 16 - 32 - 64 µg/kg), through a catheter in the *vena jugularis*. The resistance (R) of the whole respiratory system (airways, lungs and chest wall) is measured using a “snapshot” perturbation. For each concentration, 12 “snapshot” perturbations are performed. The percentage increase in airway resistance per mouse, relative to the baseline resistance, is plotted against the concentration of carbachol or 5-HT and the area under the curve (AUC) is calculated.

REFERENCES

1. Van Pottelberge GR, Bracke KR, Van den Broeck S, Reinartz SM, van Drunen CM, Wouters EF, Verleden GM, Vermassen FE, Joos GF and Brusselle GG. Plasmacytoid dendritic cells in pulmonary lymphoid follicles of patients with COPD. *Eur Respir J* 2010; 36(4):781-791.
2. Demedts IK, Morel-Montero A, Lebecque S, Pacheco Y, Cataldo D, Joos GF, Pauwels RA and Brusselle GG. Elevated MMP-12 protein levels in induced sputum from patients with COPD. *Thorax* 2006; 61(3):196-201.
3. Maes T, Provoost S, Lanckacker EA, Cataldo DD, Vanoirbeek JA, Nemery B, Tournoy KG and Joos GF. Mouse models to unravel the role of inhaled pollutants on allergic sensitization and airway inflammation. *Respir Res* 2010; 11:7.
4. Dawkins PA and Stockley RA. Animal models of chronic obstructive pulmonary disease. *Thorax* 2001; 56(12):972-977.
5. D'Hulst A I, Vermaelen KY, Brusselle GG, Joos GF and Pauwels RA. Time course of cigarette smoke-induced pulmonary inflammation in mice. *Eur Respir J* 2005; 26(2):204-213.
6. Brusselle GG, Bracke KR, Maes T, D'Hulst A I, Moerloose KB, Joos GF and Pauwels RA. Murine models of COPD. *Pulm Pharmacol Ther* 2006; 19(3):155-165.
7. Shapiro SD. Transgenic and gene-targeted mice as models for chronic obstructive pulmonary disease. *Eur Respir J* 2007; 29(2):375-378.
8. Churg A, Sin DD and Wright JL. Everything Prevents Emphysema: Are Animal Models of Cigarette Smoke-Induced COPD Any Use? *Am J Respir Cell Mol Biol* 2011.
9. Gross P, Pfitzer EA, Tolker E, Babyak MA and Kaschak M. Experimental Emphysema: Its Production with Papain in Normal and Silicotic Rats. *Archives of environmental health* 1965; 11:50-58.
10. Janoff A, Sloan B, Weinbaum G, Damiano V, Sandhaus RA, Elias J and Kimbel P. Experimental emphysema induced with purified human neutrophil elastase: tissue localization of the instilled protease. *Am Rev Respir Dis* 1977; 115(3):461-478.
11. Vernooij JH, Dentener MA, van Suylen RJ, Buurman WA and Wouters EF. Long-term intratracheal lipopolysaccharide exposure in mice results in chronic lung inflammation and persistent pathology. *Am J Respir Cell Mol Biol* 2002; 26(1):152-159.
12. Wright JL, Cosio M and Churg A. Animal models of chronic obstructive pulmonary disease. *Am J Physiol Lung Cell Mol Physiol* 2008; 295(1):L1-L15.
13. Saetta M, Shiner RJ, Angus GE, Kim WD, Wang NS, King M, Ghezzi H and Cosio MG. Destructive index: a measurement of lung parenchymal destruction in smokers. *Am Rev Respir Dis* 1985; 131(5):764-769.
14. Bracke KR, D'Hulst A I, Maes T, Demedts IK, Moerloose KB, Kuziel WA, Joos GF and Brusselle GG. Cigarette smoke-induced pulmonary inflammation, but not airway remodelling, is attenuated in chemokine receptor 5-deficient mice. *Clin Exp Allergy* 2007; 37(10):1467-1479.

Chapter 7. Role of Transient Receptor Potential channel A1 in Chronic Obstructive Pulmonary Disease

Lisa L. Dupont*, Yeranddy A. Alpizar*, Guy G. Brusselle, Ken R. Bracke, Fien M. Verhamme, Guy F. Joos, Karel Talavera[#], Tania Maes[#]. * *Both authors contributed equally to this study*
[#] *Both authors contributed equally to the study supervision*

Manuscript in preparation

Chronic obstructive pulmonary disease (COPD) is mainly caused by smoking of cigarettes and is characterized by a chronic pulmonary inflammation. Several components of cigarette smoke (CS) are activators of TRPA1. Upon activation, TRPA1 can induce the release of neuropeptides from nerve terminals, leading to neurogenic inflammation. Therefore, we studied the potential role of TRPA1 in the pathogenesis of COPD and in CS-induced inflammation.

(part on Transient Receptor Potential Channels has been removed from the electronic version of the dissertation)

Chapter 8. Investigation of 5-HT₄ receptors in bronchial hyperresponsiveness in cigarette smoke-exposed mice

Lisa L. Dupont, Ken R. Bracke, Joris H. De Maeyer, Valérie Compan, Guy F. Joos, Romain A. Lefebvre and Guy G. Brusselle

Pulmonary Pharmacology and Therapeutics 2013; 28(1):60-7

According to Genome Wide Association studies, genetic variants in the gene encoding the 5-hydroxytryptamine 4 receptor (5-HT₄R) in humans are associated with lung function, airflow obstruction and COPD. However, the *in vivo* functional role of 5-HT₄R in COPD has not yet been demonstrated. Therefore, we studied the potential role of 5-HT₄R in bronchial responsiveness in CS-exposed mice.

Investigation of 5-HT₄ receptors in bronchial hyperresponsiveness in cigarette smoke-exposed mice.

Lisa L. Dupont^a, Ken R. Bracke^a, Joris H. De Maeyer^b, Valérie Compan^{c,d,e,f}, Guy F. Joos^a, Romain A. Lefebvre^g and Guy G. Brusselle^{a,§}

^a Laboratory for Translational Research in Obstructive Pulmonary Diseases, Department of Respiratory Medicine, Ghent University Hospital, Ghent, Belgium

^b Shire-Movetis NV, Turnhout, Belgium

^c CNRS, UMR-5203, Institut de Génomique Fonctionnelle, Montpellier, France

^d INSERM, U661, Montpellier, France

^e Universités de Montpellier 1 & 2, UMR-5203, Montpellier, France

^f Université de Nîmes, Nîmes, France

^g Heymans Institute of Pharmacology, Ghent University, Ghent, Belgium

[§] Corresponding author

Email addresses:

LLD: lisa.dupont@ugent.be

KRB: ken.bracke@ugent.be

JHDM: joris.demaeyer@outlook.com

VC: valerie.compan@igf.cnrs.fr

GFJ: guy.joos@ugent.be

RAL: romain.lefebvre@ugent.be

GGB: guy.brusselle@ugent.be, Tel. +32 9 3322604

Title: 98 characters; **Words:** 3373 ; **Abstract:** 250; **Figures:** 7

Abstract

Background: Chronic obstructive pulmonary disease (COPD) arises from an interaction between genetic host factors and environmental exposures (mainly cigarette smoke (CS)). Genome Wide Association studies have demonstrated that genetic variations in the gene encoding 5-hydroxytryptamine 4 receptors (5-HT₄R), *HTR4*, were associated with measures of airway obstruction and with COPD. We hypothesised that 5-HT₄ receptors, in addition to 5-HT_{2A}R and muscarinic receptors, contribute to the pathogenesis of COPD by facilitating cholinergic bronchoconstriction.

Methods: The levels of pulmonary 5-HT₄R mRNA were measured in CS-exposed mice by qRT-PCR. We investigated the effect of CS exposure on bronchial hyperresponsiveness (BHR) to 5-HT and evaluated the contribution of 5-HT_{2A}R, muscarinic receptors and 5-HT₄R in the response to 5-HT by using the corresponding antagonists and 5-HT₄R knockout (KO) mice.

Results: The 5-HT₄R mRNA levels were significantly elevated upon acute (3 days), subacute (4 weeks) and chronic (24 weeks) CS exposure. Both acute and subacute CS exposure significantly increased BHR to 5-HT. Antagonism of 5-HT_{2A}R abolished the CS-induced BHR to 5-HT, and antagonism of muscarinic receptors significantly reduced the response to 5-HT. However, pre-treatment with GR113808, a specific 5-HT₄R antagonist, did not alter the response to 5-HT in CS-exposed mice. Accordingly, the CS-induced BHR to 5-HT was not different between wild-type and 5-HT₄R KO mice.

Conclusion: CS increased the levels of 5-HT₄R mRNA in the lungs, concomitantly with bronchial responsiveness to 5-HT. Our *in vivo* data using pharmacologic and genetic approaches suggest that 5-HT₄ receptors are not involved in the BHR to 5-HT in CS-exposed mice.

Keywords: serotonin 4 receptors, cigarette smoke, murine model, COPD

Chemical compounds studied in this article: Atropine (PubChem CID: 64663); GR113808 (PubChem CID: 119376); ketanserin (PubChem CID: 16219944); prucalopride (PubChem CID: 3052762); serotonin (PubChem CID: 5202)

Background

Chronic obstructive pulmonary disease (COPD) is a chronic respiratory disease, characterised by an abnormal inflammatory response of the lungs to harmful substances, leading to obstruction of small airways and destruction of lung parenchyma (emphysema) [1]. Both the narrowing of the small airways and the emphysematous lung destruction result in airflow limitation in COPD patients. This airflow limitation is expressed as the forced expiratory volume in one second (FEV_1) and its ratio to the forced vital capacity (FVC), and is essential for the diagnosis of COPD. The prevalence and mortality of COPD are globally increasing with cigarette smoking as main risk factor [1, 2]. Importantly, only 20% of smokers develop COPD, indicating that genetic factors are also involved in the disease. Since COPD arises from an interaction between genetic predisposition and environmental exposures, the study of gene-environment interactions is essential to elucidate the pathogenesis of this disease.

The CHARGE consortium has performed a Genome Wide Association study (GWAS) of lung function and identified eight genetic loci associated with the ratio of FEV_1 to FVC [3]. The SpiroMeta consortium independently confirmed most of these genes, including the gene encoding the 5-hydroxytryptamine 4 receptor (5-HT₄R), *HTR4*, in humans [4]. Moreover, meta-analyses of GWAS showed that genetic variants in *HTR4* were associated with the development of airflow obstruction and with COPD, emphasising the clinical relevance of this locus [5, 6].

Serotonin receptors exert a range of effects upon activation by their endogenous ligand, serotonin (5-hydroxytryptamine, 5-HT). Peripherally this amine, derived from tryptophan, is mainly produced in enterochromaffin cells of the intestine [7], but also in rodent mast cells [8] and pulmonary neuroendocrine cells [9]. After synthesis, 5-HT enters the blood stream where it is stored in blood platelets and is released during platelet aggregation. Elevated levels of 5-HT have been detected in the plasma of symptomatic asthma [10] and COPD patients [11], and in the platelets of smokers [12]. Importantly, 5-HT acts as a potent bronchoconstrictor. We have previously shown that, in rats, 5-HT has a direct effect on

airway smooth muscle and an indirect effect through the activation of postganglionic cholinergic nerves, inducing the release of acetylcholine [13]. Both direct and indirect effects lead to bronchoconstriction [13]. According to an *in vitro* study on human airways, the indirect effect of 5-HT is partially mediated by 5-HT₄R [14]. The 5-HT₄ receptors belong to one of seven serotonin receptor classes, which are widely expressed throughout the body. The mRNA encoding 5-HT₄Rs has previously been detected peripherally in human lung tissue [4], monocytes [15], mature dendritic cells [16], bronchial epithelial cells, airway smooth muscle cells and peripheral blood mononuclear cells [4].

We hypothesised that 5-HT₄ receptors contribute to the pathogenesis of COPD by facilitating the cholinergic contraction of the airways. We therefore investigated the role of 5-HT₄R in the development of inflammation and altered lung function in a well-established murine model of cigarette smoke (CS) exposure [17, 18]. Firstly, the mRNA levels of 5-HT₄R in lung tissue of mice was measured upon 3 days (acute), 4 weeks (subacute) and 24 weeks (chronic) exposure to air or CS. Secondly, the bronchial responsiveness to 5-HT was analysed upon acute and subacute CS exposure. Next, the effect of a 5-HT_{2A}R antagonist, a muscarinic receptor antagonist and a 5-HT₄R antagonist on bronchial responsiveness to 5-HT was analysed in CS-exposed mice (pharmacologic approach). Finally, the bronchial responsiveness to 5-HT was investigated in CS-exposed 5-HT₄R knockout mice and their wild-type littermates (genetic approach).

Methods

Animals

Male C57Bl/6 wild-type (WT) mice (8-9 weeks old) were obtained from The Jackson Laboratory. Male C57Bl/6 5-HT₄R KO and control mice (WT) from C57Bl/6 heterozygous breeding were obtained from V. Compan. The 129Sv heterozygous mice [19] were first mated with C57Bl/6 WT female mice from The Jackson Laboratory, and heterozygous mutant mice were bred for more than ten generations of C57Bl/6 heterozygous mutant mice at the transgenic animal facility of the “Institut de Génomique Fonctionnelle”. All mice were housed in sterilised cages with filter tops and received food and water *ad libitum*. All *in vivo* manipulations were approved by the local Ethics Committee for animal experimentation of the Faculty of Medicine and Health Sciences (Ghent University).

Smoke exposure

Male mice were exposed to cigarette smoke (CS), as described previously [17]. Briefly, groups of mice were exposed whole body to the tobacco smoke of 5 cigarettes (Reference Cigarette 3R4F without filter, University of Kentucky, Lexington, KY), four times a day with 30 minutes smoke-free intervals for 3 days (acute exposure), 4 weeks (subacute exposure) or 24 weeks (chronic exposure). During the exposure, an optimal smoke:air ratio of 1:6 was obtained. The control groups were exposed to room air. Carboxyhemoglobin in serum of smoke-exposed mice reached a non-toxic level of 8.3 ± 1.4 % (compared to 1.0 ± 0.2 % in air-exposed mice), which is similar to carboxyhemoglobin blood concentrations of human smokers [20].

Acute effect after single dosing of a 5-HT₄R antagonist, a 5-HT_{2A}R antagonist or a muscarinic receptor antagonist

C57Bl/6 WT mice were treated 15 minutes before the first lung function measurement with the muscarinic receptor antagonist atropine (6.95 mg/kg i.p.; Sigma-Aldrich, St Louis, MO, USA), the 5-HT_{2A}R antagonist ketanserin (12 mg/kg i.p.; Sigma-Aldrich) or the 5-HT₄R

antagonist GR113808 (10 mg/kg i.p.; Sigma-Aldrich), dissolved in physiological water. During this interval, mice were mechanically ventilated. Sham-treated mice were injected with physiological water.

The most optimal dose of ketanserin, atropine and GR113808 was used according to literature [21-24].

Lung function measurements

Twenty-four hours after the last CS exposure, mice were anaesthetised with an injection of pentobarbital (100mg/kg i.p.; Sanofi, Libourne, France) and bronchial hyperresponsiveness to 5-HT was measured using a forced oscillation technique (FlexiVent, SCIREQ, Montreal, Canada). This procedure has previously been described by our research group [25]. The trachea was exposed and a 19-gauge metal needle was inserted into the trachea. Mice were ventilated by a computer-controlled small animal ventilator with a breathing frequency of 150 breaths/minute. A muscle relaxant (pancuronium bromide (1mg/kg i.v., Organon, Oss, The Netherlands)) was administered to the mice, to prevent autonomous breathing. During the entire lung function experiment, the *arteria carotis* was cannulated to monitor the blood pressure.

After measurement of the baseline resistance, each mouse was injected with increasing doses (2.0 – 4.0 – 8.0 – 16.0 – 32.0 – 64.0 µg/kg i.v.) of 5-HT (Sigma-Aldrich) or increasing doses (200 – 400 – 800 – 1600 – 3200 – 6400 µg/kg i.v.) of the 5-HT₄R agonist prucalopride (provided by Shire-Movetis NV). The resistance (R) of the whole respiratory system (airways, lungs and chest wall) was measured using a “snapshot” perturbation. For each mouse, the percentage increase in airway resistance was plotted against 5-HT concentration and the area under the curve (AUC) was calculated.

Bronchoalveolar lavage (BAL)

After lung function measurements, the 19-gauge metal needle was replaced by a tracheal cannula to collect bronchoalveolar lavage (BAL) fluid as described previously [18, 26].

Briefly, lungs were lavaged by using first 3 x 300 µl HBSS, free of calcium and magnesium, but supplemented with 1% BSA, followed by 3 x 1 ml HBSS, supplemented with 0.6 mM EDTA. The 6 lavage fractions were pooled and centrifuged, and the cell pellet was finally resuspended in 200 µl buffer (PBS supplemented with 1% BSA, 5 mM EDTA and 0.1% sodium azide). The total cell count was performed in a Bürker chamber, and differential cell counts (on at least 400 cells) were performed on cytocentrifuge preparations after May-Grünwald-Giemsa staining. Discrimination of macrophages and neutrophils were obtained based on standard morphologic criteria.

Labelling of BAL cells for flow cytometry

All labelling reactions were performed on ice in FACS-EDTA buffer, using monoclonal antibodies (mAbs) from BD Pharmingen (San Diego, CA, USA). To reduce nonspecific binding, an FcR-blocking antibody was added to all cells (anti-CD16/CD32, clone 2.4G2; kindly provided by L. Boon, Bioceros Utrecht, The Netherlands). The following mAbs were used to identify mouse DC populations: APC-conjugated CD11c (HL3) and PE-conjugated anti-I-A[b] (AF6-120.1). We used the methodology described by Vermaelen and Pauwels [27] to discriminate between macrophages and DCs. Macrophages are identified as the CD11c-bright, high autofluorescent population. DCs are identified as CD11c-bright, low autofluorescent cells, which strongly express MHC class II. Mouse T cell subpopulations were identified with the following mAbs : FITC-conjugated anti-CD4 (GK1.5), PE-conjugated anti-CD8 (53-6.7) and APC-conjugated anti-CD3 (145-2C11).

Flow cytometry data acquisition was performed on a FACSCalibur™ running CellQuest™ software (BD Biosciences, San Diego, CA, USA). FlowJo software (Tree Star Inc., Ashland, OR, USA) was used for data analysis.

qRT-PCR analysis

qRT-PCR was performed on total lung tissue of untreated air-exposed and CS-exposed C57BL/6 mice (3 days, 4 weeks and 24 weeks CS exposure). Total lung RNA was extracted

with the RNeasy Mini Kit (Qiagen, Hilden, Germany). qRT-PCR results were obtained via absolute quantification, relating the PCR signal to a standard curve. Levels of 5-HT₄R mRNA (Taqman Gene Expression Assay Mm00434129_m1), relative to transferrin receptor (TFRC) and hypoxanthine guanine phosphoribosyltransferase (HPRT) mRNA, were analysed using Taqman Gene Expression Assays (Applied Biosystems, Forster City, CA, USA). qRT-PCR was performed on a LightCycler 480 Instrument (Roche Diagnostics, Basel, Switzerland) with murine leukaemia virus RTase (Applied Biosystems). Reverse transcription was performed at 48°C for 30 min, followed by 10 min incubation at 95°C for denaturation of RNA-DNA heteroduplexes, and 45 cycles of 95°C for 10 sec and 60°C for 15 sec. Monitoring of the qRT-PCR occurred in real time using a FAM/TAMRA probe.

Statistical analysis

Reported values are expressed as mean \pm SEM. Statistical analysis was performed with Sigma Stat software (SPSS 20.0, Chicago, IL, USA) using nonparametric tests (Kruskal-Wallis; Mann-Whitney U). A p value < 0.05 was considered significant.

Results

Increased 5-HT₄R mRNA levels in lung tissue upon cigarette smoke exposure

To investigate whether CS exposure affects the mRNA levels of 5-HT₄R, qRT-PCR analysis was performed on RNA extracted from total lung tissue of C57Bl/6 mice. This revealed a significant upregulation of 5-HT₄R mRNA levels upon acute (3 days), subacute (4 weeks) and chronic (24 weeks) CS exposure, compared to air-exposed controls (Fig 1).

Effect of cigarette smoke exposure on bronchial responsiveness to 5-HT

Since CS exposure significantly increased the mRNA levels of 5-HT₄R, the *in vivo* effect of 3 days and 4 weeks CS exposure on the bronchial responsiveness to 5-HT was measured. Both acute (Fig 2 A-B) and subacute (Fig 2 E-F) CS exposure induced bronchial hyperresponsiveness (BHR) to 5-HT, shown by a significantly higher increase in airway resistance in comparison to air-exposed mice .

Macrophages and neutrophils were significantly increased in the BAL fluid upon acute (Fig 2 C-D) and subacute (Fig 2 G-H) CS exposure.

Role of muscarinic receptors and 5-HT_{2A}R in bronchial responsiveness to 5-HT in CS-exposed mice

CS-exposed mice were treated with the muscarinic receptor antagonist atropine to analyse the contribution of the indirect pathway, through cholinergic nerves, in the BHR to 5-HT. Atropine induced a significant decrease in BHR to 5-HT in CS-exposed mice (3 days), compared to sham-treated CS-exposed mice (Fig 3 A-B).

In mice treated with ketanserin, an antagonist of 5-HT_{2A} receptors (located on the airway smooth muscle), the increase in airway resistance to 5-HT was nearly abolished (Fig 3 A-B). BAL fluid inflammation in CS-exposed mice was similar between mice treated with atropine or ketanserin and sham-treated mice (Fig 3 C-D).

Role of 5-HT₄R in bronchial responsiveness to 5-HT in CS-exposed mice

The potential role of 5-HT₄R in the indirect pathway was further unravelled by using a selective 5-HT₄R agonist, a selective 5-HT₄R antagonist (pharmacologic approach) and 5-HT₄R KO mice (genetic approach).

Pharmacologic approach

The role of 5-HT₄R in bronchial responsiveness to the 5-HT₄R agonist prucalopride or to 5-HT was examined in CS-exposed mice.

In a first experiment, the BHR to the 5-HT₄R agonist prucalopride was measured in air- and CS-exposed mice. Mice exposed to CS for 4 weeks showed no different response to prucalopride compared with air-exposed mice (data not shown).

In a second experiment, subacutely CS-exposed mice received an intraperitoneal injection with the selective 5-HT₄R antagonist GR113808 15 minutes before the lung function measurements. The CS-exposed mice had no altered response to 5-HT compared to sham-treated mice (Fig 4 A-B).

Administration of GR113808 had no influence on the number of macrophages (CS sham: 714946 ± 82403, CS GR113808: 756723 ± 118223; p = 0.968) and neutrophils (CS sham: 138377 ± 28341, CS GR113808: 125543 ± 28497; p = 0.780) in the BAL fluid as compared to sham-treated mice.

Genetic approach

5-HT₄R KO mice and their wild-type littermates were exposed to CS for 4 weeks, and the bronchial responsiveness and inflammation were analysed. 5-HT₄R deficiency had no effect on the bronchial responsiveness to 5-HT in CS-exposed mice (Fig 5 A-B).

Role of 5-HT₄R in CS-induced inflammation

To elucidate the potential role of 5-HT₄R in the CS-induced inflammation, the BAL fluid of 4 weeks CS-exposed 5-HT₄R WT and KO mice was analysed by cytopins and flow cytometry.

No significant differences in number of inflammatory cells in the BAL fluid (macrophages, neutrophils, dendritic cells, CD4⁺ and CD8⁺ T-cells) were observed between 5-HT₄R WT and KO mice (Fig 6 A-F).

Discussion

Since recent Genome Wide Association studies have shown that genetic polymorphisms in the gene for 5-HT₄R, *HTR4*, were associated with pulmonary function and with COPD, we tested whether these receptors contribute to bronchial responsiveness to 5-HT in mice. Interestingly, 5-HT₄R mRNA levels are significantly increased upon CS exposure. Therefore, the role of 5-HT₄R was studied in CS-exposed mice. However, in this *in vivo* mouse model, antagonism of the 5-HT₄ receptor does not decrease 5-HT-induced BHR in mice. This finding was confirmed using 5-HT₄R WT and KO mice, which responded similarly to CS-induced BHR to 5-HT. Nevertheless, 5-HT_{2A} receptors and muscarinic receptors are important in the BHR to 5-HT. No evidence was found for the involvement of 5-HT₄R in the increased inflammation upon CS exposure.

In this study, we demonstrate mRNA expression of 5-HT₄R in murine lung tissue, similar to a study in human lung tissue [4]. The protein expression of 5-HT₄R in human lung tissue is very low, according to a recent publication [28]. To our knowledge, it is not possible to verify the expression of 5-HT₄R in mice at the protein level by immunohistochemistry or Western Blot, due to the lack of commercially available specific antibodies against this receptor. The levels of 5-HT₄R mRNA are significantly amplified after smoke exposure in mice. This increase might be explained by the CS-induced inflammation, since 5-HT₄R have been reported to be expressed on human monocytes and mature dendritic cells [15, 16]. Indeed, we have previously shown that dendritic cells are not only increased upon CS exposure, but they are also in an activated state and more mature, shown by an upregulation of MHCII and the co-stimulatory molecules CD40 and CD86 [17]. This is a possible explanation for the observed increase in 5-HT₄R mRNA levels in CS-exposed mice.

Parameters of spirometry such as FEV₁/FVC, using forced expiratory maneuvers, are difficult to assess in animals. However, an association has been shown between airway hyperresponsiveness and airway calibre on the one hand and accelerated rates of decline in lung function on the other hand [29, 30]. Therefore, we use the forced oscillation technique,

which is the golden standard for measuring airway resistance in response to different concentrations of a triggering agent. In this manner, we can link the spirometric measures in humans, used as phenotypic outcome in Genome Wide Association studies, and the measurement of BHR in a murine model of CS exposure.

Previous publications have suggested a role for the serotonergic system in the pathogenesis of COPD. Nicotine, an important component of CS, has been reported to stimulate the release of 5-HT *in vitro* through platelet activation [31]. Correspondingly, plasma 5-HT levels are elevated in COPD patients, representing an important link between cigarette smoking and the presence of COPD [11]. We demonstrate that the intravenous administration of increasing doses of 5-HT, which is taken up by platelets and translocated to the lungs, causes a dose-dependent increase in airway resistance in air-exposed mice. The observed 5-HT-induced airway smooth muscle contraction is potentially established through an indirect way as well as through a direct way, similar to the situation in humans, guinea pigs and rats [13, 14, 32]. In guinea pigs, 5-HT directly activates 5-HT_{2A} receptors, located on airway smooth muscle cells, leading to bronchoconstriction. Indirectly, 5-HT stimulates the 5-HT₄ receptors present on cholinergic nerves. This causes the release of acetylcholine, which eventually results in bronchoconstriction by binding to muscarinic receptors on airway smooth muscle cells [32].

There are several reports on the 5-HT-induced contraction in murine airways, but 5-HT₄R have not been studied before in this aspect. Martin *et al.* have demonstrated that the 5-HT-induced pulmonary obstruction in C57Bl/6 mice is predominantly due to the stimulation of 5-HT₂ receptors [33]. Other 5-HT receptors (5-HT_{1A}, 5-HT_{1B}, 5-HT_{1D} and 5-HT₃) were not involved in this response. In murine models of allergic asthma, antigen-induced airway constriction in sensitized mice was abolished after treatment with atropine or ketanserin [23]. Since the specific 5-HT_{2A}R antagonist ketanserin almost abolishes the BHR to 5-HT in our murine model of CS exposure, the direct pathway seems to be the most important. Nevertheless, treatment with atropine significantly reduces the response to 5-HT, suggesting that also the indirect pathway plays a role. Similar to the *in vivo* results in mice, our lab has

previously demonstrated that atropine reduces the *in vitro* contractile response to 5-HT in murine tracheas. Moreover, tetrodotoxin, a neurotoxin, has an analogous effect as atropine on the 5-HT-induced contraction [34]. These results provide support for 5-HT inducing the release of acetylcholine from cholinergic nerve endings in mice. To study the role of 5-HT₄R in this indirect pathway, the specific 5-HT₄R antagonist GR113808 was administered to CS-exposed mice. However, in this *in vivo* mouse model, antagonism of the 5-HT₄ receptors does not decrease the response to 5-HT, implicating that 5-HT₄R do not play a role in CS-induced BHR in mice. This finding is confirmed using 5-HT₄R WT and KO mice, which respond similarly to CS-induced BHR to 5-HT. The response to the 5-HT₄R agonist prucalopride (instead of 5-HT) was also not different between air- and CS-exposed WT mice. Other 5-HT receptors located on cholinergic nerves could be more important in this phenomenon. 5-HT₂ receptors have been described on parasympathetic cholinergic nerves [8] and ketanserin almost abolished the BHR to 5-HT, implicating that members of the 5-HT₂R family could be involved. An overview of the results of this paper can be found in Figure 7.

In the current study, BHR was measured in mice exposed to CS for 3 days and 4 weeks. However, it would be interesting to analyse the BHR in a mouse model of chronic (24 weeks) CS exposure. In addition to an increased inflammatory response, chronic CS-exposed mice develop emphysema and airway remodelling. These important characteristics of COPD could have an effect on the BHR.

Since the baseline inflammatory profile of the 5-HT₄R KO mice has not been described previously, we analyzed the inflammation in the BAL fluid more thoroughly by flow cytometry. Using this technique, also dendritic cells and CD4⁺ and CD8⁺ T cells can be enumerated, in addition to macrophages and neutrophils counted by cytopins. However, no differences were observed between 5-HT₄R WT and KO mice. The administration of GR113808 to WT mice also had no effect on the amount of macrophages or neutrophils in the BAL fluid, suggesting that 5-HT₄R is not involved in the CS-induced inflammatory response.

The finding that 5-HT₄R does not seem to play a role in BHR to 5-HT is important, since 5-HT₄R agonists are on the market for treating diseases as diverse as Alzheimer disease [35] and gastro-intestinal motility disorders [7]. Our results suggest that the use of these drugs is unlikely to cause any clinical benefits in patients with COPD. Finally, a major asset of our study is that we used both pharmacologic (antagonists) and genetic (5-HT₄R KO mice) approaches to investigate the role of 5-HT₄R.

Conclusions

In conclusion, CS exposure increases pulmonary 5-HT₄R mRNA levels, and induces BHR to 5-HT *in vivo*. 5-HT₄ receptors, shown by Genome Wide Association studies to be associated with pulmonary function and with COPD, do not seem to mediate the CS-induced BHR to 5-HT. In contrast, 5-HT_{2A} receptors and muscarinic receptors are important in the BHR to 5-HT in CS-exposed mice. No evidence is found for the involvement of 5-HT₄R in the increased inflammation upon CS exposure.

List of abbreviations

5-HT: 5-hydroxytryptamine; AUC: area under the curve; BHR: bronchial hyperresponsiveness; COPD: chronic obstructive pulmonary disease; CS: cigarette smoke.

Competing interests

LLD, KRB, VC, GFJ and GGB declare that they have no competing interests.

RAL has been scientifically involved in a Research and Development Project of Shire-Movetis NV on 5-HT₄ receptor agonists, financially supported by IWT (Agency for Innovation by Science and Technology); he has performed contract studies with gastrointestinal tissue for Shire-Movetis NV. JHDM is a former employee of Shire-Movetis NV; prucalopride belongs to the portfolio of Shire-Movetis NV.

Authors' contributions

LLD carried out the design and coordination of the study, performed the murine experiments and analyses, performed all statistical analysis, and drafted the manuscript. KRB and GGB participated in the design and coordination of the study and helped to interpret the data and helped to draft the manuscript. RAL, GFJ and JHDM helped to interpret the data. JHDM provided prucalopride (Shire-Movetis NV). VC provided the 5-HT₄R KO mice. All authors critically revised the manuscript and approved the final manuscript.

Acknowledgements

The authors would like to thank Greet Barbier, Eliane Castrique, Indra De Borle, Philippe De Gryze, Katleen De Saedeleer, Anouck Goethals, Marie-Rose Mouton, Ann Neesen, Christelle Snauwaert and Evelyn Spruyt from Laboratory for Translational Research in Obstructive Pulmonary Diseases, Department of Respiratory Medicine (Ghent University Hospital, Ghent, Belgium) for their technical assistance.

This work was supported by the Concerted Research Action of the University of Ghent (BOF/GOA/01601009; Ghent, Belgium), by the Fund for Scientific Research Flanders (Project G.0195.09 and G.0194.10; Brussels, Belgium) and by the Interuniversity Attraction Poles Program/Belgian State/Belgian Science Policy (P7/30; Brussels, Belgium). L.L. Dupont is a beneficiary from a Bijzonder Onderzoeksfonds Research grant from Ghent University. K.R. Bracke is a Postdoctoral Fellow of the Fund for Scientific Research Flanders.

References

- [1] Global Initiative for Chronic Obstructive Lung Disease. Global Strategy for the Diagnosis, Management, and Prevention of Chronic Obstructive Pulmonary Disease. 2013.
- [2] Pauwels RA, Rabe KF. Burden and clinical features of chronic obstructive pulmonary disease (COPD). *Lancet*. 2004;364:613-20.
- [3] Hancock DB, Eijgelsheim M, Wilk JB, Gharib SA, Loehr LR, Marcianti KD, et al. Meta-analyses of genome-wide association studies identify multiple loci associated with pulmonary function. *Nat Genet*. 2010;42:45-52.
- [4] Repapi E, Sayers I, Wain LV, Burton PR, Johnson T, Obeidat Me, et al. Genome-wide association study identifies five loci associated with lung function. *Nat Genet*. 2010;42:36-44.
- [5] Soler Artigas M, Wain LV, Repapi E, Obeidat M, Sayers I, Burton PR, et al. Effect of five genetic variants associated with lung function on the risk of chronic obstructive lung disease, and their joint effects on lung function. *Am J Respir Crit Care Med*. 2011;184:786-95.
- [6] Wilk JB, Shrine NR, Loehr LR, Zhao JH, Manichaikul A, Lopez LM, et al. Genome-wide association studies identify CHRNA5/3 and HTR4 in the development of airflow obstruction. *Am J Respir Crit Care Med*. 2012;186:622-32.
- [7] De Maeyer JH, Lefebvre RA, Schuurkes JA. 5-HT4 receptor agonists: similar but not the same. *Neurogastroenterol Motil*. 2008;20:99-112.
- [8] Weigand LA, Myers AC, Meeker S, Udem BJ. Mast cell-cholinergic nerve interaction in mouse airways. *J Physiol*. 2009;587:3355-62.
- [9] Lauweryns JM, Van Ranst L, Verhofstad AA. Ultrastructural localization of serotonin in the intrapulmonary neuroepithelial bodies of neonatal rabbits by use of immunoelectron microscopy. *Cell Tissue Res*. 1986;243:455-9.
- [10] Lechin F, van der Dijs B, Orozco B, Lechin M, Lechin AE. Increased levels of free serotonin in plasma of symptomatic asthmatic patients. *Ann Allergy Asthma Immunol*. 1996;77:245-53.
- [11] Lau WKW, Chan-Yeung MMW, Yip BHK, Cheung AHK, Ip MSM, Mak JCW, et al. The Role of Circulating Serotonin in the Development of Chronic Obstructive Pulmonary Disease. *PLoS One*. 2012;7:e31617.
- [12] Racké K, Schwörer H, Simson G. Effects of cigarette smoking or ingestion of nicotine on platelet 5-hydroxytryptamine (5-HT) levels in smokers and non-smokers. *Clin Investig*. 1992;70:201-4.
- [13] Joos GF, Lefebvre RA, Bullock GR, Pauwels RA. Role of 5-hydroxytryptamine and mast cells in the tachykinin-induced contraction of rat trachea in vitro. *Eur J Pharmacol*. 1997;338:259-68.
- [14] Dupont LJ, Pype JL, Demedts MG, De Leyn P, Deneffe G, Verleden GM. The effects of 5-HT on cholinergic contraction in human airways in vitro. *Eur Respir J*. 1999;14:642-9.
- [15] Durk T, Panther E, Muller T, Sorichter S, Ferrari D, Pizzirani C, et al. 5-Hydroxytryptamine modulates cytokine and chemokine production in LPS-primed human monocytes via stimulation of different 5-HTR subtypes. *Int Immunol*. 2005;17:599-606.
- [16] Idzko M, Panther E, Stratz C, Muller T, Bayer H, Zissel G, et al. The serotonergic receptors of human dendritic cells: identification and coupling to cytokine release. *J Immunol*. 2004;172:6011-9.
- [17] D'Hulst A I, Vermaelen KY, Brusselle GG, Joos GF, Pauwels RA. Time course of cigarette smoke-induced pulmonary inflammation in mice. *Eur Respir J*. 2005;26:204-13.
- [18] Bracke KR, D'Hulst A I, Maes T, Moerloose KB, Demedts IK, Lebecque S, et al. Cigarette smoke-induced pulmonary inflammation and emphysema are attenuated in CCR6-deficient mice. *J Immunol*. 2006;177:4350-9.
- [19] Compan V, Zhou M, Grailhe R, Gazzara RA, Martin R, Gingrich J, et al. Attenuated response to stress and novelty and hypersensitivity to seizures in 5-HT4 receptor knock-out mice. *J Neurosci*. 2004;24:412-9.

- [20] Macdonald G, Kondor N, Yousefi V, Green A, Wong F, Aquino-Parsons C. Reduction of carboxyhaemoglobin levels in the venous blood of cigarette smokers following the administration of carbogen. *Radiother Oncol.* 2004;73:367-71.
- [21] Nagase T, Ishii S, Shindou H, Ouchi Y, Shimizu T. Airway hyperresponsiveness in transgenic mice overexpressing platelet activating factor receptor is mediated by an atropine-sensitive pathway. *Am J Respir Crit Care Med.* 2002;165:200-5.
- [22] De Bie JJ, Henricks PA, Cruikshank WW, Hofman G, Jonker EH, Nijkamp FP, et al. Modulation of airway hyperresponsiveness and eosinophilia by selective histamine and 5-HT receptor antagonists in a mouse model of allergic asthma. *Br J Pharmacol.* 1998;124:857-64.
- [23] Cyphert JM, Kovarova M, Allen IC, Hartney JM, Murphy DL, Wess J, et al. Cooperation between mast cells and neurons is essential for antigen-mediated bronchoconstriction. *J Immunol.* 2009;182:7430-9.
- [24] Kato S, Matsuda N, Matsumoto K, Wada M, Onimaru N, Yasuda M, et al. Dual role of serotonin in the pathogenesis of indomethacin-induced small intestinal ulceration: Pro-ulcerogenic action via 5-HT₃ receptors and anti-ulcerogenic action via 5-HT₄ receptors. *Pharmacological Research.* 2012;66:226-34.
- [25] Glynos C, Dupont LL, Vassilakopoulos T, Papapetropoulos A, Brouckaert P, Giannis A, et al. The Role of Soluble Guanylyl Cyclase in Chronic Obstructive Pulmonary Disease. *American Journal of Respiratory and Critical Care Medicine.* 2013;188:789-99.
- [26] Demoor T, Bracke KR, Vermaelen KY, Dupont L, Joos GF, Brusselle GG. CCR7 modulates pulmonary and lymph node inflammatory responses in cigarette smoke-exposed mice. *J Immunol.* 2009;183:8186-94.
- [27] Vermaelen K, Pauwels R. Accurate and simple discrimination of mouse pulmonary dendritic cell and macrophage populations by flow cytometry: methodology and new insights. *Cytometry A.* 2004;61:170-77.
- [28] Hodge E, Nelson C, Miller S, Billington C, Stewart C, Swan C, et al. HTR4 gene structure and altered expression in the developing lung. *Respiratory Research.* 2013;14:77.
- [29] Tashkin DP, Altose MD, Connett JE, Kanner RE, Lee WW, Wise RA. Methacholine reactivity predicts changes in lung function over time in smokers with early chronic obstructive pulmonary disease. The Lung Health Study Research Group. *Am J Respir Crit Care Med.* 1996;153:1802-11.
- [30] De Meer G, Heederik D, Postma DS. Bronchial responsiveness to adenosine 5'-monophosphate (AMP) and methacholine differ in their relationship with airway allergy and baseline FEV₁. *Am J Respir Crit Care Med.* 2002;165:327-31.
- [31] Rausch JL, Fefferman M, Ladisich-Rogers DG, Menard M. Effect of nicotine on human blood platelet serotonin uptake and efflux. *Prog Neuro-Psychoph* 1989;13:907-16.
- [32] Segura P, Vargas MH, Cordoba-Rodriguez G, Chavez J, Arreola JL, Campos-Bedolla P, et al. Role of 5-HT_{2A}, 5-HT₄ and 5-HT₇ receptors in the antigen-induced airway hyperresponsiveness in guinea-pigs. *Clin Exp Allergy.* 2009;40:327-38.
- [33] Martin TR, Cohen ML, Drazen JM. Serotonin-induced pulmonary responses are mediated by the 5-HT₂ receptor in the mouse. *J Pharmacol Exp Ther.* 1994;268:104-9.
- [34] De Swert KO, Lefebvre RA, Pauwels RA, Joos GF. Role of the tachykinin NK₁ receptor in mediating contraction to 5-hydroxytryptamine and antigen in the mouse trachea. *Pulm Pharmacol Ther.* 2007;20:588-95.
- [35] Brodney MA, Johnson DE, Sawant-Basak A, Coffman KJ, Drummond EM, Hudson EL, et al. Identification of Multiple 5-HT₄ Partial Agonist Clinical Candidates for the Treatment of Alzheimer's Disease. *Journal of Medicinal Chemistry.* 2012;55:9240-54.

Figure legends

Figure 1: Effect of exposure to air or CS on the mRNA levels of 5-HT₄R in total lung tissue.

mRNA levels of 5-HT₄R in total lung tissue upon acute (3 days), subacute (4 weeks) and chronic (24 weeks) CS exposure. Results are expressed as a ratio with hprt1 and tfrc mRNA (mean ± SEM; n = 5 per group). * p<0.05, **p<0.01

Figure 2: Effect of acute and subacute cigarette smoke exposure on bronchial responsiveness to serotonin in mice.

(A) Concentration-response curves for 5-HT in air- and CS-exposed mice upon acute (3 days) CS exposure (n = 8 per group), (B) Area under the curve (AUC) of the airway resistance of the groups depicted in (A), (C) BAL macrophages, (D) BAL neutrophils, (E) Concentration-response curves for 5-HT in air- and CS-exposed mice upon subacute (4 weeks) CS exposure (n = 9-10 per group), (F) Area under the curve (AUC) of the airway resistance of the groups depicted in (E), (G) BAL macrophages, (H) BAL neutrophils. Mean values per group ± SEM. *p<0.05, **p<0.01, ***p<0.001. Data are representative of two independent experiments.

Figure 3: Effect of atropine and ketanserin on bronchial hyperresponsiveness to serotonin in acute and subacute CS-exposed mice.

(A) Concentration-response curves for 5-HT in acute (3 days) CS-exposed mice, sham-treated, treated with the 5-HT_{2A}R antagonist ketanserin or with atropine, (B) Area under the curve (AUC) of the airway resistance of the groups depicted in (A). (C) BAL macrophages, (D) BAL neutrophils. Mean values per group ± SEM (n = 9-10 per group). *p<0.05, **p<0.01, ***p<0.001 compared with CS+sham mice.

Figure 4: Effect of the 5-HT₄R antagonist GR113808 on bronchial responsiveness to serotonin in subacute CS-exposed mice.

(A) Concentration-response curves for 5-HT in subacute (4 weeks) CS-exposed mice, sham-treated or treated with the 5-HT₄R antagonist GR113808, (B) Area under the curve (AUC) of the airway resistance of the groups depicted in (A). Mean values per group \pm SEM (n = 7-9 per group).

Figure 5: Effect of 5-HT₄R deficiency on bronchial responsiveness to serotonin in subacute CS-exposed mice.

(A) Concentration-response curves for 5-HT in subacute (4 weeks) CS-exposed 5-HT₄R WT or KO mice, (B) Area under the curve (AUC) of the airway resistance of the groups depicted in (A). Mean values per group \pm SEM (n = 8 per group).

Figure 6: Effect of 5-HT₄R deficiency on subacute CS-induced inflammation.

(A) Total BAL cells, (B) BAL macrophages, (C) BAL neutrophils, (D) BAL dendritic cells, (E) BAL CD4⁺ T-cells, (F) BAL CD8⁺ T-cells. Mean values per group \pm SEM (n = 8 per group).

Figure 7: In CS-exposed mice, the bronchoconstricting effect of 5-HT mainly acts through the direct pathway, since the antagonism of 5-HT_{2A} receptors on the airway smooth muscle by ketanserin, nearly abolished the BHR to 5-HT. However, also the indirect pathway is important, as treatment with atropine significantly decreased the BHR in CS-exposed mice. These data suggest that 5-HT indirectly favours the release of acetylcholine from nerve endings, probably by activating 5-HT receptors on these nerves. The 5-HT receptors involved are not 5-HT₄ receptors, but could potentially be members of the 5-HT₂R family.

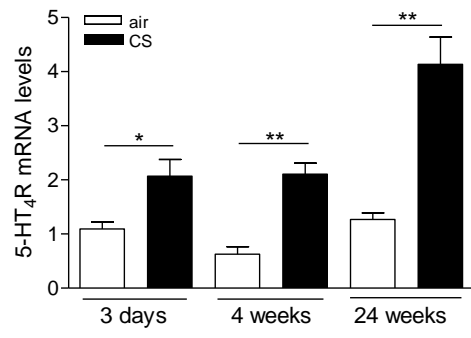
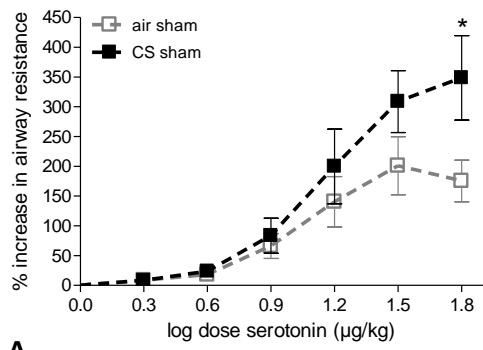
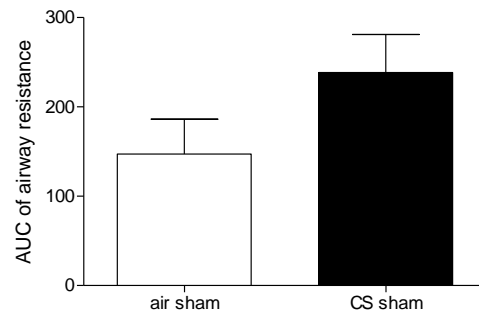


Figure 1

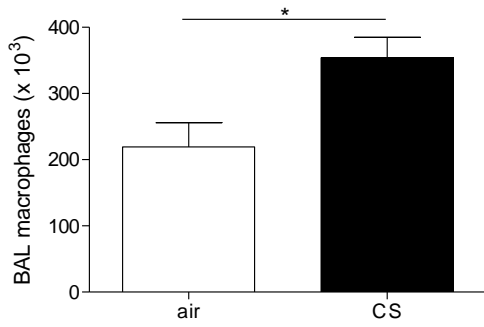
3 days CS



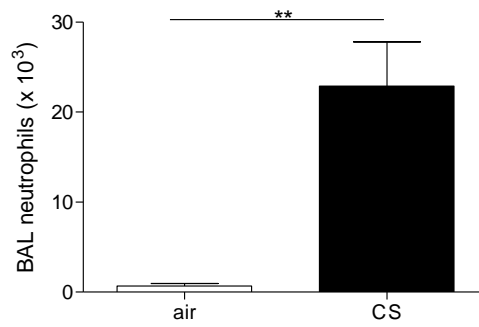
A



B

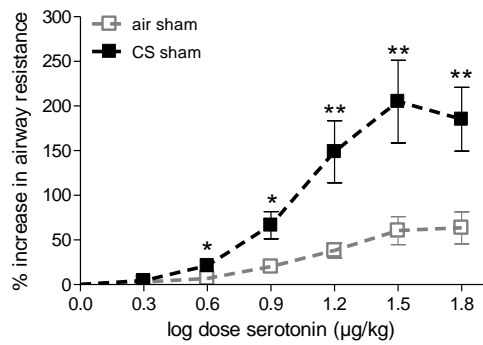


C

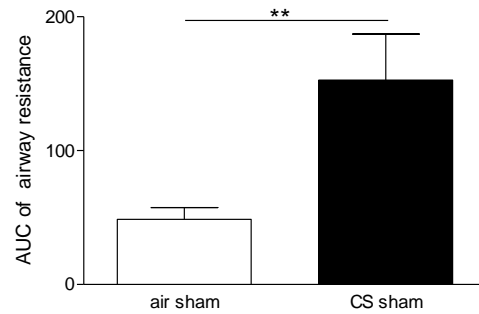


D

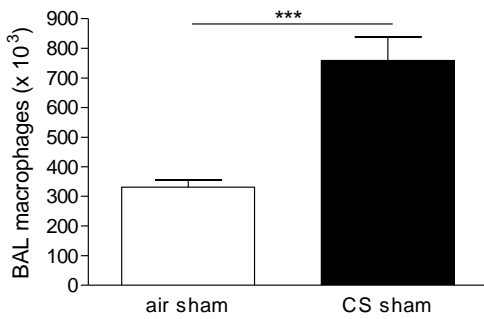
4 weeks CS



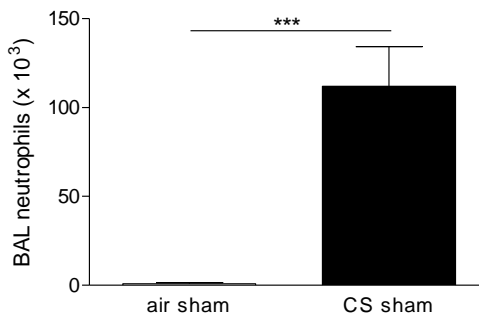
E



F

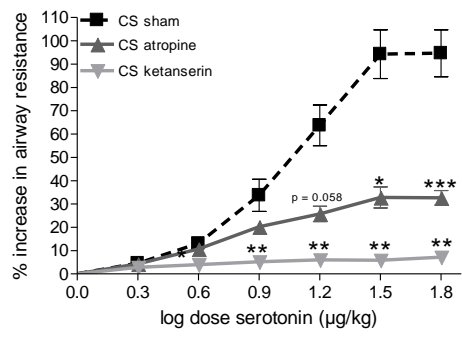


G

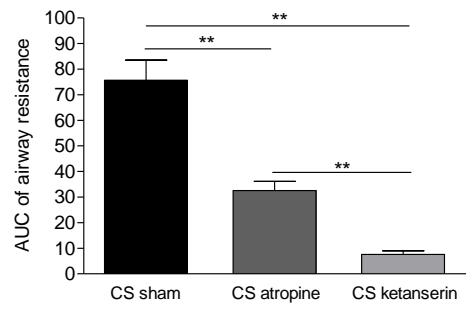


H

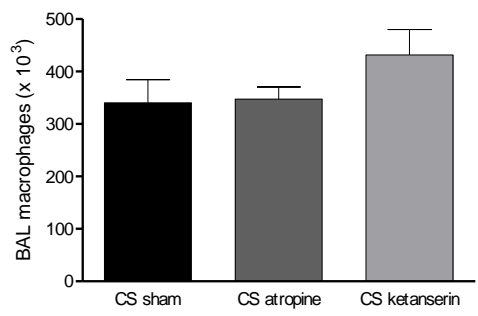
Figure 2



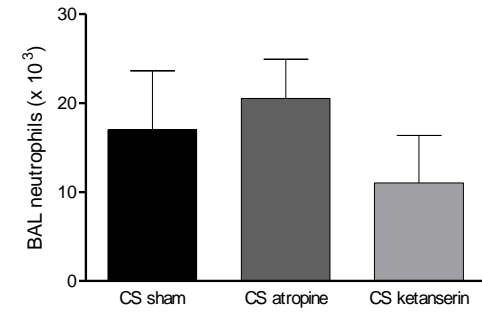
A



B

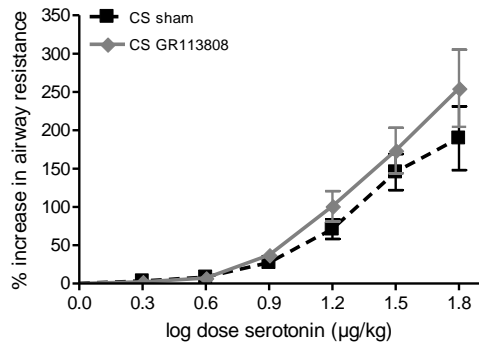


C

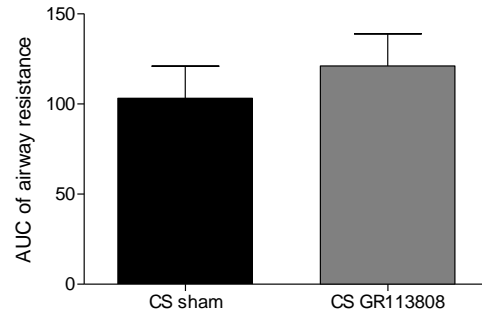


D

Figure 3

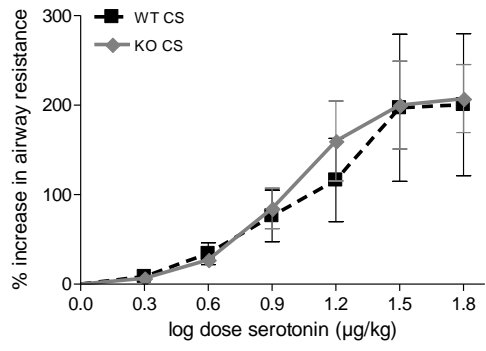


A

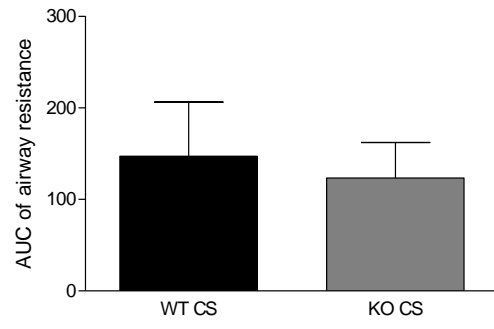


B

Figure 4

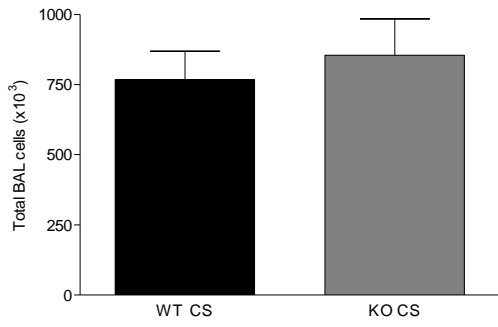


A

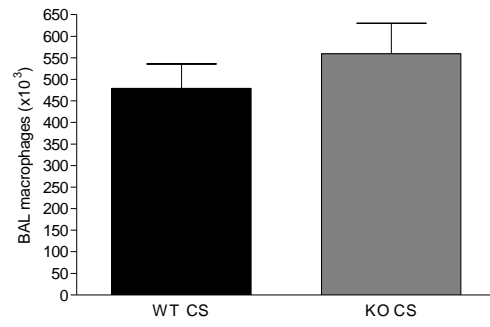


B

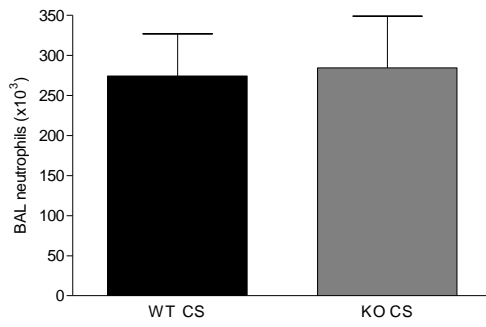
Figure 5



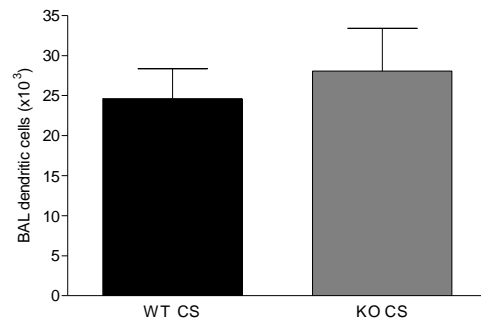
A



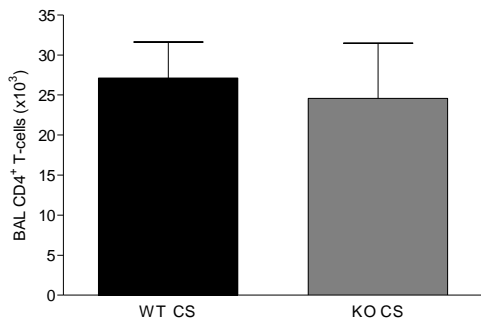
B



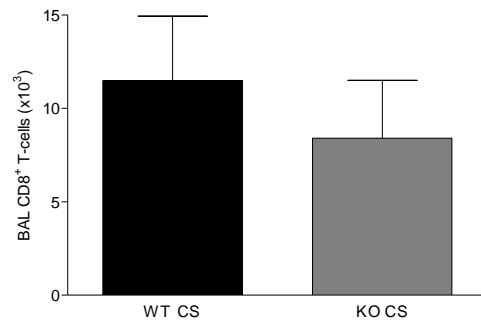
C



D



E



F

Figure 6

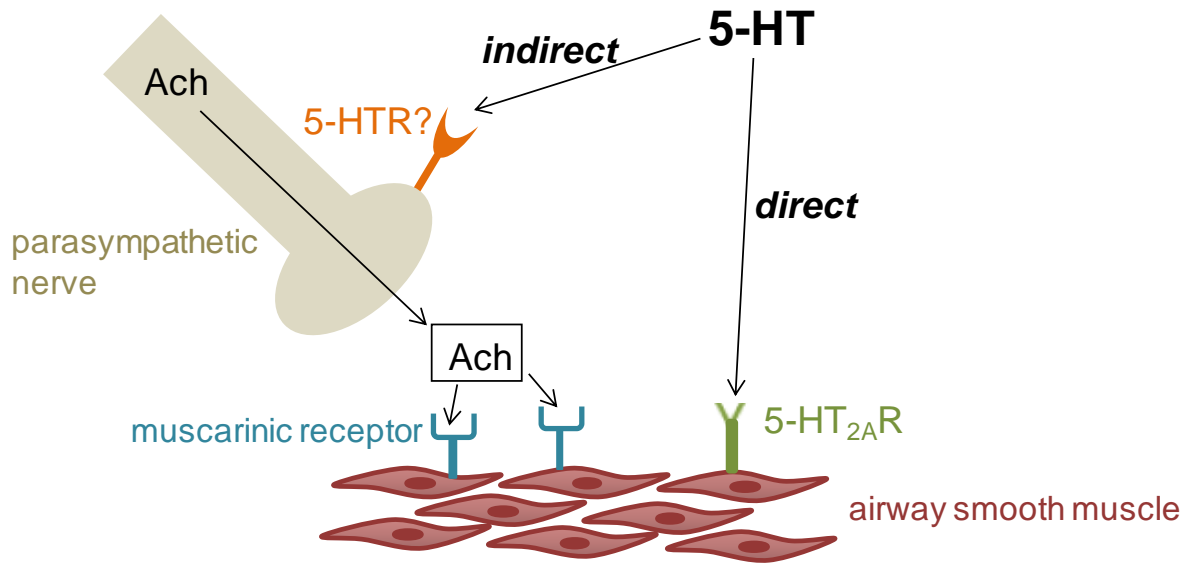


Figure 7

Chapter 9. The role of soluble guanylyl cyclase in Chronic Obstructive Pulmonary Disease

Constantinos Glynos*, Lisa L. Dupont*, Theodoros Vassilakopoulos, Andreas Papapetropoulos, Peter Brouckaert, Athanassios Giannis, Guy F. Joos, Ken R. Bracke and Guy G. Brusselle. * *equal contribution*

American Journal of Respiratory and Critical Care Medicine 2013; 188(7): 789-799

Given the elevated airway smooth muscle tone in patients with COPD and the bronchodilating effect of the NO/sGC/cGMP pathway, we analyzed the expression of sGC both in CS-exposed mice and in human patients with COPD. We investigated the functional role of sGC in CS-exposed mice using an sGC activator on the one hand and sGC α 1 KO mice on the other hand.

The role of soluble guanylyl cyclase in Chronic Obstructive Pulmonary Disease

Constantinos Glynos^{1,*}, Lisa L. Dupont^{1,*}, Theodoros Vassilakopoulos², Andreas Papapetropoulos³, Peter Brouckaert^{4,5}, Athanassios Giannis⁶, Guy F. Joos¹, Ken R. Bracke^{1,†} and Guy G. Brusselle^{1,†}.

¹Laboratory for Translational Research in Obstructive Pulmonary Diseases, Department of Respiratory Medicine, Ghent University Hospital, Ghent, Belgium.

²Department of Critical Care and Pulmonary Services, Evangelismos Hospital, University of Athens Medical School, Athens, Greece.

³Laboratory of Molecular Pharmacology, Department of Pharmacy, University of Patras, Patras, Greece.

⁴Department of Biomedical Molecular Biology, Ghent University, Ghent, Belgium.

⁵Department of Molecular Biomedical Research, VIB, Ghent, Belgium.

⁶Institut für Organische Chemie, Universität Leipzig, Leipzig, Germany.

Address Correspondence to: Guy G Brusselle, Department of Respiratory Medicine 7K12, Ghent University Hospital, Ghent B-9000, Belgium guy.brusselle@ugent.be

Running title: Role of soluble Guanylyl Cyclase in COPD

Author Contributions: * C.G and L.L.D contributed equally to this study. † G.G.B and K.R.B contributed equally to the study supervision. Conception and design: C.G, T.V and G.G.B. Analysis, technical expertise and interpretation: C.G, K.R.B, L.L.D, T.V, A.P, G.F.J and G.G.B. Research reagents, and critical review of the manuscript: A.P, A.G, G.F.J and P.B.

Funding Source: This study was supported by the Concerted Research Action of the University of Ghent (BOF/GOA/01601009; Ghent, Belgium), by the Fund for Scientific Research Flanders (Project G.0195.09 and G.0194.10; Brussels, Belgium) and by the Interuniversity Attraction Poles Program/Belgian State/Belgian Science Policy (P6/35 and

P7/30; Brussels, Belgium). C.G is a postdoctoral fellow of European Respiratory Society Long-Term Research Fellowship (LTRF175-2011). L.L.D is a beneficiary from a Bijzonder Onderzoeksfonds Research grant from Ghent University. K.R.B is a Postdoctoral Fellow of the Fund for Scientific Research Flanders. A.P and P.B are supported by the COST Action BM1005 (European Network on Gasotransmitters).

Subject Category Descriptor: 3.33

Word count text: 3599 ; ***References:*** 47; ***Figures:*** 10 ; ***Table:*** 1.

This article has an online data supplement, which is accessible from this issue's table of content online at www.atsjournals.org

At A Glance Commentary

Scientific Knowledge on the Subject

In Chronic Obstructive Pulmonary Disease (COPD), irreversible airflow limitation is progressive, despite ample amounts of the major smooth muscle relaxation factor, nitric oxide (NO). The role of the principal NO receptor, namely soluble guanylyl cyclase (sGC), in COPD is unknown.

What This Study Adds to the Field

The expression of soluble guanylyl cyclase is reduced in smokers and in patients with COPD and correlates with disease severity. In a murine model, cigarette smoke exposure induces downregulation of soluble guanylyl cyclase. Pharmacological activation of the guanylyl cyclase pathway provides a potential therapeutic strategy in COPD.

Abstract

Rationale: Soluble guanylyl cyclase (sGC), a cyclic guanosine 5'-monophosphate (cGMP) generating enzyme, regulates smooth muscle tone and exerts anti-inflammatory effects in animal models of asthma and acute lung injury. In Chronic Obstructive Pulmonary Disease (COPD), primarily caused by cigarette smoke (CS), lung inflammation persists and smooth muscle tone remains elevated, despite ample amounts of nitric oxide (NO) that could activate sGC. **Objective:** To determine the expression and function of sGC in patients with COPD and in a murine model of COPD. **Methods:** Expression of sGC α 1, α 2 and β 1 subunits was examined in lungs of never smokers, smokers without airflow limitation and patients with COPD; and in C57BL/6 mice after 3 days, 4 and 24 weeks of CS exposure. The functional role of sGC was investigated *in vivo* by measuring bronchial responsiveness to serotonin (5-HT) in mice using genetic and pharmacological approaches. **Measurements and Main Results:** Pulmonary expression of sGC, both at mRNA and protein level, was decreased in smokers without airflow limitation and in patients with COPD, and correlated with disease severity (FEV₁%). In mice, exposure to CS reduced sGC, cGMP levels and protein kinase G activity. sGC α 1^{-/-} mice exposed to CS exhibited bronchial hyperresponsiveness (BHR) to serotonin. Activation of sGC by BAY 58-2667 restored the sGC signaling and attenuated BHR in CS-exposed mice. **Conclusions:** Downregulation of soluble guanylyl cyclase due to cigarette smoke exposure might contribute to airflow limitation in COPD.

Word count: 232

INTRODUCTION

Chronic Obstructive Pulmonary Disease (COPD), is the fourth leading cause of death worldwide (1). COPD is mainly attributed to excessive oxidative stress and innate immune dysregulation (2), that interrelate and amplify lung inflammation, primarily induced by cigarette smoking (3). The chronic and exaggerated pulmonary inflammation leads to a progressive and largely irreversible airflow limitation and alveolar cell death (4). In addition to mucus hypersecretion, inflammation, and loss of alveolar attachments to peripheral airways, smooth muscle area and tone are important determinants for airflow limitation (5). However, the mechanistic concepts involved in airway muscle tone, including signaling pathways through cyclic nucleotides, are relatively unknown.

Nitric oxide (NO) synthesis and secondary signaling through activation of its “receptor”, soluble guanylyl cyclase (sGC), represents a major pathway for smooth muscle relaxation. sGC is an obligate heterodimer, composed of α and β subunits (6). Although sGC α 1 β 1 is the most prevailing heterodimeric enzyme in most tissues, with decisive influence in smooth muscle relaxation (7), sGC α 2 β 1 can also partially mediate the effect of NO on smooth muscle tone in the vasculature (8,9). Activation of sGC is followed by generation of cyclic guanosine monophosphate (cGMP), protein kinase G (PKG) phosphorylation (10) and changes in activity of other effector proteins, including phosphodiesterases, ion channels and ion pumps (11).

In COPD, despite ample amounts of NO (12) in the lung, airway smooth muscle tone is elevated, implicating suboptimal NO bioactivity (11). Increased expression of neuronal (13) and inducible (14) NO synthases (NOS), has been correlated with airflow limitation and disease severity in COPD patients. Although sGC is a key component of NO signaling, its expression and activity in COPD remain unknown. Inflammatory stimuli such as endotoxin, interleukin-1-beta (15), uncontrolled production of NO (16) and reactive oxygen species (ROS) (17) have been shown to downregulate the expression of sGC *in vitro* and *in vivo* in animal models of asthma (18) and lung injury (19,20). Since cytokines, NO and ROS are upregulated in the lung in COPD (2,4), we hypothesize a downregulation of sGC in COPD,

secondary to exposure to cigarette smoke. In addition, ROS can increase the removal of the sGC protein (21), by shifting the equilibrium from the NO-sensitive reduced state of sGC to the NO-insensitive oxidized state (22). In our study we examined the expression of sGC at mRNA and protein level in smokers and in patients with COPD. Moreover, we investigated the functional role of sGC in a well-established cigarette smoke-exposed murine model of COPD (23).

MATERIALS & METHODS

Methodological details are available in the online data supplement.

Human Subjects: Specimens of lung tissue were obtained from 64 human subjects, undergoing lung resection surgery in Ghent University Hospital. Human subjects were classified into 3 groups:(i) 11 never smokers (NS), (ii) 24 cigarette smokers, (iii) 29 patients with COPD stage II, according to the Global Initiative for COPD (GOLD) scale (24). All subjects provided written informed consent, according to protocols approved by the medical ethical committee of the Ghent University Hospital.

Animals: Eight-to-twelve-week-old male C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, USA). sGC $\alpha 1^{-/-}$ mice were provided by the SPF facility of the Department of Molecular Biomedical Research, VIB, Ghent, Belgium (25,26). All procedures were approved by the local Ethics Committee for animal experimentation of the Faculty of Medicine and Health Sciences, Ghent University.

Cigarette smoke (CS) exposure: Groups of 8 mice received mainstream CS of 5 reference cigarettes (3R4F University of Kentucky, USA) 4 times a day with 30-minute smoke-free intervals and were exposed for 3 days (acute), 4 weeks (subacute) and 24 weeks (chronic). The control groups were exposed to room air (23).

BAY 58-2667 treatment: In a pharmacological experiment of acute (3 days) CS exposure, C57BL/6 mice were treated with the sGC activator BAY 58-2667 (10 μ g/kg, i.p.) or vehicle (DMSO 10%, i.p.) one hour before lung function measurements.

Lung function measurements: Twenty-four hours after the last CS exposure, mouse lung function was measured at baseline and after serotonin (5-HT) challenge, using a forced oscillation technique (FlexiVent, SCIREQ, Canada) (27).

Bronchoalveolar lavage (BAL): Bronchoalveolar lavage was performed and total and differential cell counts were obtained (28). Flow cytometric analysis of BAL cells was performed to enumerate dendritic cells (DCs) and CD4⁺ and CD8⁺ T-lymphocytes.

Labeling of BAL cells for flow cytometry: The labeling reactions were performed to discriminate among DCs, macrophages and T-lymphocytes, according to Vermaelen and Pauwels' methodology (29).

RNA preparation and real-time RT-PCR of sGC subunits: Total RNA was extracted from lung (RNeasy Mini kit; Qiagen, Germany) and cDNA was synthesized (Transcriptor First Strand cDNA synthesis Kit; Roche, Switzerland). Target and reference genes were measured by real-time PCR using custom designed primers, in mice, or TaqMan Gene Expression assays (Applied Biosystems, USA), in humans, in a LightCycler[®]480 (Roche, Germany).

Immunoblot analysis of sGC subunits: Lung homogenates of human or murine samples were subjected to SDS-PAGE and Western blotting as previously described (18,20,30).

cGMP measurements: BAL cGMP levels were measured using a commercially available enzyme immunoassay kit (Direct cGMP Elisa Kit; Enzo Life Sciences).

Immunohistochemistry of sGC subunits: The left lung was fixed by infusion of paraformaldehyde (4%) through the tracheal cannula (23). To locate the sGC subunits expression in lung tissue, sections were stained using anti-sGC α 1 or β 1 antibody (Cayman chemicals) and isotype rabbit IgG (Abcam, UK).

Quantification of immunohistochemical staining: sGC α 1 or sGC β 1 staining was quantified in the murine airway epithelium, in a marked area between the airway lumen and the basement membrane, using AxioVision software (Zeiss, Oberkochen, Germany) as previously described (31).

Measurement of emphysema: Quantification of emphysema in chronic (24 weeks) CS-exposed mice was determined by the mean linear intercept (Lm) and the destructive index (DI), as previously described (23,28,32).

Statistical analysis: Statistical analysis using nonparametric tests, was performed with Sigma Stat software (SPSS 20.0, Chicago, IL, USA). Correlation coefficients were calculated using Spearman's rank method. The association between FEV₁% and sGC mRNA and protein levels was further analyzed by linear regression analysis. Demographic and clinical

characteristics of the study population are expressed as mean \pm SD. Reported values are expressed as mean \pm SEM. $P < .05$ was considered significant.

RESULTS

I. Translational research into the role of soluble guanylyl cyclase: *human subjects.*

Subject demographics. The demographic and clinical characteristics of the 64 patients are summarized in Table 1.

sGC mRNA expression is decreased in smokers and patients with COPD. To evaluate the effect of cigarette smoking on the expression of sGC, we determined mRNA levels of sGC α 1, α 2 and β 1 subunits in lung tissue of never smokers (n = 11), smokers (n = 24) and patients with COPD (n = 29). There was a significant reduction of sGC α 1 mRNA levels in patients with COPD (p = 0.002) compared to never smokers (Figure 1A). mRNA levels of the sGC α 2 subunit were significantly decreased both in smokers (p = 0.002) and patients with COPD (p = 0.002) compared to never smokers (Figure 1C). Similarly, mRNA levels of the sGC β 1 subunit were significantly decreased both in smokers (p = 0.014) and patients with COPD (p = 0.004) compared to never smokers (Figure 1E).

mRNA expression of sGC subunits correlates with lung function in smokers and patients with COPD. sGC α 1 mRNA levels were positively correlated with post-bronchodilator FEV₁ (% predicted): $r_s = 0.366$, p = 0.004 (Figure 1B). mRNA levels of sGC α 2 subunit were also positively correlated with FEV₁: $r_s = 0.299$, p = 0.025 (Figure 1D). In a similar way, sGC β 1 mRNA levels revealed a significant correlation with FEV₁: $r_s = 0.293$, p = 0.025 (Figure 1F). Linear regression analysis was performed to adjust for possible confounders (age, gender, pack-years, FEV₁ and drug treatment). Importantly, the association between sGC α 1 and FEV₁, and between sGC α 2 and FEV₁ remained significant, even after adjustment for these confounders (Table E2). Positive correlations were found between α 1, α 2 and β 1 subunits and FEV₁/FVC ratio or carbon monoxide diffusing capacity (DL_{CO}), but not with the corresponding Kco (see online supplement results and Figure E 1).

sGC protein expression is decreased in smokers and patients with COPD. Protein expression of the sGC subunits in the lung were measured by Western blotting, in smokers (n = 9) and patients with COPD (n = 15) compared to never smokers (n = 9). The

demographic and clinical characteristics of this subpopulation of 33 subjects are summarized in Table E1 (online supplement). More specifically, protein levels of the $\alpha 1$ subunit of patients with COPD were significantly reduced ($p = 0.017$) compared to never smokers (Figure 2A, C). Moreover, protein levels of the $\beta 1$ subunit were reduced among smokers ($p = 0.027$) and patients with COPD ($p = 0.006$) compared to never smokers (Figure 2B, D). sGC $\alpha 1$ and sGC $\beta 1$ protein levels were positively correlated with FEV₁. The association between sGC $\alpha 1$ protein levels and FEV₁ remained significant after adjustment for possible confounders (Table E2).

Immunohistochemical localization of sGC expression in human lung tissues. To evaluate the cellular type where sGC is downregulated, we performed immunohistochemical staining in peripheral lung sections of smokers and patients with COPD. In lung section of never smokers, sGC $\alpha 1$ and $\beta 1$ subunits were present at high levels in bronchial and alveolar epithelial cells and in airway smooth muscle cells (Figure 3A, B). In agreement with our results from Western blotting experiments, staining of both sGC $\alpha 1$ and $\beta 1$ subunits was reduced in lung sections of smokers (Figure 3C, D) and patients with COPD (Figure 3E, F) in bronchial and alveolar epithelial cells and in airway smooth muscle cells.

II. Translational research into the role of soluble guanylyl cyclase: *murine model.*

sGC expression is decreased in mice after CS exposure. The expression of sGC subunit mRNA and protein was determined in the lungs of mice upon CS exposure, compared to air-exposed mice. mRNA levels of the sGC $\alpha 1$ subunit were significantly reduced after subacute and chronic CS exposure (Figure 4A), while mRNA levels of the sGC $\beta 1$ subunit were reduced upon acute, subacute and chronic CS exposure (Figure 4B). At the protein level, the expression of $\alpha 1$ subunit was significantly reduced upon acute, subacute and chronic CS exposure (Figure 4C, E). In addition, a significant reduction of $\beta 1$ subunit was detected upon subacute and chronic CS exposure (Figure 4D, F). Interestingly, there was a significant reduction of the expression of sGC $\alpha 2$ at mRNA and protein level in mice, reinforcing our

notion of sGC downregulation upon acute CS exposure (Figure E2). Chronic CS exposure induced emphysema, as shown by a significantly increased mean linear intercept (Lm: air: $39.2 \pm 0.8 \mu\text{m}$ vs. CS: $42.9 \pm 1.1 \mu\text{m}$; $p < 0.05$) and destructive index (DI: air: 18.82 ± 1.08 vs. CS: 41.12 ± 2.08 ; $p < 0.01$) (Figure E3).

Immunohistochemical detection of sGC subunit expression in lung tissue after CS exposure. To evaluate the cellular type and distribution of sGC downregulation, we performed IHC staining of murine lung sections. Both $\alpha 1$ and $\beta 1$ subunit staining was detected in bronchial and alveolar epithelial cells and in airway smooth muscle cells. In control lungs, $\alpha 1$ and $\beta 1$ subunits were present at high levels after 3 days (Figure 5A, C), 4 (Figure 5E, G) and 24 weeks (Figure 5I, K) of air exposure. Using imaging software, we quantified the sGC $\alpha 1$ or sGC $\beta 1$ positive staining in the airway epithelium. CS exposure did not change the amount of sGC $\beta 1$ staining in the airway epithelium (Figure 6B, D and F). However, sGC $\alpha 1$ protein was significantly decreased within the epithelium after 3 days, 4 weeks and 24 weeks of CS exposure, which is in agreement with the results from Western blotting experiments on total lung tissue (Figure 6A, C and E).

sGC $\alpha 1$ deficiency in mice aggravates airway hyperresponsiveness upon acute CS exposure. To decipher the role of sGC downregulation in lung inflammation and respiratory system mechanics, we compared sGC $\alpha 1^{-/-}$ and WT mice after 3 days (acute) of CS exposure. sGC $\alpha 1^{-/-}$ mice subjected to acute CS exposure exhibited higher airway resistance (R) to increasing 5-HT doses, compared to acute CS exposed WT mice (Figure 7A, B). Acute CS exposure significantly increased the absolute numbers of total BAL cells, alveolar macrophages, neutrophils, DCs, CD4⁺ and CD8⁺ T lymphocytes. However, the level of increase in BAL cellularity and differentiation did not differ between the two mouse strains (Figure E4).

BAY 58-2667 administration attenuates airway hyperresponsiveness upon acute CS exposure. Acute CS exposure displayed increasing R values in a 5-HT dose-dependent manner. BAY 58-2667 attenuated airway resistance, in the CS group (Figure 8A, B). Acute CS exposure significantly increased the absolute numbers of total BAL cells, alveolar

macrophages, neutrophils, DCs, CD4⁺ and CD8⁺ T lymphocytes. However, administration of BAY 58-2667 did not affect acute CS-induced lung inflammation (Figure E5).

BAY 58-2667 administration restores the NO/sGC/cGMP pathway upon acute CS exposure. The levels of sGC α 1 and sGC β 1 protein upon acute CS exposure were analyzed by Western Blot in BAY 58-2667 treated mice. CS exposure reduced the sGC α 1 protein levels in sham-treated mice, while the levels remained similar between air- and CS-exposed mice after BAY 58-2667 treatment (data not shown). There were no differences for sGC β 1 protein levels upon acute CS exposure (data not shown). To determine the effect of CS exposure on sGC activity, we measured cGMP levels in BAL fluid in mice. Acute CS exposure resulted in significant reduction of cGMP levels (pmol/mg of protein) compared to controls. Administration of BAY 58-2667 (i.p.) in mice after acute CS exposure restored BAL cGMP levels (Figure 9A) and increased phosphorylation of VASP, a marker of PKG activity (Figure 9B). To elucidate the effect of CS exposure in cGMP signaling we determined PDE5 protein levels. Acute CS exposure significantly increased the PDE5 lung protein levels compared to the air-exposed group, whereas BAY 58-2667 given i.p. after acute CS exposure attenuated PDE5 almost to the control levels (Figure 9C).

DISCUSSION

The major findings of our study are: i) Expression of sGC is decreased in smokers and in patients with COPD; ii) in mice, acute, subacute and chronic CS exposure reduces expression of sGC; iii) genetic downregulation of sGC signaling aggravates bronchial hyperresponsiveness (BHR) to serotonin upon CS exposure; and iv) pharmacological activation of sGC attenuates CS-induced BHR to serotonin.

The expression of sGC was significantly reduced in peripheral lung tissue of smokers without airflow limitation and patients with COPD, compared to never smokers. The expression of the sGC α 1 subunit was decreased in patients with COPD, while the expression of α 2 and β 1 subunits was reduced in smokers with or without COPD. This is the first time that the presence and regulation of the α 2 subunit has been shown in relation to human respiratory disease. To determine the cell types in which the reduction of sGC expression was more pronounced, we stained human lung sections with antibodies against the most prevailing sGC α 1 and β 1 subunits. We observed a positive staining of both sGC α 1 and β 1 subunits in bronchial and alveolar epithelial cells and in airway smooth muscle cells in the lung sections of never smokers, smokers without COPD and patients with COPD. In our study, we did not evaluate the expression of the sGC α 2 subunit by Western blot analysis or by immunohistochemistry. Since sGC is an obligatory heterodimer (33), the reduction of sGC β 1 subunit expression suffices for the decline in sGC activity irrespective of any changes in the α subunits.

To investigate if the downregulation of sGC was related to lung function, we examined the expression of sGC in correlation with the corresponding spirometric parameters. Remarkably, expression of the sGC α 1, α 2 and β 1 subunits significantly correlated with disease severity, as measured by the percentage of predicted FEV₁. The association of sGC α 1 mRNA levels, sGC α 2 mRNA levels and sGC α 1 protein levels with FEV₁ remained significant after adjustment for possible confounders. To our knowledge, this is the first time

that downregulation of sGC has been shown to occur in relation to smoking and disease severity in patients with COPD.

To investigate the *in vivo* functional role of sGC, we examined the expression and function of sGC in a well-established murine model of COPD (23). The expression of sGC was reduced in mice upon acute, subacute or chronic CS exposure, both on the mRNA and the protein level. These findings are in accordance with our results in smokers and patients with COPD, and implicate a direct effect of CS on the expression of sGC. Several *in vitro* (15,17,34-36) and *in vivo* (18-20) studies have indicated that inflammatory stimuli, such as cytokines, may be involved in the attenuation of sGC mRNA stability or sGC protein expression. By performing immunohistochemistry on lung tissue of air- and CS-exposed mice, we demonstrated that sGC was mainly expressed in airway smooth muscle cells, which are relevant to airway hyperresponsiveness, and in airway and alveolar epithelial cells, conform with the human data. The decreased expression of sGC α 1 in the epithelium implicates a direct effect of environmental noxious stimuli in murine lungs. The protein expression of sGC β 1 in the airway epithelium was not changed upon CS exposure, while it was decreased in total lung tissue. Consequently, CS exposure may affect other sGC β 1-expressing cells in the lung, such as airway smooth muscle cells.

Inflammatory stimuli and oxidative stress could impair sGC signaling and promote the elevation of smooth muscle tone in CS-exposed mice. Reduced expression of sGC in the airway epithelial and smooth muscle cells has also been identified in the murine asthma model (18). In this model, oxidation of sGC and further attenuation of the enzyme activity by an sGC inhibitor, ODQ (1H-[1,2,4]oxadiazole[4,3-a]quinoxalin-1-one), was accompanied by more pronounced BHR to methacholine (18). Because available sGC inhibitors are neither enzyme- nor isoform-specific (37-39), we evaluated the role of sGC in respiratory system mechanics and lung inflammation upon acute CS exposure, using sGC α 1 knockout mice (25).

CS exposure induced BHR to serotonin in wild type mice, which was aggravated when sGC/cGMP signaling was impaired, in sGC α 1 knockout mice. Interestingly, the increased

bronchoconstriction in sGC α 1 knockout mice was not accompanied by an exaggerated inflammatory response, highlighting the importance of sGC α 1 β 1-derived cGMP in the regulation of the airway smooth muscle tone upon CS exposure. In air-exposed mice, no difference in airway responsiveness was observed between wild type mice and sGC α 1 knockout mice. Also in a murine model of hypertension, sGC α 1 knockout mice do not have a hypertensive phenotype on a C57Bl/6 background (40). Seemingly, sGC α 2 is able to provide sufficient cGMP in basal conditions. Consequently, it is probable that in the present study, sGC might be more important in COPD than in basal conditions.

To further unravel the potentially protective role of sGC, wild type mice were treated with the sGC activator BAY 58-2667. BAY 58-2667 has been shown to preferentially protect against diseases where sGC is downregulated due to oxidation, such as pulmonary hypertension or cardiovascular diseases (41,42). BAY 58-2667 activates the NO-insensitive form of sGC (after heme removal following its oxidation) and restores cGMP signaling (42). Indeed, in BAY-treated CS-exposed mice, we observed an attenuated BHR to serotonin and restored levels of sGC α 1 protein and of cGMP. This cGMP is exported out of airway epithelial cells (43) by cGMP transporters (44). Therefore, the cGMP that we detect in the BAL fluid, probably originates mainly from the airway epithelium. Moreover, phosphorylation of Vasodilator-stimulated Phosphoprotein (VASP), a marker of protein kinase G activity, was increased in BAY-treated CS-exposed mice compared to vehicle-treated mice. Phosphodiesterase (PDE)-5, which hydrolyses cGMP to GMP, was increased upon smoke exposure. Our findings suggest that both downregulation of sGC and increased expression of PDE-5, affect CS-induced BHR. Remarkably, in CS-exposed mice administration of BAY 58-2667 attenuated PDE-5 almost to control levels, implicating a negative feedback regulation. Such negative feedback regulation has also been shown to occur at the posttranscriptional level, as increased cGMP signaling leads to PKG-mediated phosphorylation on Ser-92 and inactivation of PDE-5 (45). These findings place PKG activity as an important junction in the regulation of smooth muscle tone. Our finding, concerning the bronchodilating role of sGC signaling, is consistent with several studies in isolated trachea

strips or animal models of asthma (46). In vascular diseases, potential therapeutic strategies which enhance sGC signaling have been shown to diminish oxidative stress in smooth muscle cells. Stimulation of sGC diminished production of ROS by NADPH oxidase in an ischemia/reperfusion model of acute lung injury (19). Moreover, neutrophil rolling and adhesion, which was increased in the pulmonary capillaries of eNOS $-/-$ mice, was significantly reduced by activation of sGC (47). In general, the effects of the sGC/cGMP/PKG pathway could interactively mediate a protective role in CS-induced BHR (Figure 10).

There are some limitations to this study that should be addressed. The gender ratio in the human study population is unbalanced, since the majority of never smokers is female, while all patients with COPD are male. This unbalanced gender ratio could influence sGC levels. However, gender was incorporated into a linear regression model, taking into account this possible confounding effect.

Another limitation is the relatively short exposure time to cigarette smoke of the experiment with BAY 58-2667 and with the sGC α 1 knockout mice. In this study, reduction of sGC levels was mainly a smoking effect. However, sGC α 1 mRNA and protein levels were significantly associated with FEV₁ after adjusting for possible confounders, suggesting that there is also a disease effect. To further address the role of sGC in the pathogenesis of COPD, chronic (24 weeks) CS-exposed sGC α 1 $-/-$ mice or wild type mice treated with BAY 58-2667 should be investigated.

In conclusion, we demonstrate that sGC is downregulated due to CS exposure in humans and mice, leading to BHR. Genetic ablation of sGC aggravates CS-induced BHR, whereas pharmacological activation of sGC by BAY 58-2667 has a protective effect. Our translational findings may offer a novel mechanistic concept in COPD and a new therapeutic approach.

Acknowledgements

The authors would like to thank Greet Barbier, Eliane Castrique, Indra De Borle, Katleen De Saedeleer, Anouck Goethals, Marie-Rose Mouton, Ann Neesen, Christelle Snauwaert and Evelyn Spruyt for their technical assistance (Department of Respiratory Medicine, Ghent University Hospital, Ghent, Belgium). The authors also thank dr. Tania Maes (Department of Respiratory Medicine, Ghent University Hospital, Ghent, Belgium) for critically reading the manuscript, Lies Lahousse (Department of Respiratory Medicine, Ghent University Hospital, Ghent, Belgium) for help with statistical analyses and dr. Juanita Vernooy and dr. Mieke Dentener (Department of Respiratory Medicine, Maastricht University Medical Center, Maastricht, The Netherlands) for providing human lung tissue.

Reference List

1. Lopez AD, Mathers CD, Ezzati M, Jamison DT, and Murray CJ. Global and regional burden of disease and risk factors, 2001: systematic analysis of population health data. *Lancet* 2006;367:1747-1757.
2. Brusselle GG, Joos GF, and Bracke KR. New insights into the immunology of chronic obstructive pulmonary disease. *Lancet* 2011;378:1015-1026.
3. Oswald D.N.C. and Medvei V.C. Chronic bronchitis; the effect of cigarette-smoking. *Lancet* 1955;269:843-844.
4. Churg A, Cosio M, and Wright JL. Mechanisms of cigarette smoke-induced COPD: insights from animal models. *Am J Physiol Lung Cell Mol Physiol* 2008;294:L612-L631.
5. Tiddens HA, Hofhuis W, Bogaard JM, Hop WC, de Bruin H., Willems LN, and de Jongste JC. Compliance, hysteresis, and collapsibility of human small airways. *Am J Respir Crit Care Med* 1999;160:1110-1118.
6. Mergia E, Russwurm M, Zoidl G, and Koesling D. Major occurrence of the new alpha2beta1 isoform of NO-sensitive guanylyl cyclase in brain. *Cell Signal* 2003;15:189-195.
7. Koesling D, Russwurm M, Mergia E, Mullershausen F, and Friebe A. Nitric oxide-sensitive guanylyl cyclase: structure and regulation. *Neurochem Int* 2004;45:813-819.
8. Mergia E, Friebe A, Dangel O, Russwurm M, and Koesling D. Spare guanylyl cyclase NO receptors ensure high NO sensitivity in the vascular system. *J Clin Invest* 2006;116:1731-1737.
9. Nimmegeers S, Sips P, Buys E, Brouckaert P, and Van de Voorde J. Functional role of the soluble guanylyl cyclase alpha(1) subunit in vascular smooth muscle relaxation. *Cardiovasc Res* 2007;76:149-159.
10. Friebe A and Koesling D. Regulation of nitric oxide-sensitive guanylyl cyclase. *Circ Res* 2003;93:96-105.
11. Francis SH, Busch JL, Corbin JD, and Sibley D. cGMP-dependent protein kinases and cGMP phosphodiesterases in nitric oxide and cGMP action. *Pharmacol Rev* 2010;62:525-563.
12. Ansarin K, Chatkin JM, Ferreira IM, Gutierrez CA, Zamel N, and Chapman KR. Exhaled nitric oxide in chronic obstructive pulmonary disease: relationship to pulmonary function. *Eur Respir J* 2001;17:934-938.
13. Brindicci C, Kharitonov SA, Ito M, Elliott MW, Hogg JC, Barnes PJ, and Ito K. Nitric oxide synthase isoenzyme expression and activity in peripheral lung tissue of patients with chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 2010;181:21-30.
14. Ichinose M, Sugiura H, Yamagata S, Koarai A, and Shirato K. Increase in reactive nitrogen species production in chronic obstructive pulmonary disease airways. *Am J Respir Crit Care Med* 2000;162:701-706.

15. Papapetropoulos A, Abou-Mohamed G, Marczin N, Murad F, Caldwell RW, and Catravas JD. Downregulation of nitrovasodilator-induced cyclic GMP accumulation in cells exposed to endotoxin or interleukin-1 beta. *Br J Pharmacol* 1996;118:1359-1366.
16. Papapetropoulos A, Go CY, Murad F, and Catravas JD. Mechanisms of tolerance to sodium nitroprusside in rat cultured aortic smooth muscle cells. *Br J Pharmacol* 1996;117:147-155.
17. Gerassimou C, Kotanidou A, Zhou Z, Simoes DC, Roussos C, and Papapetropoulos A. Regulation of the expression of soluble guanylyl cyclase by reactive oxygen species. *Br J Pharmacol* 2007;150:1084-1091.
18. Papapetropoulos A, Simoes DC, Xanthou G, Roussos C, and Gratziou C. Soluble guanylyl cyclase expression is reduced in allergic asthma. *Am J Physiol Lung Cell Mol Physiol* 2006;290:L179-L184.
19. Egemnazarov B, Sydykov A, Schermuly RT, Weissmann N, Stasch JP, Sarybaev AS, Seeger W, Grimminger F, and Ghofrani HA. Novel soluble guanylyl cyclase stimulator BAY 41-2272 attenuates ischemia-reperfusion-induced lung injury. *Am J Physiol Lung Cell Mol Physiol* 2009;296:L462-L469.
20. Glynos C, Kotanidou A, Orfanos SE, Zhou Z, Simoes DC, Magkou C, Roussos C, and Papapetropoulos A. Soluble guanylyl cyclase expression is reduced in LPS-induced lung injury. *Am J Physiol Regul Integr Comp Physiol* 2007;292:R1448-R1455.
21. Tsai EJ, Liu YC, Koitabashi N, Bedja D, Danner T, Jasmin JF, Lisanti MP, Friebe A, Takimoto E, and Kass DA. Pressure-Overload-Induced Subcellular Relocalization/Oxidation of Soluble Guanylyl Cyclase in the Heart Modulates Enzyme Stimulation. *Circ Res* 2012;110:295-303.
22. Evgenov OV, Pacher P, Schmidt PM, Hasko G, Schmidt HH, and Stasch JP. NO-independent stimulators and activators of soluble guanylate cyclase: discovery and therapeutic potential. *Nat Rev Drug Discov* 2006;5:755-768.
23. D'hulst AI, Vermaelen KY, Brusselle GG, Joos GF, and Pauwels RA. Time course of cigarette smoke-induced pulmonary inflammation in mice. *Eur Respir J* 2005;26:204-213.
24. Pauwels RA, Buist AS, Calverley PM, Jenkins CR, and Hurd SS. Global strategy for the diagnosis, management, and prevention of chronic obstructive pulmonary disease. NHLBI/WHO Global Initiative for Chronic Obstructive Lung Disease (GOLD) Workshop summary. *Am J Respir Crit Care Med* 2001;163:1256-1276.
25. Buys ES, Sips P, Vermeersch P, Raheer MJ, Rogge E, Ichinose F, Dewerchin M, Bloch KD, Janssens S, and Brouckaert P. Gender-specific hypertension and responsiveness to nitric oxide in sGC α 1 knockout mice. *Cardiovasc Res* 2008;79:179-186.
26. Buys ES, Cauwels A, Raheer MJ, Passeri JJ, Hobai I, Cawley SM, Rauwerdink KM, Thibault H, Sips PY, Thoonen R, Scherrer-Crosbie M, Ichinose F, Brouckaert P, and Bloch KD. sGC(α)1(β)1 attenuates cardiac dysfunction and mortality in murine inflammatory shock models. *Am J Physiol Heart Circ Physiol* 2009;297:H654-H663.
27. Levitt RC and Mitzner W. Autosomal recessive inheritance of airway hyperreactivity to 5-hydroxytryptamine. *J Appl Physiol* 1989;67:1125-1132.

28. Bracke KR, D'hulst AI, Maes T, Demedts IK, Moerloose KB, Kuziel WA, Joos GF, and Brusselle GG. Cigarette smoke-induced pulmonary inflammation, but not airway remodelling, is attenuated in chemokine receptor 5-deficient mice. *Clin Exp Allergy* 2007;37:1467-1479.
29. Vermaelen K and Pauwels R. Accurate and simple discrimination of mouse pulmonary dendritic cell and macrophage populations by flow cytometry: methodology and new insights. *Cytometry A* 2004;61:170-177.
30. Coletta C, Papapetropoulos A, Erdelyi K, Olah G, Modis K, Panopoulos P, Asimakopoulou A, Gero D, Sharina I, Martin E, and Szabo C. Hydrogen sulfide and nitric oxide are mutually dependent in the regulation of angiogenesis and endothelium-dependent vasorelaxation. *Proc Natl Acad Sci U S A* 2012;109:9161-9166.
31. Demoor T, Bracke KR, Dupont LL, Plantinga M, Bondue B, Roy MO, Lannoy V, Lambrecht BN, Brusselle GG, and Joos GF. The role of ChemR23 in the induction and resolution of cigarette smoke-induced inflammation. *J Immunol* 2011;186:5457-5467.
32. Saetta M, Shiner RJ, Angus GE, Kim WD, Wang NS, King M, Ghezzi H, and Cosio MG. Destructive index: a measurement of lung parenchymal destruction in smokers. *Am Rev Respir Dis* 1985;131:764-769.
33. Buechler WA, Nakane M, and Murad F. Expression of soluble guanylate cyclase activity requires both enzyme subunits. *Biochem biophys Res Commun* 1991;174:351-357.
34. Filippov G, Bloch DB, and Bloch KD. Nitric oxide decreases stability of mRNAs encoding soluble guanylate cyclase subunits in rat pulmonary artery smooth muscle cells. *J Clin Invest* 1997;100:942-948.
35. Kloss S, Srivastava R, and Mulsch A. Down-regulation of soluble guanylyl cyclase expression by cyclic AMP is mediated by mRNA-stabilizing protein HuR. *Mol Pharmacol* 2004;65:1440-1451.
36. Papapetropoulos A, Cziraki A, Rubin JW, Stone CD, and Catravas JD. cGMP accumulation and gene expression of soluble guanylate cyclase in human vascular tissue. *J Cell Physiol* 1996;167:213-221.
37. Feelisch M, Kotsonis P, Siebe J, Clement B, and Schmidt HH. The soluble guanylyl cyclase inhibitor 1H-[1,2,4]oxadiazolo[4,3,-a] quinoxalin-1-one is a nonselective heme protein inhibitor of nitric oxide synthase and other cytochrome P-450 enzymes involved in nitric oxide donor bioactivation. *Mol Pharmacol* 1999;56:243-253.
38. Wegener JW, Closs EI, Forstermann U, and Nawrath H. Failure of 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) to inhibit soluble guanylyl cyclase in rat ventricular cardiomyocytes. *Br J Pharmacol* 1999;127:693-700.
39. Zhao Y, Brandish PE, DiValentin M, Schelvis JP, Babcock GT, and Marletta MA. Inhibition of soluble guanylate cyclase by ODQ. *Biochemistry* 2000;39:10848-10854.
40. Buys ES, Raheer MJ, Kirby A, Mohd S, Baron DM, Hayton SR, Tainsh LT, Sips PY, Rauwerdink KM, Yan Q, Tainsh RET, Shakartzi HR, Stevens C, Decaluw E, K, Rodrigues-Machado MdG, Malhotra R, Van de Voorde J, Wang T, Brouckaert P, Daly MJ, and

- Bloch KD. Genetic modifiers of hypertension in soluble guanylate cyclase α 1-deficient mice. *J Clin Invest* 2012;122:2316-2325.
41. Chester M, Seedorf G, Tourneux P, Gien J, Tseng N, Grover T, Wright J, Stasch JP, and Abman SH. Cinaciguat, a soluble guanylate cyclase activator, augments cGMP after oxidative stress and causes pulmonary vasodilation in neonatal pulmonary hypertension. *Am J Physiol Lung Cell Mol Physiol* 2011;301:L755-L764.
 42. Stasch JP, Schmidt PM, Nedvetsky PI, Nedvetskaya TY, AK HS, Meurer S, Deile M, Taye A, Knorr A, Lapp H, Muller H, Turgay Y, Rothkegel C, Tersteegen A, Kemp-Harper B, Muller-Esterl W, and Schmidt HH. Targeting the heme-oxidized nitric oxide receptor for selective vasodilatation of diseased blood vessels. *J Clin Invest* 2006;116:2552-2561.
 43. Geary CA, Goy MF, and Boucher RC. Synthesis and vectorial export of cGMP in airway epithelium: expression of soluble and CNP-specific guanylate cyclases. *Am J Physiol* 1993;265:L598-L605.
 44. Sager G. Cyclic GMP transporters. *Neurochem Int* 2004;45:865-873.
 45. Mullershausen F, Russwurm M, Thompson WJ, Liu L, Koesling D, and Friebe A. Rapid nitric oxide-induced desensitization of the cGMP response is caused by increased activity of phosphodiesterase type 5 paralleled by phosphorylation of the enzyme. *J Cell Biol* 2001;155:271-278.
 46. Hamad AM, Clayton A, Islam B, and Knox AJ. Guanylyl cyclases, nitric oxide, natriuretic peptides, and airway smooth muscle function. *Am J Physiol Lung Cell Mol Physiol* 2003;285:L973-L983.
 47. Ahluwalia A, Foster P, Scotland RS, McLean PG, Mathur A, Perretti M, Moncada S, and Hobbs AJ. Antiinflammatory activity of soluble guanylate cyclase: cGMP-dependent down-regulation of P-selectin expression and leukocyte recruitment. *Proc Natl Acad Sci U S A* 2004;101:1386-1391.

Table 1. SUBJECT CHARACTERISTICS			
Characteristics	Never smokers n=11	Smokers n=24	COPD n=29
Age, years	59 ± 14.04	62 ± 9.4	65 ± 7.5
Gender : M/F	3/8	18/6	29/0
Smoking, pack-year	0	31.95 ± 20.6	53.36 ± 26 ^{*,#}
Smoking status: current/ex smokers	0	12/12	17/12
COPD GOLD stage	na	na	II
FEV₁ % of predicted	104.2 ± 13.4	101.6 ± 17	67.3 ± 8 ^{*,#}
FEV₁/FVC ratio	77.9 ± 4.3	76.2 ± 4.3	55.2 ± 7.4 ^{*,#}
DLco	93.1±15.3	89.3±17.6	72.9±20 ^{*,#}
K_{CO} % of predicted	104. 6 ± 18	95.6 ± 14.4	90.7 ± 23.5
Oral corticosteroids	0	1	4
Inhaled corticosteroids	0	0	12
Long acting beta-agonists	0	1	15
Tiotropium	0	2	13
Statins	0	3	4

Definition of abbreviations: na = not applicable; F= female; M= male; **FEV₁**: Forced Expiratory Volume in one second; **FVC**: Forced Vital Capacity; **DLco**= carbon monoxide diffusing capacity; **Kco**= carbon monoxide gas transfer corrected for alveolar volume; **pack-year**= number of packs of cigarettes smoked per day, multiplied by the number of years of smoking. Values are expressed as means ± SD; *: p<0.05 Vs never smokers #: p<0.05 Vs smokers.

FIGURE LEGENDS

Figure 1: sGC mRNA expression in human lungs of never smokers, smokers and patients with COPD and correlation with COPD severity. **A.** sGC α 1 mRNA levels are decreased in (n=29). **C.** sGC α 2 mRNA levels are decreased in smokers (n=24) and patients with COPD (n=29), compared to never smokers (NS) (n=11). **E.** sGC β 1 mRNA levels are decreased in smokers (n=24) and patients with COPD (n=29), compared to never smokers (NS) (n=11). Three reference genes (GADPH, HPRT1, PPIA) were used for normalization. Values are expressed as mean \pm SEM; *p< 0.05 from NS. **B. D and F.** sGC mRNA levels of α 1 (B), α 2 (D) and β 1 (F) subunit of human lung tissues were correlated with FEV₁% (% of predicted). Spearman correlation coefficient (r_s) and P value are shown.

Figure 2: sGC protein expression in human lung tissues of never smokers, smokers and patients with COPD. Western blot was performed on a total of 33 patients (9 never smokers, 9 smokers and 15 patients with COPD) **A.** Example of Western blot for sGC α 1 **B.** Example of Western blot for sGC β 1. Blots were quantified by densitometry for sGC α 1(**C**) and sGC β 1(**D**). Expression for each subunit normalized for β -actin was set at 100% for never smokers (NS). Values are expressed as mean \pm SEM; *p< 0.05 **p< 0.01 from NS.

Figure 3: Immunohistochemical localization of sGC expression in human lung tissues of never smokers, smokers and patients with COPD. Representative photomicrographs of lung sections show small airways from a never smoker (NS) (A and B), a smoker with normal lung function (C and D) and a patient with COPD stage II (E and F), immunostained for identification of α 1 and β 1 subunits respectively. sGC subunits were visualized with 3,3-diaminobenzidine, which produces a dark brown color. Lung section of a never smoker was used for isotype control staining (G and H). (magnification x 200).

Figure 4: Effect of CS exposure on sGC expression in mice: mRNA levels of sGC α 1 and β 1 subunits. **A and B.** mRNA levels of sGC α 1 (A) and β 1 (B) upon acute (3 days), subacute (4 weeks) and chronic (24 weeks) CS exposure. cDNA samples from total RNA, extracted by homogenized mice lung, were used and PCR amplifications were performed in triplicate. To calculate the relative quantity of the respective subunit, the CT method was

used; GAPDH was used for normalization. **C and D.** Protein levels of sGC α 1 or sGC β 1 upon acute (3 days), subacute (4 weeks) and chronic (24 weeks) CS exposure. **C.** Example of Western blot for sGC α 1, **D.** Example of Western blot for sGC β 1. Western blot was performed on 8 mice per group. **E and F.** Blots were quantified by densitometry. Expression for each subunit normalized for β -actin was set at 100% for air-exposed mice. Values are expressed as mean \pm SEM; n=8/group; *p< 0.05 **p< 0.01 ***p< 0.001 from air-exposed mice.

Figure 5: Immunohistochemical localization of sGC expression in mice after 3 days, 4 and 24 weeks of CS exposure. Representative photomicrographs of lung sections show airway and alveolar epithelium and airway smooth muscle after staining for α 1 or β 1 subunits upon acute (3 days; A,B and C,D), subacute (4 weeks; E,F and G,H) and chronic (24 weeks; I,J and K,L) air or CS exposure. Lung sections are immunostained for identification of sGC subunits and visualized with 3,3-diaminobenzidine, which produces a dark brown color. Lung section from 3-days air-exposed mice was used for isotype control staining (M, N). (magnification x 400).

Figure 6: Quantification of sGC α 1 and sGC β 1 staining in airway epithelium in mice after 3 days, 4 and 24 weeks of CS exposure. Quantification of sGC β 1 staining in the airway epithelium upon 3 days (B), 4 weeks (D) or 24 weeks (F) of CS exposure. Quantification of sGC α 1 staining in the epithelium after 3 days (A), 4 weeks (C) and 24 weeks (E) of CS exposure. Values are expressed as mean \pm SEM; n=8/group; *p< 0.05 **p< 0.01 from air-exposed mice.

Figure 7: sGC α 1 deficiency in mice aggravates airway hyperresponsiveness upon acute CS exposure. **A.** Effect of CS on airway resistance (R) percent increase of baseline in a dose response to serotonin (5-HT) challenge in sGC α 1^{-/-} and WT mice. **B.** Area under curve for airway resistance. Values are expressed as mean \pm SEM; n=8/group; *p< 0.05 from air-exposed groups and # p< 0.05 from CS-exposed group of WT mice.

Figure 8: BAY 58-2667 administration in acute cigarette smoke-exposed mice, ameliorates bronchoconstriction. **A.** Effect of BAY 58-2667 on airway resistance (R)

percent increase in a dose response to serotonin (5-HT) challenge. and **B**. Area under curve for airway resistance. Values are expressed as mean \pm SEM; n=8/group; *p< 0.05 from air-exposed group and # p< 0.05 from CS + BAY 58-2667 group.

Figure 9: BAY 58-2667 administration restores sGC signaling. A. cGMP levels in BAL. Administration of BAY 58-2667 in mice after CS exposure restored cGMP levels in the BAL to the control levels. **B.** Acute CS exposure reduced lung phosphorylated ser239 VASP protein levels. Administration of BAY 58-2667 in CS-exposed mice increased the levels of ser239 VASP compared to the air group. Representative Western blots for ser239 VASP and total VASP are shown. Western blot was performed on 8 mice per group. Expression for ser239 VASP was normalized for total VASP and set at 100% for air-exposed mice. **C.** PDE5 protein levels were increased upon acute CS exposure. Administration of BAY 58-2667 attenuated PDE5 levels. Blots were quantified by densitometry. Representative Western blots for PDE5 and actin are shown. Western blot was performed on 8 mice per group. Expression for PDE5 was normalized for β -actin and set at 100% for air-exposed mice. Values are expressed as mean \pm SEM; n=8/group; *p< 0.05 from air-exposed group.

Figure 10: The proposed pathway for cigarette smoke-induced bronchoconstriction involves reduced expression (\downarrow) of soluble guanylyl cyclase (sGC) and impaired activation of oxidized heme-free enzyme state, followed by reduced (\downarrow) cyclic guanosine monophosphate (cGMP) generation and reduced cGMP-dependent protein kinase (PKG) phosphorylation as reflected by attenuated phosphorylation of VASP. The cGMP signal is mainly determined by phosphodiesterases (PDE) in a negative feed-back regulation. The impaired sGC/cGMP/PKG signaling contributes to elevated smooth muscle tone and bronchoconstriction.

Figure 1

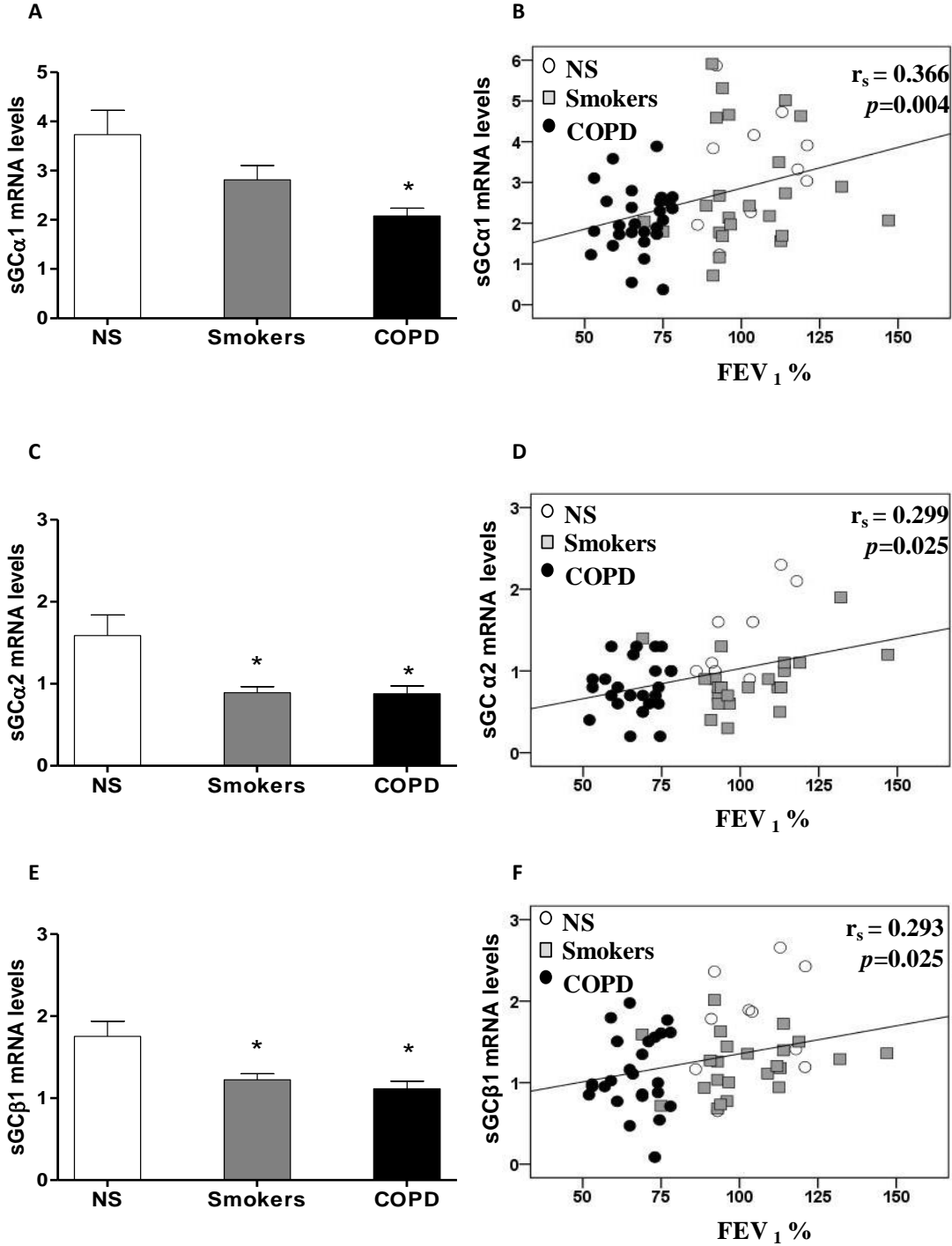


Figure 2

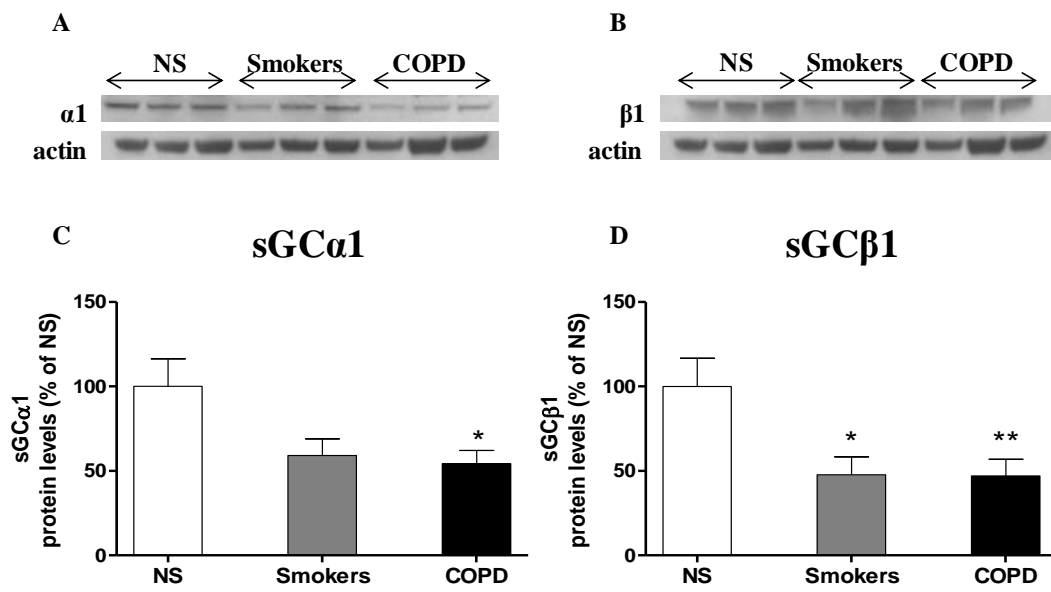


Figure 3

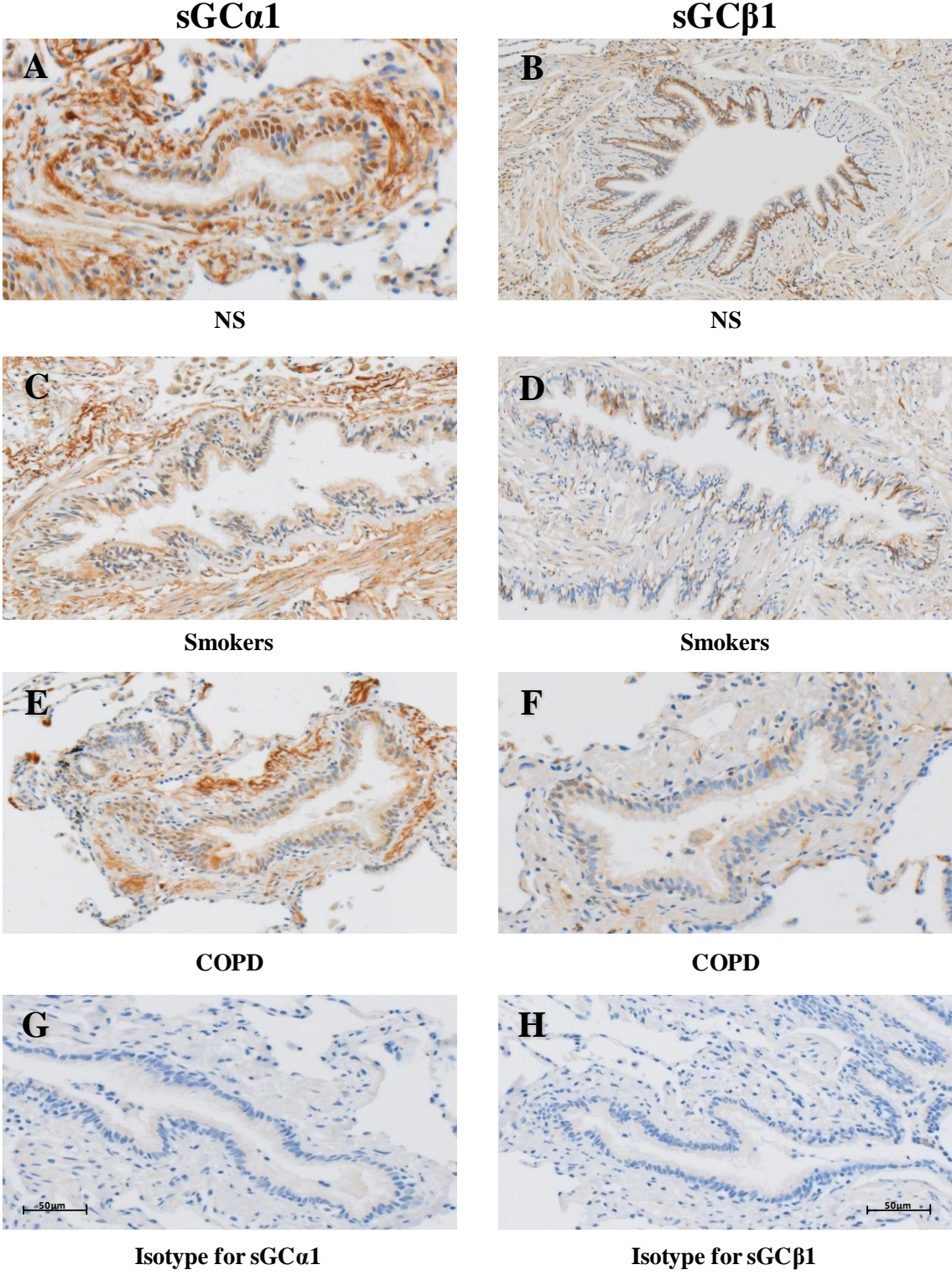


Figure 4

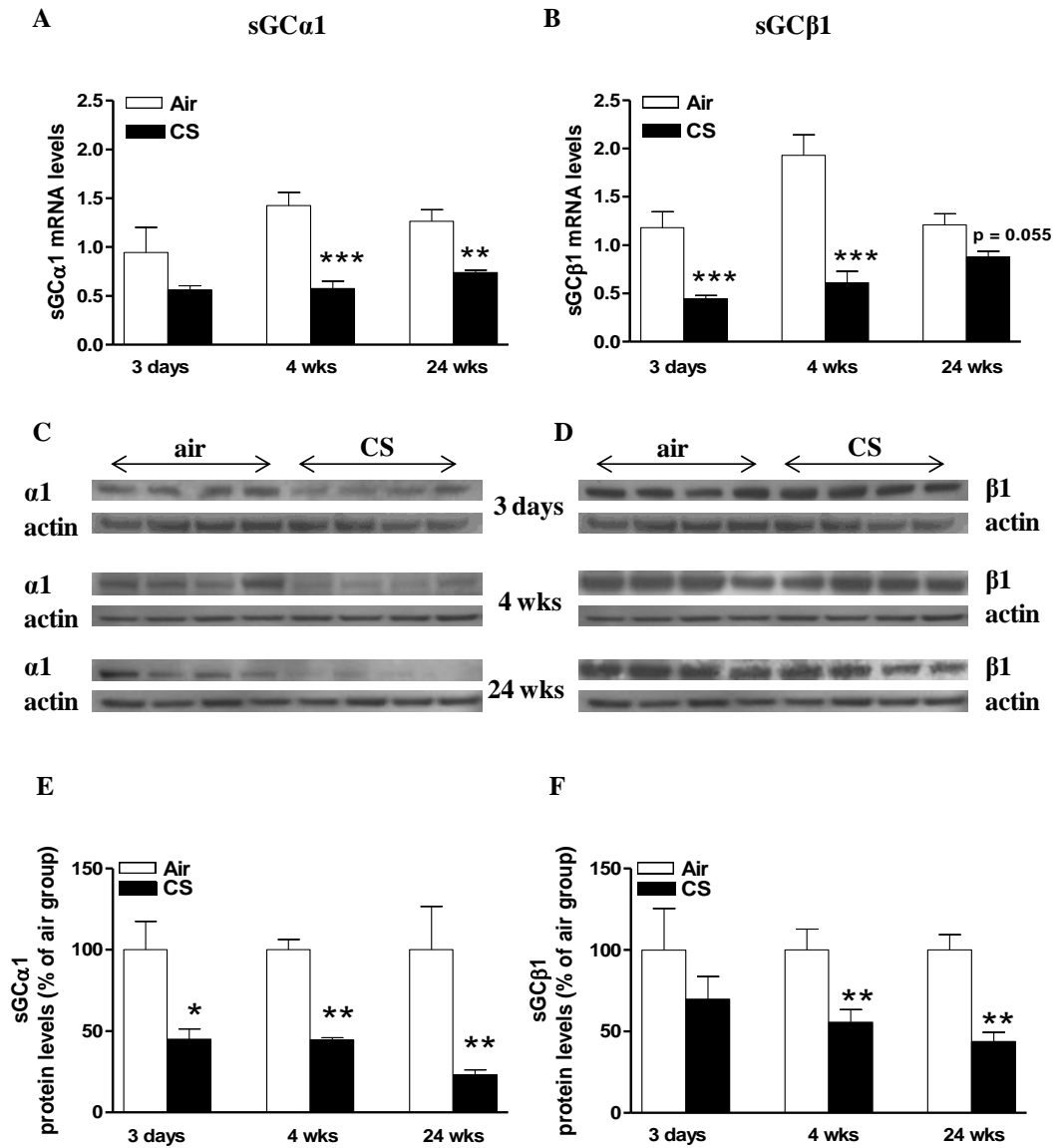


Figure 5

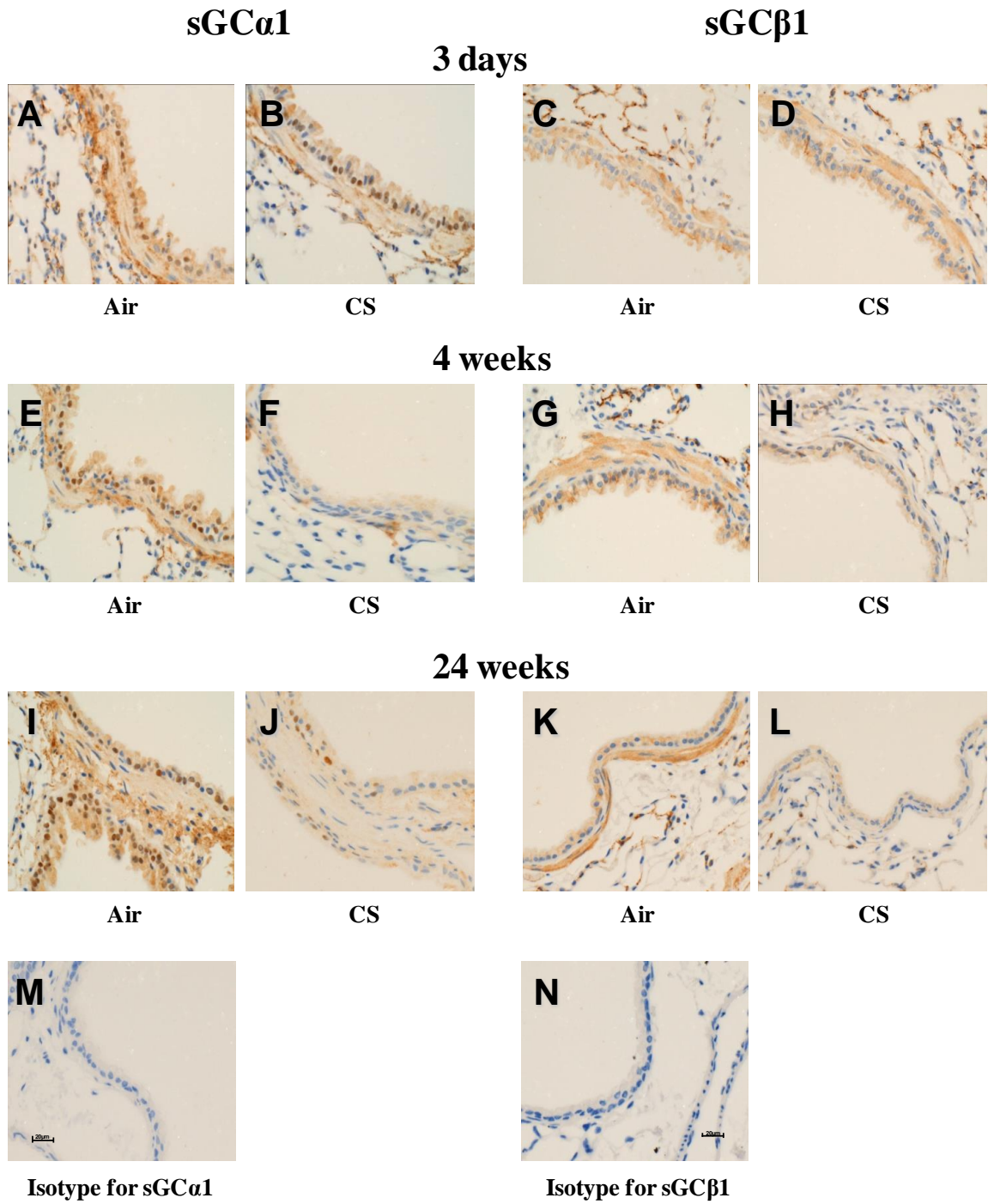


Figure 6

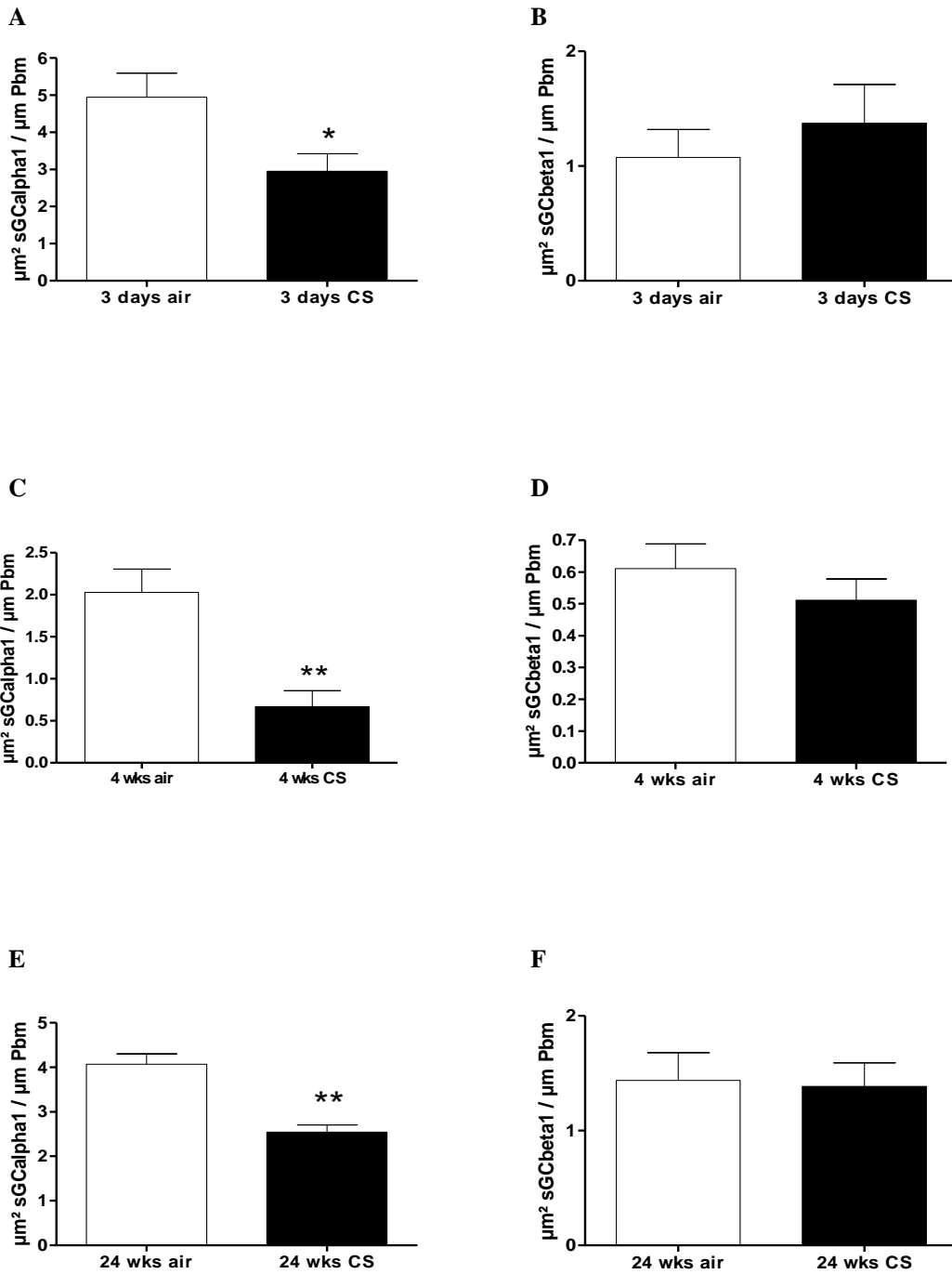
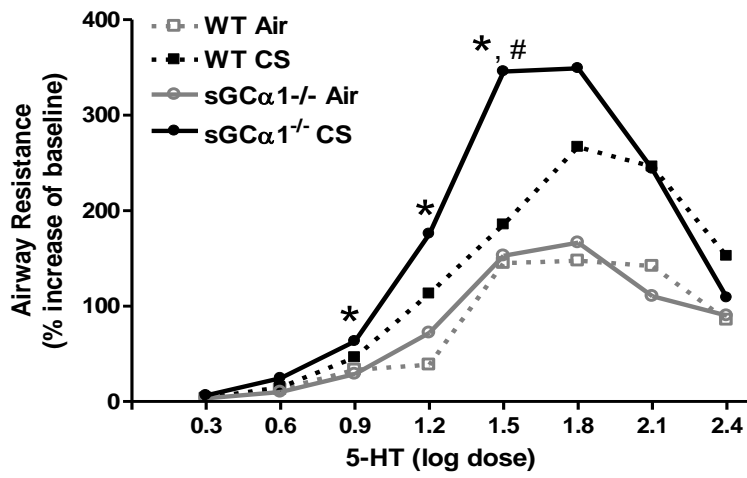


Figure 7

A



B

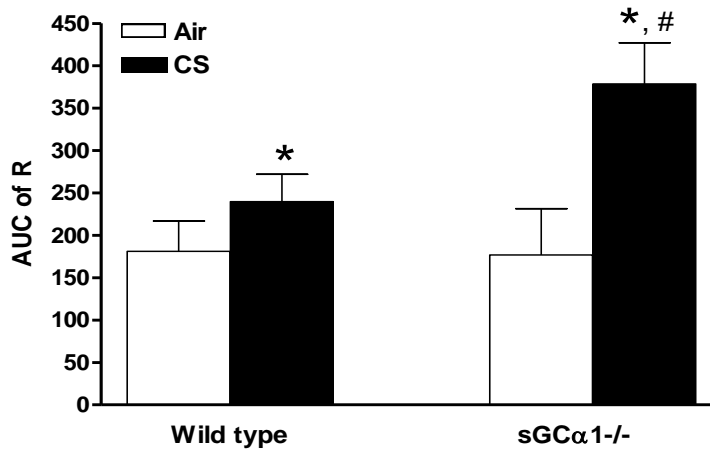
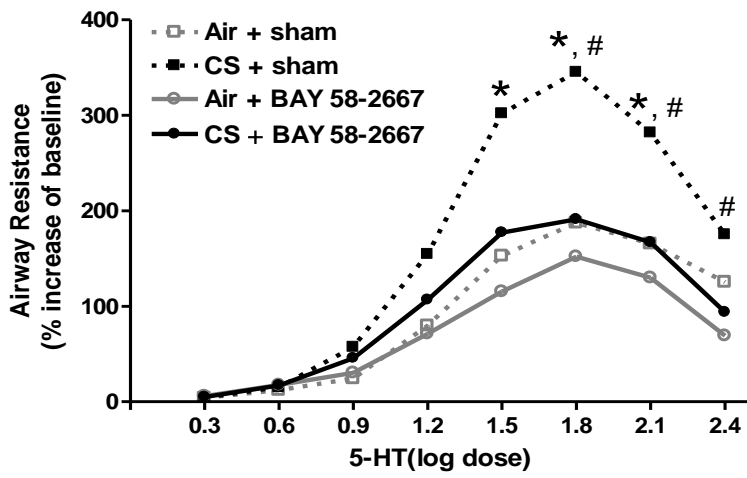


Figure 8

A



B

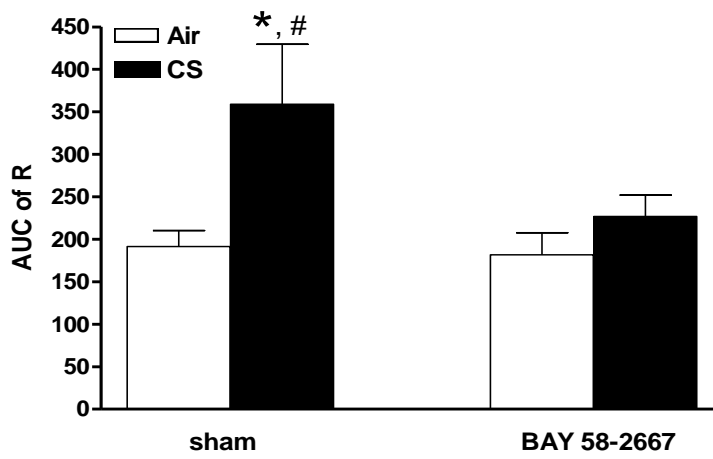


Figure 9

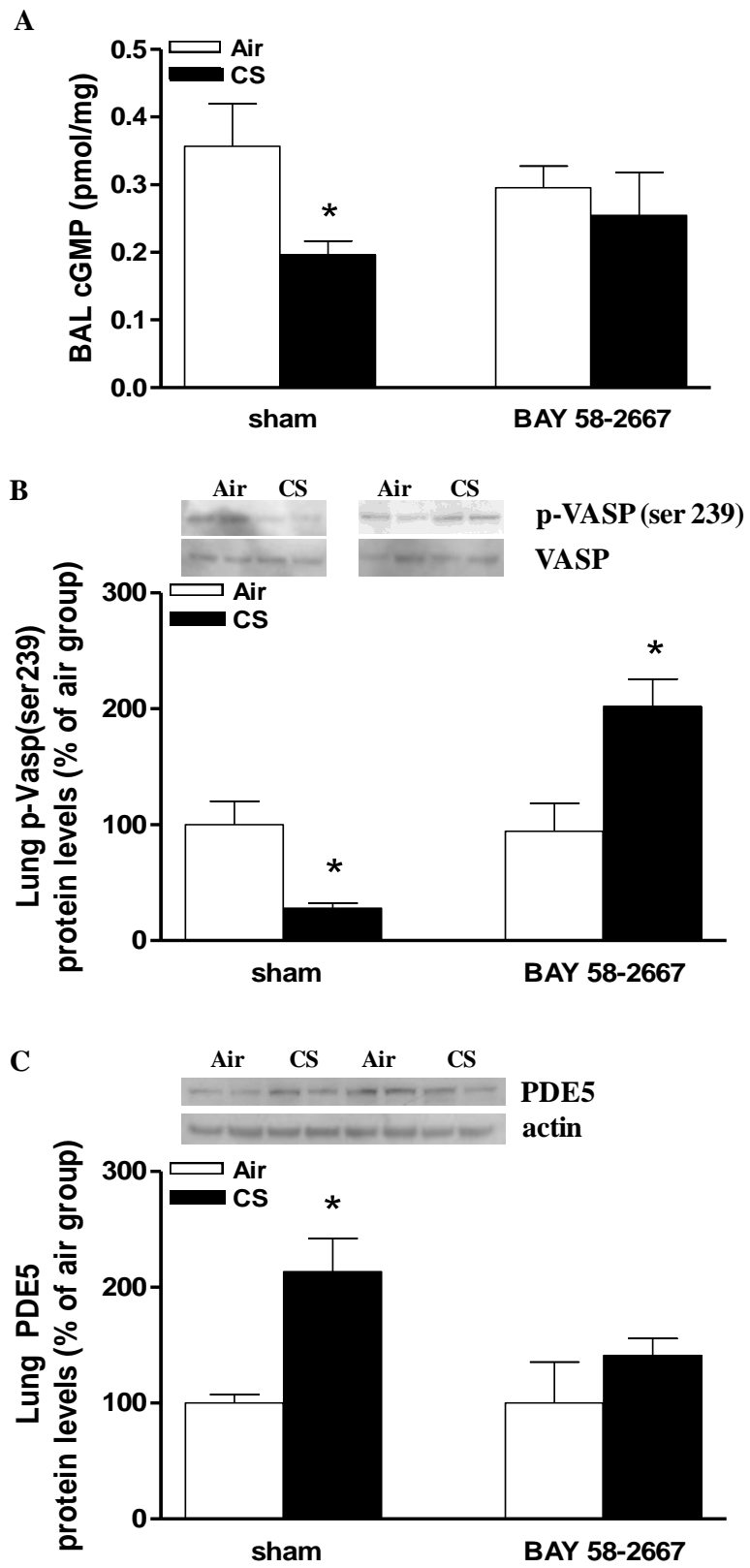
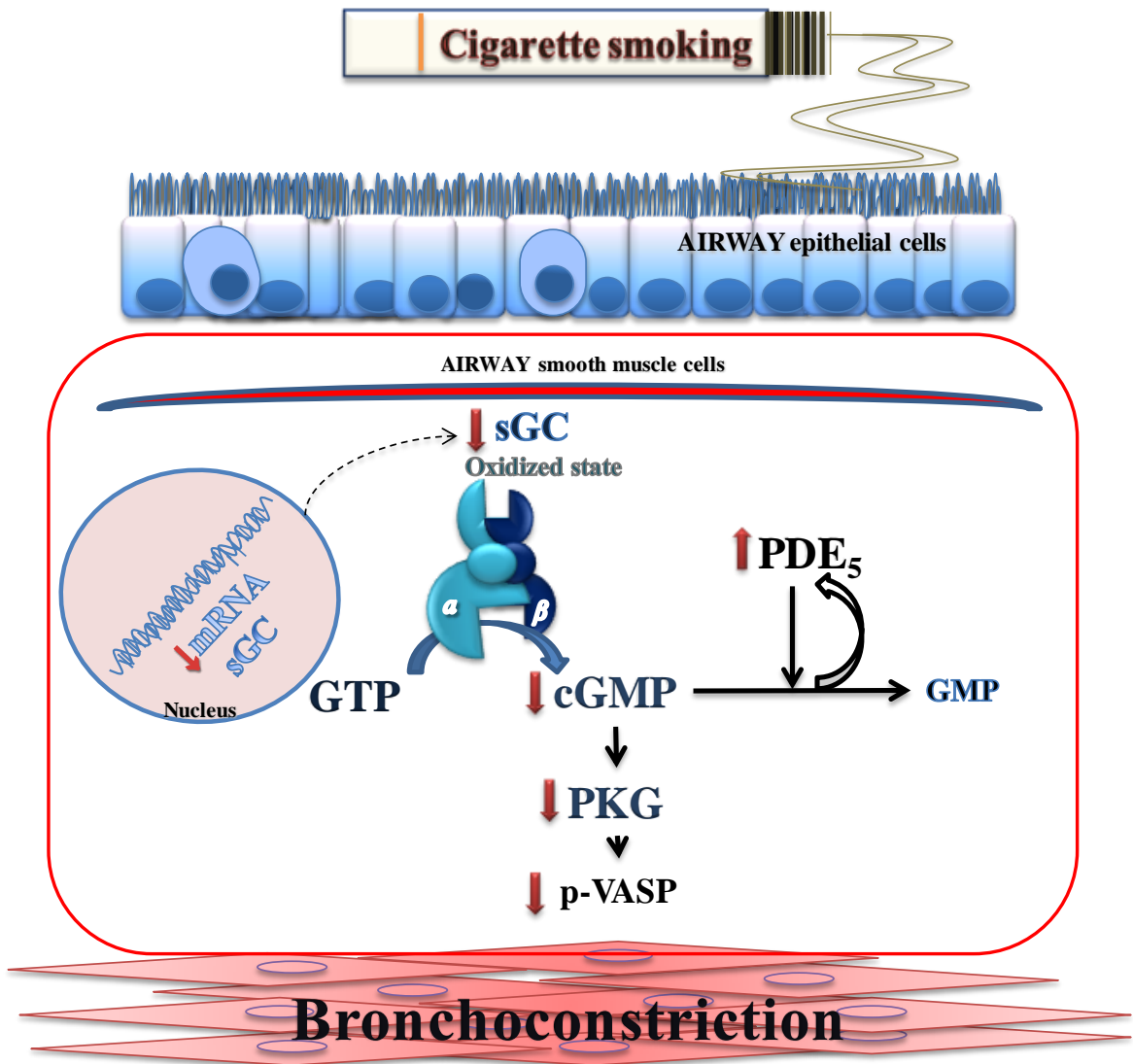


Figure 10



Chapter 10. Review: 'Role of the nitric oxide - soluble guanylyl cyclase pathway in obstructive airway diseases'

Lisa L. Dupont, Constantinos Glynos, Ken R. Bracke, Peter Brouckaert and Guy G. Brusselle

Pulmonary Pharmacology and Therapeutics 2014 29(1):1-6.

In this review we discuss the involvement of nitric oxide, nitric oxide synthases, guanylyl cyclases, cGMP and phosphodiesterase-5 in asthma and COPD and potential therapeutic approaches to modulate this pathway.

Role of the nitric oxide - soluble guanylyl cyclase pathway in obstructive airway diseases

Lisa L. Dupont^a, Constantinos Glynos^{b,c}, Ken R. Bracke^a, Peter Brouckaert^{d,e} and Guy G. Brusselle^{a,§}

^a Laboratory for Translational Research in Obstructive Pulmonary Diseases, Department of Respiratory Medicine, Ghent University Hospital, Ghent, Belgium

^b George P. Livanos and Marianthi Simou Laboratories, Evangelismos Hospital, 1st Department of Pulmonary and Critical Care, University of Athens, Athens, Greece

^c Laboratory of Molecular Pharmacology, Department of Pharmacy, University of Patras, Patras, Greece

^d Department of Biomedical Molecular Biology, Ghent University, Ghent, Belgium

^e Department of Molecular Biomedical Research, VIB, Ghent, Belgium

[§] Corresponding author

Title: 77 characters; **Words:** 2896; **Abstract:** 202 ; **Figures:** 2

Keywords: COPD, asthma, nitric oxide, soluble guanylyl cyclase

Abstract

Nitric oxide (NO) is a gaseotransmitter, which is involved in many signalling processes in health and disease. Three enzymes generate NO from L-arginine, with citrulline formed as a by-product: neuronal NO synthase (nNOS or NOS1), endothelial NOS (eNOS or NOS3) and inducible NOS (iNOS or NOS2). NO is a ligand of soluble guanylyl cyclase (sGC), an intracellular heterodimer enzyme that catalyzes the conversion of guanosine triphosphate (GTP) to cyclic GMP (cGMP). cGMP further activates protein kinase G that eventually reduces the smooth muscle tone in bronchi or vessels. Phosphodiesterase 5 (PDE₅) degrades cGMP to GMP. However, NO reacts with superoxide anion (O₂⁻), leading to formation of the proinflammatory molecule peroxynitrite.

Under physiological conditions, NO plays a homeostatic bronchoprotective role in healthy subjects.

In obstructive airway diseases, NO can be beneficial by its bronchodilating effect, but could also be detrimental by the formation of peroxynitrite. Since asthma and COPD are associated with increased levels of exhaled NO, chronic inflammation and increased airway smooth muscle tone, the NO/sGC/cGMP pathway could be involved in these highly prevalent obstructive airway diseases. Here we review the involvement of NO, NO synthases, guanylyl cyclases, cGMP and phosphodiesterase-5 in asthma and COPD and potential therapeutic approaches to modulate this pathway.

1. Nitric oxide (NO) and NO synthases

1.1 Introduction

Nitric oxide (NO) is the first identified gaseotransmitter, which is involved in many diverse signaling processes including inflammation, smooth muscle tone and neurotransmission [1, 2]. Within the respiratory tract, the main sources of NO are airway epithelial cells and endothelial cells [3, 4]. Under physiological conditions, NO is present in the exhaled breath [5].

The synthesis of NO is catalyzed by 3 NO synthase (NOS) isoforms. Neuronal NOS (nNOS or NOS1) and endothelial NOS (eNOS or NOS3) are constitutively expressed and their activity is regulated by intracellular calcium concentrations and calmodulin [6, 7]. Inducible NOS (iNOS or NOS2) is independent of calcium and is regulated by cytokines and proinflammatory stimuli [8]. However, this distinction is not that strict, since the activity of nNOS and eNOS, also referred to as “constitutive NOS (cNOS)”, can be induced by several cytokines (IL-1 β , IFN- γ , TNF- α) [9, 10]; while iNOS may be constitutively expressed at certain sites including the airway epithelium [11].

An overview of the expression of the 3 isoforms of NO synthase in human and murine lung tissue is shown in **Table 1**.

1.2 Chronic obstructive pulmonary disease

Chronic obstructive pulmonary disease (COPD) is mainly caused by smoking cigarettes, which are an exogenous source of NO. Ansarin *et al.* [12] have analyzed the exhaled nitric oxide (eNO) levels in patients with COPD, patients with asthma and healthy controls. Patients with COPD had higher eNO levels than healthy controls, but lower levels than patients with asthma. Lung function parameters such as forced expiratory volume in 1 second (FEV₁) and carbon monoxide diffusing capacity (DL_{CO}) were inversely correlated with eNO levels in patients with COPD. In contrast to patients with asthma, the use of inhaled corticosteroids had no significant influence on eNO levels in patients with COPD [12]. Brindicci *et al.* measured eNO at multiple expired flows to make a distinction between

alveolar and bronchial NO and found that COPD is associated with elevated alveolar NO [13].

In peripheral lung tissue and bronchial submucosa of patients with COPD, **iNOS** expression was significantly increased, irrespective of the GOLD stage [9, 14]. Patients with severe COPD had increased iNOS⁺ cells in the alveolar wall and most of these cells were type II pneumocytes [15]. These results are in agreement with the observed increased alveolar NO in patients with COPD [13]. In bronchial submucosa, there was also an effect of smoking, since smokers without airflow limitation had increased iNOS levels [14]. The expression of iNOS was increased in bronchial smooth muscle cells of patients with COPD and was correlated with the degree of airflow limitation [16].

The expression levels of **eNOS** showed a discrepancy between the two localizations. In peripheral lung tissue, eNOS protein levels were similar between nonsmokers, smokers without airflow limitation and patients with COPD GOLD 1, 2 and 3. Remarkably, eNOS levels were significantly decreased in patients with severe COPD GOLD 4, probably caused by the destruction of the alveolar walls [9, 17]. The levels of eNOS behaved similar to iNOS in bronchial submucosa [14].

The levels of **nNOS** were increased in peripheral lung tissue of patients with COPD GOLD stages 2, 3 and 4 compared with nonsmokers. Moreover, nNOS protein expression and disease severity (measured by FEV₁% and FEV₁/FVC) were significantly correlated [9].

Similar to the results in human peripheral lung tissue [9], the levels of iNOS in murine lung homogenate were increased after 8 months cigarette smoke (CS) exposure, while the levels of eNOS were decreased [17]. In contrast to eNOS knockout mice, iNOS knockout mice were protected against the development of CS-induced emphysema. Treating wild-type mice with an iNOS inhibitor after 8 months CS exposure even reversed the lung damage and significantly downregulated the amount of granulocytes, macrophages and T-lymphocytes in the lung [17].

A chronic, iNOS-related inflammation, hypertrophy and hyperplasia of alveolar type II cells and several abnormalities in pulmonary structure and function develop in mice deficient in

Surfactant Protein D (SP-D) [18]. SP-D and iNOS double knockout mice (DiNOS) maintained hyperplasia of alveolar type II cells, but they had a reduced inflammation, correction of the alveolar structural abnormalities and a restored lung function, compared with SP-D single KO mice. So by producing NO under pathological conditions, iNOS is involved in inflammation, development of structural abnormalities and lung function [18]. These findings and the observed increased iNOS expression in patients with COPD suggest that the inhibition of iNOS may be a potential therapy for patients with COPD [17, 18]. However, in a murine model of elastase-induced emphysema [19], with increased expression of iNOS and eNOS, inhibition of iNOS decreased the amount of protein nitration, but had no effect on inflammation or development of emphysema. Inhibition of eNOS had overall no effect [19].

Table 1. Lung expression of the 3 isoforms of NO synthase (nNOS, iNOS and eNOS) in human subjects and murine models.

patients/model	tissue	nNOS	iNOS	eNOS	reference
<u>HUMAN</u>					
patients with COPD / smokers / nonsmokers	peripheral lung tissue	↑ GOLD 2,3,4 (M+P)	↑ GOLD 2,3,4 (M) ↑ GOLD 1, 2,3,4 (P)	↓ GOLD 3, 4 (M) ↓ GOLD 4 (P)	[9]
patients with COPD / smokers / nonsmokers	bronchial submucosa	ND	↑ smokers, COPD (P)	↑ smokers, COPD (P)	[14]
patients with severe COPD / smokers	alveolar wall	ND	↑ severe COPD (P) (mainly type II pneumocytes)	ND	[15]
<u>MICE</u>					
after 8 months CS	lung homogenate	ND	↑ (P)	↓ (P)	[17]
elastase-induced emphysema	lung homogenate day 1-3-7-20	↓ at d1 and d7 (M)	↑ (M+P)	↑ (M+P)	[19]

ND = not determined, M: mRNA, P:protein, CS: cigarette smoke

1.3 Asthma

In patients with asthma, especially in allergic asthmatics with eosinophilic airway inflammation, the levels of nitric oxide in exhaled air are significantly elevated compared with healthy subjects [20-22]. Moreover, lung function parameters such as FEV₁ and DL_{CO} were inversely correlated with exhaled NO levels [12]. This increased flux of NO from the airways was significantly decreased after inhalation of aminoguanidine, a relatively selective iNOS inhibitor [23]. The expression of iNOS was increased in airway epithelial cells and inflammatory cells from patients with asthma compared with healthy subjects, suggesting that this enzyme plays an important role in the production of NO in pathological conditions [24, 25]. Increased FeNO levels are a predictor of response to inhaled corticosteroids in patients with asthma [26]. iNOS levels in corticosteroid-treated asthmatics were significantly decreased compared to non-corticosteroid treated asthmatics [27]. Treatment of asthma patients with a selective iNOS inhibitor, GW274150, significantly reduced FeNO levels. However, this reduction in FeNO levels did not lead to a change in early or late responses to allergen challenge, or to a change in numbers of inflammatory cells in bronchoalveolar lavage (BAL) [28]. This suggests that therapeutic treatment with an iNOS inhibitor would not be beneficial in patients with asthma.

In a murine model of allergic asthma, the pharmacological inhibition of iNOS by L-NAME or aminoguanidine significantly decreased the number of eosinophils and lymphocytes in BAL fluid in OVA-challenged mice compared with non-treated mice [29]. Also airway hyperreactivity (AHR) and mucus secretion were significantly decreased in OVA-challenged mice after treatment with an iNOS inhibitor. In the same study, number of BAL cells, AHR and mucus secretion were not different between iNOS knockout and wild-type mice, suggesting that the lack of iNOS in these constitutive knockout animals is probably compensated by other mechanisms. In another study using the OVA model of allergic asthma, no differences in AHR were observed in iNOS knockout mice compared with wild-type mice. Although iNOS knockout mice developed a similar degree of inflammation as the

wild-type controls, the deficiency of iNOS resulted in reduced eosinophils in BAL and in peripheral blood [30].

2. Oxidative/nitrative stress in obstructive airway diseases

Reactive oxygen species (ROS) are unstable molecules with an unpaired electron that can be generated endogenously by mitochondrial electron transport during respiration or during activation of inflammatory cells, and exogenously by cigarette smoke or air pollutants. These small reactive signaling molecules can oxidize proteins, lipids or DNA, leading to cell dysfunction and cell death. Also reactive nitrogen species (RNS), such as the highly proinflammatory molecule peroxynitrite, can cause tissue injury in various organs. Normally, they are counterbalanced by antioxidants and rapidly removed from the body. An imbalance between ROS/RNS and antioxidants leads to oxidative/nitrative stress [31-33]. Both oxidative and nitrative stress have been linked with inflammatory, obstructive airway diseases, including asthma and COPD [34, 35].

Activated inflammatory cells such as macrophages and neutrophils produce increased levels of NO and ROS (superoxide ($O_2^{\cdot-}$) and hydrogen radical (HO^{\cdot})) (**Figure 1**). NO rapidly reacts with $O_2^{\cdot-}$ to form the proinflammatory molecule peroxynitrite. Peroxynitrite alters the function of proteins by nitration of tyrosine residues. Currently, 3-nitrotyrosine is measured as a footprint of peroxynitrite release. Using a new noninvasive technique, Osoata *et al.* were able to measure peroxynitrite in exhaled breath condensate [36]. The levels of peroxynitrite were significantly higher in patients with COPD compared with smokers and healthy controls [36].

3. Soluble guanylyl cyclase

Guanylyl cyclases (GCs), members of the family of nucleotide cyclizing enzymes, are widely distributed signal-transduction enzymes that catalyze the conversion of GTP to cGMP.

Both transmembrane and soluble forms of guanylyl cyclases exist. The transmembrane, particulate GC (pGC) acts as a receptor for hormones such as atrial, brain (B-type) and C-

type natriuretic peptides. For further information on this transmembrane form of GC, we refer the reader to an excellent review [37].

Soluble GC (sGC) is an intracellular receptor for gaseous ligands (NO and CO) and is able to associate with the plasma membrane through protein-protein interactions in a Ca^{2+} -dependent manner [38]. sGC is a heterodimer, consisting of an α -subunit and a β -subunit. There are 2 forms of the α -subunit ($\alpha 1$ and $\alpha 2$) and of the β -subunit ($\beta 1$ and $\beta 2$). $\alpha 1\beta 1$ and $\alpha 2\beta 1$ are equally present in the brain, while $\alpha 1\beta 1$ is the most prevalent form in other tissues such as the lung [39]. Both forms have a similar catalytic rate and sensitivity towards NO. The C-terminal catalytic domains of both isoforms are required to form a catalytic active centre. The β -subunit has an amino-terminal haem-binding domain. A haem moiety that interacts with the haem-binding domain, is essential for the sensing of NO, increasing the cGMP production from GTP [40]. The haem moiety is a large heterocyclic organic ring with a central metal ion (Fe). sGC is activated by nanomolar concentrations of NO in the presence of the reduced Fe^{2+} (ferrous) haem moiety, while oxidized, Fe^{3+} (ferric) haem is insensitive to NO (**Figure 2**). Moreover, the oxidized haem is more prone to ubiquitination, leading to loss of the haem-group. Similar to oxidized haem, haem-deficient sGC is unresponsive to NO. Oxidation is induced by exogenous molecules, such as ODQ (1H-[1,2,4]oxadiazolo-[4, 3-a]quinoxalin-1-one), and by endogenous molecules, including reactive oxygen species (ROS) and reactive nitrogen species (RNS) [38].

Activation of sGC induces the generation of cyclic guanosine monophosphate (cGMP), phosphorylation of protein kinase G (PKG) and changes in activity of effector proteins such as phosphodiesterases (PDE), ion channels and ion pumps [41]. This pathway eventually leads to dilation of bronchi (bronchodilation) or vessels (vasodilation).

In patients with asthma, bronchodilation is impaired, despite the presence of large amounts of NO in the airways that could activate sGC and cause relaxation of the smooth muscle. In a

murine model of allergic asthma, the levels of sGC α 1, α 2 and β 1 were reduced in the lungs, both on mRNA and protein level [42]. Mice treated with the selective sGC inhibitor ODQ had increased airway reactivity to methacholine compared with sham-treated mice [42]. This finding suggests that sGC could be inhibited in patients with asthma, leading to the observed airway hyperresponsiveness. Also in patients with COPD, the lungs contain ample amounts of NO, but the airway tone remains elevated [12]. Patients with COPD have decreased pulmonary mRNA and protein levels of sGC which are correlated with disease severity [43]. The levels of sGC are also decreased in CS-exposed mice [43]. CS-exposed mice deficient for the sGC α 1 subunit had a significantly higher airway resistance compared with CS-exposed wild-type mice (**Figure 1**). These results indicate that sGC downregulation due to CS exposure in humans and mice, emerges as an alternative pathophysiological mechanism of the airway hyperresponsiveness.

4. Phosphodiesterase 5 (PDE₅)

Phosphodiesterase 5 (PDE₅) degrades cGMP to GMP; thereby impairing the downstream effects of cGMP (**Figure 1**). Sildenafil, a short-acting inhibitor of PDE₅, is already on the market for erectile dysfunction and induces smooth muscle relaxation. Tadalafil is a long-acting inhibitor of PDE₅. The effect of PDE₅ inhibition was analyzed in guinea pigs exposed to lipopolysaccharide (LPS) and in sensitized guinea pigs exposed to ovalbumin [44]. Pretreatment with sildenafil inhibited the LPS-induced airway hyperreactivity, influx of leukocytes and generation of NO. Exposure to ovalbumin caused early- and late-phase asthma responses which were not affected by sildenafil. However, AHR to histamine, leukocyte influx in BAL and increased NO metabolites in BAL were significantly attenuated in OVA-exposed mice after treatment with sildenafil [44].

In a rat model of acrolein (a component of cigarette smoke) exposure, sildenafil suppressed the acrolein-induced airway inflammation and mucus production [45]. CS-exposed mice have increased PDE₅ protein levels in the lung compared with air-exposed mice [43]. These

results suggest that PDE₅ inhibitors have a therapeutic potential in airway diseases such as asthma and COPD. However, in contrast to OVA-challenged guinea pigs [44], OVA-challenged mice treated with sildenafil did not affect airway inflammation [46].

PDE₅ inhibitors have not yet been tested in asthma or COPD for its anti-inflammatory properties. In contrast, PDE₅ inhibitors have been investigated in patients with COPD and (concomitant) pulmonary hypertension. Treating patients with COPD-associated pulmonary hypertension with sildenafil or tadalafil did not improve exercise capacity or quality of life [47, 48].

5. Therapy

Potential therapeutic approaches to modulate the NO/sGC/cGMP pathway are activation of sGC by NO donors, sGC stimulators and sGC activators; or inhibition of the inflammation-induced formation of NO by iNOS inhibitors.

5.1 Nitric oxide donors

A reduced bioavailability and/or responsiveness to endogenously produced NO contributes to the development of several pathologies, including pulmonary diseases.

NO-donors, such as organic nitrates, release NO by spontaneous decomposition or bioconversion, thereby activating the enzyme sGC. However, the use of NO-donors is limited because of the potential lack of response, the development of tolerance and the non-specific interactions of NO with biomolecules (such as superoxide, leading to formation of peroxynitrite) [38]. Moreover, in the airways of patients with COPD or asthma, there are already large amounts of NO present that could activate sGC and induce smooth muscle relaxation.

5.2 Inducible NO synthase inhibitors

The research on the role of iNOS using animal models has given conflicting results. Given the species differences in the expression and regulation of iNOS, translational research is

required to fully elucidate the function of iNOS in obstructive airway diseases. Treating asthma patients with the selective iNOS inhibitor GW274150 did not lead to a change in early or late responses to allergen challenge, or to a change in numbers of inflammatory cells in BAL [28].

In healthy subjects, physiological levels of NO are produced by eNOS and nNOS. In inflammatory conditions, NO levels increase mainly due to increased iNOS activity. By inhibiting iNOS, both the beneficial effects of NO through sGC and the pro-inflammatory effect of NO through formation of peroxynitrite are inhibited. The iNOS inhibitor can reduce the levels of NO, but ROS such as superoxide remain elevated. These ROS can react with the 'constitutively' produced NO, leading to the observed unchanged 3-nitrotyrosine (3-NT) levels in patients with asthma [28]. Indeed, an *in vitro* study using human alveolar epithelial cells of patients with severe asthma, has shown that not only nitrite, produced by iNOS, but also H₂O₂, produced by dual oxidases, are important in the formation of 3-NT [49].

Activation of the sGC/cGMP pathway on the one hand, and/or inhibition of the NO-induced formation of pro-inflammatory molecules on the other hand could be more beneficial.

5.3 Soluble guanylyl cyclase activators and stimulators

In several pathologies, including asthma and COPD, the NO/sGC/cGMP pathway can be compromised by the oxidized state of sGC, making it unresponsive to both endogenous and exogenous NO. Therefore, an NO-independent treatment could be recommended in these diseases [38]. Both sGC stimulators and sGC activators are potential therapies. sGC stimulators stimulate sGC directly and enhance the sensitivity of the reduced enzyme to low levels of bioavailable NO (**Figure 2**). While sGC stimulators are haem-dependent, sGC activators activate the NO-unresponsive, haem-oxidized or haem-free enzyme (**Figure 2**). The efficacy of the sGC stimulator riociguat has already been shown in patients with pulmonary arterial hypertension [50]. In the same line, a recent study demonstrated that

treatment of two different CS-exposed animal models with sGC stimulators riociguat (BAY 63-2521) or BAY 41-2272 prevent CS-induced pulmonary hypertension and emphysema [51].

BAY58-2667 (or cinaciguat) is a potent NO-independent sGC activator, replacing the weakly bound oxidized haem of sGC, leading to activation of the enzyme; while reduced haem is unresponsive to BAY58-2667 [38]. Treating CS-exposed mice with BAY58-2667 restored the sGC/cGMP pathway and significantly attenuated the CS-induced AHR compared with sham-treated mice [43], denoting sGC as a promising pharmaceutical target of obstructive airway diseases (**Figure 2**).

6. Concluding remarks

The use of NO donors and iNOS inhibitors as a treatment in obstructive airway diseases has not been successful. The specific activation of the sGC/cGMP pathway by treating patients with an sGC activator or stimulator may be a new therapeutic approach in obstructive lung diseases. The results of using sGC activators and sGC stimulators in animal models of asthma and COPD are promising, however further research is needed. Treatment by the inhaled route should be investigated to limit potential side-effects of systemic drug administration.

Competing interests

LLD, CG, KRB, PB and GGB declare that they have no competing interests.

Acknowledgements

This work was supported by the Concerted Research Action of the University of Ghent (BOF 14-GOA-027; Ghent, Belgium), by the Fund for Scientific Research Flanders (Project G.0195.09 and G.0194.10; Brussels, Belgium) and by the Interuniversity Attraction Poles Program/Belgian State/Belgian Science Policy (P7/30; Brussels, Belgium). L.L. Dupont is a beneficiary from a Bijzonder Onderzoeksfonds Research grant from Ghent University. K.R.

Bracke is a Postdoctoral Fellow of the Fund for Scientific Research Flanders. C. Glynos was supported by EU grant (Call: FP7-REGPOT2011-1): “establishment of a centre of excellence for structured-based drug target characterization: strengthening the research capacity of South-Eastern Europe” (SEE-DRUG, www.seedrug.upatras.gr). P. Brouckaert is supported by the COST Action BM1005 (European Network on Gasotransmitters).

References

- [1] Szabo C. Gaseotransmitters: new frontiers for translational science. *Science translational medicine*. 2010;2:59ps4.
- [2] Mustafa AK, Gadalla MM, Snyder SH. Signaling by gasotransmitters. *Science signaling*. 2009;2:re2.
- [3] van der Vliet A, Eiserich JP, Cross CE. Nitric oxide: a pro-inflammatory mediator in lung disease? *Respir Res*. 2000;1:67-72.
- [4] Redington AE. Modulation of nitric oxide pathways: therapeutic potential in asthma and chronic obstructive pulmonary disease. *Eur J Pharmacol*. 2006;533:263-76.
- [5] Gustafsson LE, Leone AM, Persson MG, Wiklund NP, Moncada S. Endogenous Nitric-Oxide Is Present in the Exhaled Air of Rabbits, Guinea-Pigs and Humans. *Biochem Bioph Res Co*. 1991;181:852-7.
- [6] Lucas KA, Pitari GM, Kazeronian S, Ruiz-Stewart I, Park J, Schulz S, et al. Guanylyl cyclases and signaling by cyclic GMP. *Pharmacol Rev*. 2000;52:375-414.
- [7] Bredt DS. Nitric oxide signaling specificity - the heart of the problem. *Journal of Cell Science*. 2003;116:9-15.
- [8] Asano K, Chee CB, Gaston B, Lilly CM, Gerard C, Drazen JM, et al. Constitutive and inducible nitric oxide synthase gene expression, regulation, and activity in human lung epithelial cells. *Proc Natl Acad Sci U S A*. 1994;91:10089-93.
- [9] Brindicci C, Kharitonov SA, Ito M, Elliott MW, Hogg JC, Barnes PJ, et al. Nitric oxide synthase isoenzyme expression and activity in peripheral lung tissue of patients with chronic obstructive pulmonary disease. *Am J Respir Crit Care Med*. 2010;181:21-30.
- [10] Förstermann U, Boissel J-p, Kleinert H. Expressional control of the 'constitutive' isoforms of nitric oxide synthase (NOS I and NOS III). *The FASEB Journal*. 1998;12:773-90.
- [11] Guo FH, Deraeve HR, Rice TW, Stuehr DJ, Thunnissen FBJM, Erzurum SC. Continuous Nitric-Oxide Synthesis by Inducible Nitric-Oxide Synthase in Normal Human Airway Epithelium in-Vivo. *P Natl Acad Sci USA*. 1995;92:7809-13.
- [12] Ansarin K, Chatkin JM, Ferreira IM, Gutierrez CA, Zamel N, Chapman KR. Exhaled nitric oxide in chronic obstructive pulmonary disease: relationship to pulmonary function. *Eur Respir J*. 2001;17:934-8.
- [13] Brindicci C, Ito K, Resta O, Pride NB, Barnes PJ, Kharitonov SA. Exhaled nitric oxide from lung periphery is increased in COPD. *Eur Respir J*. 2005;26:52-9.
- [14] Ricciardolo FL, Caramori G, Ito K, Capelli A, Brun P, Abatangelo G, et al. Nitrosative stress in the bronchial mucosa of severe chronic obstructive pulmonary disease. *J Allergy Clin Immunol*. 2005;116:1028-35.
- [15] Maestrelli P, Páska C, Saetta M, Turato G, Nowicki Y, Monti S, et al. Decreased haem oxygenase-1 and increased inducible nitric oxide synthase in the lung of severe COPD patients. *Eur Respir J*. 2003;21:971-6.
- [16] Tadié J-M, Henno P, Leroy I, Danel C, Naline E, Faisy C, et al. Role of nitric oxide synthase/arginase balance in bronchial reactivity in patients with chronic obstructive pulmonary disease. *Am J Physiol*. 2008;294:L489-L97.
- [17] Seimetz M, Parajuli N, Pichl A, Veit F, Kwapiszewska G, Weisel Friederike C, et al. Inducible NOS Inhibition Reverses Tobacco-Smoke-Induced Emphysema and Pulmonary Hypertension in Mice. *Cell*. 2011;147:293-305.
- [18] Knudsen L, Atochina-Vasserman EN, Guo CJ, Scott PA, Haenni B, Beers MF, et al. NOS2 Is Critical to the Development of Emphysema in Sftpd Deficient Mice but Does Not Affect Surfactant Homeostasis. *PLoS One*. 2014;9:e85722.
- [19] Boyer L, Plantier L, Dagouassat M, Lanone S, Goven D, Caramelle P, et al. Role of nitric oxide synthases in elastase-induced emphysema. *Lab Invest*. 2011;91:353-62.
- [20] Alving K, Weitzberg E, Lundberg JM. Increased amount of nitric oxide in exhaled air of asthmatics. *Eur Respir J*. 1993;6:1368-70.
- [21] Persson MG, Zetterstrom O, Agrenius V, Ihre E, Gustafsson LE. Single-breath nitric oxide measurements in asthmatic patients and smokers. *Lancet*. 1994;343:146-7.
- [22] Kharitonov SA, Yates D, Robbins RA, Logan-Sinclair R, Shinebourne EA, Barnes PJ. Increased nitric oxide in exhaled air of asthmatic patients. *Lancet*. 1994;343:133-5.
- [23] Brindicci C, Ito K, Barnes PJ, Kharitonov SA. Effect of an inducible nitric oxide synthase inhibitor on differential flow-exhaled nitric oxide in asthmatic patients and healthy volunteers. *Chest*. 2007;132:581-8.

- [24] Saleh D, Ernst P, Lim S, Barnes PJ, Giaid A. Increased formation of the potent oxidant peroxynitrite in the airways of asthmatic patients is associated with induction of nitric oxide synthase: effect of inhaled glucocorticoid. *FASEB J*. 1998;12:929-37.
- [25] Hamid Q, Springall DR, Riveros-Moreno V, Chanez P, Howarth P, Redington A, et al. Induction of nitric oxide synthase in asthma. *Lancet*. 1993;342:1510-3.
- [26] Smith AD, Cowan JO, Brassett KP, Filsell S, McLachlan C, Monti-Sheehan G, et al. Exhaled nitric oxide: a predictor of steroid response. *Am J Respir Crit Care Med*. 2005;172:453-9.
- [27] Redington AE, Meng Q-H, Springall DR, Evans TJ, Créminon C, Maclouf J, et al. Increased expression of inducible nitric oxide synthase and cyclo-oxygenase-2 in the airway epithelium of asthmatic subjects and regulation by corticosteroid treatment. *Thorax*. 2001;56:351-7.
- [28] Singh D, Richards D, Knowles RG, Schwartz S, Woodcock A, Langley S, et al. Selective inducible nitric oxide synthase inhibition has no effect on allergen challenge in asthma. *Am J Respir Crit Care Med*. 2007;176:988-93.
- [29] Landgraf RG, Russo M, Jancar S. Acute inhibition of inducible nitric oxide synthase but not its absence suppresses asthma-like responses. *Eur J Pharmacol*. 2005;518:212-20.
- [30] Xiong Y, Karupiah G, Hogan SP, Foster PS, Ramsay AJ. Inhibition of Allergic Airway Inflammation in Mice Lacking Nitric Oxide Synthase 2. *J Immunol*. 1999;162:445-52.
- [31] Bargagli E, Olivieri C, Bennett D, Prasse A, Muller-Quernheim J, Rottoli P. Oxidative stress in the pathogenesis of diffuse lung diseases: a review. *Respir Med*. 2009;103:1245-56.
- [32] Nathan C, Cunningham-Bussell A. Beyond oxidative stress: an immunologist's guide to reactive oxygen species. *Nat Rev Immunol*. 2013;13:349-61.
- [33] Kirkham PA, Barnes PJ. Oxidative stress in COPD. *Chest*. 2013;144:266-73.
- [34] Holguin F. Oxidative Stress in Airway Diseases. *Annals of the American Thoracic Society*. 2013;10:S150-S7.
- [35] Kharitonov SA, Barnes PJ. Exhaled markers of pulmonary disease. *Am J Respir Crit Care Med*. 2001;163:1693-722.
- [36] Osoata GO, Hanazawa T, Brindicci C, Ito M, Barnes PJ, Kharitonov S, et al. Peroxynitrite elevation in exhaled breath condensate of copd and its inhibition by fudosteine. *Chest*. 2009;135:1513-20.
- [37] Hamad AM, Clayton A, Islam B, Knox AJ. Guanylyl cyclases, nitric oxide, natriuretic peptides, and airway smooth muscle function. *Am J Physiol Lung Cell Mol Physiol*. 2003;285:L973-83.
- [38] Evgenov OV, Pacher P, Schmidt PM, Hasko G, Schmidt HH, Stasch JP. NO-independent stimulators and activators of soluble guanylate cyclase: discovery and therapeutic potential. *Nat Rev Drug Discov*. 2006;5:755-68.
- [39] Mergia E, Russwurm M, Zoidl G, Koesling D. Major occurrence of the new alpha2beta1 isoform of NO-sensitive guanylyl cyclase in brain. *Cell Signal*. 2003;15:189-95.
- [40] Friebe A, Koesling D. Regulation of nitric oxide-sensitive guanylyl cyclase. *Circ Res*. 2003;93:96-105.
- [41] Francis SH, Busch JL, Corbin JD, Sibley D. cGMP-dependent protein kinases and cGMP phosphodiesterases in nitric oxide and cGMP action. *Pharmacol Rev*. 2010;62:525-63.
- [42] Papapetropoulos A, Simoes DC, Xanthou G, Roussos C, Gratziou C. Soluble guanylyl cyclase expression is reduced in allergic asthma. *Am J Physiol Lung Cell Mol Physiol*. 2006;290:L179-84.
- [43] Glynos C, Dupont LL, Vassilakopoulos T, Papapetropoulos A, Brouckaert P, Giannis A, et al. The role of soluble guanylyl cyclase in chronic obstructive pulmonary disease. *Am J Respir Crit Care Med*. 2013;188:789-99.
- [44] Toward TJ, Smith N, Broadley KJ. Effect of Phosphodiesterase-5 Inhibitor, Sildenafil (Viagra), in Animal Models of Airways Disease. *Am J Resp Crit Care Med*. 2004;169:227-34.
- [45] Wang T, Liu Y, Chen L, Wang X, Hu XR, Feng YL, et al. Effect of sildenafil on acrolein-induced airway inflammation and mucus production in rats. *Eur Respir J*. 2009;33:1122-32.
- [46] Clayton R, Dick C, Mackenzie A, Nagasawa M, Galbraith D, Hastings S, et al. The effect of selective phosphodiesterase inhibitors, alone and in combination, on a murine model of allergic asthma. *Respir Res*. 2004;5:4.
- [47] Goudie AR, Lipworth BJ, Hopkinson PJ, Wei L, Struthers AD. Tadalafil in patients with chronic obstructive pulmonary disease: a randomised, double-blind, parallel-group, placebo-controlled trial. *Lancet Respir Med*. 2014;2:293-300.
- [48] Blanco I, Gimeno E, Munoz PA, Pizarro S, Gistau C, Rodriguez-Roisin R, et al. Hemodynamic and Gas Exchange Effects of Sildenafil in Patients with Chronic Obstructive Pulmonary Disease and Pulmonary Hypertension. *Am J Resp Crit Care Med*. 2010;181:270-8.

- [49] Voraphani N, Gladwin MT, Contreras AU, Kaminski N, Tedrow JR, Milosevic J, et al. An airway epithelial iNOS-DUOX2-thyroid peroxidase metabolome drives Th1/Th2 nitrate stress in human severe asthma. *Mucosal Immunol*. 2014.
- [50] Ghofrani HA, Galie N, Grimminger F, Grunig E, Humbert M, Jing ZC, et al. Riociguat for the treatment of pulmonary arterial hypertension. *N Engl J Med*. 2013;369:330-40.
- [51] Weissmann N, Lobo B, Pichl A, Parajuli N, Seimetz M, Puig-Pey R, et al. Stimulation of Soluble Guanylate Cyclase Prevents Cigarette Smoke-induced Pulmonary Hypertension and Emphysema. *Am J Respir Crit Care Med*. 2014;189:1359-73.

Figure legends

Figure 1: The NO/sGC/cGMP signaling pathway in obstructive airway diseases. Bronchial and alveolar epithelial cells produce NO, which activates sGC under normal conditions. However, decreased sGC levels in COPD and asthma lead to an impaired downstream pathway; while upregulated PDE₅ levels further decrease cGMP levels. Activated inflammatory cells such as macrophages and neutrophils also release NO and reactive oxygen species such as O₂⁻. NO and O₂⁻ form ONOO⁻, leading to protein nitration.

NO: nitric oxide, sGC: soluble guanylyl cyclase, GTP: guanosine triphosphate, cGMP: cyclic guanosine monophosphate, PDE₅: phosphodiesterase 5, PKG: protein kinase G, O₂⁻: superoxide, ONOO⁻: peroxynitrite.

Figure 2: Soluble guanylyl cyclase (sGC). Under physiological conditions, there is a balance between the reduced, NO-sensitive sGC and the oxidized, NO-insensitive sGC. Oxidative stress and reactive oxygen species shift the balance to the oxidized form, resulting in an impaired sGC/cGMP pathway. The enzyme can even lose the haem group (haem-free sGC). sGC stimulators enhance the sensitivity of the reduced enzyme to low levels of bioavailable NO, while sGC activators activate the NO-unresponsive, haem-oxidized or haem-free enzyme. Treating smoke-exposed mice with the sGC activator BAY58-2667 reactivates the sGC/cGMP pathway, demonstrated by increased cGMP and PKG levels, and decreased PDE-5 levels; leading to a normalized smooth muscle tone [43].

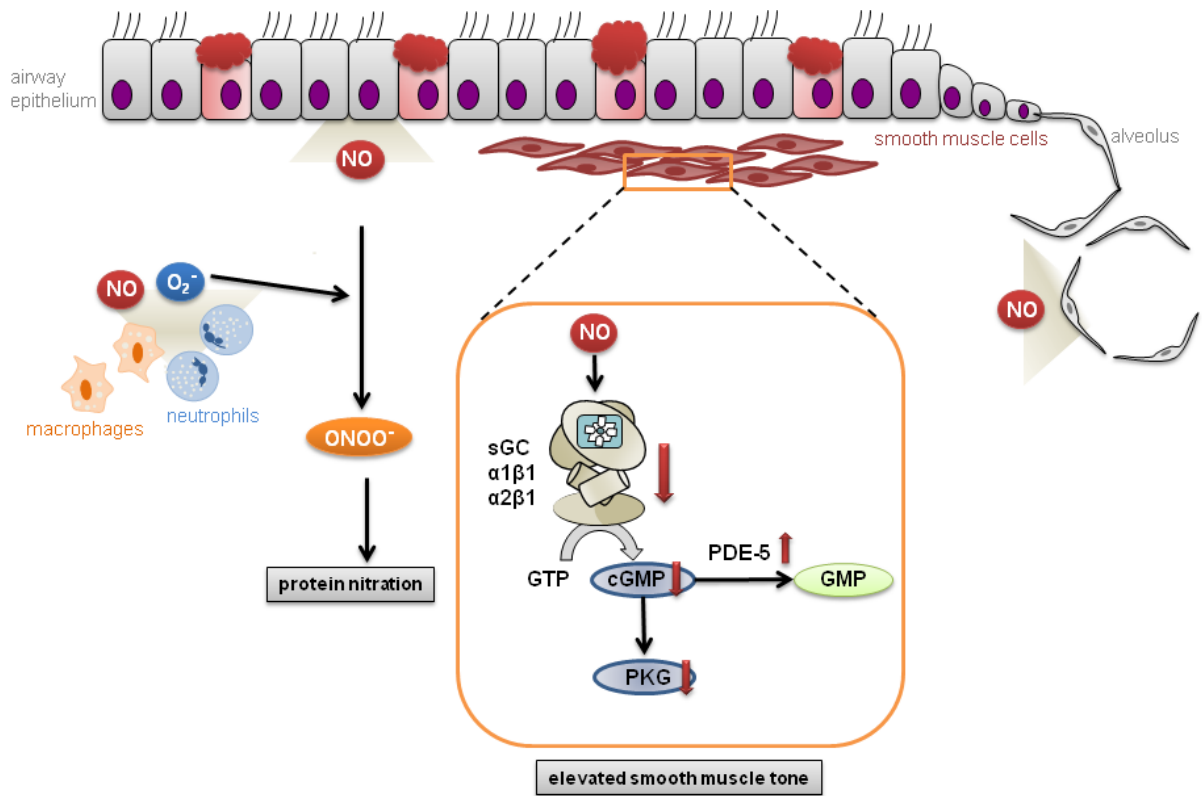


Figure 1

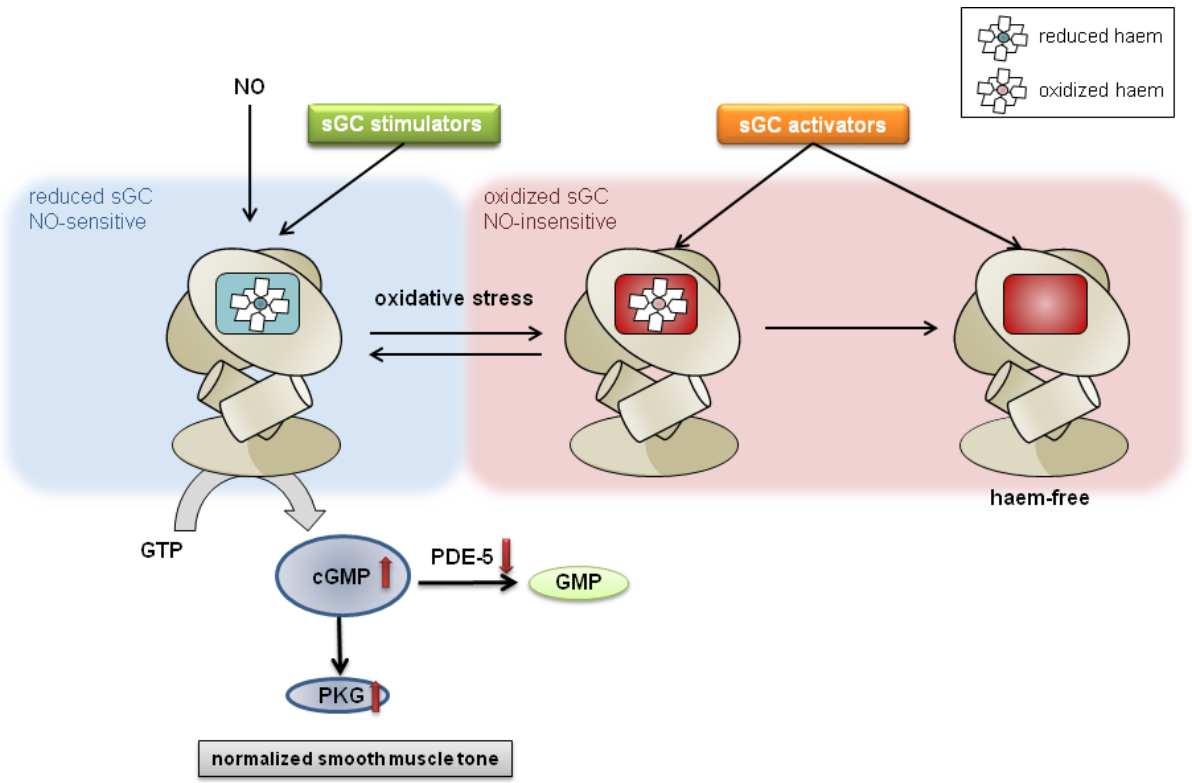


Figure 2

Chapter 11. Discussion and future perspectives

Chronic obstructive pulmonary disease (COPD) is mainly caused by smoking of cigarettes. Cigarette smoke (CS) contains more than 4500 components, including reactive oxygen species, nicotine, acrolein and endotoxin (lipopolysaccharide (LPS))^{1,2}. Several of these compounds bind to pattern recognition receptors (PRR) expressed on alveolar macrophages, dendritic cells and epithelial cells, leading to the release of proinflammatory cytokines and chemokines and further attracting inflammatory cells to the lungs¹. This 'inflammatory pathway' has already been thoroughly investigated and described by our and other research groups^{1,3}.

11.1 Transient Receptor Potential channels

(part on Transient Receptor Potential Channels has been removed from the electronic version of the dissertation)

11.2 Serotonin receptor 4 (5-HT₄R)

Since genetic polymorphisms in the gene for 5-HT₄R were associated with pulmonary function and with COPD^{20,21}; and postganglionic 5-HT receptors have been shown to be implicated in the release of acetylcholine²², we hypothesized that 5-HT₄ receptors contribute to the pathogenesis of COPD by facilitating the cholinergic contraction of the airways. In lung tissue of CS-exposed mice (3 days, 4 weeks and 24 weeks CS exposure), mRNA levels of 5-HT₄R were significantly elevated compared to air-exposed mice. The administration of increasing doses of 5-HT caused a dose-dependent increase in airway resistance in air-exposed mice and CS exposure further increased this response. We have shown that this 5-HT-induced airway smooth muscle contraction is established through a direct way, since inhibition of 5-HT_{2A}R located on airway smooth muscle cells almost abolished the bronchial hyperresponsiveness (BHR) to 5-HT; and through an indirect way by stimulation of receptors on nerve endings, since inhibition of muscarinic receptors by atropine reduces the response

to 5-HT. To elucidate the role of 5-HT₄ receptors in this response, mice were treated with GR113808, a 5-HT₄R specific antagonist. However, antagonism of the 5-HT₄R did not decrease the response to 5-HT, implicating that 5-HT₄ receptors are not involved in the CS-induced BHR to 5-HT. We also examined CS-exposed 5-HT₄R WT and KO mice. Both genotypes had a similar BHR to 5-HT, confirming our pharmacological findings. Inhibition of 5-HT₄R in WT mice or deficiency of 5-HT₄R had no effect on inflammatory cells in BAL fluid. Although 5-HT₄R mRNA levels were significantly increased after CS exposure, these receptors do not seem to play a role in CS-induced inflammation or BHR to 5-HT. The observed increase in pulmonary 5-HT₄R mRNA levels might be explained by an increase in 5-HT₄R-positive inflammatory cells, such as monocytes²³ and mature dendritic cells²⁴. We previously demonstrated that CS exposure induces the activation and maturation of dendritic cells, shown by an upregulation of MHCII and the co-stimulatory molecules CD40 and CD86^{25,26}.

5-HT receptors, other than 5-HT₄R and 5-HT_{2A}R, could also mediate the BHR. Segura *et al.* found that activation of 5-HT₄R and 5-HT₇ receptors in guinea pig tracheas increased the contractile cholinergic responses to electrical field stimulation²⁷. In human airways, the 5-HT-induced facilitation of cholinergic contraction was significantly inhibited by both 5-HT₃ and 5-HT₄R antagonists; while high concentrations of selective 5-HT₃ and 5-HT₄R agonists mimicked the effect of 5-HT on cholinergic contraction²⁸. In our *in vivo* murine model of CS exposure, inhibition (or genetic deficiency) of 5-HT₄R did not affect the BHR to 5-HT. Other 5-HT receptors, such as 5-HT₃ or 5-HT₇ could be implicated in this response. Therefore, the experiments should be repeated using antagonists against a broader scale of 5-HT receptors.

Raemdonck *et al.* demonstrated that the neural response was abolished in rats after ketamine and xylazine anesthesia²⁹. However, another study in anesthetized mice, showed that dissection of the vagal nerve abolished the 5-HT-induced increase in airway resistance. This finding indicates that the neural response is still present after anesthesia in our study,

and is able to release acetylcholine. It is possible that acetylcholine is not only released from nerve endings, but also from bronchial epithelial cells or inflammatory cells³⁰.

We have analyzed the potential role of 5-HT₄ receptors in acute (3 days) and subacute (4 weeks) smoke-exposed mice. However, it would be interesting to investigate 5-HT₄ receptors in mice after chronic (24 weeks) smoke exposure, since mice then also develop emphysema and airway remodeling, in addition to an increased inflammatory response. These characteristics of COPD could have an effect on the BHR.

Agonists of 5-HT₄R are currently on the market for the treatment of Alzheimer disease and gastrointestinal motility disorders. Our results suggest that the use of these drugs is not likely to cause clinical benefits in patients with COPD.

11.3 Soluble guanylyl cyclase (sGC)

Impairment of the NO/sGC/cGMP pathway is also implicated in bronchoconstriction (**Figure 23**). In patients with COPD, elevated levels of NO are present in the exhaled breath. Under physiological conditions, sGC catalyzes the enzymatic conversion of GTP to cGMP, which activates protein kinase G, eventually leading to bronchodilation. However, patients with COPD have impaired bronchodilation, although there are ample amounts of NO present that could activate sGC. We have shown that in patients with COPD, mRNA and protein levels of sGC were decreased compared to healthy controls. Moreover, sGC levels were correlated with the severity of COPD (measured by FEV₁%).

In our murine model of CS exposure, sGC levels were also decreased after acute, subacute and chronic smoke exposure. In murine models of OVA-induced allergic asthma and of acute lung injury, a similar decrease of sGC levels was observed^{31,32}. Inflammatory stimuli, such as cytokines, may be involved in the attenuation of sGC mRNA stability or sGC protein expression, as shown by several *in vitro* studies³³⁻³⁶. Smoke exposure led to an increased airway resistance to serotonin compared to air-exposed mice. Mice deficient in the sGC α 1 subunit had higher responsiveness to serotonin after CS exposure compared to WT mice. Treating CS-exposed WT mice with the sGC activator BAY58-2667 (cinaciguat) restored the

sGC/cGMP pathway and significantly decreased the CS-induced BHR compared with untreated mice, making this compound a promising drug target for the treatment of COPD. BAY58-2667 is a potent NO-independent sGC activator, which replaces the weakly bound oxidized haem of sGC, leading to activation of the enzyme; in contrast, reduced haem is unresponsive to BAY58-2667³⁷.

The decreased levels of sGC in humans in our study were mainly an effect of smoking, although sGC α 1 mRNA and protein levels were significantly associated with FEV₁. To further investigate the function of sGC in the pathogenesis of COPD, WT mice treated with the sGC activator; or sGC α 1^{-/-} mice should be exposed to CS for 6 months. Our *in vivo* results are obtained in mice exposed to 3 days CS, while our chronic model develops more COPD-like pathologies such as emphysema, airway remodeling and lymphoid neogenesis.

Recently, another research group analyzed the role of the sGC-cGMP pathway in lung emphysema using the sGC stimulator riociguat³⁸. Similar to our results, they observed a downregulation of sGC β 1 subunit in patients with COPD, and in guinea pigs and mice after chronic CS exposure. Mice developed emphysema after 6 months CS exposure, which was prevented by treating the mice with the sGC stimulator riociguat. These findings highlight the therapeutic potential of the sGC-cGMP pathway for treatment of COPD, as we already suggested.

NO can be beneficial by its bronchodilating effect, but could also be detrimental by the formation of peroxynitrite. Activated inflammatory cells such as macrophages and neutrophils produce increased levels of NO and reactive oxygen species (superoxide (O₂⁻) and hydrogen radical (HO[•])). NO rapidly reacts with O₂⁻ to form the proinflammatory molecule peroxynitrite, which alters the function of proteins by nitration of tyrosine residues. The levels of peroxynitrite were significantly higher in exhaled breath condensate of patients with COPD compared with smokers and healthy controls³⁹. Activation of the sGC/cGMP pathway on the one hand, and/or inhibition of the NO-induced formation of pro-inflammatory molecules on the other hand could be more beneficial, and should be further investigated in patients with COPD.

Phosphodiesterase 5 (PDE₅) degrades cGMP to GMP; thereby impairing the downstream effects of cGMP. Sildenafil, which is already on the market for erectile dysfunction and pulmonary arterial hypertension, is an inhibitor of PDE₅ and induces smooth muscle relaxation. The effect of PDE₅ inhibition was analyzed in a rat model of acrolein (a component of cigarette smoke) exposure. Sildenafil suppressed the acrolein-induced airway inflammation and mucus production⁴⁰. In our murine model of smoke exposure, PDE₅ levels are increased in CS-exposed mice. These results suggest that PDE₅ inhibitors could have a therapeutic potential in airway diseases such as COPD. However, in contrast to OVA-challenged guinea pigs⁴¹, OVA-challenged mice treated with sildenafil did not show reduced airway inflammation⁴². Testing PDE₅ inhibitors in COPD for its anti-inflammatory properties may be of interest. In contrast, PDE₅ inhibitors have been investigated in patients with COPD and (concomitant) pulmonary hypertension. Treating patients with COPD-associated pulmonary hypertension with the short-acting PDE₅ inhibitor sildenafil or the long-acting tadalafil did not improve exercise capacity or quality of life^{43,44}.

General conclusion

The expression of TRPA1 channels is increased in nodose/jugular and trigeminal ganglia after CS exposure, while TRPA1 expression in airway non-neuronal cells is not affected by CS. The functional role of TRPA1 will be further unraveled using TRPA1 KO mice.

CS exposure induces bronchial hyperresponsiveness to serotonin (5-HT). Although pulmonary 5-HT₄R mRNA levels are increased in CS-exposed mice, 5-HT₄R does not seem to play a role in the CS-induced BHR to 5-HT. Importantly, soluble guanylyl cyclase, which is downregulated in CS-exposed mice, in smokers and patients with COPD, is involved in BHR and is a promising drug target for the treatment of COPD. Local delivery of sGC activators/stimulators via the inhaled route should be envisaged in COPD to limit potential systemic side-effects.

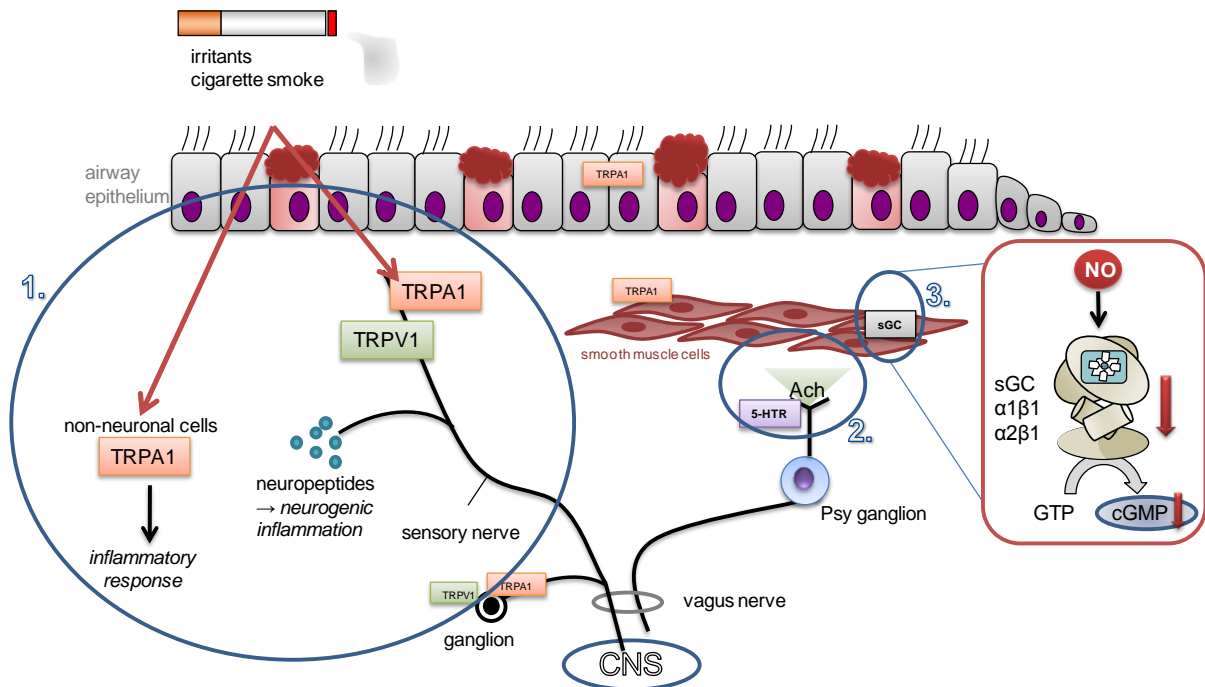


Figure 23. Schematic overview of our work. **(1)** (part on Transient Receptor Potential Channels has been removed from the electronic version of the dissertation) **(2)** Efferent fibers, initiating in the brain or spinal cord, send the appropriate reaction back to the airways, also via ganglia. Postganglionic cholinergic nerves release acetylcholine (Ach), which binds to muscarinic receptors on airway smooth muscle, causing contraction and subsequent bronchoconstriction. We have shown that 5-hydroxytryptamine (5-HT) 4 receptors (5-HT₄R) do not seem to play a role in the 5-HT-induced bronchial responsiveness. **(3)** Both in CS-exposed mice, smokers without airflow limitation and patients with COPD, the levels of soluble guanylyl cyclase (sGC) are decreased, leading to an impaired bronchodilation. Down-regulation of sGC, induced by CS exposure, might contribute to airflow limitation in patients with COPD. NO: nitric oxide; GTP: guanosine triphosphate; cGMP; cyclic guanosine monophosphate; Psy: parasympathetic.

REFERENCES

1. Brusselle GG, Joos GF and Bracke KR. New insights into the immunology of chronic obstructive pulmonary disease. *Lancet* 2011; 378(9795):1015-1026.
2. Stampfli MR and Anderson GP. How cigarette smoke skews immune responses to promote infection, lung disease and cancer. *Nat Rev Immunol* 2009; 9(5):377-384.
3. Barnes PJ. Immunology of asthma and chronic obstructive pulmonary disease. *Nat Rev Immunol* 2008; 8(3):183-192.
4. Bessac BF and Jordt S-E. Sensory Detection and Responses to Toxic Gases. *Proceedings of the American Thoracic Society* 2010; 7(4):269-277.
5. Grace MS, Dubuis E, Birrell MA and Belvisi MG. Pre-clinical studies in cough research: role of Transient Receptor Potential (TRP) channels. *Pulm Pharmacol Ther* 2013; 26(5):498-507.
6. Geppetti P, Nassini R, Materazzi S and Benemei S. The concept of neurogenic inflammation. *BJU international* 2008; 101:2-6.
7. Joos GF, De Swert KO, Schelfhout V and Pauwels RA. The role of neural inflammation in asthma and chronic obstructive pulmonary disease. *Ann N Y Acad Sci* 2003; 992:218-230.
8. Talavera K, Gees M, Karashima Y, Meseguer VM, Vanoirbeek JA, Damann N, Everaerts W, Benoit M, Janssens A, Vennekens R, Viana F, Nemery B, Nilius B and Voets T. Nicotine activates the chemosensory cation channel TRPA1. *Nat Neurosci* 2009; 12(10):1293-1299.
9. Taylor-Clark TE, McAlexander MA, Nassenstein C, Sheardown SA, Wilson S, Thornton J, Carr MJ and Udem BJ. Relative contributions of TRPA1 and TRPV1 channels in the activation of vagal bronchopulmonary C-fibres by the endogenous autacoid 4-oxononenal. *J Physiol* 2008; 586(14):3447-3459.
10. Nassini R, Pedretti P, Moretto N, Fusi C, Carnini C, Facchinetti F, Viscomi AR, Pisano AR, Stokesberry S, Brunmark C, Svitacheva N, McGarvey L, Patacchini R, Damholt AB, Geppetti P and Materazzi S. Transient Receptor Potential Ankyrin 1 Channel Localized to Non-Neuronal Airway Cells Promotes Non-Neurogenic Inflammation. *PLoS One* 2012; 7(8):e42454.
11. Fernandes ES, Fernandes MA and Keeble JE. The functions of TRPA1 and TRPV1: moving away from sensory nerves. *Br J Pharmacol* 2012; 166(2):510-521.
12. Hatano N, Itoh Y, Suzuki H, Muraki Y, Hayashi H, Onozaki K, Wood IC, Beech DJ and Muraki K. Hypoxia-inducible factor-1alpha (HIF1alpha) switches on transient receptor potential ankyrin repeat 1 (TRPA1) gene expression via a hypoxia response element-like motif to modulate cytokine release. *J Biol Chem* 2012; 287(38):31962-31972.
13. Chao LK, Hua KF, Hsu HY, Cheng SS, Lin IF, Chen CJ, Chen ST and Chang ST. Cinnamaldehyde inhibits pro-inflammatory cytokines secretion from monocytes/macrophages through suppression of intracellular signaling. *Food Chem Toxicol* 2008; 46(1):220-231.
14. Caceres AI, Brackmann M, Elia MD, Bessac BF, del Camino D, D'Amours M, Witek JS, Fanger CM, Chong JA, Hayward NJ, Homer RJ, Cohn L, Huang X, Moran MM and Jordt SE. A sensory neuronal ion channel essential for airway inflammation and hyperreactivity in asthma. *Proc Natl Acad Sci U S A* 2009; 106(22):9099-9104.
15. Hox V, Vanoirbeek JA, Alpizar YA, Voedisch S, Callebaut I, Bobic S, Sharify A, De Vooght V, Van Gerven L, Devos F, Liston A, Voets T, Vennekens R, Bullens DMA, De Vries A, Hoet P, Braun A, Ceuppens JL, Talavera K, Nemery B and Hellings PW. Crucial Role of Transient Receptor Potential Ankyrin 1 and Mast Cells in Induction of Nonallergic Airway Hyperreactivity in Mice. *Am J Respir Crit Care Med* 2013; 187(5):486-493.
16. Glynos C, Dupont LL, Vassilakopoulos T, Papapetropoulos A, Brouckaert P, Giannis A, Joos GF, Bracke KR and Brusselle GG. The role of soluble guanylyl cyclase in chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 2013; 188(7):789-799.
17. Dupont LL, Bracke KR, De Maeyer JH, Compan V, Joos GF, Lefebvre RA and Brusselle GG. Investigation of 5-HT4 receptors in bronchial hyperresponsiveness in cigarette smoke-exposed mice. *Pulm Pharmacol Ther* 2014; 28(1):60-67.
18. Zhu G, Gulsvik A, Bakke P, Ghatta S, Anderson W, Lomas DA, Silverman EK and Pillai SG. Association of TRPV4 gene polymorphisms with chronic obstructive pulmonary disease. *Hum Mol Genet* 2009; 18(11):2053-2062.
19. Zhao L, Sullivan MN, Chase M, Gonzales AL and Earley S. Calcineurin/Nuclear factor of activated T cells-coupled vanilloid transient receptor potential channel 4 ca(2+) sparklets stimulate airway smooth muscle cell proliferation. *Am J Respir Cell Mol Biol* 2014; 50(6):1064-1075.
20. Hancock DB, Eijgelsheim M, Wilk JB, Gharib SA, Loehr LR, Marcianti KD, Franceschini N, van Durme YM, Chen TH, Barr RG, Schabath MB, Couper DJ, Brusselle GG, Psaty BM, van Duijn CM, Rotter JI, Uitterlinden AG, Hofman A, Punjabi NM, Rivadeneira F, Morrison AC, Enright PL, North KE, Heckbert SR, Lumley T, Stricker BH, O'Connor GT and London SJ. Meta-analyses of genome-

wide association studies identify multiple loci associated with pulmonary function. *Nat Genet* 2010; 42(1):45-52.

21. Wilk JB, Shrine NR, Loehr LR, Zhao JH, Manichaikul A, Lopez LM, Smith AV, Heckbert SR, Smolonska J, Tang W, Loth DW, Curjuric I, Hui J, Cho MH, Latourelle JC, Henry AP, Aldrich M, Bakke P, Beaty TH, Bentley AR, Borecki IB, Brusselle GG, Burkart KM, Chen TH, Couper D, Crapo JD, Davies G, Dupuis J, Franceschini N, Gulsvik A, Hancock DB, Harris TB, Hofman A, Imboden M, James AL, Khaw KT, Lahousse L, Launer LJ, Litonjua A, Liu Y, Lohman KK, Lomas DA, Lumley T, Marcianti KD, McArdle WL, Meibohm B, Morrison AC, Musk AW, Myers RH, North KE, Postma DS, Psaty BM, Rich SS, Rivadeneira F, Rochat T, Rotter JI, Artigas MS, Starr JM, Uitterlinden AG, Wareham NJ, Wijmenga C, Zanen P, Province MA, Silverman EK, Deary IJ, Palmer LJ, Cassano PA, Gudnason V, Barr RG, Loos RJ, Strachan DP, London SJ, Boezen HM, Probst-Hensch N, Gharib SA, Hall IP, O'Connor GT, Tobin MD and Stricker BH. Genome-wide association studies identify CHRNA5/3 and HTR4 in the development of airflow obstruction. *Am J Respir Crit Care Med* 2012; 186(7):622-632.
22. Joos GF, Lefebvre RA, Bullock GR and Pauwels RA. Role of 5-hydroxytryptamine and mast cells in the tachykinin-induced contraction of rat trachea in vitro. *Eur J Pharmacol* 1997; 338(3):259-268.
23. Durk T, Panther E, Muller T, Sorichter S, Ferrari D, Pizzirani C, Di Virgilio F, Myrtek D, Norgauer J and Idzko M. 5-Hydroxytryptamine modulates cytokine and chemokine production in LPS-primed human monocytes via stimulation of different 5-HTR subtypes. *Int Immunol* 2005; 17(5):599-606.
24. Idzko M, Panther E, Stratz C, Muller T, Bayer H, Zissel G, Durk T, Sorichter S, Di Virgilio F, Geissler M, Fiebich B, Herouy Y, Elsner P, Norgauer J and Ferrari D. The serotonergic receptors of human dendritic cells: identification and coupling to cytokine release. *J Immunol* 2004; 172(10):6011-6019.
25. D'Hulst A I, Vermaelen KY, Brusselle GG, Joos GF and Pauwels RA. Time course of cigarette smoke-induced pulmonary inflammation in mice. *Eur Respir J* 2005; 26(2):204-213.
26. Maes T, Bracke KR, Vermaelen KY, Demedts IK, Joos GF, Pauwels RA and Brusselle GG. Murine TLR4 is implicated in cigarette smoke-induced pulmonary inflammation. *Int Arch Allergy Immunol* 2006; 141(4):354-368.
27. Segura P, Vargas MH, Cordoba-Rodriguez G, Chavez J, Arreola JL, Campos-Bedolla P, Ruiz V, Garcia-Hernandez LM, Mendez C and Montano LM. Role of 5-HT(2A), 5-HT(4) and 5-HT(7) receptors in the antigen-induced airway hyperresponsiveness in guinea-pigs. *Clin Exp Allergy* 2009; 40:327-338.
28. Dupont LJ, Pype JL, Demedts MG, De Leyn P, Deneffe G and Verleden GM. The effects of 5-HT on cholinergic contraction in human airways in vitro. *Eur Respir J* 1999; 14(3):642-649.
29. Raemdonck K, de Alba J, Birrell MA, Grace M, Maher SA, Irvin CG, Fozard JR, O'Byrne PM and Belvisi MG. A role for sensory nerves in the late asthmatic response. *Thorax* 2012; 67(1):19-25.
30. Wessler IK and Kirkpatrick CJ. The non-neuronal cholinergic system: An emerging drug target in the airways. *Pulmonary Pharmacology & Therapeutics* 2001; 14(6):423-434.
31. Glynos C, Kotanidou A, Orfanos SE, Zhou Z, Simoes DC, Magkou C, Roussos C and Papapetropoulos A. Soluble guanylyl cyclase expression is reduced in LPS-induced lung injury. *Am J Physiol Regul Integr Comp Physiol* 2007; 292(4):R1448-1455.
32. Papapetropoulos A, Simoes DC, Xanthou G, Roussos C and Gratziau C. Soluble guanylyl cyclase expression is reduced in allergic asthma. *Am J Physiol Lung Cell Mol Physiol* 2006; 290(1):L179-184.
33. Papapetropoulos A, Abou-Mohamed G, Marczin N, Murad F, Caldwell RW and Catravas JD. Downregulation of nitrovasodilator-induced cyclic GMP accumulation in cells exposed to endotoxin or interleukin-1 beta. *Br J Pharmacol* 1996; 118(6):1359-1366.
34. Gerassimou C, Kotanidou A, Zhou Z, Simoes DC, Roussos C and Papapetropoulos A. Regulation of the expression of soluble guanylyl cyclase by reactive oxygen species. *Br J Pharmacol* 2007; 150(8):1084-1091.
35. Filippov G, Bloch DB and Bloch KD. Nitric oxide decreases stability of mRNAs encoding soluble guanylate cyclase subunits in rat pulmonary artery smooth muscle cells. *J Clin Invest* 1997; 100(4):942-948.
36. Kloss S, Srivastava R and Mulsch A. Down-regulation of soluble guanylyl cyclase expression by cyclic AMP is mediated by mRNA-stabilizing protein HuR. *Mol Pharmacol* 2004; 65(6):1440-1451.
37. Evgenov OV, Pacher P, Schmidt PM, Hasko G, Schmidt HH and Stasch JP. NO-independent stimulators and activators of soluble guanylate cyclase: discovery and therapeutic potential. *Nat Rev Drug Discov* 2006; 5(9):755-768.

38. Weissmann N, Lobo B, Pichl A, Parajuli N, Seimetz M, Puig-Pey R, Ferrer E, Peinado VI, Dominguez-Fandos D, Fysikopoulos A, Stasch JP, Ghofrani HA, Coll-Bonfill N, Frey R, Schermuly RT, Garcia-Lucio J, Blanco I, Bednorz M, Tura-Ceide O, Tadele E, Brandes RP, Grimminger J, Klepetko W, Jaksch P, Rodriguez-Roisin R, Seeger W, Grimminger F and Barbera JA. Stimulation of Soluble Guanylate Cyclase Prevents Cigarette Smoke-induced Pulmonary Hypertension and Emphysema. *Am J Respir Crit Care Med* 2014; 189(11):1359-1373.
39. Osoata GO, Hanazawa T, Brindicci C, Ito M, Barnes PJ, Kharitonov S and Ito K. Peroxynitrite elevation in exhaled breath condensate of copd and its inhibition by fudosteine. *Chest* 2009; 135(6):1513-1520.
40. Wang T, Liu Y, Chen L, Wang X, Hu XR, Feng YL, Liu DS, Xu D, Duan YP, Lin J, Ou XM and Wen FQ. Effect of sildenafil on acrolein-induced airway inflammation and mucus production in rats. *Eur Respir J* 2009; 33(5):1122-1132.
41. Toward TJ, Smith N and Broadley KJ. Effect of Phosphodiesterase-5 Inhibitor, Sildenafil (Viagra), in Animal Models of Airways Disease. *Am J Resp Crit Care Med* 2004; 169(2):227-234.
42. Clayton R, Dick C, Mackenzie A, Nagasawa M, Galbraith D, Hastings S and MacKenzie S. The effect of selective phosphodiesterase inhibitors, alone and in combination, on a murine model of allergic asthma. *Respir Res* 2004; 5(1):4.
43. Goudie AR, Lipworth BJ, Hopkinson PJ, Wei L and Struthers AD. Tadalafil in patients with chronic obstructive pulmonary disease: a randomised, double-blind, parallel-group, placebo-controlled trial. *Lancet Respir Med* 2014; 2(4):293-300.
44. Blanco I, Gimeno E, Munoz PA, Pizarro S, Gistau C, Rodriguez-Roisin R, Roca J and Barberà JA. Hemodynamic and Gas Exchange Effects of Sildenafil in Patients with Chronic Obstructive Pulmonary Disease and Pulmonary Hypertension. *Am J Resp Crit Care Med* 2010; 181(3):270-278.

Dankwoord

Een doctoraat houdt veel zelfstandig werk in, maar gelukkig zijn er ook veel mensen die voor een helpende hand, een kritische blik en een goede begeleiding zorgen. Daarom wil ik eindigen met het bedanken van iedereen die op één of andere manier bijgedragen heeft bij het tot stand komen van dit boekje.

In de eerste plaats wil ik mijn promotor professor Brusselle bedanken, om mij de kans te geven om na mijn masterthesis een doctoraat te starten in het labo Longziekten. U maakte wekelijks tijd voor mij vrij om de nieuwste resultaten te bespreken en om mij weer op het goede spoor te zetten als het onderzoek wat minder goed ging. U gaf ook telkens op een constructieve manier feedback over mijn schrijfsels en presentaties, waardoor ik veel bijgeleerd heb. Bedankt voor uw goede begeleiding!

Professor Joos, ik herinner mij nog uw enthousiasme toen ik serotonine, één van uw stokpaardjes, en later ook neuropeptiden zou onderzoeken. Bedankt voor de interesse in mijn werk en de ondersteuning van het labo.

Hierbij wil ik ook de leden van de examencommissie bedanken voor hun kritische blik op mijn werk, en in het bijzonder de leden van de leescommissie voor het grondig nalezen van mijn thesis.

Ook een dankuwel aan professor Lefebvre, lid van mijn begeleidingscommissie, voor uw bijdrage aan de serotonine paper en voor uw uitleg over farmacologische begrippen.

Ken, je was er altijd om mij raad te geven over experimenten, de opbouw van artikels en het nalezen van verschillende abstracts en artikels. Ik denk dat iedereen die hier vertrekt een betere 'figuren-maker' geworden is door jou, omdat niemand wil onderdoen voor jouw artistieke talent. Een dikke West-Vlaamse 'merci'! ;)

Tania, ik ben pas later intensief met jou beginnen samenwerken, maar ik heb er heel wat van opgestoken. Bedankt voor het sturen van het TRP-project en je kritische kijk over de resultaten. Hopelijk leidt onze samenwerking binnenkort nog tot een mooie paper!

Sharen, Fien, Smitha, Griet, Leen, Katrien en Elise: ik vond het zeer leuk om jullie collega te mogen zijn. Bedankt om een handje toe te steken bij de experimenten, voor de fijne babbels en de leuke sfeer. Fien, bedankt voor het vele werk bij het verwerken van de humane stalen. Ik ben er zeker van dat je een goede postdoc zal worden, ik duim mee! De anderen wens ik ook nog heel veel succes bij het behalen van jullie doctoraat. Ook al zit het soms eens tegen, het zal jullie lukken, daar ben ik van overtuigd! I would also like to thank Constantinos 'Costas' Glynos for his enthusiasm and passion for research. It was nice to work with you on the sGC-project.

Ik wil ook mijn voorgangers bedanken: Tine, Nele en Ellen. Het is leuk dat ik af en toe nog eens iets van jullie hoor via e-mail of als we elkaar toevallig ontmoeten. Jullie waren allemaal een voorbeeld voor mij toen ik hier pas begon. Bedankt voor jullie goede raad.

Lies, Kevin en Bibi uit het verre K12, het was altijd aangenaam om jullie te ontmoeten op recepties, meetings en congressen. Lies, proficiat met je indrukwekkende publicatielijst en met je pas behaalde doctoraat! Kevin en Bibi, nog veel succes met jullie onderzoek!

I would also like to thank Yeranddy and Karel from Leuven for the collaboration on the TRP project.

Uiteraard ook bedankt aan alle laboranten (Greet, Eliane, Lien, Indra, Philippe, Katleen, Anouck, Marie-Rose, Ann, Christelle en Evelyn), voor de hulp bij de rookblootstellingen, de talloze eindpunten, kleuringen, het opnemen van beelden, cytopins tellen, ELISA's, PCRs, genotyperingen, ... Zonder jullie had ik er nog een aantal jaar langer over gedaan om dit boekje af te krijgen. Het is ongelooflijk wat een geoliede machine jullie vormen, vooral bij het uitvoeren van de eindpunten. Het was ook altijd zeer amusant om tijdens de pauze te luisteren naar de amoureuze escapades, verhalen over (klein)kinderen, grappige anekdotes uit de geschiedenis van het labo en natuurlijk ook de samenvattingen van 'Thuis'.

Bedankt aan Bart, Annie en Chantal uit K12 om alle administratie in orde te brengen, onze bestellingen uit te voeren, congressen te regelen, ...

Bedankt aan alle medewerkers van het animalarium voor de goede zorgen voor onze muisjes en voor de administratie die ermee gepaard gaat.

Ik wil ook graag mijn nieuwe collega's bij Emtex bedanken, waar ik opnieuw uitstekend begeleid word. Ik hoop dat ik mijn opgebouwde kennis goed kan gebruiken en een goede medical writer zal worden!

Bedankt aan mijn familie: Marraine, Marianne en Erwin, Thomas, Julie, Cédric en partners; Chris, Charlotte, Xavier, Roos en Bo voor de gezellige familiefestjes tussen het schrijven en studeren door;

Aniek en Dries, Mattias en Armel: jullie hebben zich wellicht dikwijls afgevraagd wat dat 'klein grut' in het grote Gent allemaal uitspookte; en veel opheldering zal dit boekje niet brengen vrees ik. Merci voor de goede zorgen voor jullie kleine zusje en de aangename etentjes (want vreemd genoeg komt er toch altijd eten aan te pas als we elkaar zien...). Ine, bedankt om van hierboven te helpen om alles te relativiseren, niet teveel te stressen om onbenulligheden en om te genieten van kleine dingen.

Loeka, Noa, Milo en Robbe: bedankt om mij al sinds jullie geboorte af en toe wat verstrooiing te bieden en het kind in mij naar boven te brengen. Ik sta vaak versteld van wat jullie allemaal al weten. Robbe, het is een eer om jouw meter te mogen zijn. Met je ongelooflijke kennis van de dierenwereld, schuilt er ongetwijfeld ook een klein wetenschappertje in jou. Ik beloof dat ik binnenkort naar je voetbalmatch kom kijken ;)

Ik wil graag mijn vrienden van de biomedische wetenschappen bedanken: Ellen, Linde, Nathalie, Thary en Hannah. Ook al heeft iedereen het druk met werken, doctoreren, bouwen, gezin, ... toch vinden we af en toe tijd om samen af te spreken en gezellig bij te babbelen.

Sofie, ik heb altijd al opgekeken naar je wiskundige 'brain'. Ik ben blij dat je een leuke job gevonden hebt. Geniet van je uitstapjes naar de bergen!

Julie, een dikke merci voor het mooie design van de uitnodiging en dit boekje! Je hebt ervoor gezorgd dat mijn 5 jaar werk door een mooie, unieke verpakking omhuld wordt.

Stefanie, mijn beste maatje, bedankt voor je steun tijdens mijn doctoraat, om mij de nodige ontspanning te bieden, om mij te kalmeren indien nodig en om mij te motiveren: ik kan het! ;)

Als laatste wil ik graag mijn ouders bedanken. Ma en pa, bedankt voor de jarenlange financiële en morele steun, jullie interesse, de geregelde aanvoer van vitamientjes en de gezellige etentjes ☺

Bedankt iedereen om mee te schrijven aan dit hoofdstuk, op naar het volgende!

Lisa, november 2014

Curriculum Vitae – Lisa Dupont

Personal details

Home

Munkelostraat 49

8750 Wingene

E-mail: lisa.dupont@ugent.be

Phone: +32 (0) 474 62 85 12

Date, place of birth: August 24 1986, Bruges

Nationality: Belgian

Office

Department of Respiratory Medicine

Ghent University Hospital

Blok B, 4th floor

De Pintelaan 185

9000 Ghent

Phone: +32 (0) 9 332 59 60

Education – Work experience

- Medical Writer at Emtex, Sint-Gillis-Waas (October 2014)

- PhD student in Medical Sciences (2009 – 2014)

Department of Respiratory Medicine, Ghent University Hospital

- Master of Biomedical Sciences, major Degeneration and Regeneration (2004-2009)

Ghent University, Faculty of Life Sciences and Medicine

Master Thesis (2007-2009): *Onderzoek naar lymfoïd neogenese in een experimenteel model van Chronisch Obstructief Bronchiaal Lijden (COPD).*

Promotor: Prof. Dr. G. Brusselle.

- Mathematics and Sciences (1998-2004)

De Bron, Tielt

Additional Courses

2012: Academic English: Writing Skills (Ghent University, UCT)

Clinical studies (Ghent University)

2010-2011: SPSS basic and advanced course (Ghent University)

2010: qPCR experiment design and data-analysis (Biogazelle, Ghent University)

2009: Training on Flow Cytometry (BD Biosciences, Belgium)

Peer-reviewed publications

1. Glynos C*, **Dupont LL***, Vassilakopoulos T, Papapetropoulos A, Brouckaert P, Giannis A, Joos GF, Bracke KR and Brusselle GG. The role of soluble guanylyl cyclase in chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 2013; 188(7):789-799.
* equal contribution
2. **Dupont LL**, Bracke KR, De Maeyer JH, Compan V, Joos GF, Lefebvre RA and Brusselle GG. Investigation of 5-HT₄ receptors in bronchial hyperresponsiveness in cigarette smoke-exposed mice. *Pulm Pharmacol Ther* 2014; 28(1):60-67.
3. **Dupont LL***, Alpizar YA*, Brusselle GG, Bracke KR, Verhamme FM, Joos GF, Talavera K and Maes T. Role of Transient Receptor Potential channel A1 in Chronic Obstructive Pulmonary Disease. (manuscript in preparation) * equal contribution
4. **Dupont LL**, Glynos C, Bracke KR, Brouckaert P and Brusselle GG. Role of the nitric oxide - soluble guanylyl cyclase pathway in obstructive airway diseases. *Pulm Pharmacol Ther* 2014; 29(1):1-6.
5. Pauwels NS, Bracke KR, **Dupont LL**, Van Pottelberge GR, Provoost S, Vanden Berghe T, Vandenabeele P, Lambrecht BN, Joos GF, Brusselle GG. Role of IL-1alpha and the Nlrp3/caspase-1/IL-1beta axis in cigarette smoke-induced pulmonary inflammation and COPD. *Eur Respir J* 2011; 38(5): 1019-1028.
6. Demoor T, Bracke KR, **Dupont LL**, Plantinga M, Bondue B, Roy MO, Lannoy V, Lambrecht BN, Brusselle GG, Joos GF. The role of ChemR23 in the induction and resolution of cigarette smoke-induced inflammation. *J Immunol* 2011; 186(9): 5457-5467.
7. Demoor T, Bracke KR, Vermaelen KY, **Dupont L**, Joos GF, Brusselle GG. CCR7 modulates pulmonary and lymph node inflammatory responses in cigarette smoke-exposed mice. *J Immunol* 2009; 183(12): 8186-8194.

Conference abstracts

- LL. Dupont, T. Demoor, K. Bracke, M.-O. Roy, V. Lannoy, B. Lambrecht , G. Brusselle and G. Joos
ChemR23-signaling Modulates Cigarette Smoke-induced Pulmonary Inflammation In Mice.
American Thoracic Society 2011 (Denver) *poster presentation*
- LL. Dupont, G. Joos, G. Brusselle and K. Bracke
Role of ADAM19 and neuregulin-1 in Muc5ac expression in lungs of cigarette smoke-exposed mice
BVP meeting 2012 (GSK awards in Pneumology) (Brussels) *oral presentation*
- LL. Dupont, G. Joos, G. Brusselle and K. Bracke
Role of ADAM19 and neuregulin-1 in Muc5ac expression in lungs of cigarette smoke-exposed mice
American Thoracic Society 2012 (San Francisco) *poster presentation*
- LL. Dupont, C. Glynos, T. Vassilakopoulos, A. Papapetropoulos, P. Brouckaert, A. Giannis, G. Joos, K. Bracke, G. Brusselle.
The role of soluble guanylyl cyclase in Chronic Obstructive Pulmonary Disease
BVP meeting 2013 (GSK awards in Pneumology) (Brussels) *oral presentation*
- LL. Dupont, K. Bracke, J. De Maeyer, G. Joos, R. Lefebvre, G. Brusselle
Role of 5-HT₄R in bronchial hyperresponsiveness in cigarette-smoke exposed mice
American Thoracic Society 2013 (Philadelphia) *poster presentation*
- LL. Dupont, C. Glynos, T. Vassilakopoulos, A. Papapetropoulos, P. Brouckaert, A. Giannis, G. Joos, K. Bracke, G. Brusselle.
The role of soluble guanylyl cyclase in Chronic Obstructive Pulmonary Disease
European Respiratory Society 2013 (Barcelona) *oral presentation*
- LL. Dupont, Y. Aguiar Alpizar, GG Brusselle, KR Bracke, K Talavera, GF Joos, T Maes.
Expression of Transient Receptor Potential (TRP) channels in a murine model of cigarette smoke exposure
American Thoracic Society 2014 (San Diego) *poster presentation*

Awards/grants

European Respiratory Society Young Investigator grant (2013)

Bijzonder Onderzoeksfonds Research grant from Ghent University (2010-2014)