Association of platelet serotonin, plasma brain-derived neurotrophic factor (BDNF) and Val66Met BDNF gene polymorphism with asthma severity

Sreter, Katherina Bernadette

UNIVERSITY OF ZAGREB
SCHOOL OF MEDICINE

Katherina Bernadette Sreter

Association of platelet serotonin, plasma brain-derived neurotrophic factor (BDNF) and Val66Met *BDNF* gene polymorphism with asthma severity

DISSERTATION

Zagreb, 2022.

UNIVERSITY OF ZAGREB
SCHOOL OF MEDICINE

Katherina Bernadette Sreter

Association of platelet serotonin, plasma brain-derived neurotrophic factor (BDNF) and Val66Met *BDNF* gene polymorphism with asthma severity

DISSERTATION

Zagreb, 2022.

This doctoral dissertation describes research carried out at the following institutions in Zagreb, Croatia:

(1) Clinic for Lung Diseases Jordanovac, University Hospital Centre Zagreb;
(2) Laboratory for Molecular Neuropsychiatry, Division of Molecular Medicine, Ruđer Bošković Institute; and
(3) Croatian Institute for Transfusion Medicine.

Parts of the presented work in this dissertation have been published in:

Sreter KB, Popovic-Grle S, Lampalo M, Konjevod M, Tudor L, Nikolac Perkovic M, et al. Plasma brain-derived neurotrophic factor (BDNF) concentration and *BDNF*/*TrkB* gene polymorphisms in Croatian adults with asthma. J Pers Med 2020;10:189.

Permission from the journal publisher has been obtained to include material in this PhD thesis from the aforementioned scientific paper (i.e., copyright is retained by the authors of the published article).

Mentors:

Professor Sanja Popović-Grle, MD, PhD
Clinic for Lung Diseases Jordanovac, University Hospital Centre Zagreb;
University of Zagreb, School of Medicine, Zagreb, Croatia

Associate Professor Dubravka Švob Štrac, BSc, PhD, Senior Research Associate
Laboratory for Molecular Neuropsychiatry, Division of Molecular Medicine,
Ruđer Bošković Institute, Zagreb, Croatia

This doctoral thesis was submitted to the University of Zagreb, School of Medicine, on September 12th, 2022, to fulfil the requirements of the PhD Programme (in English) in Biomedicine and Health Sciences.

ACKNOWLEDGMENTS

- I would like to express my deepest gratitude to my mentors, Prof. Sanja Popović-Grle and Assoc. Prof. Dubravka Švob Štrac, for their professional guidance, constructive feedback and continued support during my doctoral journey and the preparation of this dissertation. I am sincerely thankful to Prof. Popović-Grle for inspiring me to spearhead this PhD research in the field of adult asthma. A special thanks also to Assoc. Prof. Švob Štrac for her help with the statistical data analysis.
- I gratefully acknowledge the nursing staff at the Clinic for Lung Diseases Jordanovac, University Hospital Centre Zagreb, for their contributions during the recruitment process, blood collection and diagnostic testing of the asthma patients. I am very thankful to all the patients who volunteered to take part in the study and to Asst. Prof. Marina Lampalo for her assistance with the initial data collection.
- It was an honour and pleasure to work with Assoc. Prof. Švob Štrac's entire staff (Marcela Konjevod, Lucija Tudor, Matea Nikolac Perković, Gordana Nedić Erjavec, Snježana Juler) and Prof. Nela Pivac, Senior Scientist (Permanent Position), at the Laboratory for Molecular Neuropsychiatry, Ruđer Bošković Institute. I am truly appreciative of their time, effort and dedication during the processing of the blood samples. Thank you for generously sharing your expertise in carrying out the various laboratory techniques and procedures. I am so grateful for this invaluable experience and especially to Prof. Pivac for championing this PhD research from the outset.
- I would also like to express my sincere appreciation to the healthcare team (Irena Jukić, Jasna Bingulac-Popović, Hana Safić Stanić) at the Croatian Institute for Transfusion Medicine for assisting with the recruitment of the healthy controls and collection of their blood samples. Many thanks to the healthy volunteer blood donors for their kind participation.
- I am profoundly grateful to the Croatian Thoracic Society and Novartis for their financial contribution to a part of this doctoral work, namely covering the laboratory costs associated with the BDNF research. I am thankful for the research opportunity this funding has provided. In particular, I would like to extend my appreciation to Prof. Miroslav Samaržija, Full Member of the Croatian Academy of Sciences and Arts, for helping to facilitate the funding process as well as for his encouragement and support of this PhD project during my pulmonology residency under his supervision.
- Many thanks to the Laboratory for Molecular Neuropsychiatry for donating surplus laboratory materials from other collaborative scientific projects to accomplish the serotonin research.
- My heartfelt thanks to Prof. Jasenka Markeljević for all her support and constant encouragement throughout my PhD studies.
- I would like to dedicate this doctoral thesis to my beautiful family. Your patience, encouragement, tremendous love, uplifting spirit, and unwavering belief in me and my potential remain the driving force of all my personal and professional achievements, including the successful culmination of this dissertation. I am truly blessed and eternally grateful to you for everything. I love you, Mom, Dad and brother, Matthew, with all my heart, more than words could ever say!

TABLE OF CONTENTS

LIST OF TABLES AND FIGURES

TABLES

FIGURES

LIST OF SYMBOLS AND ABBREVIATIONS

4-HOQ	4-hydroxyquinoline
5-HIAA	5-hydroxyindol-3-ylacetic acid, also 5-hydroxyindoleacetic acid
5-HIAL	5-hydroxyindol-3-ylacetaldehyde, also 5-hydroxyindoleacetaldehyde
5-HT	5-hydroxytryptamine or serotonin
$5\text{-HT}_{2A/2B/2C}$	5-hydroxytryptamine (serotonin) receptor 2A/2B/2C
5-HTT	serotonin transporter, also known as SERT
ABG	arterial blood gas
ABS	acid-base status
ACD	acid-citrate-dextrose
ACQ	Asthma Control Questionnaire
ACT	Asthma Control Test
ADP	adenosine diphosphate
AERD	aspirin-exacerbated respiratory disease
AHR	airway hyperresponsiveness
ANOVA	analysis of variance
ASM	airway smooth muscle
BBB	blood-brain barrier
BDNF	brain-derived neurotrophic factor
BMI	body mass index
BSA	bovine serum albumin
Ca^{2+}	calcium ion
CAMP	cyclic adenosine monophosphate
CD4+	cluster of differentiation 4
cm	centimetre
CNS	central nervous system
COPD	chronic obstructive pulmonary disease
COVID-19	coronavirus disease 2019
CT	computed tomography
$CuSO_4$	copper sulphate
CXCL	chemokine (C-X-C) motif ligand
CXCR-3	C-X-C chemokine receptor type 3
DALYs	disability-adjusted life years

DLCO	diffusing capacity of the lung for carbon monoxide
DNA	deoxyribonucleic acid
EC	enterochromaffin cells
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
EMA	European Medicines Agency
EU	European Union
Eurostat	European Statistical Office (EU's Statistics Agency)
FDA	Food and Drug Administration
FeNO	fractional exhaled nitric oxide
FEV1	forced expiratory volume in the first second
FEV1/FVC	the ratio of FEV1 to FVC (also called Tiffeneau-Pinelli index)
FVC	forced vital capacity
g	gravitational force
GERD	gastroesophageal reflux disease
GBD	Global Burden of Disease
GI	gastrointestinal
GINA	Global Initiative for Asthma
GSDMB	gasdermin B
GWAS	genome-wide association studies
h	hour
HCl	hydrochloric acid
H_2O	water
H_2O_2	hydrogen peroxide
HRP	horseradish peroxidase
HWE	Hardy-Weinberg equilibrium
ICS	inhaled corticosteroids
IgE	immunoglobulin E
IKZF3	Ikaros family zinc finger protein 3
IL	interleukin
ILC2	type 2 innnate lymphoid cells
Inc.	incorporated
IQR	interquartile range
ISAAC	International Study of Asthma and Allergy in Childhood

IU	international units
kDa	kilodalton
K-Na tartrate	potassium-sodium tartrate
kg	kilogram
kg/m^2	kilogram per square metre
kHz	kilohertz
kU	kilounit
L	litre
LABA	long-acting beta-agonist
LAMA	long-acting muscarinic antagonist
LSD	lysergic acid diethylamide
LTRA	leukotriene receptor antagonist
m	metre
M	molar (concentration) or number of moles of a solute per litre of solution
M3	muscarinic acetylcholine receptor subtype 3
MAO	monoamine oxidase
mBDNF	mature BDNF
µg	microgram
µL	microlitre
µM	micromolar
min	minute
mg	milligram
$MgCl_2$	magnesium chloride
mL	millilitre
mm	millimetre
mmHg	millimetre of mercury
mM	millimolar
n	total number
N	normality of the solution
NaCl	sodium chloride
Na_2CO_3	sodium carbonate
NADPH	nicotinamide adenine dinucleotide phosphate
NaOH	sodium hydroxide
NOX4	NADPH oxidase 4

ng	nanogram
nm	nanometre
nM	nanomolar
nmol	nanomole
NSAID	non-steroidal anti-inflammatory drugs
NT	neurotrophin
NTRK2	neurotrophin receptor tyrosine kinase 2
OPT	ortho-phthalaldehyde
ORMDL3	orosomucoid 1-like protein 3
PCR	polymerase chain reaction
PD20	provocative dose of methacholine causing a 20% drop in FEV1
PEF	peak expiratory flow
PF4	platelet factor 4; also known as CXCL4
PFT	pulmonary function test
pg	picogram
pH	power of hydrogen
PKA	protein kinase A
pp	percentage points
ppb	parts per billion
PPP	platelet-poor plasma
PRP	platelet-rich plasma
p	probability value
Q1	25^{th} percentile
Q3	75^{th} percentile
QALYs	quality-adjusted life years
R&D	research and development
(R)-DOI	(R)-2,5-dimethoxy-4-iodoamphetamine
RLB	red blood cell lysis buffer
RNA	ribonucleic acid
RD1S	assay diluent (buffered protein base)
RD6P	calibrator diluent (calibration standard solution of animal serum for dilution)
ROS	reactive oxygen species
rpm	revolutions per minute
RT-PCR	real-time polymerase chain reaction

s	second
SABA	short-acting beta-agonist
SAMA	short-acting muscarinic antagonist
SARS-CoV-2	severe acute respiratory syndrome coronavirus 2
SDS	sodium dodecyl sulphate
SERT	serotonin transporter, also known as 5-HTT
SNP	single nucleotide polymorphism
SPT	skin prick testing
T cells	“thymus”-dependent lymphocytes
T2	type 2 (when referring to T2-high and T2-low asthma, or T2 inflammation)
TGF	transforming growth factor
Th2	T helper 2
TMB	tetramethylbenzidine
TNF-α	tumour necrosis factor alpha
TPH	tryptophan hydroxylase
Tris	tris(hydroxymethyl)aminomethane
TrkB	tropomyosin-related kinase B (official name – NTRK2)
TSLP	thymic stromal lymphopoietin
UK	United Kingdom
USA	United States of America
Val66Met	valine (Val) to methionine (Met) substitution at codon 66
VMAT	vesicular monoamine transporter
WHO	World Health Organization
YLD	years of life lived with disability
$ZnSO_4$	zinc sulphate
ZPBP2	zona pellucida binding protein 2
~	approximately
$\chi 2$	chi-square
°C	degree Celsius
%	percentage
®	registered trademark
™	trademark

1. INTRODUCTION AND BACKGROUND

1.1. Asthma

Asthma is a complex area of respiratory medicine that has seen considerable advances in research over the past few decades. In particular, the last twenty years have witnessed the development of significant initiatives in precision medicine for asthma therapy [1]. Furthermore, important strides have been made towards better understanding the genetic and molecular mechanisms that contribute to its heterogeneity. Translating this new-found knowledge into routine clinical care in order to improve the quality of life of asthma patients has been quite challenging, especially in those with the most severe forms of asthma [2]. The goal of providing personalized or individualized medicine in asthma, as a means to ensure better outcomes for patients, has unfortunately lagged somewhat behind other areas of respiratory medicine, such as lung cancer, mainly due to limitations in fully understanding the intricacies of asthma pathophysiology and heterogeneity [3]. In recent years, the characterization of different clinical phenotypes has paved the way for the currently emerging opinion that endotyping asthma using novel specific biomarkers is key to tailoring the best therapy for the right patient [4]. Treatment schemes, therefore, should not only reflect disease severity, but also the different mechanisms (i.e., biological pathways) driving the pathogenesis of asthma [3]. A focus on further research to delve more deeply into various aspects of asthma, such as those presented in this work, should address the many unmet needs and highlight the importance of applying a precision medicine approach to the diagnosis and treatment of asthma patients.

1.1.1. Definition of asthma

Defining asthma simplistically is rather difficult given its complex pathophysiology [5]. More than a hundred years ago, asthma was described only as a single disease [6]. Over the years, however, there have been several different definitions of asthma [7]. For example, in 1892, Sir Willam Osler published in his textbook of medicine (Principles and Practice of Medicine) that asthma had “a strong neurotic element”, as quoted in Peebles & Aronica (2019) [7]. Currently, it is widely acknowledged that asthma is a chronic inflammatory disorder of the airways that results physiologically in hyperreactivity and clinically in recurrent episodes of wheezing, chest tightness, or coughing, in addition to its varied presentations, disease severities, treatment outcomes, and underlying mechanisms [5, 8].

The essential features of this common respiratory disorder are emphasized in the latest guidelines by the Global Initiative for Asthma (GINA) in 2022, with asthma being described as:

1) “a heterogeneous disease, usually characterized by chronic airway inflammation”, and

2) “defined by the history of respiratory symptoms such as wheeze, shortness of breath, chest tightness and cough that vary over time and in intensity, together with variable expiratory airflow limitation” [9].

GINA produces a clinically oriented, evidence-based strategy document on an annual basis to support the development of practice guidelines on asthma management and prevention in different countries and regions [10]. Since 1993, GINA has been championed and driven by the National Heart, Lung, and Blood Institute, National Institutes of Health, United States of America (USA), and the World Health Organization (WHO), with the goal of creating a global strategy on the optimal management of asthma in both adults and children [10]. Suprisingly, the heterogeneous nature of asthma has only recently been recognized by GINA, starting in 2016 [5]. The current GINA definition of asthma was reached by consensus through the consideration of typical characteristics that distinguish asthma from different respiratory conditions [9]. Asthma is now widely accepted as an umbrella term, such as anemia, arthritis and cancer, to describe a clinical spectrum consisting of multiple distinct subgroups (i.e., phenotypes and endotypes) [11].

1.1.2. Epidemiology of asthma

1.1.2.1. Epidemiology of asthma worldwide – general trends

Chronic respiratory diseases occur in more than a billion people globally, and asthma is a major contributor to this group of non-communicable diseases [12]. Although it affects people of all ages, asthma is the most common chronic disease in children [13]. The exact number of individuals with asthma worldwide, however, is unknown, and estimates of asthma prevalence vary greatly [14]. For example, the WHO published a report in 2017 in which asthma was estimated to affect around 235 million people [13]. On the other hand, the latest available epidemiological data from the Global Burden of Disease (GBD) Study 2016 approximated the prevalence of asthma globally at more than 339 million people [12, 15]. Furthermore, temporal trends in the epidemiological data for asthma vary across different parts of the world, with some country-specific data reporting an increasing prevalence, such

as in the USA, whereas in other countries, such as in Europe and Asia, asthma epidemiology is noted to be stable or decreasing [16]. The rates of asthma prevalence also vary greatly among different ethnic groups, with the United Kingdom (UK) and Canada providing most of the international data on ethnic-related differences [17].

A recently published study based on worldwide epidemiological information on asthma from electronic searches in the Global Health Data Exchange database (containing data from 1990 to 2016 and 195 different countries and territories) has reported an update for the year 2017 [18]. Asthma prevalence and mortality are recorded as 272.68 million cases (3.57%) and 0.49 million deaths (0.006%), respectively, and the incidence as 43.12 million new cases per year (0.56%) [18]. Although some studies have shown that the number of asthma cases seems to have significantly increased during the last 25 years [14, 18], the first data compilation of the Global Asthma Network Phase I Surveillance (2017-2020) is expected to provide results for future analyses and publications that will fill in the knowledge gaps with fresh, reliable worldwide epidemiological data on asthma trends and prevalence in both children and adults [14, 19]. It is presently accepted that the prevalence of both childhood and adult asthma has peaked or even subsided in some geographical areas, predominantly in high-income countries, whereas low and mid-income countries seem to be experiencing a continual increase [20-21]. Any reduction in asthma prevalence is likely due to improved asthma control through increased medication use owing to more widespread prescribing habits and personalized case management, in addition to better compliance (i.e., adherence to treatment) as a result of improved patient education and health literacy [20].

Besides the geographical variation in asthma incidence, prevalence and severity, a gender disparity has been reported to exist across the lifespan [20]. Although childhood asthma (incidence, prevalence and hospitalization rate) has a predilection for prepubertal boys, this trend reverses during adolescence, and females continue to have a higher burden of asthma (increased asthma prevalence and severity) well into the fifth decade of life [22]. At around the time of menopause, a shift occurs and a decrease in asthma prevalence in women is noted [23]. The severity of asthma symptoms is known to be impacted by the phase of the menstrual cycle, with 30-40% of women reporting pre- or peri-menstrual worsening; however, the exact role of ovarian hormones (e.g., estrogen and progesterone) and the molecular mechanisms involved in airway inflammation remain unclear [24].

1.1.2.2. Epidemiology of asthma in Europe

Most recent online data from the European Union (EU)'s statistics agency, Eurostat, support a slight increase in self-reported asthma prevalence in the EU population in 2019 (5.7%) when compared to 2014 (5.4%) [25]. The Eurostat data for asthma in people aged 15 years and over among EU countries for 2014 and 2019 are summarized graphically on the Eurostat website [25]. According to Eurostat, in 2019, Finland reported the highest proportion of people with asthma (9.4%), whereas Romania (1.5%) and Bulgaria (2.2%) reported the lowest [25-26]. The largest increase in the number of people reporting to have asthma in 2019, compared to 2014, was registered by Germany (+1.9 percentage points (pp)), Croatia (+1.8 pp) and Belgium (+1.5 pp) [26]. The reverse trend was noted for Ireland, Greece and France, with the greatest decline in asthma cases at -1.7 pp, -1.1 pp and -1.0 pp, respectively [26].

1.1.2.3. Epidemiology of asthma in Croatia

With respect to Croatia, the Eurostat data confirm a rise in asthma reporting (i.e., asthma prevalence) from 3.0% in 2014 to 4.8% in 2019 [25]. The most recent Eurostat morbidity statistics for Croatia, published by the Croatian Institute for Public Health in 2022, is based on data collected between 01/03/2019 and 01/09/2020 (with some EU countries extending the project deadline due to the coronavirus disease 2019 [COVID-19] pandemic) for those aged 0-95+ years [27]. The incidence and prevalence of asthma in Croatia was found to be 3.0/1000 (or 3.0%) and 5.048/1000 (or 5.0%) population (derived from data on the estimated total population in Croatia in 2017), respectively [27].

The prevalence of asthma in Croatia, especially the assessment of regional diversity and temporal trends, has previously been estimated using local epidemiological data based on the International Study of Asthma and Allergy in Childhood (ISAAC) validated questionnaire and conducted on pediatric populations of elementary school children and adolescents from different regions [28-33]. However, accurately assessing the prevalence of asthma in Croatian adults, particularly those with severe symptoms, remains an ongoing challenge given the lack of a national registry [34]. Using an estimated asthma prevalence of 3.0% (in 2014) and the Dutch report on severe asthma [35], the number of severe asthmatics in Croatia was approximated to range from 1000 to 4000 [34].

1.1.3. Burden of asthma

1.1.3.1. Burden of asthma worldwide

The WHO recognizes the major public health and economic burden of asthma [12]. Its impact on quality of life is substantial, especially in those with severe disease [20]. The GBD – Causes of Death collaboration estimated that a total of 420,000 deaths occurred worldwide from asthma in 2016, or more than 1000 per day, as noted in the Global Asthma Report 2018 [12]. Asthma is also listed as one of the top 20 causes of years of life lived with disability (YLD), and in 2016, ranked 16th globally (or 13.2 million YLD) [12]. In addition, 23.7 million disability-adjusted life years (DALYs), an index of burden of disease, were lost across all ages in 2016 because of asthma [12]. With respect to the trends of asthma burden, reports suggest that DALYs and deaths have remained stable or are in constant decline over the past 25 years [18].

It is important to remember that asthma is a rather uncommon cause of mortality worldwide, contributing less than 1% of all deaths in most countries and occurring predominantly in those with severe cases and in adults after middle age [12, 20]. Global trends in asthma mortality rates over time (1993-2012) in the 5 to 34-year age group, from the online WHO Mortality Database of 46 countries, show no appreciable change from 2006 to 2012 [36]. This is in contrast to the marked reduction in estimated global asthma mortality from 0.44 deaths per 100,000 people in 1993 to 0.19 deaths per 100,000 people in 2006 [36]. International mortality statistics, however, are limited only to those countries that report detailed causes of death, and therefore, are not all-inclusive [12]. In addition, international time trends in asthma mortality strongly reflect management practices and the use of particular drug regimens [36]. Thus, it is widely acknowledged that the huge health and economic burden of asthma is due to the large proportion of patients lacking proper or sustained asthma control despite advancements in research and the availability of effective treatments [37].

Asthma-related costs continue to rise at an alarming rate and the reported values vary within and between country-specific studies. For example, in the USA alone, where there are approximately 25 million patients (in 2019) with asthma [38], related annual costs are in the billions, ranging from $18 billion, as quoted in an earlier study [39], to $81.9 billion in 2013 [40]. The future 20-year (2019 to 2038) direct costs associated with uncontrolled asthma in American adolescents and adults are expected to climb to $300.6 billion, or a total economic burden of $963.5 billion with the addition of indirect costs [37]. Moreover, an estimated 15.46

million quality-adjusted life years (QALYs) are projected to be lost because of uncontrolled asthma in this group of asthmatic patients [37].

1.1.3.2. Burden of asthma in Europe

The impact of lung diseases in Europe is considerable, with one-eighth of all deaths attributed to respiratory diseases and lung conditions [41]. Within the EU, 5.2 million DALYs were reported to be lost annually at an estimated cost of €300 billion [41]. The prevalence of asthma in the EU was previously estimated to be 8.2% in adults, affecting almost 10 million people under the age of 45 years [42]. Taking these numbers into consideration, the annual direct costs (i.e., healthcare) within the EU were calculated to be €19.5 billion, with indirect costs (i.e., loss of productivity) at €14.4 billion and a monetised value of DALYs lost at €38.3 billion, totalling €72.2 billion [41].

1.1.3.3. Burden of asthma in Croatia

According to the latest WHO data (last updated in November 2021), asthma deaths in Croatia in 2017 reached 1.2 per 100 000 population (age-standardized death rate), compared to 1.34 deaths per 100 000 population for the EU region in the same year (range 0.12-3.25 deaths per 100 000 population) [43].

1.1.4. Heterogeneity of asthma

1.1.4.1. Etiology of asthma

The exact cause of asthma has not yet been fully elucidated [44]. Asthma is now used widely as an umbrella term for a very heterogeneous disease with many different pathophysiological processes [45]. However, whether asthma involves a collection of various clinical conditions or is a single condition with multiple mechanisms and phenotypes is still not well understood [46]. Current evidence suggests that asthma is a complex multifactorial disorder involving interactions between genetic susceptibility, host factors and environmental exposures [20]. The numerous possible combinations of environmental (e.g., air pollution; pollen, mould, house dust mite, and other aeroallergens; weather), host (e.g., obesity, nutritional factors, infections, allergic sensitization) and genetic (e.g., asthma susceptibility loci on genes) risk factors contribute to the heterogeneity present across the asthma spectrum [20]. In fact, daily clinical practice has proven time and again that no two asthmatics are completely alike [47].

The importance of gene-environment interactions in asthma is well recognized [44]. Over the past 40 years, initially linkage analysis and candidate-gene association studies, and later,

genome-wide association studies (GWAS), have been used extensively to study the genetic basis of asthma [48]. Several studies support the role of genetics in the heritability of asthma, with figures ranging from 35% to 95% [49-50]. It has long been known that children of asthmatic patients have an increased risk of developing asthma, particularly those with asthmatic mothers [51]. However, twin studies have demonstrated that genetics alone are unable to entirely explain asthma or its susceptibility given the discordance between identical twins [52].

Since the first report of genetic linkage of chromosome 11q13 with atopy in 1989 [53], over a thousand studies on the genetics of asthma and other allergic diseases have been published [54]. In the last few years, genetic investigations in asthma have concentrated on GWAS due to the advantage of being able to scan the entire human genome without any prior conditioning hypothesis [55], thereby enabling the simultaneous exploration of hundreds of thousands of single nucleotide polymorphisms (SNPs) [48]. Major gene alterations that influence the genetic heterogeneity in asthma have been identified through these studies [summarized in 44]. It is important to mention, however, that it has been incredibly challenging to determine which polymorphisms are actually "causal" in nature given the marked variability of clinical phenotypes in asthma, the involvement of multiple molecular mechanisms, insufficiently powered samples (i.e., small sizes and reduced number of genetic variants tested), and underrepresented populations [56].

The first GWAS in asthma was published in 2007 and highlighted the chromosome 17q21 locus as an asthma susceptibility locus, later confirmed to be the most widely replicated and significant asthma locus, involving four genes: orosomucoid 1-like protein 3 (*ORMDL3*), gasdermin B (*GSDMB*), zona pellucida binding protein 2 (*ZPBP2*), and Ikaros family zinc finger protein 3 (*IKZF3*) [57-58]. In their review, Bunyavanich and Schadt (2015) summarized the various GWAS on the genetic susceptibility loci of asthma [58]. Vicente et al. (2017) outlined the GWAS from 2007 to 2016, revealing a total of 39 common SNPs independently associated with asthma risk [59]. Hernandez-Pacheco and colleagues (2019) expanded on these earlier GWAS as well as later studies from 2016 to 2018, including those focused on the characterization of multiple genetic variants associated with therapeutic responsiveness to asthma medications [48].

In their recent comprehensive review, Ntontsi et al. (2021) presented the latest scientific advances of genetic and epigenetic studies regarding asthma susceptibility [60]. The most

common epigenetic mechanisms identified, including deoxyribonucleic acid (DNA) methylation, post-translational histone modifications and micro-ribonucleic acid (micro-RNA) expression, have been shown to play a regulatory role in immune responses and gene expression in asthma [60]. In addition to the aforementioned genetic studies, transcriptome profiling using oligonucleotide microarrays to compare RNA transcription differences between individuals with and without asthma, or between asthma patients before and after taking steroids, have implicated hundreds of genes [44]. These studies provide further evidence that environmental exposures and physiological heterogeneity can alter clinical expression of asthma [44].

Another precision medicine approach that has increased our understanding of the complexity of asthma is microbiome analysis [6]. The respiratory and gastrointestinal (GI) tracts house large numbers of microbial communities that are essential for human health, and may play a unique role in disease [6]. Barcik et al. (2020) provided a remarkably detailed overview about the explosion in research over the past couple of decades regarding lung-gut microbiota, and described the interplay between microbial dysbiosis and altered immune development leading to inappropriate inflammatory responses in asthma [6].

1.1.4.2. Pathogenesis and pathophysiology of asthma

The precise pathophysiological basis of asthma is not entirely clear, but seems to involve a multifaceted network of interacting factors, including various bioactive mediators [61-62]. There is growing evidence that multiple biological pathways drive the immune and inflammatory responses associated with disease heterogeneity in asthma [4, 61, 63]. The pathogenesis of asthma can be divided into three chief disease domains with overlapping features: airway inflammation (Type 2 [T2]-high and T2-low), airway hyperresponsiveness (AHR) and airway remodelling [61].

1.1.4.2.1. Airway inflammation

Airway inflammation is a hallmark of asthma, and the inflammatory response in asthmatic airways involves a complex interplay between the respiratory epithelium and the immune system [62]. The overall T2 inflammatory response is now considered to result from activation of molecular pathways of both innate and adaptive immune responses that are likely differentially expressed between the distinct phenotypes of asthma [63]. The innate response is orchestrated by natural killer cells and type 2 innate lymphoid cells (ILC2s), while cluster of differentiation 4 “thymus” cells (CD4+ T cells) contribute to the adaptive response [7].

The T2-biased airway inflammation is observed in 50% of mild-to-moderate asthma patients [64], but only 37% of patients with severe asthma [65-66], even though previously it was thought likely to be higher than 50% in those with the most severe forms of asthma [67-68]. T2 inflammation is not only found in asthma, but also frequently occurs in allergic diseases, eosinophilic disorders and parasite infections [44]. Airway inflammation is now generally dichotomized as eosinophilic ("T2-high") or non-eosinophilic ("T2-low" or non-T2) [69]. Eosinophilic asthma inflammation can be further broken down into non-atopic and atopic subgroups given that 50% of patients have T2 inflammation (eosinophils) in the absence of allergy, and nonallergic noneosinophilic phenotypes of asthma have also been identified [63, 68]. Russell and Brightling (2017) provide a comprehensive illustration that helps explain the heterogeneity of asthma immunopathology by showing the divisions of airway inflammation into three main groups: eosinophilic asthma (T2-high), non-eosinophilic asthma (T2-low) and mixed granulocytic asthma, including their corresponding subgroups, mechanisms and pathological features [61].

Previously, asthma had been considered a T helper 2 (Th2)-cell-mediated disease based largely on studies using mouse models in the 1990s showing that animals genetically lacking Th2 cell cytokines (interleukin [IL]-4, IL-5 and/or IL-13) were devoid of or ameliorated many of the key features of asthma, such as airway eosinophilia, immunoglobulin E (IgE) synthesis, bronchial hyperresponsiveness, mucus overproduction, and airway remodelling [70-74]. In fact, this concept of Th2-cell-mediated immunity to allergens driving asthma pathogenesis in mice and humans had remained the dominant thinking for the past 30 years, pushing forward treatments with biological agents that target the Th2 cell cytokines [75]. With the advancement of science and the development of "-omics" technologies to study asthma, other innate immune cells such as basophils, mast cells and ILC2, in addition to the Th2 cells, have been found to produce Th2-cell-associated cytokines involved in asthma [7, 74]. Furthermore, studies have revealed that a form of neutrophilic asthma, with concomitant T helper type 17 (Th17) cell involvement, also exists [76-77]. As a result of these new insights, a gradual shift in terminology has occurred from "Th2-cell-high" asthma to the currently employed "T2-high" asthma [68, 74].

The most commonly used biomarkers in routine clinical practice to recognize T2-inflammatory responses in asthma include blood and sputum eosinophils, fractional exhaled nitric oxide (FeNO) and IgE [4]. The gold standard to distinguish T2-high from T2-low disease is the level of eosinophils detectable in the airways (i.e., sputum cell count) [4, 69]. In

addition, T2-high asthma is highly sensitive to anti-inflammatory corticosteroids or specific eosinophilic treatment, whereas T2-low asthma is not [64, 69]. This highlights the importance of phenotyping asthma to personalize treatments given that asthmatics without a strong T2-inflammatory response often do not respond to corticosteroids, and thus, can be difficult to manage [44].

Asthma severity and AHR are directly related to the degree of airway inflammation [62]. Apart from the T2-high versus T2-low classification, asthmatic airway inflammation can be categorized into three subtypes based on the acuity of presentation: acute, subacute and chronic [62]. Acute asthmatic inflammation involves the early recruitment of cells into the airways and subacute inflammation is the activation of these recruited cells [62]. Chronic asthmatic inflammation is characterized by cellular damage [62]. The acute inflammatory reactions and accompanying clinical symptoms typically occur intermittently owing to the underlying chronic inflammation, despite the lack of a continous allergen exposure [62]. The drive for a chronic inflammatory response initiates with the production of bioactive mediators from airway epithelium that attract, activate and recruit inflammatory cells into lung airways [62]. Infiltrated cells augment the inflammatory response through the release of other biochemical mediators [62].

1.1.4.2.2. Airway hyperresponsiveness (AHR)

AHR is a consequence of increased or dysfunctional narrowing of the airway lumen due to indirect or direct stimuli causing airway smooth muscle (ASM) contraction [61]. Both T2-high and T2-low inflammation intensify AHR [61]. Mast cells infiltrate into the ASM to a greater extent in asthma patients, and the degree of mast cell infiltration correlates with bronchial reactivity as measured using the provocative dose (PD20) during the methacholine challenge test [61, 78]. Firstly, mast cells in ASM directly cause AHR by releasing mediators (e.g., histamine, prostaglandin D2 and cysteinyl leukotrienes) that induce contraction of the ASM [61, 78-79]. Moreover, mast cells located in the ASM bundle also release cytokines IL-4 and IL-13 that are not found in healthy individuals or in those with eosinophilic bronchitis [80].

External stimuli such as oxidative stress and inhaled environmental pollutants also augment AHR in ASM [61]. Increased oxidative stress acts on ASM through increased nicotinamide adenine dinucleotide phosphate (NADPH) oxidase 4 (NOX4) expression [81]. There is also evidence for the roles of epithelial-derived IL-33 in maintaining AHR during allergen

challenge [82] and high mobility group box-1 protein (HMGB1), released during damage of the airways, in amplifying ASM hypercontractility via activation of Toll-like receptor-4 [83]. Recruitment of mast cells to the ASM appears to be primarily mediated by the chemokine CXCL10 (expressed on ASM cells) interacting with the chemokine receptor CXCR3 (expressed on the surface of mast cells) [79]. CXCL10 and CXCR3 show markedly increased expression in smooth muscle cells and mast cells located in the region of the ASM of asthmatics [79].

1.1.4.2.3. Airway remodelling

Acute and chronic allergic inflammatory responses in asthmatic lungs result in various alterations in the structural cells and tissues of airway walls, collectively referred to as airway remodelling, including: epithelial changes (hyperplasia, metaplasia, and shedding), goblet cell hyperplasia, submucosal gland hypertrophy, subepithelial fibrosis in peribronchial interstitial tissue, subepithelial collagen layer thickening, increased submucosal matrix deposition, angiogenesis, dysfunctioning of bronchial blood vessels, hypertrophy and hyperplasia of ASM, and mast cell localization and degranulation in the ASM bundle [61-62, 74, 81, 84-85]. Although other inflammatory conditions such as chronic obstructive pulmonary disease (COPD) are associated with structural remodelling in the lungs, albeit with notable differences, a unique pathology to asthma is thickening of the reticular basement membrane of the bronchial epithelium [86]. An illustration of the aforementioned changes involved in the remodelling process in the lung of an asthma patient compared to a healthy individual can be found in the review by Lambrecht and colleagues (2019) [74].

Airway wall thickness has been shown to be correlated with disease severity in asthma [87]. Macro-level changes of bronchial wall thickening have been observed on computed tomography (CT) scans of severe asthmatics and micro-level remodelling changes have been described on bronchial biopsy samples [61, 88]. Furthermore, the degree of epithelial hyperplasia and metaplasia increases according to asthma severity as shown on bronchial biopsy samples from mild, moderate and severe asthmatics, versus healthy subjects [61, 89].

The airway epithelium is obviously central to both the normal functioning of the lungs and the pathogenesis of asthma [90]. Inflammation markedly impairs its structure and function [90]. Epithelial damage leads to 1) ciliary dysfunction and 2) reduced barrier function [61]. Ciliary dysfunction contributes to defective sputum clearance from the airways and mucous plugging, features of severe asthma and often found in fatal asthma exacerbations [61]. Reduced barrier

function results in an increase in susceptibility to inhaled pathogens, allergens and pollutants, thereby triggering the inflammatory cascades [61]. In Th2-driven models of asthma, thickening of the basement membrane and increased volume of ASM are associated with increases in growth factors, including transforming growth factor (TGF)-β1 and platelet-derived growth factor [62]. In addition, changes in asthmatic airways are associated with increased expression or overexpression of Th2 interleukins, especially IL-4, IL-5 and IL-13 [62]. For instance, IL-4 and IL-5 cause airway eosinophilia, mucus metaplasia and subepithelial fibrosis, whereas IL-13 leads to subepithelial fibrosis, mucus metaplasia and infiltration of eosinophils and macrophages [62].

1.1.4.3. Classification of asthma

A simple classification of asthma does not exist due to its complex heterogeneity. In the first instance, asthma can be classified as either intermittent or persistent according to some published literature [5]. However, it is noteworthy that GINA 2022 guidelines state that GINA does not distinguish between intermittent and mild persistent asthma because this historical distinction was arbitrary and erroneously based on the assumption that the so-called intermittent group would not benefit from therapy with inhaled corticosteroids (ICS) [9]. Instead, GINA 2022 has defined mild asthma as asthma that remains well controlled with low-dose ICS or as-needed ICS-formoterol (i.e., still commonly referred to as Step 1 or 2 treatment), and now recommends that if the term mild asthma is used in clinical practice, it should be qualified with a reminder that patients with infrequent or mild asthma symptoms can still be at risk of severe or fatal exacerbations [9]. Due to differing opinions about the specific criteria to define mild asthma, GINA continues to review the concept of asthma severity and the definition of mild asthma, and an update is awaited in 2023 [9]. In the interim, the subgroups of persistent asthma remain divided by degree of disease severity into mild, moderate or severe disease [5, 9]. According to earlier, as well as the latest recommendations from GINA, asthma severity (i.e., GINA Steps 1 to 5 – most recently; GINA Steps 1 to 4 – previously) is based on the levels or "steps" of therapy needed to achieve good control of the disease (i.e., reduce the risk of exacerbations and control the symptoms), as assessed retrospectively after at least several months of treatment [9]. Asthma can be also be divided into uncontrolled, partially controlled and controlled groups depending on how well symptoms are managed [91].

In accordance with the above criteria for asthma severity, treatment regimens are administered to decrease symptoms [92]. Asthma symptom control can be easily and quickly assessed

using tools such as the Asthma Control Questionnaire (ACQ) and Asthma Control Test (ACT) [91]. It is also important to remember that clinical presentations in asthma patients, including asthma control and consequently severity, may be complicated and worsened by the presence of comorbidities that also need careful consideration and optimal treatment strategies [93-94].

Asthma and its symptoms can initially appear at any age, even in older adults [91]. Thus, asthma can also be broadly categorized into early-onset asthma (generally under 12 years of age) or adult-onset asthma [95]. At the point of disease onset, asthma can be mild, moderate or severe, and a progression of severity over the lifespan does not necessarily occur [96]. Both children and adults with asthma may have relapsing and remitting periods of their symptoms [61]. However, even during periods of relative disease stability, AHR and/or airway remodelling may persist in patients, thus monitoring and ongoing treatment should be emphasized [96]. In addition, asthma is commonly divided into groups based on the presence or absence of atopy into allergic versus non-allergic asthma [5].

1.1.5. Diagnosis of asthma

The diagnosis of asthma is often difficult because no one symptom, sign or test is pathognomonic [69]. The GINA 2022 guidelines provide comprehensive step-by-step recommendations as well as diagnostic criteria for asthma in adults, adolescents, and children 6-11 years old [9]. In general practice, the diagnosis of asthma is largely clinical, relying on a detailed history and physical examination, combined with diagnostic tests that provide objective evidence of underlying pathophysiology via assessments of bronchial hyperreactivity and lung mechanics [5, 91]. In terms of history, patients usually present with similar symptoms of cough, wheezing, chest tightness, or shortness of breath [97-98]. However, some patients may complain of atypical symptoms that could lead to a misdiagnosis [91]. Physical examination often, but not always, reveals wheezing, hyperinflation, prolonged expiratory time, and increased labour of breathing [99]. Normal physical examinations are also quite common as patients are often in between exacerbations that are triggered by a variety of factors (e.g., exercise, cold air, viral infections, stress, inhaled allergens, irritants, etc...) [99]. Chest radiography in asthma is usually normal, but bronchial thickening is often observed [91]. The presence of atopy is determined using skin prick tests of a variety of common environmental allergens in addition to serum levels of total and specific IgE [100]. Allergy testing, however, is not specific to the diagnosis of asthma, but may identify allergic asthma in individuals with asthmatic symptoms when exposed to a confirmed allergen [98].

Conventional diagnostic tests to support the diagnosis of asthma consist of pulmonary function tests (PFTs) such as spirometry and methacholine challenge (also known as bronchoprovocation test) [9]. The cornerstone of asthma diagnostic testing in both children and adults is spirometry with pre- and postbronchodilator responses [98]. Spirometry can be easily performed in the office setting to document expiratory airway obstruction and variable expiratory airflow limitation, two important criteria for the diagnosis of asthma [9, 91, 98, 100]. Lung function measurements in spirometry include: 1) forced vital capacity (FVC) or the total volume of air (in litres) an individual can forcefully exhale from their lungs in one breath; 2) forced expiratory volume in one second (FEV1) or the volume of air (in litres) forcibly expelled from the lungs in the first second of expiration; and 3) the ratio of FEV1 to FVC (FEV1/FVC) [91, 98].

In order to diagnose asthma, evidence of airway obstruction using the FEV1/FVC ratio is required [9, 98]. The cutoff point for an obstructive defect varies between professional societies and caution is advised with the use of a fixed ratio as it may result in false-positive diagnoses in older adults and false-negative diagnoses in younger adults [98]. According to the GINA guidelines, when FEV1 is decreased, a reduced FEV1/FVC ratio compared with the lower limit of normal (usually > 0.75-0.80 in adults) demonstrates airflow obstruction in adults [9]. It is important to note that obstruction may not be present at all times in the patient and spirometry should be repeated if a clinical suspicion for asthma remains [98].

There are two important concepts in spirometry that must be considered when evaluating for asthma: variability and reversibility. Following confirmation of an obstructive defect, variability in airway obstruction should be sought at subsequent clinical visits [9]. Variability is defined as the improvement and/or deterioration in symptoms and lung function [9]. Reversibility (or responsiveness) is a general term for rapid improvements in FEV1 measured 10 to 15 min after inhalation of a rapid-acting bronchodilator, or more sustained improvement at subsequent clinic visits after the introduction of an effective controller treatment such as ICS [9, 101]. A significant (or positive) bronchodilator reversibility test in asthma is defined as an increase in FEV1 of > 12% and > 200 mL from baseline FEV1 measured 10-15 min following administration of 200-400 μg (i.e., two to four puffs) of a short-acting beta-agonist such as salbutamol (albuterol) [9, 91].

In case respiratory symptoms, spirometry, or response to therapy are atypical, a methacholine challenge test can be used to evaluate the change in lung function with airway hyperreactivity

(or hyperresponsiveness), and thereby, help assess the likelihood of an asthma diagnosis [102]. Methacholine is a synthetic derivative of the neurotransmitter acetylcholine [103]. Inhaled methacholine causes bronchoconstriction (or airway narrowing) by directly binding to and stimulating ASM muscarinic receptors, specifically the muscarinic acetylcholine receptor subtype 3 (M3), and triggering a cascade of intracellular signals followed by calcium release and ASM contraction [102-103]. The resulting airway narrowing is measured as a decrease in FEV1 on spirometry assessment [103]. Individuals inhale progressively increasing standard doses of methacholine as FEV1 is serially measured [91, 103]. A positive bronchial challenge test is declared when a reduction in FEV1 from baseline of $\geq$ 20% occurs at the effective delivered dose of methacholine known as the PD_{20} [9, 102]. Values of PD_{20} between 1 and 16 mg/mL are indicative of bronchial hyperresponsiveness, but not necessarily asthma, whereas PD_{20} greater than 16 mg/mL (i.e., a normal or negative methacholine challenge) essentially rules out asthma [102].

Airway inflammation, categorized generally as eosinophilic (T2-high) or non-eosinophilic (T2-low), is a central component of asthma that can be measured using FeNO [69]. Nitric oxide is produced in the lungs as a result of eosinophilic inflammation through the action of pro-inflammatory cytokines such as interleukin IL-4, IL-5 and IL-13 that upregulate inducible nitric oxide synthase [69, 104]. Therefore, FeNO serves as an indirect surrogate biomarker of T2-high eosinophilic airway inflammation in asthma [69]. Nevertheless, the FeNO test is not universally recommended in the guidelines for confirmation of an asthma diagnosis, but rather is considered a complementary tool to help guide asthma management [9, 69, 98]. A positive FeNO test may lend support to a diagnosis of asthma, however, a negative one does not necessarily rule it out [98].

1.1.6. Treatment of asthma

Asthma treatment is largely based on the most up-to-date international guidelines (i.e., GINA asthma strategy) that focus on disease severity and prescribing the most appropriate medical therapy to control symptoms and reduce the risk of exacerbations [9, 105]. Despite optimal medical therapy, a select group of patients continues to experience poor disease control, irrespective of asthma severity, due to a number of different triggering factors [105]. The mainstay of asthma treatment is predominantly anti-inflammatory in nature, with the newest biological therapies (monoclonal antibodies) directed against the T2 inflammatory cytokines IgE, IL-5, IL-13, and IL-4 for severe, treatment-refractory asthma [as reviewed in 106-109]. The success of these newest treatments, however, is dependent on different asthma

phenotypes, with some patients experiencing ongoing symptoms that negatively impact quality of life, asthma morbidity and healthcare costs [110].

An appreciation of these unmet needs in asthma management has led to a broader search for the mechanisms underpinning its pathophysiology, resulting in a better understanding of neuronal regulation in asthma. Several current therapies are thus appropriately focused against the pathways involved in neuronal dysregulation in asthma [111]. For instance, muscarinic receptor antagonists (anticholinergics) inhibit effects of the primary parasympatheic neurotransmitter acetylcholine in the airways, thus, underpinning the relevance of increased cholinergic tone and muscarinic M3-mediated bronchoconstriction in the pathophysiology of asthma [112]. As add-on therapies in moderate and severe asthma, long-acting anticholinergics are effective in improving lung function and reducing exacerbations through bronchodilation and diminished mucus secretion [113-114]. The latest research has demonstrated the involvement of cholinergic pathways in airway remodelling and inflammation, highlighting the wider effects of anticholinergics in exerting positive outcomes in patients with asthma or COPD [112].

The treatment of asthma includes pharmacologic and non-pharmacologic therapeutic strategies that should be individualized to each patient, with the goals of long-term symptom control (i.e., absence of daytime symptoms, nighttime waking, reliever use, and functional limitation), improving quality of life, and prevention of future exacerbations and deterioration in lung function [9, 115-116]. In addition, treatments are aimed to prevent fatalities, hospitalizations, emergency visits, and adverse effects from medications [117]. It should be carefully noted that patient education, adherence and proper inhaler technique are of paramount importance to ensure the full benefit of asthma treatment [91]. Although the cause of uncontrolled asthma is multifactorial, it is widely accepted that poor adherence to treatment is one of the major factors leading to treatment failure [116, 118].

Pharmacologic therapies in asthma have traditionally been classified as controllers (i.e., maintenance medications) and relievers [9, 116]. Controller medications are meant to be taken regularly to control the underlying airway inflammation in persistent asthma [116]. Reliever or rescue medications are rapid-acting and should be used only when needed to provide symptom relief [9, 116]. The most commonly used asthma medications include short-acting beta-agonists (SABA), long-acting beta-agonists (LABA), short-acting anticholinergics or muscarinic antagonists (SAMA), long-acting muscarinic antagonists (LAMA), leukotriene

receptor antagonists (LTRA), ICS – the cornerstone of maintenace asthma therapy, systemic glucocorticoids, methylxanthines, allergen immunotherapy, and biologic therapy (in severe asthma) [9, 91]. Step therapies as described in GINA are meant to be used as guides, with treatments tailored to each patient, stepping-up or -down as appropriate according to the clinical situation [91]. The newest 2022 GINA strategy document provides easy-to-follow steps and diagrams that summarize asthma management, including a detailed chart that is split into two tracks based on the reliever therapy (i.e., preferred versus alternative) being used along with the controller [9].

A brief rationale about the different therapies in asthma, including their mechanisms of action, is discussed here. For over 40 years, the use of ICS has been the cornerstone of asthma therapy [116, 119]. Since the 1970s and early 1980s, ICS has been used as maintenance therapy in moderate to severe asthma, and later, in mild asthma as well [120]. Systemic glucocorticoids are needed in uncontrolled asthma and during asthma exacerbations, but are associated with numerous unwanted side-effects when used long-term [91]. Corticosteroids decrease inflammation and remodelling of the lungs by increasing the production of the anti-inflammatory cytokine interleukin IL-10 from alveolar macrophages and inhibiting the release of pro-inflammatory cytokines, T-cell activation and different white blood cells such as mast cells and eosinophils [121-122].

Beta2-agonists are also a mainstay of asthma management, acting as both a controller (LABA in combination with ICS) for long-term therapy and a reliever (SABA) to quickly reverse bronchoconstriction in an acute asthma attack [123]. Beta2-agonists cause dilatation of the bronchi by activating the beta2-adrenoreceptors in the respiratory tract (i.e., ASM, lung epithelial and endothelial cells, Type II cells, and mast cells) [123-124]. Activation of beta2-adrenoreceptors is mediated by increases in intracellular cyclic adenosine monophosphate (cAMP) [123]. The exact mechanism by which cAMP induces ASM cell relaxation is not fully understood, but it has been shown that it catalyses the activation of protein kinase A (PKA) which phosphorylates proteins involved in the control of muscle tone [123-124]. In addition, relaxation of the ASM results from cAMP inhibiting calcium ion (Ca^{2+}) release from intracellular stores, reducing Ca^{2+} entry into cells, and inducing the sequestration of intracellular Ca^{2+} [124].

Muscarinic antagonists or anticholinergics, particularly LAMA, serve as add-on therapy in uncontrolled asthma patients on existing therapies with ICS and LABA [125].

Anticholinergics reduce the acetylcholine-induced inflammatory response by diminishing cytokine release and recruitment of inflammatory cells [126]. Muscarinic antagonists (SAMA and LAMA) are usually combined with a beta-agonist or glucocorticoid, or as a fixed triple therapy (LABA-LAMA-ICS) in asthma patients for a synergistic effect and greater benefit than single bronchodilation, given the different mechanisms of action [9, 92, 126].

The cysteinyl leukotrienes, potent inflammatory lipid metabolites derived from arachidonic acid, enhance inflammatory cytokine cascades and serve as crucial regulators of airway inflammation and remodelling in asthma [127]. The LTRAs are listed by current guidelines as an alternative controller to ICS in the management of chronic asthma, and are widely prescribed in clinical practice [9, 127]. However, the clinician is cautioned that daily oral treatment with LTRA is less effective than monotherapy with ICS in adults and children, particularly for exacerbations, and therefore, ICS therapy is the preferred option [9, 127-128].

Methylxanthines had been used for decades for asthma treatment due to their bronchodilator effects as well as potential anti-inflammatory and immunomodulatory actions [129]. However, methylxanthines are no longer recommended by international guidelines to be used in the management of acute asthma due to their poor efficacy and risk of adverse events, including nausea, vomiting, seizures, and arrhythmias [9, 129]. Moreover, add-on treatment with methylxanthines, such as intravenous aminophylline, during severe asthma exacerbations does not improve outcomes compared to SABA alone [9, 130].

In allergic asthma patients sensitized to inhaled allergens, including asthma with allergic rhinoconjunctivitis, allergen-specific immunotherapy as add-on therapy has been shown to reduce asthma symptoms and rescue medication use [9, 131]. Both subcutaneous and sublingual allergen immunotherapy seem to be effective in allergic asthma, but additional studies are needed [9, 131]. GINA is currently reviewing the evidence on allergen immunotherapy and will be updating the international guidelines for 2023 [9].

Although ICS is recommended as first-line therapy for asthma in the current clinical guidelines, either as monotherapy or in combination with LABAs, or alternatively, LTRA, often the severe forms of asthma require add-on therapy (i.e., oral corticosteroids or LAMA) to achieve disease control [9, 67, 107]. Despite these measures, some severe asthma patients continue to have suboptimally controlled disease, and more effective strategies, such as biologic therapies, must be adopted [67, 107]. Biologic therapies are antibodies (i.e., anti-IgE, anti-IL-5, anti-IL-5-receptor (R), and anti-IL-4R) directed against specific key players in

inflammatory pathways (i.e., IgE, IL-5, IL-4, and IL-13) [107]. The first biological agent, omalizumab (anti-IgE), was approved for severe allergic asthma almost twenty years ago, in 2003, by the Food and Drug Administration (FDA), followed by the European Medicines Agency (EMA) in 2005 [67, 107]. Four more biologic agents (i.e., mepolizumab, reslizumab, benralizumab, dupilumab) have been approved since then, and new ones are currently in development [9, 107]. A comprehensive review of these biologic therapies and their mechanisms of action with detailed summary tables is found in McGregor et al. (2019) [107].

Briefly, anti-IgE biologic agents are recombinant humanized monoclonal antibodies that bind to free IgE, thus preventing IgE from binding to its high-affinity receptors (FCεR1) found on mast cells and basophils, resulting in the dampening of cellular responses to allergens [67, 107]. Inflammation is further reduced by the downregulation of the IgE receptors on mast cells and basophils [67, 107]. Anti-IL-5 biologics are monoclonal antibodies that bind either directly to IL-5 (i.e., mepolizumab ane reslizumab) or to the alpha subunit of the IL-5 receptor (benralizumab) on eosinophils and basophils [107]. IL-5 is the primary cytokine involved in the recruitment, activation, and survival of eosinophils [107]. Therefore, anti-IL-5 biologics reduce eosinophilic airway inflammation in severe eosinophilic asthma patients who have an increase in sputum and/or blood eosinophils despite treatment with corticosteroids [107]. By targeting the IL-4 alpha receptor, the most recently approved biologic agent for severe uncontrolled asthma, dupilumab, is able to block signalling from both IL-4 and IL-13, and thereby, reduce production of IgE and recruitment of inflammatory cells, as well as modulate AHR and airway remodelling [107]. Although the currently approved biologics target downstream pathways of T2 inflammation, new investigational biologic agents are focused on various upstream targets, including IL-25, IL-33 and thymic stromal lymphopoietin (TSLP) as well as small molecule antagonists targeting kinases (e.g., Janus kinase pathways) that are further downstream to the T2 cytokines [107].

When choosing the type of medication, device and dose for both controller and reliever inhalers, the clinician must consider several factors, including symptom control, risk factors and triggers, patient preference, and practical issues (e.g., cost, ability to use the device and adherence) [9]. GINA treatment recommendations have been updated for 2022 based on a thorough review of the evidence regarding clinical practice for Steps 1 to 5, and are summarized in a treatment figure showing preferred treatment options for each step [9]. The most fundamental change in asthma treatment strategy occurred in April 2019 when the annual GINA report advised that SABA should not be used alone in adolescents and adults

with asthma, but rather ICS use was deemed necessary and should be taken on either a symptom-driven (mild asthma only) or regular daily basis to reduce the risk of serious exacerbations [132-133].

Non-pharmacologic therapeutic interventions in asthma include smoking cessation, physical activity, weight reduction for obese patients, breathing exercises, avoidance of triggers, and bronchial thermoplasty [9]. Bronchial thermoplasty may be considered as an alternative treatment for some adult patients with severe uncontrolled asthma despite optimal pharmacologic therapies, but evidence is limited and long-term effects are unknown [9]. Some studies have highlighted the promising role of pulmonary rehabilitation as an additional nonpharmacologic therapy in asthma, but more research is required to further characterize rehabilitation programmes in order to improve clinical care [115]. A multifaceted and multidisciplinary approach to asthma management, simultaneously combining several different action plans, including non-drug interventions, seems most likely to be effective in optimizing clinical outcomes, though further research is needed for these recommendations to be successfully implemented in daily practice [134].

Another important consideration when reviewing the patient's current treatment and disease control is to address the comorbidities (coexisting or interacting conditions) associated with asthma that may impact outcomes and make asthma difficult to control, especially if treatment effects remain inadequate [135]. The most common comorbidities of asthma include gastroesophageal reflux disease (GERD), laryngopharyngeal reflux, upper airway diseases (i.e., rhinitis, sinusitis, rhinosinusitis), sleep apnea, and recurrent respiratory infections [117, 135]. Rhinosinusitis and GERD are common triggers of cough due to postnasal drip and heartburn, respectively, and may exacerbate asthma [117]. Lifestyle modification and the use of proton pump inhibitors may benefit patients with these comorbidities and difficult-to-control asthma [117].

1.1.7. Phenotypes and endotypes of asthma

1.1.7.1. Asthma Phenotypes

Since Wenzel’s seminal paper in 2006, in which potential phenotypic categories were proposed, many studies have attempted to classify the diverse group of asthma patients into specific phenotypes [47, 136]. A disease phenotype (i.e., clinical presentation) is defined as any observable characteristic or trait of a disease (i.e., clinical, demographic or physiological features, trigger-related or inflammatory processes) without necessarily implying an

underlying mechanistic pathway [4, 68]. For many years, asthma was believed to consist of only two major phenotypes: 1) non-atopic or “intrinsic”, and 2) atopic or “extrinsic” asthma [66]. Over the past two decades, additional asthma phenotypes have been defined based on a hypothesis-based approach, and later, a systems biology or cluster analysis approach. The hypothesis-based approach relies on classifying patients into broad categories based on a single variable, including disease severity, symptom triggers, age at onset, inflammatory patterns, exacerbations, and airflow obstruction [66, 137-138]. A major limitation of this approach, however, is a high occurrence of phenotype overlap so that the groups cannot be adequately distinguished from each other [66]. Furthermore, the principle studies on this topic involved different numbers and classifications of asthma phenotypes, thus making final conclusions challenging [47]. The more phenotypes are identified in the studies, the greater the likelihood of finding instances of overlap, thus potentially diminishing the meaning and interpretation of the data [47]. In addition, longitudinal studies have found that phenotypes are not stable over time, with those determined by biomarkers showing less stability than those defined by physiological variables, especially in severe asthma patients [47, 139].

The systems biology methodology diminishes the impact of preconceived biases through the use of clustering algorithms applied to large datasets consisting of clusters of traits, instead of focusing on simple single-feature phenotypes [4, 47, 66]. Unbiased cluster analyses are capable of integrating the effect of multiple interacting components of a wide range of “observable” variables or clinical parameters (e.g., demographics, lung function, BMI, atopy, and eosinophils) in large patient cohorts (i.e., Severe Asthma Research Program (SARP) [140], the Unbiased Biomarkers for the Prediction of Respiratory Disease Outcome (U-BIOPRED) [141], and Airways Disease Endotyping for Personalized Therapeutics (ADEPT) [142] in order to objectively characterize and predict clinical phenotypes of asthma [4, 66]. Despite significant differences between the cluster studies, particular subsets have nevertheless led to the identification of several different phenotypes, as summarized in Pérez de Llano, 2021 [4]. The major asthma phenotypes are early-onset allergic asthma, late-onset eosinophilic asthma and late-onset non-eosinophilic asthma [4]. The other phenotypic groups are based on symptoms (exacerbation prone, cough variant, obesity-related asthma, asthma with persistent airflow obstruction), triggers (exercise-induced, occupational asthma, seasonal asthma, aspirin-induced asthma), biomarkers (eosinophilic asthma, neutrophilic asthma, paucigranulocytic asthma, mixed granulocytic asthma), and treatments (steroid-resistant asthma) [4]. How well these phenotypes actually reflect specific biologic pathways is

debatable given the heterogeneity of each phenotype cluster, the significant overlapping between the clusters, their variability over time, and the different methodologies used and cohorts examined in the studies [4]. It is noteworthy that most of these cluster studies have been conducted only in severe asthmatics, thereby limiting the generalizability of phenotypes across the severity spectrum [4].

Most recently, a newer approach to phenotyping asthma that involves the assessment and targeting of "treatable traits" has emerged [47, 143]. This new paradigm for the management of airway diseases, particularly complex diseases, aims to apply personalized medicine to each asthma patient in order to improve outcomes, and therefore, limits the analysed phenotypes to clinically relevant, identifiable characteristics, which are modifiable with treatment [47, 143]. This concept is already being employed in clinical practice (e.g., targeted therapies of severe eosinophilic or allergic asthma) [46-47], however, the current guidelines, including GINA 2021, reserve its use for the most severe patients, with the "one-size-fits-all approach" remaining the current paradigm in the management of the majority of asthma patients [47]. The "treatable traits" approach seems to be the way forward to novel asthma management with potential benefits for all asthma patients, regardless of disease severity (i.e., mild, moderate and severe) [47].

With respect to international recommendations, the GINA 2022 guidelines define asthma phenotypes as recognizable clusters of demographic, clinical and/or pathophysiological characteristics [9]. The following are listed by GINA 2022 as the most common clinical phenotypes: allergic asthma, non-allergic asthma, adult onset (late-onset) asthma, asthma with persistent airflow limitation, and asthma with obesity [9]. Although some phenotype-guided treatments are available for the most severe asthma patients, GINA advises that further studies are necessary to determine the utility of phenotyping asthma in clinical practice given that asthma phenotypes have not been shown to correlate with specific pathological processes or treatment responses [9].

The results of an international collaboration between five countries (United Kingdom, New Zealand, Brazil, Ecuador, and Uganda), known as the World Asthma Phenotypes (WASP) study, are awaited and highly anticipated [144]. Initiated in 2016, the WASP study aims to evaluate detailed biomarker and clinical information in order to better understand asthma phenotypes and their distribution across the socioeconomic and prevalence spectrum, compare phenotype characteristics including clinical severity, and assess risk factors for each

phenotype [144-145]. In addition, this study should provide a standardised protocol for other centres around the world to conduct similar analyses which will provide even more data to enhance our comprehension of asthma phenotypes [144].

1.1.7.2. Asthma Endotypes

From the above discussion, it is quite evident that phenotyping asthma is far from an easy task, and requires an understanding of the underlying mechanisms that cannot be obtained simply from an analysis of the clinical and laboratory (e.g., physiologic or inflammatory) parameters associated with asthma phenotypes [4]. The term endotype was proposed in 2008 as a conceptual framework to shift the focus from phenotypes to identifying precise molecular pathways (endotypes) driving disease, and thereby, helping to demystify the molecular heterogeneity of asthma [68]. More precisely, it was suggested that each endotype is a disease subtype that is defined by a distinct functional or pathophysiological mechanism [68]. The main advantages of classifying asthma into specific groups of endotypes are the therapeutic and prognostic implications of tailoring treatments specific to the causative molecular mechanisms, thus moving even closer towards a personalized medicine approach to managing asthma [66].

This concept of endotyping asthma seems quite straightforward in theory, but is actually a highly demanding and challenging process [4]. As a result, no "true" endotypes have been described with complete confidence to date, and endotyping asthma remains a continuous work in progress [4]. In their review, Pérez de Llano and co-authors (2021) summarized the main reasons for the difficulties in endotyping asthma as follows: 1) similar symptoms and signs ("observable features") can arise through different pathophysiological mechanisms, 2) different endotypes may have similar, or even the same phenotype, and 3) similar molecular mechanisms may be present in different asthma phenotypes [4]. Optimal stratification of asthma "mechanism-based treatment targeting" is the ultimate goal of genuine precision medicine, and can only be achieved when true endotypes of asthma and their underlying mechanisms are confirmed [4, 66].

A molecular characterization of the clinical differences and pathobiological processes in a range of asthma patients has only recently emerged owing to the evolution of various "-omics" platforms, improved statistical analyses, and integration of data from animal models and clinical trials of targeted biologic therapies [146]. Differences between patients with and without increased levels of markers of type 2 immune and inflammatory pathways in the

airways, and compared with the normal reference range of healthy controls, have led to the confirmation of a broad categorization of T2-high asthma [66, 68, 146]. As a result, two major endotypes or "molecular phenotypes" of asthma, although incompletely defined, have been distinguished thus far based on Th2 inflammation (or T2 inflammation, as clarified above): 1) "T2-high" (defined by increased type 2 cytokine release or epithelial gene expression compared to a reference population) and 2) "T2-low" (or non-T2) asthma (much less well defined; with low or no T2 inflammation; without clear biomarkers; usually identified by excluding a T2-high profile) [4, 64, 146].

Biomarkers are essential tools for the discovery of endotypes and are defined as "measurable indicators linking an underlying pathway to a phenotype of a disease" [4]. The most routinely used biomarkers in the clinical evaluation of asthma endotypes include: blood eosinophils, induced sputum cell count (sputum eosinophils), FeNO, and IgE [4]. The most commonly used biomarkers for the recognition of T2 inflammatory response in asthma patients are blood and sputum eosinophils, with the latter currently recognized as the gold standard for identifying the T2-high endotype [4]. However, each biomarker has its advantages and disadvantages, and a more appropriate gold standard for T2 inflammation is needed, especially given the fact that only 50% of asthma patients are identified with T2 airway inflammation and there is a paucity of biomarkers for T2-low (or non-T2) asthma (i.e., in the majority of cases, only induced sputum cell count) [4].

According to a review by Khalaf and co-authors, asthma is thought of as an "endotypic range" of airway inflammation with one end of the spectrum being eosinophilic (typically complex T2-high) and the other, neutrophilic (typically complex T2-low or non-T2) [94, 147]. The existence of a "mixed" endotype consisting of an overlap of these syndromes has also been described as a possible presentation in patients [94, 148]. The eosinophilic phenotypes include early onset allergic asthma, late onset eosinophilic asthma, non-steroidal anti-inflammatory drugs (NSAIDs) exacerbated respiratory disease, and exercise-induced bronchoconstriction, while the non-eosinophilic phenotypes (non-atopic neutrophilic asthma) consist of paucigranulocytic asthma and non-atopic neutrophilic asthma (smokers, obesity and elderly) [94].

A similar characterization of the phenotypes of T2-high and T2-low endotypes has been provided by Kuruvilla and co-authors (2019) [66]. The T2-high phenotypes are classified into three groups: early-onset allergic (or atopic) asthma, late-onset eosinophilic asthma and

aspirin-exacerbated respiratory disease (AERD) [66]. The T2-low phenotypes are classified according to clinical characteristics that include obesity, smoking and age [66]. These authors provide a more detailed and interesting overview of the molecular endotypes T2-high and non-T2 (or T2-low), and their corresponding asthma phenotypes, clinical characteristics, molecular mechanisms, existing biomarkers, and natural histories [66]. In a more recent review by Gülşen, possible T2-high phenotypes are characterized by clinical presentation and biomarkers [145].

Unlike T2-high asthma, the T2-low endotype is less well understood, but is generally identified by different levels of non-eosinophilic inflammation characterized by 1) the presence of other cells such as neutrophils (i.e., T1/T17 neutrophilic inflammation), or 2) very few inflammatory cells (i.e., paucigranulocytic) [69]. T2-low asthma is classified as having levels of T2-inflammation in the airways that are comparable to the normal reference range of healthy controls [64]. Unlike T2-high asthma, T2-low asthma is associated with fewer treatment options, poor disease control and more exacerbations [69]. There are no defined biomarkers for T2-low asthma, so it is therefore mainly identified by excluding a T2-high profile (i.e., the absence of T2-high biomarkers such as eosinophilia) [4, 145]. Proposed biomarkers for T2-low asthma include blood and sputum neutrophilia, but the clinical relevance of these increased neutrophils is still unknown as they may be a byproduct of the local inflammatory response or secondary to unrelated causes such as concomitant high-dose corticosteroid therapy, exposure to environmental pollution or cigarette smoke, or concurrent bacterial infection [66].

1.2. Serotonin (5-HT)

1.2.1. Discovery and sources of central and peripheral 5-HT

Serotonin (5-hydroxytryptamine, 5-HT) is a small, ubiquitous biogenic amine found in nearly all eukaryotes [149]. First discovered by the Italian pharmacologist Vittorio Erspamer in 1937, this vasoconstrictor was initially termed “enteramine” given that it was extracted from enterochromaffin gut cells in mammals [150]. Over a decade later, the American physiologist Irvine Page and his colleagues discovered a serum-derived vasoconstrictor that they named “serotonin” [151-152]. In 1949, the chemical structure of “serotonin” was determined to be 5-hydroxytryptamine (5-HT) [153]. It was subsequently confirmed that peripheral enteramine and 5-HT were in fact the same substance [154-155]. Besides its peripheral localization, 5-HT was found by Dr. Betty Mack Twarog also to exist centrally in the mammalian brain [156-

157]. Since the discovery of this "happy" hormone, as referred to by Barnes (2011), extensive research has provided evidence showing that 5-HT modulates a wide range of physiological processes and pathological states in different tissues throughout the body via the action of specific enzymes, transporters and multiple subtypes of 5-HT receptors, with varying expression depending on their location, that affects local 5-HT levels and neurotransmission [56, 158-162].

Central 5-HT, produced by the serotonergic neurons in the central nervous system (CNS), accounts for only approximately 1-2% of the total amount of 5-HT in the body [163]. Because 5-HT is highly charged at physiological pH, it can neither cross the blood-brain barrier (BBB) nor passively diffuse into cells from the extracellular space [164-165]. This impermeability of the BBB to 5-HT means that the central and peripheral serotonergic systems are largely functionally independent [161, 166]. By the same token, the central and peripheral stores of 5-HT are separate entities [167].

Most 5-HT is located in the periphery, outside of the CNS [162]. The principal source of peripheral 5-HT is the gut, where most of the total body 5-HT production (~95%) occurs, or more specifically, the enterochromaffin cells (EC) of the GI mucosa [162-163, 166]. The majority of peripheral 5-HT synthesized by the EC is rapidly sequestered into platelets (also known as thrombocytes) via the 5-HT reuptake transporter (SERT or 5-HTT) to be stored in dense granules [162, 166]. Hence, thrombocytes are the main circulating reservoir of peripheral 5-HT (~90%), with a minor fraction of the total peripheral 5-HT pool locally produced by and distributed to different cells and tissues throughout the body [158, 161, 163, 168]. In healthy individuals, only a very small percentage of 5-HT circulates freely in the blood, but an increase of 1000-fold can be detected following platelet activation, for example, in response to inflammation [162].

1.2.2. Roles of central and peripheral 5-HT in physiological and pathological states

Exerting multifaceted effects, 5-HT serves as a hormone, neurotransmitter, neuromodulator, and immunomodulator, and plays important regulatory roles in multiple physiological and cell responses, including pulmonary function [149, 155, 169-171]. Although best known as a neurotransmitter or signalling molecule in the CNS, mediating a wide range of neuronal activities and neuropsychiatric disorders, the additional actions of 5-HT in the periphery (i.e., as a hormone or autocrine/paracrine factor or immunomodulator) extend to numerous

nonneuronal processes through a complex network of receptor signalling in different tissues [155, 158, 161, 163, 168]. Clearly, 5-HT physiology is extensive and very complex [169].

In fact, 5-HT is involved in the regulation of almost every physiological function, biological process and behaviour in humans, including body temperature (thermoregulation), breathing rhythm and respiratory drive (respiratory system), heart rate and vascular resistance (cardiovascular function), digestion, appetite, body weight/food intake and bowel motility (GI system), ejaculatory latency (reproduction system), bladder control, muscle contraction and relaxation (locomotion), sleep-wake cycle, pain and sensory perception, stress responses, memory, emotion, cognition, mood, platelet function and aggregation (hemostasis), mammary gland development and homeostasis, metabolism, hepatic lipid balance, adipogenesis, insulin secretion, and energy homeostasis [158-162, 172-174]. More recently, it has emerged that 5-HT is also an essential mediator of immune responses in the peripheral immune system [175-176].

Dysfunction of the central and peripheral serotonergic systems has been implicated in a diversity of pathological states, such as irritable bowel syndrome, colitis, celiac disease, metabolic disease, obesity, carcinoid diarrhea, restless legs syndrome, sudden infant death syndrome, autism, headache (primarily migraine), chemotherapy-induced emesis, insomnia, anxiety, alcoholism, depression, anorexia, schizophrenia, Parkinson's disease, Alzheimer's disease, autoimmune disease (e.g., rheumatoid arthritis), and epilepsy [159, 161-162, 165, 177-180]. There is now increasing evidence regarding the contribution of 5-HT also to asthma pathogenesis [149, 181-182].

Many pharmacological agents that are used to treat some of the aforementioned disorders target the 5-HT receptor subtypes of the serotonergic systems, such as anxiolytics and antidepressants (for example, tricyclic and tetracyclic antidepressants, and selective 5-HT reuptake inhibitors (SSRIs)), setron antiemetics, triptans to relieve migraine, atypical neuroleptics, and antiepileptics (e.g., fenfluramine) [159, 162, 165, 179-180]. Psychoactive substances, also known as serotonergic psychedelics, such as lysergic acid diethylamide (LSD), tryptamines (e.g., psilocybin), phenethylamines (e.g., mescaline), cocaine, and amphetamines, alter 5-HT functions by acting on specific 5-HT receptor subtypes and monoamine transporters [159, 162, 183-184].

1.2.3. Distribution of 5-HT neurons in the central nervous system (CNS)

In the CNS, neurons located in the raphe nuclei of the brainstem produce and release 5-HT [158]. The serotonergic cell bodies of these neurons, originally classified by Dalhstroem and Fuxe (1964), mainly reside in the dorsal and median raphe nuclei [159, 185-186]. The axons of these brainstem 5-HT neurons extend diffusely to almost all brain structures, sending ascending projections that terminate in cortical, limbic, midbrain, and hindbrain regions [158, 187]. The descending projections of these brainstem 5-HT neurons are sent into the spinal cord to modulate nociceptive (pain) signalling [158]. During neuronal depolarization, action potentials travelling down the axons cause release of 5-HT into the synapse where it can bind to pre- and postsynaptic receptors that are coupled to different signal transduction mechanisms [187]. The individual neurons and all human brain regions express a variety of 5-HT receptors in specific subtype patterns [158]. This widespread innervation of the 5-HT system throughout the whole neuraxis accounts for the participation of 5-HT in a plethora of functions, as listed above [187].

1.2.4. Synthesis of 5-HT in the CNS and periphery

In humans, the synthesis of 5-HT in the periphery and centrally is dependent on the enzyme tryptophan hydroxylase (TPH) that exists in two different isoforms [155]. Tryptophan hydroxylase-1 (TPH-1) is involved in the generation of 5-HT in the peripheral organs, such as the EC of the GI tract, whereas tryptophan hydroxylase-2 (TPH-2) synthesizes 5-HT in neurons [155, 166]. Platelets are unable to synthesize 5-HT themselves because they lack TPH, and thus, act solely as carriers of 5-HT in the circulation [166, 188]. However, other cells in the blood, such as mast cells, monocytes/macrophages and T cells, can partly participate in 5-HT production [163].

In both the CNS and peripheral tissues, 5-HT is synthesized from L-tryptophan (or tryptophan), an essential amino acid obtained from food [162-163]. Once ingested, L-tryptophan circulates in the blood throughout the body, primarily bound to albumin (75-95%), but also freely [189-190]. Unlike 5-HT, L-tryptophan can cross the BBB by the action of a cognate L-type amino acid transporter [162, 186, 191]. The free, unbound L-tryptophan is readily available for transport across the BBB, but albumin-bound L-tryptophan, with a higher affinity for the BBB transporter than albumin, will first dissociate from the albumin in close proximity to the BBB, in order to be taken up by the brain [190].

The synthesis of 5-HT occurs in two enzymatic steps [162-163]. The first step involves the hydroxylation of L-tryptophan by TPH to yield 5-hydroxytryptophan (5-HTP), the rate-limiting enzymatic step [163, 192]. The second step results in the conversion of 5-HTP to 5-HT through decarboxylation by aromatic L-amino acid decarboxylase [162-163].

In the CNS, as soon as 5-HT is synthesized, it is packaged into synaptic vesicles via the action of the vesicular monoamine transporter 2 (VMAT-2), a (H+)-dependent carrier, later to be released into the synapse following an action potential [159]. In the periphery, 5-HT produced in EC cells is rapidly taken up by platelets and compartmentalized into dense granules by VMAT-2, later to be released upon platelet activation [193]. The quick sequestration of 5-HT into synaptic vesicles and dense granules, respectively, prevents its rapid degradation by the enzyme monoamine oxidase (MAO) [162]. This uptake of 5-HT into presynaptic terminals in the CNS and platelets in the periphery is mediated by SERT [162, 194]. It is worth noting that SERT is also expressed in pulmonary and peripheral blood vessels and the GI tract [158].

1.2.5. Metabolism of 5-HT

Most 5-HT undergoes metabolism in the outer mitochondrial membrane by the enzyme MAO, in particular the A isoform (MAO-A) due to its high affinity, but also conceivably the B isoform (MAO-B) (i.e., if MAO-A is deficient or absent) [161, 195]. The two-step process of enzymatic degradation of 5-HT begins with MAO catalyzing the oxidative deamination of 5-HT into 5-hydroxyindol-3-ylacetaldehyde (5-HIAL) [163, 196]. The 5-HIAL intermediate is then transformed under normal conditions into the main 5-HT metabolite called 5-hydroxyindol-3-ylacetic acid (5-HIAA; also known as 5-hydroxyindoleacetic acid) via oxidation by an aldehyde dehydrogenase [163, 196]. The 5-HIAL can also enter an alternative metabolic route, but to a much lesser extent normally, and be converted to a minor metabolite, 5-hydroxytryptophol (5-HTOL), through a minor reductive pathway catalysed by an aldehyde reductase [196]. Both the major 5-HIAA and minor 5-HTOL metabolites are eliminated from the body by the kidneys in urine; the latter, primarily in conjugated form with glucuronic acid [163, 197]. The metabolism of 5-HT mainly takes place in the liver and the pulmonary vascular endothelium [163, 198-199]. Platelets can also degrade granular 5-HT by MAO [161, 200].

Another important pathway of 5-HT metabolism also occurs in the human body under normal conditions. In the pineal gland, 5-HT is metabolized to melatonin as a result of two enzymatic reactions [161, 196]. Initially, N-acetylation of 5-HT by arylalkylamine N-acetyltransferase (also

known as 5-HT N-acetyltransferase) converts 5-HT to the intermediate N-acetyl-5-hydroxytryptamine (N-acetyl-5-HT) [161, 196, 201]. Subsequently, O-methylation of this intermediate by hydroxyindole-O-methyltransferase results in the production of 5-methoxy-N-acetyltryptamine (i.e., melatonin) [161, 196, 201-202]. Melatonin is involved in a broad range of physiological functions, including the circadian rhythm, reproduction, detoxification of free radicals, antioxidant actions as well as cardiovascular, immune system and body mass regulation, among others [202].

1.2.6. The 5-HT receptors and 5-HT transporter (SERT)

There are seven classes or families of 5-HT receptors (5-HT_1 to 5-HT_7); all are G-protein coupled receptors except for 5-HT_3, a ligand-gated ion channel [203-204]. In total, there are 15 different 5-HT receptor subtypes [155]. However, in mammals, 5-HT acts on 14 distinct receptor subtypes [149]. The subtypes of 5-HT receptors within each family are labelled with letters (e.g., 5-HT_{1A}, 5-HT_{2A}) [190]. Therefore, the 5-HT receptor subtypes are classified as follows: 5-HT_1 (5-HT_{1A}, 5-HT_{1B}, 5-HT_{1D}, 5-HT_{1E}, 5-HT_{1F}), 5-HT_2 (5-HT_{2A}, 5-HT_{2B}, 5-HT_{2C}), 5-HT_3, 5-HT_4, 5-HT_5 (5-HT_{5A}, 5-HT_{5B}), 5-HT_6, and 5-HT_7 [187]. In the human brain, large sets of 5-HT receptors are found in the substantia nigra, the hippocampal formation, the hypothalamus, the amygdala, the striatum, and the frontal cortex, with different distributions and varying densities [159].

The processing of 5-HT after synthesis in the CNS depends on the subtype and location of the 5-HT receptors as well as its reuptake via SERT. Neuronal depolarization triggers the extracellular release of 5-HT from secretory vesicles into the synaptic cleft, with subsequent binding to either postsynaptic or presynaptic 5-HT receptors [190]. The amount of 5-HT released into the synaptic cleft is controlled by the binding of 5-HT to the presynaptic 5-HT autoreceptors localized in the cell bodies and dendrites (5-HT_{1A} receptors) of the dorsal raphe nuclei or the axon terminals (5-HT_{1B} receptors), creating a negative feedback inhibition against further firing activity of the serotonergic neurons and release of 5-HT into the synapse [190, 205-206]. The 5-HT_{1A} autoreceptors affect firing rates, whereas the 5-HT_{1B} autoreceptors sense the extracellular 5-HT concentration, and decrease synthesis and release of 5-HT when it accumulates extracellularly [186]. Moreover, the binding of 5-HT to somatodendritic 5-HT_{1D} autoreceptors may also negatively regulate the release of 5-HT inside the raphe nuclei [169, 207].

When extraneuronal 5-HT interacts with and binds to postsynaptic receptors (including 5-HT_{2A} and 5-HT_{2C}, among others), a signal is transmitted from one cell to the next [169]. Thus, 5-HT participates both in one-to-one neural (or neuron-to-neuron) signalling and as a neuromodulator of the effects of other neurotransmitters in the extracellular space via volume transmission (or nonsynaptic diffusion neurotransmission) [186, 208]. The latter may involve neural transmission that occurs at sites outside of the synaptic cleft by the diffusion of transmitters to distant target receptors [208].

Another important mechanism of regulation of 5-HT signalling and modulation of extracellular 5-HT concentrations is the clearance of 5-HT from the extracellular space [209]. Removal of 5-HT from the synapse is mediated by the transport of 5-HT into the presynaptic neuron via SERT (5-HTT), the 5-HT specific transporter located on the presynaptic axonal plasma membrane [205, 210]. Acting as a key mediator of neurotransmission, SERT also serves as a major molecular target for pharmacological treatments of neuropsychiatric disorders, such as the selective 5-HT reuptake inhibitors (SSRIs) that exert their antidepressive effects by blocking 5-HT reuptake, thereby, increasing 5-HT levels in the synaptic cleft, and further enhancing 5-HT signalling [169, 211-212]. Therefore, the reuptake of 5-HT by SERT not only terminates the extracellular effects of 5-HT, the subsequent repacking of 5-HT into the synaptic vesicles ensures that 5-HT is recycled and made available for reuse during the next serotonergic neurotransmission [169, 209]. Any 5-HT remaining in the cytosol of the neuron is degraded by MAO-A, as explained above, thus regulating intracellular 5-HT levels [169].

1.2.7. Platelets and 5-HT

It is now widely accepted that platelets are an important reservoir of 5-HT in peripheral blood [213]. Much interest lately has been directed towards the regulation of plasma 5-HT levels by platelets, and in turn, the modulation of platelet function by 5-HT itself, including the impact of such disruption on multiple disease states [214]. Although 5-HT in the human body is mainly synthesized in the periphery (>95%) by intestinal EC, most of it is rapidly taken up from the blood by resting platelets via the 5-HT specific transporter SERT [176]. In addition to platelets, other inflammatory cells such as basophils and mast cells also express SERT and enzymes involved in 5-HT metabolism (i.e., MAO), and therefore, contain high concentrations of 5-HT [155, 215-216]. Nevertheless, approximately 99% of the total 5-HT in

blood is stored in platelets [217]. In fact, 5-HT is concentrated in platelets at a level approximately 1000 times higher than in plasma [218].

This finding of 5-HT in platelets, including their remarkable ability to act as a major storage reservoir for circulating 5-HT, was first identified in the late 1950s [219-220]. Since then, research has provided evidence showing that platelets contribute to the regulation of circulating 5-HT levels within the plasma [see review by 214]. One important mechanism involves the activation of platelets and the release of 5-HT from their dense granules into the blood [221]. Acting as a "helper agonist", 5-HT itself dose-dependently augments platelet activation induced by adenosine diphosphate (ADP) and especially thrombin, the most potent platelet activator [222-223].

1.2.8. Rationale for using platelet 5-HT versus plasma or free 5-HT levels

Given that human blood platelets possess several components similar to those of central 5-HT synaptosomes, including MAO-B and different receptors, such as SERT and 5-HT_2 receptors, and that platelet 5-HT concentration is altered in various neuropsychiatric conditions such as alcoholism, autism and depression, platelets are assumed to represent a convenient peripheral model to study different aspects of the central 5-HT system [217, 224-226]. A similar conjecture can be made regarding platelets and asthma based on the currently available literature as follows: 1) several studies have established 5-HT as an important neuromodulator of breathing, with direct actions in the neuronal circuits of the CNS [227]; 2) platelets have been observed to migrate into the lung tissue of patients with asthma and into the lungs of allergen challenged sensitised animals, possibly contributing directly to alterations in lung function in patients with asthma [228]; 3) the level of 5-HT in the lungs is closely related to that of platelets in the blood [229-230]; 4) the concentration of 5-HT in the lungs of allergic asthma patients is closely correlated with the concentration of platelet 5-HT in the blood [213, 231-232]; and 5) animal models and many studies to date provide evidence supporting platelet involvement in asthma [for eg., as reviewed in 228, 233].

Based on the aforementioned discussion, platelet 5-HT concentration might serve as a suitable and easily accessible peripheral biomarker of asthma. Moreover, an important advantage of using platelet 5-HT instead of plasma 5-HT is that the half-life of the former (33 to 48 hr) is considerably longer than that of the latter [234]. Under normal physiological conditions, very low levels of free 5-HT actually exist in the plasma owing to both the metabolism of plasma 5-HT in the liver by MAO-A and its quick uptake by platelets for transportation to the lungs

where degradation by MAO-B occurs inside endothelial and smooth muscle cells [234-235]. Thus, the total blood 5-HT pool is composed of 95-97% platelet 5-HT and 3-5% free 5-HT [232]. In addition, two independent studies found that platelet 5-HT maintains intrapersonal stability over time, with no change on repeated venepuncture over nine weeks [236], and a high intra-individual correlation of 5-HT levels in human platelets measured three months apart [237], suggesting that platelet 5-HT levels are a reasonable marker of long-term status [226]. As a result, platelet 5-HT could represent a more robust measurement parameter of 5-HT levels in the circulation than plasma / free 5-HT [234].

1.2.9. Asthma and 5-HT

The 5-HT receptors have in recent times attracted attention for their potential actions in inflammatory diseases such as asthma [149]. For instance, in studies based on murine models of allergic asthma, ASM cells have been shown to express 5-HT receptors, more specifically 5-HT classes 2A, 3, 4, and 7 that mediate bronchoconstriction through receptor activation on parasympathetic acetylcholine-containing neurons by causing release of acetylcholine [238-241]. In addition, the inflammatory mediator, tumour necrosis factor alpha (TNF-α), has been found to upregulate 5-HT_{2A} receptor mediated contractions in murine ASM [242].

Among the 5-HT receptors, class 2 seems to be rather important in asthma. The 5-HT_{2A} receptor, although primarily known for mediating complex cognitive behaviours within the CNS and physiological processes in the periphery [243-244], is likely also a notable player, though still not completely defined, in inflammatory processes [149]. For instance, the 5-HT_{2A} receptor mRNA is expressed at higher levels than other 5-HT receptor subtypes (i.e., 5-HT_{1A}, 5-HT_{1D}, 5-HT_{2C}, 5-HT_{4}, 5-HT_{5A}, and 5-HT_{5B}) in immune-related tissues, such as spleen, thymus and peripheral-circulating lymphocytes [245]. More specifically, with respect to the involvement of 5-HT in asthma, a number of cells associated with the pathophysiology of this inflammatory lung disease have been found to functionally express the 5-HT_{2A} receptors, including activated $CD4^+$ T cells, alveolar macrophages, eosinophils, and lung epithelial and smooth muscle cells [149, 246-250].

Early studies using 5-HT receptor antagonists in both animal models and humans with asthma have pointed to the potential significance of 5-HT and its receptor in asthma [251]. For instance, in some strains of rat and guinea pig, 5-HT antagonists were shown to markedly reduce bronchospasm [252-253]. Similarly, in a small study of asthma patients (n=8), improved pulmonary function was noted after administration of the 5-HT_2 receptor blocker

ketanserin [254]. These results suggested that 5-HT may act as an important mediator of adenosine-induced bronchoconstriction [251]. In a murine study by De Bie and colleagues (1998) using selective histamine and 5-HT_2 receptor antagonists, it was concluded that both histamine and 5-HT contribute to antigen-induced AHR and eosinopilia [255].

Lending further support to the involvement of 5-HT_2 receptors in asthma, Kang and associates (2013) have recently discovered that migration of eosinophils in allergic asthma is dependent on 5-HT_{2A} receptor activation [249], while Dürk and colleagues (2013) showed that 5-HT_2 receptors are implicated in platelet function relevant to allergic asthma [149, 176]. Most recently, Nau et al. (2015) and Flanagan et al. (2019) further emphasized the importance of the 5-HT_2 receptors in allergic airway disease using a 5-HT_2 receptor selective agonist [(R)-2,5-dimethoxy-4-iodoamphetamine [(R)-DOI]] in animal models of acute and chronic asthma, respectively [149, 256].

These studies give strength to the therapeutic potential of 5-HT_2 receptor agonism (i.e., (R)-DOI as a possible novel small-molecule based therapy) and 5-HT_2 receptor activation for the treatment of asthma. For example, Nau et al. (2015) demonstrated that inhaled (R)-DOI blocks AHR, recruitment of eosinophils to the lung, mucus hyperproduction, and inflammatory airway remodelling in a mouse model of acute asthma [149]. In their earlier published work, Nau et al. (2013) showed that systemic administration of (R)-DOI blocks the systemic effects of TNF-α in the whole animal through activation of the 5-HT_{2A} receptor, including the blockade of TNF-α-induced expression of pro-inflammatory cell adhesion (*Icam-1*, *Vcam-1*), cytokine (*IL-6, IL-1β*), and chemokine (*Mcp-1, Cx3cl1*) genes, and expression of VCAM-1 protein in the intestine, as well as prevention of the TNF-α-induced increase of circulating IL-6 [257]. Most recently, Flanagan et al. (2019) presented data showing that 5-HT_2 activation via intranasal (R)-DOI administration attenuates elevated AHR to methacholine, diminishes pulmonary inflammation and mucus production, and reduces airway structural remodelling and collagen deposition by nearly 70% in a mouse model of persistent chronic asthma [256].

Several studies support an association between peripheral 5-HT and asthma, strongly implicating the role of the serotonergic system in asthma. For instance, there is growing evidence that asthma exacerbations are triggered by high levels of free 5-HT in blood plasma [258-259]. Asthma patients after allergen provocation, as well as those with stable asthma, have elevated levels of 5-HT in the bronchoalveolar lavage fluid compared to healthy controls

[176]. However, allergen provocation is also associated with a significant decrease in serum 5-HT levels in asthmatics, and according to the authors, this suggests that 5-HT translocates from platelet stores into the lungs after allergen provocation [176]. An increase in the levels of free 5-HT in plasma was noted in symptomatic asthma patients compared to asymptomatic patients, and FEV1 was significantly lower in the former than the latter [259]. Therefore, free 5-HT is positively correlated with clinical severity and negatively with pulmonary function [259].

Given the above findings, it has been postulated that reducing the plasma concentration of free 5-HT could be a useful strategy for the treatment of asthma patients [241]. In fact, the use of pharmacological agents that modify 5-HT levels in the blood provides further support for the pathophysiological role of 5-HT in acute asthma [181, 260]. Research has indicated that asthma medications containing corticosteroids can lower 5-HT levels in children [261]. In two research papers on asthmatic children by Lechin and authors [262-263], tianeptine, a drug which reduces plasma free 5-HT and enhances uptake by platelets, provoked a dramatic and sudden decrease of both clinical rating and free 5-HT plasma levels, leading to abrupt suppression of asthma exacerbations and an increase in pulmonary function. In contrast to asthmatic children, tianeptine did not affect platelet or serum 5-HT concentrations significantly in depressed patients [264]. Conversely, buspirone, a drug which displays 5-HT agonist effect, increases plasma free 5-HT and triggers asthma attacks [265-266]. Furthermore, 5-HT is known to induce bronchoconstriction via peripheral and central pathways by increasing cholinergic activity and histamine release [267]. This provides additional support for the pathogenic role of 5-HT in asthma.

As explained above, most published studies to date have focused on the association between 5-HT and asthma using free 5-HT levels in blood serum or plasma, and mainly in children. Although platelets contain the majority of blood 5-HT, few known published reports in the literature have focused on clarifying the role of platelet 5-HT concentration in asthma, and even fewer in adults [268-269]. The application of knowledge gained from pursuing research in this area may help identify novel asthma phenotypes and promising therapeutic strategies to treat asthma.

1.3. Monoamine oxidases (MAOs)

1.3.1. MAO-A and MAO-B

The MAOs (enzyme nomenclature EC 1.4.3.4) are enzymes located in the mitochondrial outer membrane that degrade catecholamines (e.g., dopamine, epinephrine and norepinephrine) and other monoamine neurotransmitters (e.g., 5-HT and histamine) by catalyzing their oxidative deamination [270-271]. Historically, MAO was known as tyramine oxidase following its discovery by Mary Hare in 1928 in the rabbit liver where it was found to catalyze the oxidation of the monoamine tyramine [272]. The location of MAO in the outer mitochondrial membrane was later ascertained by Schnaitman and colleagues in 1967 [273]. The MAOs belong to the protein family of flavin-containing amine oxidoreductases [274]. The two distinct isoforms of this flavoprotein, namely MAO-A and MAO-B, are differently expressed across species, organs and cell types [275-279]. In humans, MAO-A and MAO-B are encoded by separate structural genes on the short arm of the X chromosome (Xp11.23) [271, 275, 280-281].

The MAOs exist in most tissues and cells of the human body, both neuronal and non-neuronal (peripheral) [274, 281]. However, the MAO isotypes show differences in expression and tissue distribution across the various organs [282]. In humans, MAO-A is found in the highest concentrations in the GI tract, liver, placenta, and lungs, corresponding to the enzyme's ontogenetic detoxifying function, and in moderately higher levels in the heart, while higher concentrations of MAO-B are found in glial cells in the brain and blood platelets [281-282]. In the CNS and peripheral tissues, the MAOs perform their physiological role of metabolizing endogenous monoaminergic neurotransmitters and exogenous biogenic amines ingested in the diet [278]. These two isoenzymes differ in their substrate specificity, though there is overlap depending on the conditions, and the MAOs contrast even more in their inhibitor selectivity or affinity [281]. MAO-A preferentially metabolizes the catecholamines (norepinephrine and epinephrine), 5-HT and melatonin, while MAO-B prefers phenylethylamine and benzylamine [278]. Both MAO-A and MAO-B catalyze the deamination of tyramine, dopamine, octopamine, and tryptamine [278, 283]. MAO-A is inhibited by low concentrations of clorgyline, whereas MAO-B is irreversibly inhibited by low levels of deprenyl [281].

1.3.2. MAO-B in platelets

Platelets are the most readily available source of MAO in humans [200]. Interestingly, MAO-B is the only MAO found in human platelets and the primary one in the brain [271]. Given

that the amino acid sequences of MAO-B in platelets and the brain are identical [284], and its biochemical and pharmacological characteristics are also similar [285], platelet MAO-B has been adopted as a peripheral marker of central MAO activity, and therefore, is a useful and convenient surrogate model in studies of different aspects of central neuronal function [286]. As a result, platelet MAO-B has been extensively investigated over the years in various neurological and psychiatric disorders [for example: 286-289], cigarette smoking [290-291], personality and psychological disturbances [292], alcoholism [293-294], and Alzheimer's disease [295], among others. Consequently, MAO-A, MAO-B and combination MAO-A/B inhibitors have traditionally been prescribed for the treatment of CNS-associated disorders [296-298].

1.3.3. MAO activity and inhibition

Concerning MAO activity in non-CNS-associated disorders such as inflammatory diseases, research indicates that MAO-B is induced in peripheral chronic inflammatory diseases, whereas MAO inhibition alleviates disease severity [298]. Carradori and colleagues (2018) have recently reviewed the innovative therapeutic potential of newly proposed MAO inhibitors in the management of different non-CNS disorders such as inflammation, obesity/diabetes, hair growth, cancer, cardiovascular damage, ocular diseases, and muscular dystrophies by analysing data collected from patents filed between 2002 and 2017 [298-299]. Accumulating evidence from a number of *in vitro* and *in vivo* disease models suggests that MAO inhibitors reduce mediators of inflammation and tissue destruction [282]. The repurposing of existing, and development of novel, MAO inhibitors with reduced BBB permeability have been proposed for the treatment of non-CNS chronic inflammatory environments as well as cancer, cardiovascular disease and muscular dystrophy [298-302].

In general, MAO-A (e.g., moclobemide), MAO-B (e.g., deprenyl and RG0216) and MAO-A/B (e.g., phenelzine) inhibitors have been reported to reduce proinflammatory (e.g., TNF-α, IL-1β, IL-8, IL-6, monocyte chemoattractant protein-1, cytokine-induced neutrophil attractant-1), and induce anti-inflammatory (e.g., IL-10), cytokine expression in a number of cell lines [282, 303-307]. For example, in a diabetic cardiomyopathy model, treatment of mice with pargyline (MAO-B inhibitor) reduced mast cell degranulation and cardiac fibrosis, and normalized diastolic function [308]. In a lipopolysaccharide-induced rat model of chronic inflammatory periodontal disease, MAO-B was identified as one of the ten most significantly up-regulated genes in the epithelium of diseased tissue, and inhibition of MAO-A/B activity

with phenelzine effectively reduced disease progression by decreasing hydrogen peroxide (H_2O_2), tumour necrosis factor alpha (TNF-α) and markers of disease worsening: epithelial cell proliferation, migration and bone loss [282, 306]. Like phenelzine, deprenyl (i.e., selegiline; irreversible MAO-B inhibitor) also decreased lipopolysaccharide-induced TNF-α protein expression in epithelial cell cultures [282]. Interestingly, treatment of Crohn's disease or rheumatoid arthritis with MAO-A/B inhibitors (phenelzine, tranylcypromine) was associated with disease remission [282, 309-310]. More specifically, in the case report by Kast (1998), the patient with Crohn's disease was originally prescribed phenelzine for her depression, resulting in the stabilization of her inflammatory bowel disease that reactivated six weeks after stopping phenelzine treatment [310].

With respect to the lungs, tobacco smoking is widely recognized as a potent driver of inflammation in the mucosa of the respiratory tract [282]. Activation of bronchial epithelial cells by cigarette smoke gives rise to a wide variety of proinflammatory cytokines and chemokines [311]. Likewise, whole blood samples of smokers contain higher levels of a number of cytokines and chemokines than non-smokers [312]. Moreover, the MAO-B inhibitor deprenyl reduces the expression of IL-8 in airway epithelial cell cultures [305] and reverses the cigarette smoke-induced elevation of MAO-B activity [313]. In addition, deprenyl significantly decreases bronchoalveolar lavage levels of proinflammatory mediators and increases the expression of the anti-inflammatory cytokine IL-10 [313]. Regarding chronic inflammatory respiratory diseases, increased levels of the chemokine IL-8, a potent neutrophil recruiting and activating factor in the airway lumen, have been observed in clinical samples of both patients with asthma and COPD [314-315].

The therapeutic potential of MAO inhibitors has also been investigated in asthma given the importance of psychological factors in asthma patients and the participation of MAO in the metabolism of amines that is seemingly connected with the pathogenesis of allergy [316-317]. In the work by Mathov in 1959 and 1963, asthma patients did not improve upon administration of non-selective MAO inhibitors (i.e., iproniazid, nialamide, catron, phenelzine), and unexpectedly, approximately a third of them reported worsening symptoms [316-317]. This unfavourable effect of MAO inhibition on human asthma was also confirmed by Cortés and co-authors [318]. Since then, no other clinical papers on MAO in asthma, specifically MAO-B, have been published. Moreover, to the best of our knowledge, there are no known published studies to date on platelet MAO-B activity in asthma. Further research is

therefore warranted to elucidate the possible role of platelet MAO-B in asthma, an inflammatory lung disease, based on the aforementioned discussion regarding MAO-B and its inhibition in chronic peripheral inflammatory diseases. The role of *MAO-B* gene polymorphism (e.g., genetic variants *MAO-B* rs1799836 and rs6651806) in the susceptibility to asthma is also unclear and remains to be explored.

1.4. Brain-derived neurotrophic factor (BDNF)

1.4.1. Discovery and roles of BDNF

Brain-derived neurotrophic factor (BDNF; synonym: MGC34632) is a member of the class of fundamental growth factors known as neurotrophins that are expressed by neurons of both the peripheral and central nervous systems, and are essential for neuronal remodelling [319-320]. In addition to BDNF, the neurotrophin family comprises nerve growth factor, neurotrophin-3 (NT-3), NT-4/5, and NT-6 [321]. BDNF is initially synthesized in the endoplasmic reticulum as a ~ 32-35 kDa precursor protein (proBDNF) that moves through the Golgi apparatus and trans-Golgi network, undergoes sorting by vesicles, and is eventually secreted by post-synaptic dendrites outside the plasma membrane in its ~ 13 kDa biologically active, mature BDNF (mBDNF) form following cleavage of its terminal domain by a convertase enzyme [321]. The highly conserved BDNF protein is composed of 119 amino acids in its mature form across species (i.e., pig, mouse, rat, and human BDNF are identical) [322].

Since its discovery in 1982 [323], BDNF has become the most extensively studied neurotrophin in view of its many different roles in healthy and pathological conditions, as summarized in several publications [e.g., 320-321, 324-325]. For instance, BDNF serves as a neurotransmitter modulator, supports neuronal development and survival, mediates synaptic plasticity, exerts a neuroprotective effect under adverse conditions, and regulates energy homeostasis [321]. Studies have revealed altered levels of BDNF in psychiatric disorders, such as major depression, bipolar disorder and schizophrenia [e.g., 326-334], and neurodegenerative diseases, such as Parkinson's disease, multiple sclerosis, Alzheimer's disease, and Huntington's disease [321, e.g., 335-342]. Moreover, BDNF levels are affected by psychiatric medications (e.g., antidepressants, mood stabilizers and antipsychotics) [326]. Considering these lines of evidence, BDNF concentration in the blood has been suggested as a potential biomarker of diagnosis and/or disease activity in a number of psychiatric disorders [326, 330].

Besides its many aforementioned roles in the nervous system, BDNF is expressed in a number of non-neuronal peripheral tissues, including the lung [343]. It is also present in human blood [344], with platelets acting as the main reserve pool of peripheral BDNF (90%) [345]. BDNF is therefore a major contributor not only to the pathogenesis of depression and other neuropsychiatric disorders [346], but is also implicated in the pathogenesis of airway diseases such as asthma, or more specifically, allergic bronchial asthma [347].

1.4.2. Peripheral circulating BDNF

The obvious challenge of measuring BDNF levels in the brain tissue (central BDNF) of humans prompted the discovery of noninvasive sources of BDNF in the periphery (peripheral BDNF) that are easily accessible, readily detectable, and can serve as surrogate measures [348-349]. Fortunately, circulating BDNF protein levels can be assessed in both the plasma and serum of human blood [348, 350]. For practical reasons, therefore, BDNF in humans is usually quantified based on serum or plasma levels, as evidenced by the numerous published studies on this topic. It is important to keep in mind, however, that serum and plasma BDNF levels are not correlated despite these terms being utilized interchangeably in the human literature [348]. It is generally acknowledged that plasma BDNF better reflects free BDNF concentrations due to the smaller amount of platelet-associated BDNF in plasma as well as the available modifications that can be applied to the methodologies to minimize any potential influence of platelet BDNF on plasma BDNF levels [348].

1.4.3. *BDNF* gene and *BDNF* genetic variation

The human *BDNF* gene was initially identified by Maisonpierre and colleagues in 1991 [322], but its location was revised a year later to chromosome 11 band p13-14 by Hanson and researchers [351]. The *BDNF* gene (MIM: 113505) spans ~ 70 kb and contains 11 exons [352]. Many genetic variants have been detected within the *BDNF-TrkB* system and are described in the population genetics literature [320]. Most variants fall within non-coding regions, however, a few common *BDNF* coding variants (all within the *BDNF* prodomain sequence) have been described, including Thr2Ile (rs8192466), Val66Met (rs6265), Gln75His (rs1048218), Arg125Met (rs1048220), and Arg127Leu (rs1048221) polymorphisms [320].

The most well-known and extensively studied *BDNF* coding variant is the *BDNF* Val66Met (rs6265) polymorphism [320]. A nonsynonymous G to A SNP at position 196 (G196A) of exon 2 (dbSNP number rs6265) of the *BDNF* gene results in a valine (Val) to methionine (Met) substitution at codon 66 (Val66Met), changing the 5' proregion of the human BDNF

protein [353]. This functional *BDNF* SNP, resulting in the substitution of an essential amino acid within the BDNF prodomain, interferes with a sortilin binding site that disrupts intracellular packaging of pro-BDNF, its axonal transport, and in turn, activity-dependent secretion of BDNF at the synapse [320, 354-356]. The Val66Met polymorphism also affects the binding of translin to *BDNF* mRNA, providing a pretranslational disruption of endogenous *BDNF* transcripts targeted to dendrites [357].

Numerous phenotypes are associated with the Val66Met polymorphism, including psychological states and psychiatric conditions such as major depression, schizophrenia, anxiety, unipolar and bipolar disorder [320, 353]. Considering the fact that BDNF is highly expressed in the hippocampus, the principal phenotype associated with the Val66Met phenotype is a disruption in hippocampal function [320], notably the altering of memory function and hippocampal volumes in human [354, 358-360] and mouse studies [361], though the results of various studies are inconsistent and controversial [320].

1.4.4. Tropomyosin receptor kinase B (TrkB) and *TrkB* gene (*NTRK2*)

Tropomyosin receptor kinase B (TrkB), the primary target receptor of mBDNF, is activated by BDNF with high potency and specificity [362-363]. TrkB is encoded by the neurotrophic receptor tyrosine kinase 2 (*NTRK2)* gene that consists of 24 exons [364]. The human *TrkB* gene (*NTRK2*) spans ~ 400 kbp and is localized to chromosome 9q22.1 [364-365]. TrkB exists in three different forms: the full-length receptor (fl. TrkB), discovered in 1989 by Klein & colleagues (1989), and two truncated isoforms, found a year later [320, 366-368]. In humans, these isoforms are designated as tr. $TrkB^{1}$ and TrkB-Shc [320, 369], and are the product of alternative *NTRK2* splicing that removes the tyrosine kinase domain [367].

Briefly, binding of mBDNF to its high affinity receptor fl. TrkB initiates a cascade of events beginning with the dimerization of fl. TrkB and autophosphorylation of several tyrosine residues located within the transmembrane tyrosine kinase domain [320, 369]. Activation of the TrkB receptor by BDNF triggers signal transduction via the mitogen activated protein kinase (MAPK), phosphoinositide 3-kinase (P13K) and phospholipase C gamma (PLCγ) pathways [320, 370]. These mBDNF-TrkB signalling mechanisms are extensively reviewed in a number of published articles [e.g., 369, 371-374].

1.4.5. Asthma and BDNF

Research has pointed to neurotrophins such as BDNF as candidate molecules in the regulation of the interplay between the peripheral nervous system and immune system [375]. The neuroimmune interactions underlying the pathophysiology of asthma are described by Manti and colleagues [376]. Briefly, neurotrophins are reported to act on a variety of immune cells involved in the pathogenesis of allergic disease that can themselves produce neurotrophins under certain conditions [377]. It is noteworthy that the levels and activities of neurotrophins are strongly upregulated in allergic states [377].

There is now mounting evidence in the literature showing that extensive communication occurs between neurons and immune cells in the lungs and airways of asthmatic patients [375]. The exploration of these novel mechanisms of neuro-immune crosstalk continues to be an important focus of asthma research [375]. In the past few decades, many biologically active neuropeptides have been identified in the lung, thus potentiating interest in the role of neurogenic inflammation in asthma [40]. For instance, asthmatic patients have increased levels of neurotrophins in the blood as well as locally in the lung [378]. Neuronal dysfunction and dysregulation have been shown to contribute to the pathogenesis of allergic asthma [377, 379].

The contribution of neurotrophin signalling to normal lung development is well supported in the literature [380]. Of all the neurotrophin family members, BDNF is of particular relevance to asthma pathogenesis as evidenced in the literature [reviewed in 111]. For instance, nerve terminals express Trk receptors, including the high-affinity BDNF receptor TrkB [111]. In addition, different types of cells in the airways (i.e., neurons, epithelium, ASM, lymphocytes, macrophages, and mast cells) release neurotrophins [111, 381]. Specifically, with respect to BDNF, there is now strong evidence that BDNF and its receptors are expressed in different components of the respiratory system such as the nasal and bronchial epithelium, ASM cells, nerves, and immune cells [380]. Local BDNF production by resident airway cells of the lungs contributes to normal airway structure and function as well as AHR and remodelling in pulmonary diseases such as asthma [343]. Furthermore, emerging clinical data indicate that altered BDNF levels and function are associated with pulmonary pathologies, including developmental lung disease, allergy and inflammation (e.g., rhinitis/sinusitis, neonatal and adult asthma), influenza, lung fibrosis, and even lung cancer [343]. BDNF is therefore an exciting, major investigative area of interest in asthma as a possible novel therapeutic target that may suppress several key aspects of asthma pathophysiology [343].

1.4.6. Neuronal plasticity and asthma

The concept of asthma as a “disease of the nervous system”, also termed neurogenic asthma or neurogenic inflammation, is not new, having been proposed many decades ago given the observation of altered neuronal regulation associated with the inflammatory response in asthmatic airways [40, 111, 382-386]. Indeed, Walter & Holtzman's review of the centennial history of research on asthma pathogenesis is a good reminder that the clinical description of asthma dates back to ancient times, with multiple paradigms for asthma pathogenesis (i.e., bronchoconstrictor, allergic, mediator, Th2-style inflammatory, innate immune, genetic, and nervous system) having been proposed and then revised over the years [387]. The neurogenic hypothesis was originally described in 1684 by Thomas Willis who regarded asthma as a nervous condition “stirr'd up by the default partly of the Lungs ill-fram'd and partly by default of the Nerves and nervous Fibers appertaining to the breathing parts” [387-389]. Later, in 1868, Henry Hyde Salter popularized the notion of a “perverted nervous action” as the underlying pathophysiologic mechanism of airway constriction [387]. Substantial work in the field of respiratory neurobiology over the last century has fueled ongoing interest in the neurogenic paradigm, owing to pivotal discoveries showing that the lungs are innervated by the sympathetic, parasympathetic and nonadrenergic noncholinergic nervous systems [387, 390]. All things considered, the importance of expanding research on different asthma paradigms to include central and peripheral neurological mechanisms as potential targets for asthma treatment cannot be overstated, especially in light of previous reports supporting the neurogenic paradigm of asthma, including the beneficial effects of anti-epileptic drugs and neuropathic pain drugs in subtypes of patients with asthma [386, 391-392].

Interestingly, it is now well recognized that airway diseases such as asthma are associated with altered neuronal structure and function (i.e., neuronal plasticity or remodelling) in the respiratory tract [111, 393-394]. Strong evidence suggests that neuronal plasticity underlies symptoms in asthma, including AHR [reviewed by 111]. Whether asthma causes or results from an altered neuronal regulation, however, remains uncertain [111]. Nevertheless, it is now apparent that neuronal plasticity in asthma is directly and/or indirectly influenced by different inflammatory mediators and growth factors such as neurotrophins [111].

Although neurotrophin antagonism in asthma has not yet been investigated, levels of BDNF and other neurotrophins have been shown to be altered by current therapies [111]. For instance, neurotrophin levels are decreased by inhaled corticosteroids [344, 395], whereas

BDNF levels are increased by long-acting β-agonists [396]. No studies to date have explored the effect of anticholinergics on neurotrophin levels in asthma despite evidence supporting the role of acetylcholine in the pathophysiology of asthma, including airway inflammation and remodelling [126, 397].

While it is known that neurotrophins contribute to ASM contractility under normal circumstances, BDNF has also been implicated in abnormalities of ASM contractility (i.e., exaggerated airway narrowing or obstruction) under airway inflammation (such as that induced by TNF-α) [241, 398-399]. Studies support the actions of neurotrophins such as BDNF in enhancing the effects of inflammation on bronchoconstriction (via ASM calcium and epithelial nitric oxide) and airway remodelling (via matrix metalloproteinases and extracellular matrix proteins such as collagen) [398, 400]. Furthermore, increased activity of cholinergic neurons has previously been shown to mediate AHR in asthma, and by extension, recent research has demonstrated novel insights into the mechanisms of cholinergic neuroplasticity, namely that remodelling of the cholingeric neural network in asthma is driven by BDNF-TrkB signalling [401]. Clearly, this is an area of continued scientific interest, and new knowledge should provide the foundation for better understanding the role of BDNF in airway diseases such as asthma.

1.4.7. *BDNF* Val66Met (rs6265) gene polymorphism and asthma

Genetic variants of *BDNF* have now also been associated with asthma, such as in Han Chinese patients [346], and more specifically severe asthma, such as in the study on large groups of German children [402]. There is also a paucity of reports on the association between genetic variations in the *BDNF* gene and various allergic diseases, including allergic asthma, mainly in children, and generally, producing conflicting results. Jesenak and colleagues studied the functional polymorphism Val66Met in the *BDNF* gene in a group of Slovakian asthmatic children versus healthy controls [403]. They found a significant association of the Met/Met variant genotype with asthmatic children and a positive association between the rs6265 polymorphism and bronchial asthma [403]. A similar finding of a strong association for the rs6265 polymorphism in the *BDNF* gene was also found in Chinese Han children with asthma [404].

1.4.8. *NTRK2* (*TrkB* gene) and asthma

Genetic variations in the *NTRK2* locus may be relevant to asthma and other lung diseases. For instance, genetic modification of the TrkB or p75 receptor alters neuronal innervation and

airway responses [as reviewed in 111]. Recently, Dragunas and colleagues (2020) found increased expression of the *NTRK2* gene in bronchial biopsies of asthma patients compared to healthy subjects and reported SNPs in the *NTRK2* and *BDNF* genes that correlated with asthma susceptibility [401]. The latter suggests that genetic variation in these genes may contribute to asthma susceptibility and thus, changes in this pathway could predispose individuals to developing asthma [401]. However, no studies in the literature so far have analysed the rs1439050 SNP of the *NTRK2* gene in asthma patients.

1.4.9. Rationale for BDNF research in adult asthma

To date, only a few published papers exist on the association of BDNF and asthma, with the majority based on the concentrations of platelet or serum BDNF and/or in children with asthma, and even fewer are focused on plasma BDNF in adult asthmatics. For example, Müller and colleagues found that in children (6-15 years old) with persistent asthma, the moderate to severe cases had significantly elevated plasma BDNF levels than both mild asthmatics and controls [405]. Lommatzsch and co-authors found that BDNF levels were elevated in serum, platelet and plasma in a small cohort of recently diagnosed adult patients with allergic asthma as compared with age- and sex-matched control subjects [344].

BDNF and its receptor TrkB are expressed by diverse cell types in the lungs, such as ASM cells, fibroblasts, airway epithelium, neurons, and immune cells [343, 406]. The majority of vagal (sensory) neurons innervating the lungs are BDNF-dependent and can affect the functions of motor neurons [403, 407-408]. BDNF expression and TrkB signalling, as well as their significance in the normal physiological and pathophysiological processes in the lungs, continue to be explored [343]. Specifically, various stimuli affecting intra- and extracellular BDNF concentration have been analysed with a view to understand the relevance of major variations of BDNF levels in human blood and tissue [409-410]. A number of studies thus far have sought to investigate the role of BDNF in the airways by assessing its concentration locally and in the peripheral circulation, as well as by determining the association between selected polymorphisms in the *BDNF* and *NTRK2* (*TrkB*) genes and the development of asthma [401].

Discrepancies between studies in asthma patients, however, highlight the need for further research in this area [344, 405, 408, 410-411]. The functional *BDNF* Val66Met (rs6265) variant seems to be particularly important given that the Met allele has been associated with abnormal cellular trafficking and packaging of pro-BDNF, resulting in a decreased production

of mature BDNF protein in neurons [354, 412]. On the other hand, the *NTRK2* rs1439050 is an intronic polymorphism, and therefore, does not have a specific reported role [413]. Nevertheless, it may still be associated with asthma, albeit not as a vulnerability factor per se (i.e., directly explaining the phenotype), but rather in linkage disequilibrium with a nearby unidentified functional mutation in the *TrkB* gene [413].

Few reports in the literature up to this point have evaluated the possible influence of *BDNF* and *NTRK2* polymorphic variants on serum and plasma BDNF levels in patients with asthma. However, recent studies conducted mainly on the pediatric population across several different countries and ethnicities have produced conflicting results [401-404, 414-416]. These may be due to differences in the methodological approaches and types of samples used, for instance, blood (plasma, serum, or platelets), sputum, or bronchoalveolar lavage fluid.

In summary, our current understanding of BDNF's effect on asthma in humans, particularly in adults, is limited. There is also a paucity of research on the TrkB. Both *BDNF* and *NTRK2* are suggested as candidate genes in asthma, but future studies should expand the scope of the investigation to other *BDNF* and *NTRK2* genetic variants in asthma. In addition, larger samples are warranted to replicate findings in this dissertation and earlier studies.

2. HYPOTHESIS

The concentrations of platelet 5-HT and plasma BDNF are significantly altered in asthmatic patients versus healthy controls, while a SNP [rs6265 (Val66Met)] in the *BDNF* gene is strongly associated with risk of severe asthma in Caucasian adults.

3. AIMS AND PURPOSE OF THE RESEARCH

3.1. General aim

To investigate the association of platelet 5-HT and plasma BDNF concentrations, as well as Val66Met *BDNF* gene polymorphism, with asthma severity, thus contributing to the understanding of the pathophysiology of asthma.

3.2. Specific aims

(1) To compare platelet 5-HT and plasma BDNF concentrations in adult asthmatic patients versus healthy controls, as well as in allergic versus non-atopic asthmatics, and to assess the associations of these concentrations with severity of disease.

(2) To determine the potential association between the Val66Met *BDNF* gene variants with allergic asthma and disease severity in Caucasian adults.

4. MATERIALS AND METHODS

This research was conducted at the Clinic for Lung Diseases Jordanovac, University Hospital Centre Zagreb, Zagreb, Croatia, under the mentorship of Professor Sanja Popović-Grle, MD, PhD, and the Laboratory for Molecular Neuropsychiatry, Division of Molecular Medicine, Ruđer Bošković Institute, Zagreb, Croatia, under the co-mentorship of Associate Professor Dubravka Švob Štrac, BSc, PhD, in collaboration with the Croatian Institute for Transfusion Medicine, Zagreb, Croatia. The BDNF portion of this work was financially supported by Novartis and the Croatian Thoracic Society. The 5-HT section of this research was carried out using laboratory materials left over from other collaborative scientific projects at the Laboratory for Molecular Neuropsychiatry.

4.1. Subjects

In this prospective observational study, 120 Caucasian Croatians treated for asthma as outpatients at the Clinic for Lung Diseases Jordanovac, University Hospital Centre Zagreb, and 120 healthy Caucasian Croatians from the Croatian Institute for Transfusion Medicine were recruited from February 2014 to April 2015 and July 2017 to February 2018. All 240 enrolled subjects of both genders were unrelated, aged 18 years or older, and had normal platelet counts (reference range: 158-424 $x10^9$/L).

4.2. Inclusion and exclusion criteria

4.2.1. Asthma patients

Adult patients who satisfied the following inclusion criteria were eligible for enrolment: an established diagnosis of asthma according to the GINA criteria [417-418], with no acute exacerbation of asthma at the time of outpatient visit. Asthma patients with the following exclusion criteria were not included in the study: diagnosis of depression or other psychiatric or neurologic disorders; history of asthma exacerbation or respiratory tract infection within the previous four weeks; diagnosis of co-morbid airway disease (i.e., COPD) or obstructive sleep apnea; diagnosis of immune, infectious, malignant, cardiovascular, or GI diseases; poorly controlled co-morbidities such as arterial hypertension, diabetes mellitus, chronic kidney disease, etc.; pre-existing coagulopathy; and treatment with antidepressants, antipsychotics, anticonvulsants, mood stabilizers, antineoplastics, antiplatelet therapy (i.e., aspirin and clopidogrel), anticoagulants, estrogen replacement therapy, and NSAIDs.

4.2.2. Healthy controls

The control group consisted of healthy adults, free of somatic and psychiatric disorders. Healthy controls were volunteer blood donors who were initially screened for good general health and an unremarkable medical history as part of the eligibility criteria for blood donation. Healthy controls were recruited consecutively during the routine blood donation procedures by the healthcare personnel at the Croatian Institute for Transfusion Medicine. Exclusion criteria were as follows: use of any medications; alcohol or other substance dependence or abuse; allergic diseases; history of asthma or other airway diseases; respiratory symptoms; and recent infections or inflammation.

4.3. Ethical considerations

The study protocol was approved by the local Ethics Committees of the University Hospital Centre Zagreb, Croatian Institute for Transfusion Medicine, and University of Zagreb, School of Medicine. All participants provided written informed consent (in Croatian) prior to enrolment and before any study-related procedures were performed. This study was conducted in accordance with the ethical standards of the Declaration of Helsinki [419].

4.4. Data collection

4.4.1. Asthma patients

Before blood sample collection, a thorough demographic data and medical history was recorded, including age, gender, body mass index (BMI), smoking habits, alcohol use, co-morbidities, and list of medications (in particular, aspirin, clopidogrel and antidepressants), followed by a thorough physical examination, including heart and lung auscultation, and diagnostic work-up. The BMI, an indicator of relative obesity, was calculated (kg/m^2) using measured weight (kg) and height (cm) recorded during the pulmonary function testing. According to the WHO criteria, subjects with a BMI of 30 and above were considered obese.

The PFTs consisted of spirometry with reversibility test (following administration of 4 inhalations of the bronchodilator salbutamol, 100 µg per metered dose), single-breath diffusing capacity of lung for carbon monoxide (DLCO) and FeNO measurement. These PFTs, as well as arterial blood gas (ABG) status, were performed by experienced pulmonary function technicians using standardized procedures. Spirometry and DLCO were performed using the Master-Screen PFT analyser (Version 5.0, Jaeger GmbH, Hoechberg, Germany) according to the specified guidelines. FEV_1, FVC and peak expiratory flow (PEF) were calculated from the flow-volume loop as the outcome measures, using internally derived

references. Predicted values for spirometry and DLCO are reference values of the European Community for Coal and Steel [420-421]. The FeNO measurement (parts per billion, ppb) was determined using Denox 88 Module (Eco Medics AG, Duernten, Switzerland). The ABG analysis was done with the blood gas analyzer ABL5 (Radiometer, Copenhagen, Denmark).

Skin prick testing (SPT) was carried out with common airborne allergens (Stallergen allergenic extracts for SPT with control solution, Paris, France). The following standard set of inhalant allergen extracts was used: *Dermatophagoides pteronyssinus*, cat dander, dog dander, *Aspergillus fumigatus, Alternaria alternata, Cladosporium herbarum*, pollen mixture from the *Betullacae* family, pollen mixture from the *Cupressaceae* family, grass pollen mixture, and *Ambrosia elatior* pollen, as well as *Artemisia vulgaris* pollen, as representatives of weed pollen, with positive (histamine) and negative (saline) controls.

The asthma patients were subdivided into non-severe (i.e., mild-to-moderate) and severe asthma groups based on the GINA guidelines [418]. Further subdivisions of the asthma patients were made according to the various asthma phenotypes into non-allergic vs. allergic, eosinophilic vs. non-eosinophilic, and T2-high vs. T2-low asthma. Patients were grouped into non-allergic and allergic asthma based on the patient's clinical history, physical examination, and SPT or serum-specific extract-based IgE testing [422]. Asthma patients with (≥ 300 cells/μL) or without (< 300 cells/μL) high blood eosinophil count were classified as patients with or without eosinophilic asthma, respectively [423]. Subjects with asthma were categorized into T2-high and T2-low groups according to their phenotypic characteristics [64, 424-425]. T2-high asthma patients were defined as follows: allergic (i.e., positive skin prick test and/or increased total IgE [>120 kU/L] or positive specific IgE), had eosinophilic asthma (i.e., high blood eosinophil count of ≥ 300 cells/μL), increased FeNO > 25 ppb, or exercise-induced asthma; T2-low asthma patients lacked these disease features, and were smokers or obese [426]. Additionally, patients with the combination of asthma, recurrent nasal polyps and sensitivity to aspirin or other NSAIDs were classified as having aspirin-exacerbated respiratory disease (AERD), also known as Samter's Triad [427], and compared to asthma patients without AERD.

4.4.2. Healthy controls

Prior to blood sampling, healthy blood donors underwent an initial interview in order to determine their eligibility, record their demographic and clinical data (gender, age, smoking

habits, alcohol consumption, weight, height, medications, and medical history), and to calculate their BMI.

4.5. Blood sample collection

For all subjects, standard venipuncture technique with aseptic precautions was used to draw peripheral venous blood from a cubital vein. Whole blood samples were obtained in the morning (between 7 and 9 a.m.), following an overnight fast to minimize possible circadian variations. Each blood sample was collected into an 8.5 mL yellow-top glass tube (BD Vacutainer®) containing 1.5 mL of acid-citrate-dextrose (ACD) anticoagulant solution to prevent unwanted platelet activation and blood coagulation. The specimens were immediately refrigerated at a temperature of 4^{o}C and transported the same morning to the Laboratory for Neuropsychiatry, Division of Molecular Medicine, at the Ruđer Bošković Institute.

For asthma patients, at the time of sampling, additional blood specimens were collected in ethylenediaminetetraacetic acid (EDTA) tubes for routine laboratory studies (complete blood count, biochemistry and coagulation tests) and total serum IgE antibodies. The eosinophil, neutrophil and platelet counts were obtained from automated complete blood counts, and reported in total numbers (x 10^9/L). These samples were immediately sent for analysis to the Department of Laboratory Diagnostics at the University Hospital Centre Zagreb. Total serum IgE levels were measured using enzyme amplified chemiluminescent immunoassays on an automated analyser (Immulite® 2000XPi, Siemens Healthcare Diagnostics Erlangen, Germany) according to the manufacturer's instructions.

4.6. Isolation of platelets and plasma from whole blood samples

The glass tubes were placed on a tube roller mixer for gentle mixing of the whole blood samples for 15 min. Platelet concentrates were prepared by differential (serial) centrifugation of the blood samples at 3000 revolutions per minute (rpm) for 3 min at 4^{o}C using an automated benchtop centrifuge (Sorvall Legend RT). The resulting supernatant platelet-rich plasma (PRP) was transferred into another set of sterile centrifugation tubes. The bottom layer containing red blood cells was discarded. The platelets were further concentrated by centrifugation at 5000 rpm for 15 min at 4^{o}C, generating platelet pellets at the bottom of the tubes. The supernatant with platelet-poor plasma (PPP) was collected for determination of plasma BDNF concentration and stored at -20^{o}C. The platelet pellet was washed with 1.5 mL of saline, shortly vortexed to detach the platelets from the bottom of the tube, and

centrifugated at 5000 rpm for 15 min at 4°C. The sedimented platelets were stored at -20°C prior to being assayed for 5-HT concentration.

4.7. Determination of platelet 5-HT concentration

The platelet samples were disrupted by sonication (20 KHz, amplitude 8 x 10^{-3} mm for 30 s). Platelet 5-HT concentration was determined using the spectrofluorometric method, as described previously by Pivac and co-authors (2001 and 2009) [428-429]. The chemical agents used in this process are shown below.

Chemicals for the determination of platelet 5-HT concentration:

- HCl (Kemika, Croatia; 37%, p.a.): 0.1 M solution in reH_2O and 1 M solution in reH_2O
- $ZnSO_4$ (Gram-Mol, Croatia): 5% solution in reH_2O
- NaOH (Kemika, Croatia): 1 M solution in reH_2O
- L-cysteine (Sigma Aldrich, USA): 0.1% solution in 0.1 M HCl
- Ortho-phthalaldehyde (OPT; Sigma Aldrich, USA): 0.05% solution in 1 M HCl
- 5-HT (Sigma Aldrich, USA)

Triplicates (1.2 mL) of the standards of different 5-HT concentrations (2000 ng/mL, 500 ng/mL, 250 ng/mL, 125 ng/mL, 63 ng/mL), blank samples (water) and platelet sonicates were mixed with 1 mL of 10% zinc sulphate ($ZnSO_4$) and 0.5 mL of 1N sodium hydroxide (NaOH), incubated at room temperature for 5 min and centrifuged 15 min at 5087 x gravitational force (g) at 4°C to precipitate the proteins. The deproteinized supernatant was transferred to a new glass tube to which 0.2 mL of 0.1% L-cysteine and 1.2 mL of 0.05% ortho-phthalaldehyde (OPT) were added to prepare the fluorophore. After boiling for 10 min, the measurement of 5-HT fluorescence was performed on a Varian Spectrophotofluorometer Cary Eclipse (Agilent Technologies, USA) at 345 nm excitation and 485 nm emission wavelengths. The detection limit of the method was 10.0 ng per sample, and intra- and inter-assay coefficients of variation were 3.66% and 8.69%, respectively. The concentration of 5-HT in the platelet samples was calculated according to the 5-HT standards of known concentrations and was expressed in nmol per mg of platelet proteins, whose concentration was determined by the Lowry method, described in detail below [430].

4.8. Determination of platelet MAO-B activity

Platelet MAO-B activity was determined in the platelet sonicates using kynuramine as a substrate by modification of the method of Krajl (1965) [431], as previously described [264]. The chemical agents used in this process are shown below.

Chemicals for the determination of platelet MAO-B activity:

- 0.736 mM kynuramine (Sigma Aldrich, USA)
- 2 mM 4-hydroxyquinolone (Sigma Aldrich, USA)
- Acid phosphate buffer (0.5 M NaH_2PO_4 x H_2O) (Kemika, Croatia)
- Alkaline phosphate buffer (0.5 M NaH_2PO_4 x $2H_2O$) (Kemika, Croatia)
- 1 M NaOH (Kemika, Croatia)

The platelet pellet was thawed at room temperature and the platelets were destroyed by sonication (20 kHz, amplitude 8 x 10-3 mm for 60 s). Standards of different 4-hydroxyquinoline (4-HOQ) concentrations (5 nM, 2.5 nM, 1.25 nM, 0.562 nM and 0.313 nM) and blank samples (sonicated platelets) were prepared in duplicate. To each tube 100 µl of sonicated platelet pellet (sample and blank) or 100 µl of standard of different concentration was pipetted. The 800 µL of phosphate buffer (acid phosphate buffer adjusted to pH 7.4 with alkaline phosphate buffer) was added to the platelet samples and blanks, and 900 µl of phosphate buffer was added to the standards. After mixing, 100 µL of kynuramine (final concentration 73.6 mM) was added to platelet samples and all samples were incubated for 1 hr at 37°C. The 100 µL of kynuramine was subsequently added to the blanks. The reaction was stopped with 2 mL of cold 1M NaOH. The final reaction product, 4-HOQ, was measured spectrofluorometrically on the Varian Spectrophotofluorometer Cary Eclipse (Agilent technologies, USA) at the 310 nm excitation and 380 nm emission wavelengths. Platelet MAO-B activity was calculated according to the fluorescence of known concentrations of 4-HOQ in the standards. The result was expressed as the amount of 4-HOQ produced by the degradation of kynuramine over 1 hr relative to the total platelet protein concentration determined by the Lowry method [430].

4.9. Determination of total platelet protein concentration

To determine the protein concentration by the method of Lowry et al. (1951) [430], we first prepared an ABC solution and standards of different concentrations. The chemical agents used in this process are shown below.

Chemicals for the determination of protein concentration:

- HCl (Kemika, Croatia; 37%, p.a.): 0.1 M solution in reH_2O
- Bovine serum albumin (BSA; Sigma Aldrich, USA)
- ABC solution:
  Na_2CO_3 (Kemika, Croatia): 2% solution in reH_2O
  $CuSO_4$ (Kemika, Croatia): 1% solution in reH_2O
  K-Na tartrate (Kemika, Croatia): 2% solution in reH_2O
- Folin-Ciocalteu's phenol reagent (Sigma Aldrich, USA)

The ABC solution was prepared by mixing 140 mL of 2% Na_2CO_3, 1.4 mL of 2% K-Na tartrate and 1.4 mL of 1% $CuSO_4$. Different concentrations (2.50 mg/mL, 1.25 mg/mL, 0.63 mg/mL) of bovine serum albumin (BSA) were used as standards and 0.1M HCl was used as a blank. To make the measurement as accurate as possible, all platelet samples, standards and blank samples were prepared in triplicates. The 10 μL of platelet sample, standard (BSA) sample or blank (0.1M HCl) sample was placed in each tube and 2 mL of ABC mixture was added, mixed and incubated for 10 min at room temperature. Then, 0.2 mL of Folin-Ciocalteau reagent (diluted with water in a ratio of 1:1) was added to the mixture and incubated for 30 min at room temperature. The absorbance was measured spectrophotometrically at a wavelength of 700 nm. Protein concentration was calculated according to the optical density of known BSA concentrations as standards.

4.10. Determination of plasma BDNF concentration

Plasma BDNF concentrations were determined by enzyme-linked immunosorbent assay (ELISA) using a commercial kit (Quantikine® ELISA Human Free BDNF Immunoassay, R&D Systems, Minneapolis, Minnesota, USA) and an ELISA microplate reader (Thermo Labsystems Multiskan EX Microplate Reader, USA), as previously described [432]. All reagents, working standards, and plasma samples were prepared according to the manufacturer's guidelines.

Chemicals for the determination of plasma BDNF concentration:

Commercially available ELISA kit (R&D Systems, Inc., USA and Canada) – Quantikine® ELISA Human BDNF Immunoassay:

- BDNF Microplate: polystyrene plate with 96 microwells precoated with a mouse monoclonal antibody specific for human free BDNF

- BDNF Standard: lyophilized recombinant human BDNF protein
- BDNF Conjugate: mouse monoclonal antibody specific for human free BDNF conjugated to horseradish peroxidase (HRP)
- Assay Diluent RD1S: buffered protein base
- Calibrator Diluent RD6P: calibration standard solution of animal serum for dilution
- Wash Buffer Concentrate: 25x concentrated solution of buffered surfactant
- Color Reagent A: stabilized H_2O_2
- Color Reagent B: stabilized chromogen tetramethylbenzidine (TMB)
- Stop Solution: 2N sulfuric acid

All plasma samples were additionally centrifuged at 10,000 g (4°C) for 10 min to remove possible platelet residues. The samples were then diluted 4 times using the RD6P dilution calibration solution that was part of the ELISA kit. Microplates (96 wells) coated with a monoclonal antibody that binds to BDNF from plasma samples were used to determine BDNF concentration. 100 μL of RD1S kit diluent was first added to each well, followed by 50 μL of plasma samples or 50 μL of previously prepared BDNF standard dilutions (4000 pg/mL, 2000 pg/mL, 1000 pg/mL, 500 pg/mL, 250 pg/mL, 125 pg/mL, 62.5 pg/mL). Standards and all plasma samples were added to the wells in duplicate. Negative control (50 μL RD6P dilution calibration solution) was added to the two wells. The microplate was then covered with adhesive foil and incubated for 2 hr at room temperature to obtain antibody-BDNF protein complex. After 2 hr, 100 μL of BDNF conjugate was added to each well. Namely, a monoclonal antibody that specifically recognizes the BDNF protein and to which horseradish peroxidase (HRP) is bound was added to the resulting antibody-BDNF protein complex (Figure 1).

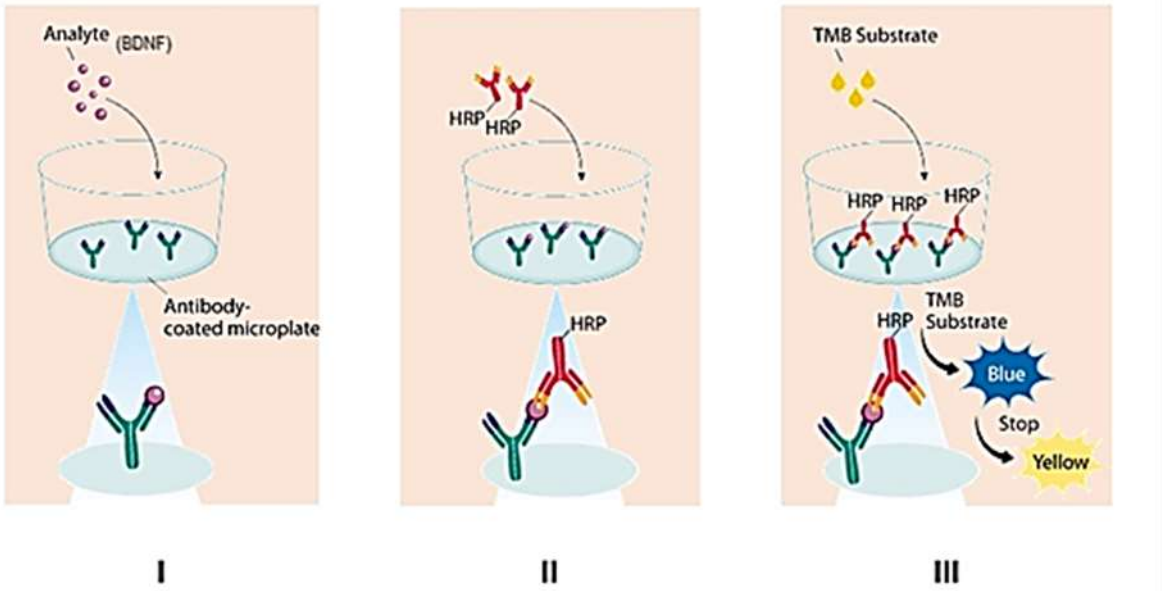

Figure 1. Schematic illustration of the determination of plasma brain-derived neurotrophic factor (BDNF) concentration using commercially available enzyme-linked immunoassay (ELISA) kits. **I.** Binding of plasma BDNF to the monoclonal antibody coating the microwells. **II.** Binding of the horseradish peroxidase (HRP) conjugated to the immobilized monoclonal antibody-BDNF complex in the microwells. **III.** Addition of tetramethylbenzidine (TMB) substrate solution produces a blue colour proportionally to the concentration of the bound plasma BDNF. A stop solution is used to oxidize TMB, terminating the reaction and resulting in a yellow colour change. The absorbance of the sample in each well is measured at 450 nm wavelength. Diagram taken and modified from R&D Systems, Inc. [433].

After incubation for 1 hr at room temperature, excess monoclonal antibody from each well was rinsed 3 times with 400 µL of diluted wash buffer, and the wells were dried by decantation. The TMB was added to the microplate in equal proportions and incubated in the dark for 30 min at room temperature. After 30 min, 50 µL of the reaction stop solution (2 N sulfuric acid) was added to each well and the absorbance of the samples was measured at 450 nm, with correction at 570 nm, using an ELISA reader (Thermo Labsystems Multiskan EX Microplate Reader, USA). Subsequently, using known concentrations of BDNF standards, the concentration of BDNF in individual wells was determined. The intra- and inter-assay coefficients of variations were less than 10%.

4.11. Extraction of blood deoxyribonucleic acid (DNA)

Genomic DNA was isolated from peripheral blood leukocytes using a simple salting-out method [434]. The chemical agents used in this protocol are shown below.

Chemicals for DNA isolation:

- Red blood cell lysis buffer (RLB), pH 7.6:
  10 mM Tris (Sigma Aldrich, USA)
  5 mM $MgCl_2$ (Kemika, Croatia)
  10 mM NaCl (Kemika, Croatia)
- Sodium EDTA (SE) buffer, pH 8.0:
  75 mM NaCl (Kemika, Croatia)
  25 mM Na_2EDTA (Fluka, USA)
  NaCl (Kemika, Croatia): 5 mM solution in redistilled water (reH_2O)
  Proteinase K (TaKaRa, Japan, 20 mg/mL)
- Sodium dodecyl sulphate (SDS) (Sigma Aldrich, USA): 10% solution, pH=7.2
- Ethanol (Gram-Mol, Croatia, min. 99.5%, p.a.): 75% solution in reH_2O
- Isopropanol (Gram-Mol, Croatia, min. 99.5%, p.a.)
- Tris-EDTA (TE) buffer, pH 7.6:
  10 mM Tris (Sigma Aldrich, USA)
  1 mM EDTA (Fluka, USA)

The 900 µl of red blood cell lysis buffer (RLB) was added to the 300 µl of whole blood sample, mixed and incubated on ice for 10 min. After centrifugation for 2 min at 13400 x g (4°C), the supernatant was discarded. The pellet was resuspended in the RLB and centrifuged 2 min at 13400 x g three more times. The 300 µl of Na-EDTA buffer, 30 µl of 10% sodium dodecyl sulphate (SDS) and 1.5 µl of proteinase K were added to the purified precipitate and incubated for 2 hr at 56°C to lyse the leukocytes. After incubation, the samples were cooled down at room temperature, and 160 µl of 5 mM NaCl was added, mixed for 10 s and centrifuged for 5 min at 13400 x g (20°C). The supernatant with DNA was transferred to a new tube, and 800 µL of cold isopropanol was added, which with gentle stirring caused precipitation of DNA. The sample was centrifuged for 2 min at 12000 x g (20°C). The supernatant was decanted and 250 µL of 75% ethanol was added to the precipitate. After centrifugation for 2 min at 12000 x g (20°C), the supernatant was discarded and the precipitate was allowed to air dry for 30 min. By adding 100 µL of Tris-EDTA buffer, with shaking for 1 hr at 37°C, the isolated DNA was dissolved and stored at -20°C. The concentration and purity of the obtained DNA samples were determined spectrophotometrically using a NanoDrop 2000c UV-Vis Spectrophotometer (Thermo Scientific) at wavelengths of 280 nm and 260 nm, respectively.

4.12. Genotyping

Genotyping of Val66Met (rs6265, G196A) and rs1439050 polymorphisms in the *BDNF* and *NTRK2* genes, respectively, was performed according to the manufacturer's instructions (Applied Biosystems®, Foster City, CA, USA), and the chemical agents used are shown below.

Chemicals for genotyping:

Commercially available kits (Applied Biosystems®, USA):

- TaqMan® Pre-designed SNP Genotyping Assay-
  Reference SNP Assay ID for Applied Biosystems®: C_11592758_10 (rs6265)
- TaqMan® Pre-designed SNP Genotyping Assay-
  Reference SNP Assay ID for Applied Biosystems®: C_7424004_10 (rs1439050)
- TaqMan® Genotyping Master Mix

Samples of the isolated DNA were thawed and dilutions with redistilled water (reH_2O) were prepared so that each sample contained 20 ng of DNA. The 4.5 μL of diluted DNA sample and 5.5 μL of reaction mixture, consisting of TaqMan Genotyping Master Mix (with free deoxyribonucleotide triphosphates and AmpliTaq Gold DNA polymerase) and TaqMan SNP Genotyping Assay (combination of two primers to amplify a target DNA sequence labeled with different fluorescent dyes, VIC® and FAM™, to distinguish two alleles), were placed in each of the 96 wells on the reaction plate. The reaction plate was covered with foil, centrifuged for 2 min at 5000 rpm and placed in an ABI Prism® 7000 Sequence Detection System apparatus (ABI, Foster City, USA). In the initial step, the device heats the plates at 95°C for 10 min, then at 92°C for 15 sec to denature the DNA, and then lowers the temperature to 60°C for 1 min, to bind the primers and elongate with AmpliTaq Gold polymerase. Cycles from DNA denaturation to amplification were repeated 40 times. A summary of the reaction conditions and steps involved in genotyping using real-time polymerase chain reaction (RT-PCR) is found in Table 1.

Table 1. Reaction conditions for genotyping using real-time polymerase chain reaction (RT-PCR)

RT-PCR steps	Temperature	Time
Initial step	95°C	10 min
	40 cycles	
Denaturation	92°C	15 s
Primer hybridization and DNA extension	60°C	60 s

This method of allelic discrimination is based on the use of fluorogenic assays that bind to a particular allele and are detected by DNA amplification in a RT-PCR, as depicted in Figure 2. The assay consists of a single-stranded oligonucleotide with a bound fluorescent dye (reporter) at the 5' end and a quencher dye at the 3' end. If the probe is complementary to the DNA sequence, their hybridization occurs, and during the PCR reaction, the Taq DNA polymerase with its 5' nuclease activity releases fluorescent dye, which is detected as an increase in fluorescence intensity. Thus, the formation of the PCR product can be monitored in real time.

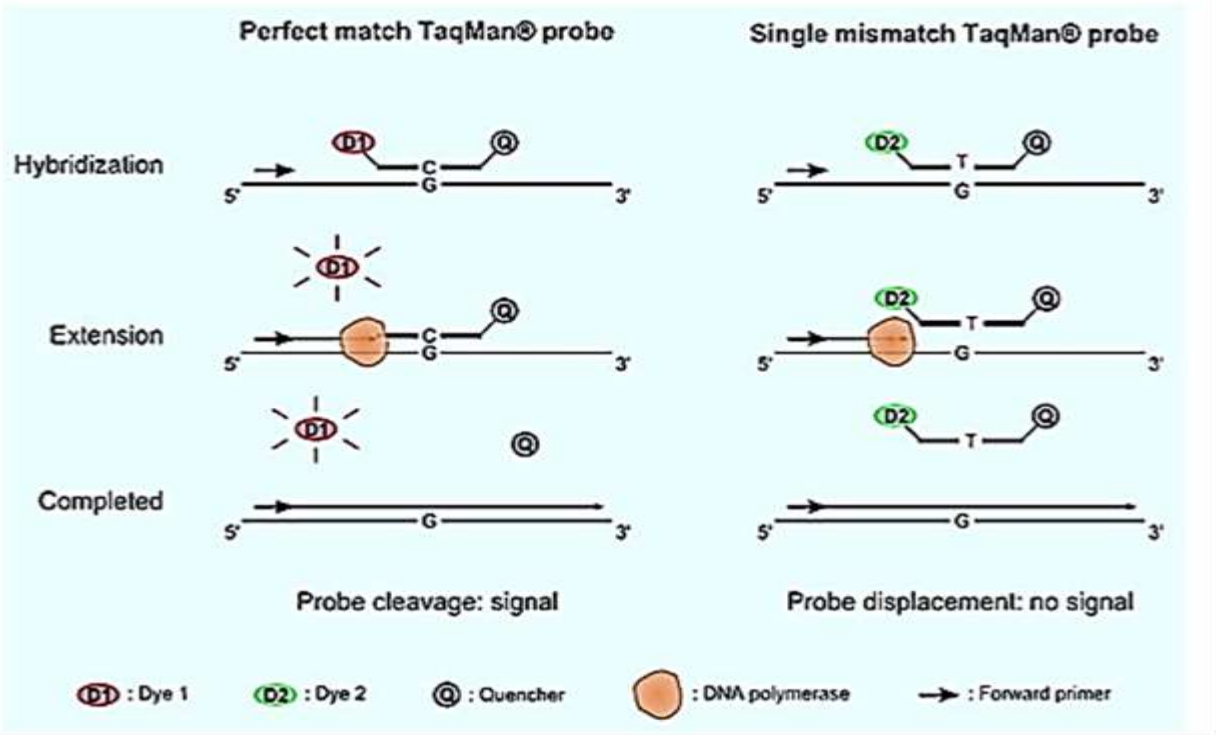

Figure 2. Schematic diagram of real-time polymerase chain reaction (RT-PCR). Diagram taken and modified from TaqMan® SNP genotyping technology [435].

The genotyping results of the RT-PCR amplification data were then analysed using computer-generated scatter plots of allelic discrimination of the fluorescence signals for each individual DNA sample, as shown in Figure 3.

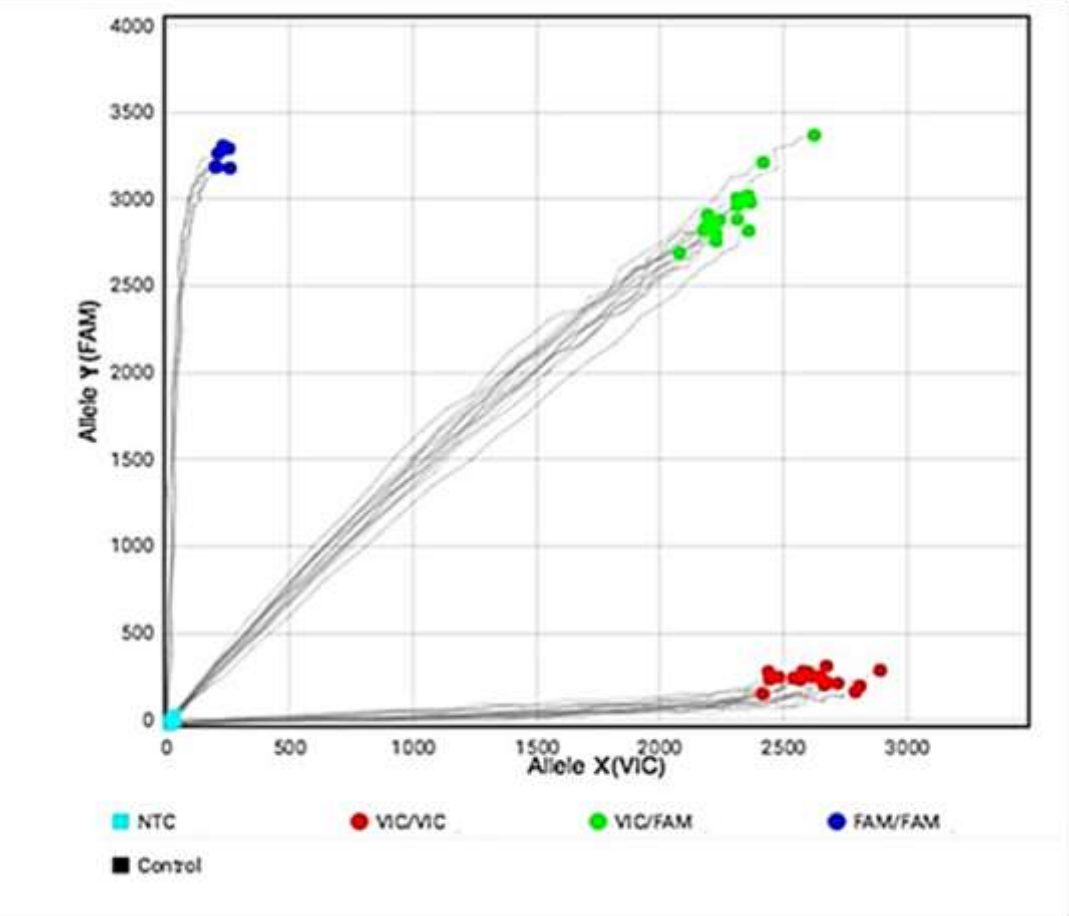

Figure 3. Scatter plot of allele X versus allele Y illustrating the genotyping results generated with real-time polymerase chain reaction (RT-PCR).
(NTC= non-template control; VIC/VIC = XX; VIC/FAM = XY; FAM/FAM = YY)
The values of the VIC® and FAM™ fluorescence signals are marked along the X-axis and Y-axis, respectively. The probe for allele X was labeled with VIC® fluorescent dye and that for allele Y with FAM™. The fluorescent signal for each individual DNA sample (i.e., in each microwell) is represented as an individual data point. Homozygotes (XX) for allele X are clustered in red and homozygotes (YY) for allele Y are clustered in dark blue. Heterozygotes (XY) are clustered in green. The NTCs are clustered in light blue and oriented to the origin. Diagram taken and modified from Thermo Fisher Scientific Inc. [436].

4.13. Statistical analysis

All statistical analyses were performed using GraphPad Prism version 4.00 for Windows (GraphPad Software, Inc., San Diego, CA, USA). The data were expressed as number (n) and percentage (%) for categorical data or as median with 25th (Q1) and 75th (Q3) percentiles for numerical (continuous) data. All examined parameters failed the assumption of normality of distribution according to the D'Agostino & Pearson omnibus test. Therefore, non-parametric analyses were used to compare the independent cohorts. Continuous variables were compared using Mann-Whitney U test (for comparison of two groups) or Kruskal-Wallis test with post-hoc Dunn's multiple comparisons test (for comparison of three or more groups). Correlations were assessed with non-parametric Spearman correlation. Possible deviations from the Hardy-Weinberg equilibrium (HWE) were tested using the chi-square ($\chi 2$) goodness-of-fit test. Genotype and allele frequencies were evaluated by a $\chi 2$ test of independence and Fisher exact test, respectively.

The obtained results were corrected for multiple testing (five comparisons of asthma phenotypes: non-severe vs. severe; T2-high vs. T2-low; non-allergic vs. allergic; non-eosinophilic vs. eosinophilic; non-AERD vs. AERD) using Bonferroni correction, and statistical significance was defined as p-value less than 0.01. A priori sample size and achieved power calculations were conducted using the G*Power 3 Software (Version 3.1.9.2.; a free program written by Franz Faul, University of Kiel, Kiel, Germany). For Mann-Whitney test, at medium effect size 0.425, power 0.8, and statistical significance set at 0.01, an adequate total sample size was determined to be 236. For Kruskal-Wallis test (three groups) at medium effect size 0.25, power 0.8, and statistical significance set at 0.01, an adequate sample size was determined to be 228. For $\chi 2$-test (df=2), at small-to-medium effect size 0.25, power 0.8, and statistical significance set at 0.01, an adequate sample size was determined to be 223, whereas for Fisher exact test (df=1) at small-to-medium effect size 0.25, power 0.8, and statistical significance set at 0.01, an adequate sample size was determined to be 187. Given that the actual total sample size was 240, the power analysis confirmed the appropriate sample size and statistical power of the study.

5. RESULTS

5.1. General and clinical characteristics of the study population

A total of 240 subjects (120 asthma patients and 120 healthy controls) were recruited for the study. The general characteristics of the study population are presented in Table 2. Healthy individuals were significantly younger than their asthmatic counterparts (p<0.0001). There were significantly more males in the control group (n=73, 60.83%) than in the asthma group (p<0.0001), with females predominating (n=79, 65.83%) among the asthma patients. More current smokers (p=0.0005) were present among healthy subjects (34.17%) than asthma patients (8.33%), but in both of these groups, there were significantly more (p<0.0001) non-smokers (never and past smokers). No significant differences in BMI (p=0.64) were detected between asthma cases and healthy controls, even across six different BMI categories (p=0.48), including underweight, normal weight, overweight, and three categories of obesity, or when the subjects were divided into obese and non-obese individuals (p=0.43), as shown in Table 2.

Table 2. General characteristics of healthy subjects and asthma patients enrolled in the study

Parameter	**Healthy Subjects (n=120)**	**Asthma Patients (n=120)**	**Statistical Analysis**
Age (years) Median (IQR: 25%-75%)	42.00 (33.25-51.00)	58.00 (40.25-67.00)	p<0.0001*, U=3912.00, Mann-Whitney test
Males n (%)	73 (60.83)	41 (34.17)	p<0.0001*, Fisher's exact test
Current smokers n (%)	41 (34.17)	10 (8.33)	p=0.0005*, Fisher's exact test
BMI (kg/m^2) Median (IQR: 25%-75%)	26.20 (23.33-28.85)	26.65 (23.03-29.80)	p=0.64, U=6949.00, Mann-Whitney test
BMI category: Normal weight (18.5-24.9 kg/m^2), n (%)	43 (35.83)	44 (36.67)	p=0.48, $\chi 2$=4.52, $\chi 2$ test
BMI category: Obesity (≥30 kg/m^2), n (%)	22 (18.33)	28 (23.33)	p=0.43, Fisher's exact test

*statistically significant (p<0.01); BMI = body mass index; IQR = interquartile range (25th to 75th percentile); n = total number of observations in the data.

The age difference observed between healthy subjects and asthma patients is attributed to the significantly older individuals (median 61.00 years, IQR: 53.00-67.50) in the group of severe asthma patients. As presented in Table 3, additional analysis using Kruskal-Wallis test followed by Dunn's multiple comparisons test confirmed the significantly older ages of the severe asthma patients (n=61) compared to both the non-severe asthma patients (n=59, 47.00 years, IQR: 35.00-67.00; p=0.005) and the control group (42.00 years, IQR: 33.25-51.00; p<0.0001). There was a statistically significant difference in the age (p=0.022, U=1366, Mann-Whitney test) between non-severe and severe asthma patients.

Table 3. General characteristics of the healthy subjects and asthma patients stratified according to severity into non-severe (mild-to-moderate) and severe asthma patients

Parameter	**Heathy Control Subjects (n=120)**	**Non-severe Asthma Patients (n=59)**	**Severe Asthma Patients (n=61)**	**Statistical Analysis**
Age (years) Median (IQR: 25%-75%)	42.00 (33.25-51.00)	47.00 (35.00-67.00)	61.00 (53.00-67.50)	p<0.0001*, Kruskal-Wallis test; p<0.0001*, control vs severe asthma; p=0.005*, non-severe vs severe asthma Dunn's multiple comparisons test
Males n (%)	73 (60.83)	23 (38.98)	18 (29.51)	p=0.0001*, $\chi2$=18.19, $\chi2$ test
Current Smokers n (%)	41 (34.17)	7 (11.86)	3 (4.92)	p=0.0006*, $\chi2$=14.92, $\chi2$ test
BMI (kg/m^2) Median (IQR: 25%-75%)	26.20 (23.33-28.85)	25.80 (23.00-29.80)	27.10 (23.55-30.10)	p=0.79, Kruskal-Wallis test
BMI category: Normal weight (18.5-24.9 kg/m^2), n (%)	43 (35.83)	25 (42.37)	19 (31.15)	p=0.51, $\chi2$=8.552, $\chi2$ test
BMI category: Obesity (≥30 kg/m^2), n (%)	22 (18.33)	13 (22.03)	15 (24.59)	p=0.60, $\chi2$=1.028, $\chi2$ test

*statistically significant (p<0.01); BMI = body mass index; IQR = interquartile range (25th to 75th percentile); n = total number of observations in the data.

On the other hand, both non-severe and severe asthma groups had a higher ratio of females (p=0.0001), lower percentage of smokers (p=0.0006) and an equal share of subjects in different BMI categories (p=0.51) in comparison to healthy individuals (Table 3).

Specifically, non-severe and severe asthma patients did not significantly differ in their gender distribution (p=0.34, Fisher's exact test), smoking status (p=0.23, $\chi 2$=2.90, $\chi 2$ test) or BMI (p=0.67, U=1718, Mann-Whitney test), even across six different BMI categories (p=0.6988, $\chi 2$=3.008, $\chi 2$ test), or when the subjects were divided into obese and non-obese individuals (p=0.8303, Fisher's exact test). Asthma patients demonstrated high heterogeneity in their clinical symptoms and phenotypes as evidenced by the data in their medical records, and presented in Table 4.

Table 4. Clinical characteristics of the asthma patients

Clinical Characteristics	**Median (IQR: 25%-75%)**
Total Serum IgE (IU / mL)	191.00 (43.00-398.00)
Blood eosinophils (x 10^9 / L)	0.215 (0.10-0.40)
Blood neutrophils (x 10^9 / L)	4.560 (3.62-6.30)
FeNO (ppb)	34.00 (14.28-67.75)
FEV_1 (% of predicted value)	74.90 (54.28-90.08)
FVC (% of predicted value)	88.50 (75.08-101.80)
PEF (% of predicted value)	77.05 (57.25-95.52)
DLCO (%)	83.30 (73.68-95.35)
Duration of disease (years)	13.50 (8.00-29.00)
Comorbidities (n)	2.00 (1.25-4.00)
Clinical Characteristics	**n (%)**
Penicillin allergy	25 (20.83)
Nutritive allergy	12 (10.00)
Animal dander and feather allergy	18 (15.00)
Dust allergy	51 (42.50)
Pollen allergy	53 (44.17)
Fungal / mould allergy	9 (7.50)
Early onset asthma (age < 12 years)	21 (17.50)
History of pneumonia	21 (17.50)
Emergency intervention (ever)	89 (74.17)
Hospitalisation for asthma exacerbation (ever)	58 (48.33)
Nasal polyps	27 (22.50)
Aspirin sensitivity	11 (9.17)
Allergen specific immunotherapy	15 (12.50)
Oral corticosteroid therapy	30 (25.00)
Biological therapy	20 (16.67)

DLCO = diffusing capacity of lung for carbon monoxide; FeNO = fractional cxhaled nitric oxide; FEV_1 = forced expiratory volume in one second; FVC = forced vital capacity; IgE = immunoglobulin E; IQR = interquartile range (25^{th} to 75^{th} percentile); IU = international units; n = total number of observations in the data; PEF = peak expiratory flow; ppb = parts per billion.

5.2. Platelet 5-HT concentration and platelet MAO-B activity

5.2.1. Platelet 5-HT concentration and platelet MAO-B activity in the study population

As displayed in Table 5 and Figure 4A, platelet 5-HT concentrations were significantly lower in the asthma patients (0.81 nmol/mg of protein, IQR: 0.62-1.09) compared to the healthy subjects (0.97 nmol/mg of protein, IQR: 0.72-1.65; p=0.0008, U=5388.00, Mann-Whitney test). On the other hand, platelet MAO-B activity was significantly higher in asthma patients (43.05 nmol/mg of protein/h, IQR: 34.39-58.21) in comparison to the healthy control subjects (33.32 nmol/mg of protein/h, IQR: 25.71-51.26); (p<0.0001, U=5056.00, Mann-Whitney test) (Table 5 and Figure 4B).

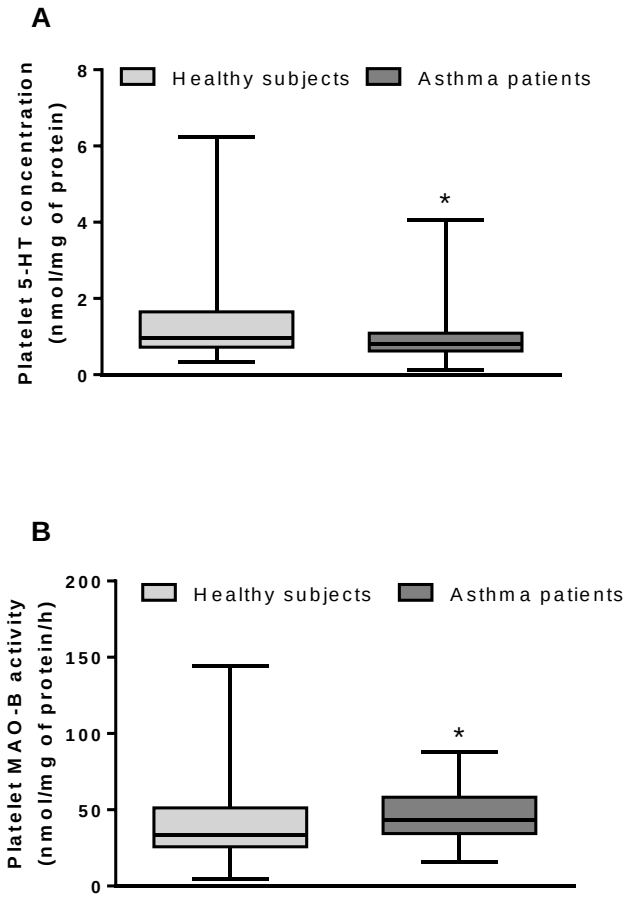

Figure 4. A) Platelet 5-HT concentrations (nmol/mg of protein) and **B)** platelet MAO-B activity (nmol/mg of protein/hour) in asthma patients (n=120) compared to healthy subjects (n=120). The central horizontal line inside each box of the box-and-whisker plot represents the median. The lower and upper ends of the boxes are the 25th and 75th percentiles, respectively; the error bars are the minimum and maximum values. *statistically significant (p<0.01); p<0.001 vs control, Mann-Whitney test.

Table 5. Platelet 5-HT concentration and platelet MAO-B activity in healthy subjects and asthma patients

Parameter	Healthy Subjects (n=120)	Asthma Patients (n=120)	Statistical Analysis
Platelet 5-HT Concentration (nmol/mg of protein) Median (IQR: 25%-75%)	0.97 (0.72-1.65)	0.81 (0.62-1.09)	p=0.0008*, U=5388.00, Mann-Whitney test
Platelet MAO-B Activity (nmol/mg of protein/h) Median (IQR: 25%-75%)	33.32 (25.71-51.26)	43.05 (34.39-58.21)	p<0.0001*, U=5056.00 Mann-Whitney test

*statistically significant (p<0.01); 5-HT = serotonin; IQR = interquartile range (25th to 75th percentile); MAO-B = monoamine oxidase B.

When we further analysed the 5-HT levels and MAO-B activity in platelets of enrolled subjects, we observed no significant correlation between platelet 5-HT concentrations (p=0.20, r=-0.12) and age in asthma patients. Likewise, platelet MAO-B activity (p=0.12, r=0.14) was not significantly correlated with age in this same group of patients. Moreover, platelet 5-HT concentrations (p=0.10, r=-0.15) and platelet MAO-B activity (p=0.42, r=0.07) were not significantly correlated with age in healthy control subjects (Spearman correlation).

There was also no significant correlation between platelet 5-HT concentrations and BMI in asthma patients (p=0.62, r=-0.04) or healthy subjects (p=0.23, r=-0.11). In addition, there was no significant correlation between BMI and platelet MAO-B activity in the patients with asthma (p=0.81, r=-0.02). However, as demonstrated in Figure 5, there was a significant negative correlation between BMI and platelet MAO-B activity in the healthy control subjects (p=0.001, r=-0.30).

No significant differences were found in platelet 5-HT concentrations between smokers and non-smokers in the control (p=0.80, U=1574.00, Mann-Whitney test) or asthma (p=0.34, U=448.00, Mann-Whitney test) groups. On the other hand, platelet MAO-B activity differed significantly between smoking and non-smoking control subjects (p=0.008, U=1143.00, Mann-Whitney test) as well as between smoking and non-smoking asthma patients (p=0.01, U=287.00, Mann-Whitney test), suggesting lower platelet MAO-B activity in smoking subjects in both groups (Figure 6).

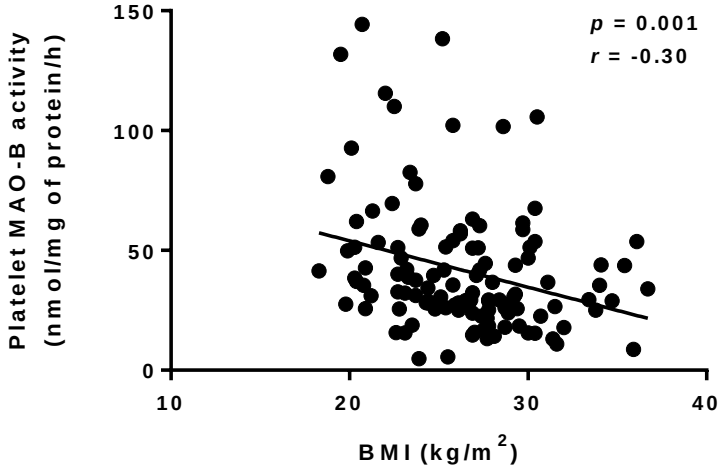

Figure 5. Significant negative correlation (p=0.001, r=-0.30, Spearman correlation) between BMI and platelet MAO-B activity in healthy subjects. BMI = body mass index.

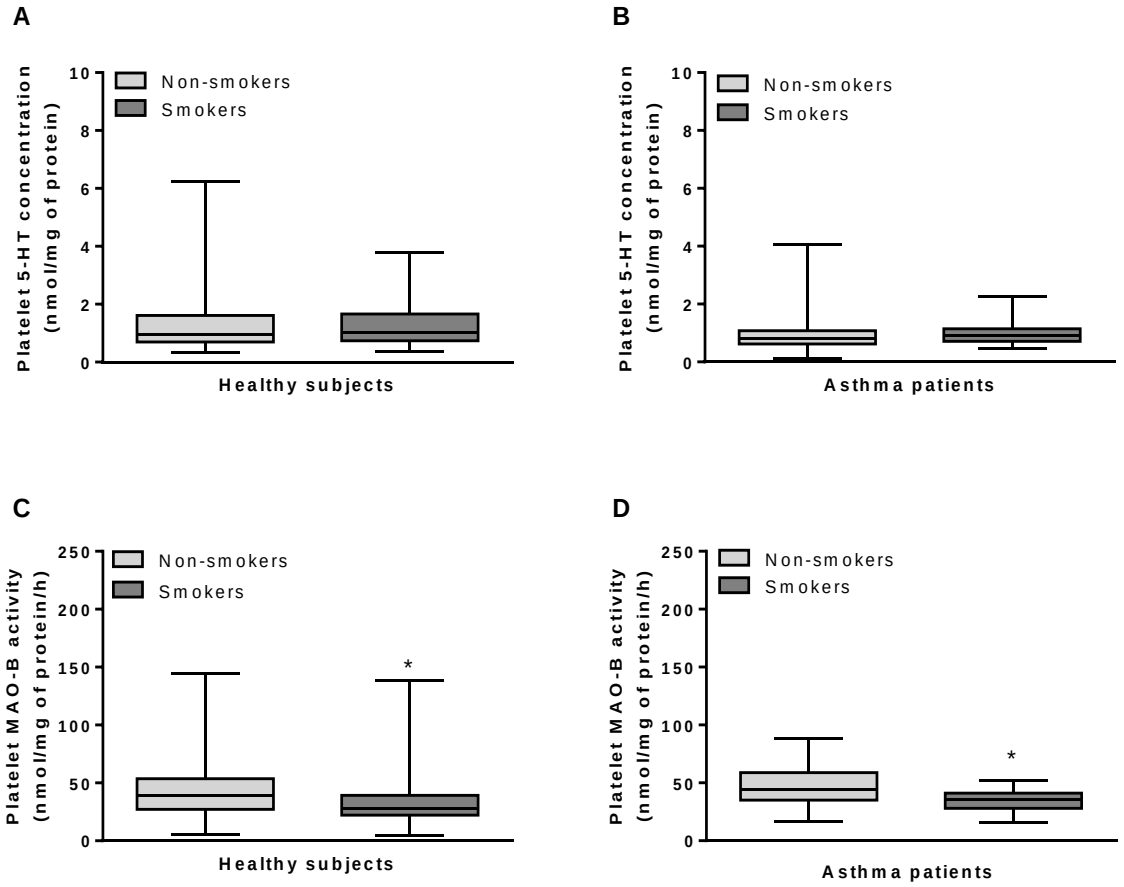

Figure 6. The 5-HT concentration **(A-B)** and MAO-B activity **(C-D)** in platelets of healthy subjects and asthma patients, subdivided according to smoking status into smokers and non-smokers. *statistically significant (p<0.01), Mann-Whitney test.

When the control subjects were subdivided according to gender, we observed no significant differences in platelet 5-HT concentrations between male and female healthy subjects (p=0.13, U=1437, Mann-Whitney test). Similarly, the platelet 5-HT concentrations did not differ significantly between male and female asthma patients (p=0.30, U=1434.00, Mann-Whitney test). However, there were significant differences in platelet MAO-B activity between male and female healthy control subjects (p<0.0001, U=827.00, Mann-Whitney test) as well as in the group of asthma patients (p=0.009, U=1149, Mann-Whitney test), suggesting that female subjects in both groups have higher platelet MAO-B activity.

5.2.2. Platelet 5-HT concentration and platelet MAO-B activity according to asthma severity

As depicted in Figure 7A, there were no significant differences in platelet 5-HT concentrations between the non-severe asthma patients (0.85 nmol/mg of protein, IQR: 0.64-1.10) and the severe asthma patients (0.75 nmol/mg of protein, IQR: 0.62-1.04); (p=0.38, U=1632.00, Mann-Whitney test). Moreover, platelet MAO-B activity was not significantly different between the non-severe asthma patients (44.22 nmol/mg of protein/h, IQR: 34.65-56.74) and the severe asthma patients (43.00 nmol/mg of protein/h, IQR: 33.84-60.34); (p=0.80, U=1750.00, Mann-Whitney test) (Figure 7B).

This finding was confirmed by further analysis with Kruskal-Wallis test followed by Dunn's multiple comparisons test (Table 6). As shown in Table 6, platelet 5-HT concentrations were significantly different only between the severe asthma patients and healthy control subjects (p=0.004). There were no significant differences in platelet 5-HT concentrations between the non-severe and the severe asthma patients (p>0.99). In addition, platelet MAO-B activity was significantly higher in both non-severe (p=0.006) and severe asthma patients (p=0.002) in comparison to healthy control subjects (Table 6). However, there were no significant differences in platelet MAO-B activity between the non-severe and the severe asthma patients (p>0.99).

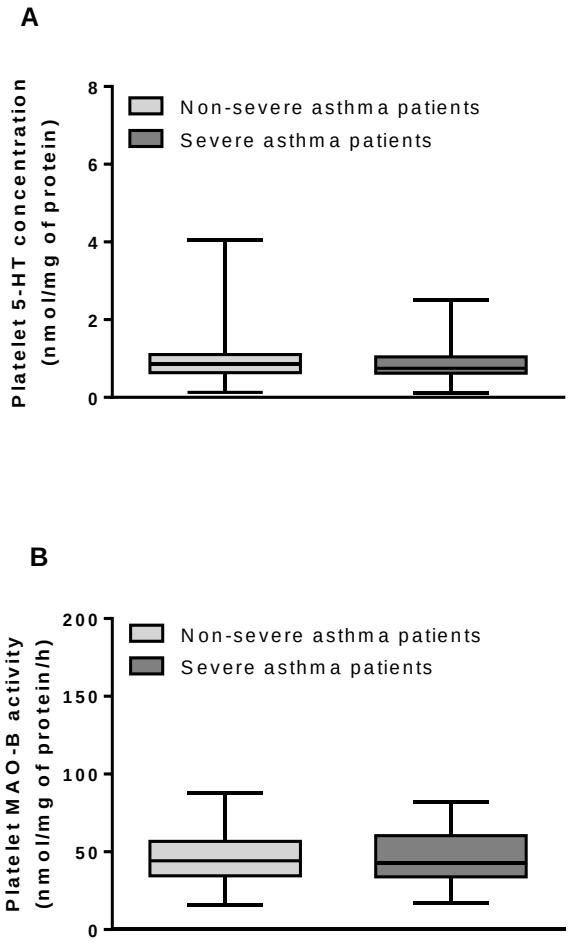

Figure 7. A) Platelet 5-HT concentration (nmol/mg of protein) and **B)** platelet MAO-B activity (nmol/mg of protein/hour) in non-severe asthma patients (n=59) compared to severe asthma patients (n=61). The central horizontal line inside each box of the box-and-whisker plot represents the median; the lower and upper ends of the boxes are the 25th and 75th percentiles, respectively; the error bars are the minimum and maximum values.

Table 6. Platelet 5-HT concentration and platelet MAO-B activity in healthy subjects and asthma patients stratified according to severity into non-severe (mild-to-moderate) and severe asthma patients

Parameter	Healthy Control Subjects (n=120)	Non-severe Asthma Patients (n=59)	Severe Asthma Patients (n=61)	Statistical Analysis
Platelet 5-HT Concentration (nmol/mg of protein) Median (IQR: 25%-75%)	0.97 (0.72-1.65)	0.85 (0.64-1.10)	0.75 (0.62-1.04)	p=0.0026*, Kruskal-Wallis test; p=0.0042* control vs severe asthma Dunn's multiple comparisons test
Platelet MAO-B Activity (nmol/mg of protein/h) Median (IQR: 25%-75%)	33.32 (25.71-51.26)	44.22 (34.65-56.74)	43.00 (33.84-82.15)	p=0.0003*, Kruskal-Wallis test; p=0.0061 control vs non-severe asthma; p= 0.0019 control vs severe asthma Dunn's multiple comparisons test

*statistically significant (p<0.01); Kruskal-Wallis and Dunn's multiple comparisons tests; 5-HT = serotonin; IQR = interquartile range (25^{th} to 75^{th} percentile); MAO-B = monoamine oxidase B.

5.2.3. The association of platelet 5-HT concentration and platelet MAO-B activity with clinical characteristics of asthma patients

The association of platelet 5-HT concentration and platelet MAO-B activity with clinical characteristics in asthma patients was analysed (Table 7). A positive correlation (p=0.02, r=0.21) was observed between platelet MAO-B activity and the number of blood neutrophils in asthma patients (Table 7), although this result was not significant after correcting for multiple testing. On the other hand, asthma patients with pollen allergy (46.68 nmol/mg of protein/h, IQR: 38.70-59.84) had nominally higher platelet MAO-B activity (p=0.02), as shown in Table 7, in comparison to asthma patients without pollen allergy (40.92 nmol/mg of protein/h, IQR: 31.99-56.73). There were no associations of other clinical parameters with platelet 5-HT concentration or platelet MAO-B activity of asthma patients.

Table 7. The association of platelet 5-HT concentration and platelet MAO-B activity with clinical characteristics of asthma patients

Clinical characteristics	Platelet 5-HT concentration (nmol/mg of protein)	Platelet MAO-B activity (nmol/mg of protein/h)	Statistical test
Total Serum IgE (IU / mL)	*p*=0.52, r=0.06	*p*=0.66, r=0.04	***Spearman correlation***
Blood eosinophils (x 10^9 / L)	*p*=0.63, r=0.04	*p*=0.39, r=-0.08	
Blood neutrophils (x 10^9 / L)	*p*=0.52, r=0.06	*p*=0.02, r=0.21	
FeNO (ppb)	*p*=0.99, r=0.001	*p*=0.70, r=-0.04	
FEV_1 (% of predicted value)	*p*=0.92, r=0.01	*p*=0.60, r=0.05	
FVC (% of predicted value)	*p*=0.28, r=0.10	*p*=0.21, r=0.11	
PEF (% of predicted value)	*p*=0.20, r=-0.12	*p*=0.73, r=-0.03	
DLCO (%)	*p*=0.20, r=-0.12	*p*=0.78, r=-0.03	
Duration of disease (years)	*p*=0.99, r=-0.001	*p*=0.63, r=0.04	
Comorbidities (n)	*p*=0.88, r=-0.01	*p*=0.61, r=0.05	
Clinical characteristics	**Platelet 5-HT concentration (nmol/mg of protein)**	**Platelet MAO-B activity (nmol/mg of protein/h)**	
Penicillin allergy	*p*=0.28, U=1018.00	*p*=0.86, U=1159.00	***Mann-Whitney test***
Nutritive allergy	*p*=0.69, U=602.0	*p*=0.43, U=556.00	
Animal dander / feather allergy	*p*=0.71, U=866.50	*p*=0.08, U=678.00	
Dust allergy	*p*=0.66, U=1676.00	*p*=0.12, U=1469.00	
Pollen allergy	*p*=0.30, U=1578.00	*p*=0.02, U=1345.00	
Fungal / mould allergy	*p*=0.34, U=401.50	*p*=0.86, U=481.50	
Early onset asthma (age < 12 years)	*p*=0.99, U=1039.00	*p*=0.90, U=1021.00	
History of pneumonia	*p*=0.77, U=997.5	*p*=0.57, U=957.5	
Emergency intervention (ever)	*p*=0.82, U=1341.00	*p*=0.55, U=1280.00	
Hospitalisation for asthma (ever)	*p*=0.16, U=1529.00	*p*=0.73, U=1732.00	
Nasal polyps	*p*=0.38, U=1104.00	*p*=0.62, U=1164.00	
Aspirin sensitivity	*p*=0.27, U=472.50	*p*=0.90, U=580.00	
Allergen specific immunotherapy	*p*=0.89, U=770.50	*p*=0.71, U=740.00	
Oral corticosteroid therapy	*p*=0.59, U=1262.00	*p*=0.09, U=1067.00	
Biological therapy	*p*=0.08, U=751.50	*p*=0.33, U=860.50	

DLCO = diffusing capacity of lung for carbon monoxide; FeNO = fractional exhaled nitric oxide; FEV_1 = forced expiratory volume in one second; FVC = forced vital capacity; 5-HT = serotonin; IgE = immunoglobulin E; IU = international units; MAO-B = monoamine oxidase B; n = total number of observations in the data; PEF = peak expiratory flow; ppb = parts per billion.

5.2.4. Platelet 5-HT concentration and platelet MAO-B activity according to asthma phenotype

In addition, we investigated the platelet 5-HT concentration and platelet MAO-B activity in various asthma phenotypes. As noted in Table 8, we found no statistically significant differences in the platelet 5-HT concentration between asthma patients with T2-high and T2-low (p=0.73), non-allergic and allergic (p=0.77), eosinophilic versus non-eosinophilic asthma (p=0.39) phenotypes, or patients with AERD and those without AERD (p=0.42). Platelet MAO-B activity was not significantly different between T2-high and T2-low (p=0.60), eosinophilic versus non-eosinophilic asthma (p=0.57) phenotypes, or patients with and without AERD (p=0.71). However, platelet MAO-B activity was nominally significantly lower in patients with non-allergic asthma in comparison to patients with allergic asthma (p=0.047).

Table 8. Platelet 5-HT concentration and platelet MAO-B activity in patients with different asthma phenotypes

Asthma phenotypes	Platelet 5-HT concentration (nmol/mg of protein), Median (IQR: 25%-75%)	Statistical Analysis (Mann-Whitney test)	Platelet MAO-B activity (nmol/mg of protein/h), Median (IQR: 25%-75%)	Statistical Analysis (Mann-Whitney test)
T2-high (n=94)	0.78 (0.62-1.09)	p=0.73, U=1168.00	42.98 (35.30-58.48)	p=0.60, U=1140.00
T2-low (n=26)	0.91 (0.60-1.09)		44.27 (31.81-58.98)	
Non-allergic (n=42)	0.85 (0.62-1.18)	p=0.77, U=1585.00	37.79 (31.67-57.17)	p=0.047, U=1278.00
Allergic (n=78)	0.77 (0.62-1.08)		44.87 (36.73-58.60)	
Non-eosinophilic (n=73)	0.81 (0.62-1.05)	p=0.39, U=1554.00	41.91 (33.63-59.14)	p=0.57, U=1609.00
Eosinophilic (n=47)	0.86 (0.64-1.19)		44.61 (35.32-57.49)	
Non-AERD (n=111)	0.81 (0.62-1.08)	p=0.42, U=413.50	43.15 (34.07-58.60)	p=0.71, U=457.00
AERD (n=9)	0.82 (0.69-1.31)		36.50 (33.93-57.21)	

AERD = aspirin-exacerbated respiratory disease; 5-HT = serotonin; IQR = interquartile range (25^{th} to 75^{th} percentile); MAO-B = monoamine oxidase B; T2 = type 2.

The comparison with healthy control subjects revealed that platelet 5-HT levels in patients with allergic asthma were significantly lower than in healthy controls (p=0.0059) (Dunn's multiple comparisons test following Kruskal-Wallis ANOVA test). In contrast, there were no significant differences in platelet 5-HT levels between non-allergic asthma patients and healthy control subjects (p=0.0699) (Dunn's multiple comparisons test following Kruskal-Wallis ANOVA test).

5.3. Plasma BDNF concentration, *BDNF* Val66Met (rs6265) and *NTRK2* (*TrkB* gene) rs1439050 polymorphisms

5.3.1. Plasma BDNF concentration in the study population

Asthma patients had significantly higher (p<0.0001) plasma BDNF concentrations (0.89 pg/mL, IQR: 0.59-1.22) than healthy controls (0.59 pg/mL, IQR: 0.45-0.92), as illustrated in Figure 8 and Table 9.

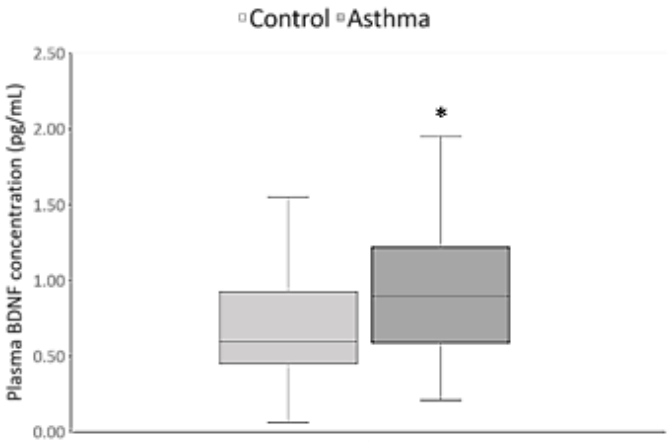

Figure 8. Plasma BDNF concentration (pg/mL) in healthy subjects (n=120) compared to asthma patients (n=120). The central horizontal line inside each box of the box-and-whisker plot represents the median; the lower and upper ends of the boxes are the 25th and 75th percentiles, respectively; the error bars are the minimum and maximum values.
*statistically significant (p<0.01); p<0.0001, U=4902.00, Mann-Whitney test

Table 9. Plasma BDNF concentration in healthy subjects and asthma patients

Parameter	Healthy Subjects (n=120)	Asthma Patients (n=120)	Statistical Analysis
Plasma BDNF Concentration (pmol/mL) Median (IQR: 25%-75%)	0.59 (0.45-0.92)	0.89 (0.59-1.22)	p<0.0001*, U=4902.00, Mann-Whitney test

*statistically significant (p<0.01); BDNF = brain-derived neurotrophic factor; IQR = interquartile range (25th to 75th percentile).

No significant correlation was found between plasma BDNF levels and age in asthma patients (p=0.49, r=0.06) or control group (p=0.84, r=-0.02). The concentration of BDNF in plasma was not significantly correlated with the BMI of asthma patients (p=0.17, r=0.13) or healthy

subjects (p=0.54, r=0.06). In addition, plasma BDNF levels did not differ significantly between smokers and non-smokers in the control (p=0.23) or asthma (p=0.07) groups. When the asthma patients and control subjects were subdivided according to gender, no significant differences were observed in plasma BDNF levels between male and female asthma patients (p=0.67). Healthy males, however, had nominally higher BDNF concentrations in plasma compared to healthy females (p=0.04), but this difference was not significant after applying the correction for multiple testing.

5.3.2. The distribution of *BDNF* Val66Met (rs6265) genetic variants in the study population

The *BDNF* Val66Met (rs6265) genotype distributions were in HWE for both the control group (p=0.38) and asthma patients (p=0.26). The *BDNF* Val66Met genotype (p=0.54) and allele (p=0.35) frequencies did not differ significantly between healthy subjects and asthma patients (Table 10). A comparison of the carriers of GG homogenous genotype and A allele carriers revealed no significant difference in their distribution (p=0.34) between healthy individuals and asthma patients. Moreover, the G versus AA carrier frequencies were not significantly different (p=1.00) between these two groups (Table 10).

Table 10. Distribution of genotypes, alleles and carriers of *BDNF* Val66Met polymorphism in healthy subjects and asthma patients

***BDNF* rs6265 Polymorphism**		**Healthy Subjects, n (%)**	**Asthma Patients, n (%)**	**Statistical Analysis**
Genotypes	**AA**	2 (1.67)	3 (2.50)	p=0.54, $\chi2$=1.23, $\chi2$ test
	AG	36 (30.00)	43 (35.83)	
	GG	82 (68.33)	74 (61.67)	
Alleles	**A**	40 (16.67)	49 (20.42)	p=0.35, Fisher's exact test
	G	200 (83.33)	191 (79.58)	
Carriers	**A**	38 (31.67)	46 (38.33)	p=0.34, Fisher's exact test
	GG	82 (68.33)	74 (61.67)	
	G	118 (98.33)	117 (97.50)	p=1.00, Fisher's exact test
	AA	2 (1.67)	3 (2.50)	

BDNF = brain-derived neurotrophic factor; n = total number of observations in the data; Val66Met = valine (Val) to methionine (Met) substitution at codon 66.

5.3.3. Plasma BDNF concentration in subjects carrying different *BDNF* Val66Met genotypes and alleles

When the asthma patients and healthy individuals were subdivided according to *BDNF* Val66Met genotype, a difference in the plasma BDNF concentrations of the control subjects was observed (Table 11). Healthy carriers of the AG genotype were found to have nominally lower BDNF concentrations (p=0.03) than healthy carriers of the GG genotype (Table 11). Moreover, plasma BDNF concentrations in the control group were significantly decreased (p=0.008) among carriers of the A allele when compared to GG carriers. There was no significant difference in the plasma BDNF concentrations (p=0.64), however, between the healthy carriers of the G allele and the AA genotype carriers.

Table 11. Plasma BDNF concentration in asthma patients (n=120) and healthy subjects (n=120) carrying different *BDNF* Val66Met genotypes and alleles

***BDNF* rs6265 Polymorphism**		**Healthy Subjects**		**Asthma Patients**	
		Plasma BDNF Concentration (pg/mL), Median (IQR: 25%-75%)	**Statistical Analysis**	**Plasma BDNF Concentration (pg/mL), Median (IQR: 25%-75%)**	**Statistical Analysis**
Genotypes	**AA**	0.56 (0.47-0.65)	p=0.03, Kruskal-Wallis test†	0.45 (0.43-1.24)	p=0.52, Kruskal-Wallis test
	AG	0.51 (0.32-0.73)		0.81 (0.52-1.35)	
	GG	0.68 (0.49-1.05)		0.94 (0.62-0.94)	
Carriers	**A**	0.51 (0.35-0.70)	p=0.008*, U=1089.00, Mann-Whitney test	0.80 (0.46-1.34)	p=0.39, U=1544.00, Mann-Whitney test
	GG	0.68 (0.49-1.05)		0.94 (0.62-1.20)	
	G	0.59 (0.45-0.93)	p=0.64, U=94.50, Mann-Whitney test	0.91 (0.61-1.22)	p=0.64, U=94.50, Mann-Whitney test
	AA	0.56 (0.47-0.65)		0.45 (0.43-1.24)	

*statistically significant (p<0.01); †AG vs. GG, p=0.03, Dunn's multiple comparisons test; BDNF = brain-derived neurotrophic factor; IQR = interquartile range (25^{th} to 75^{th} percentile); n = total number of observations in the data; Val66Met = valine (Val) to methionine (Met) substitution at codon 66.

As demonstrated in Table 11, no significant differences in plasma BDNF concentrations (p=0.52) were noted among asthma patients split into groups based on *BDNF* Val66Met genotype. In addition, plasma BDNF concentrations did not differ between asthma patients carrying the A allele and GG genotype (p=0.39), or between asthmatic carriers of the G allele and AA genotype (p=0.64).

5.3.4. The distribution of *NTRK2* (*TrkB*) rs1439050 genetic variants in the study population

As was the case for the *BDNF* Val66Met polymorphism, the *NTRK2* rs1439050 genotype distributions were also in HWE for both the control group (p=0.34) and the individuals with asthma (p=0.40). The healthy participants and asthma patients did not differ significantly in their *NTRK2* rs1439050 genotype (p=0.86) or allele (p=0.68) frequencies (Table 12). The G versus TT carrier frequencies did not differ significantly (p=1.00) between these groups. There were also no significant differences (p=0.70) in the distribution of GG genotype or T allele carriers between healthy individuals and those with asthma (Table 12).

Table 12. Distribution of genotypes, alleles and carriers of *NTRK2* rs1439050 polymorphism in asthma patients and healthy subjects

***NTRK2* rs1439050 Polymorphism**		**Healthy Subjects, n (%)**	**Asthma Patients, n (%)**	**Statistical Analysis**
Genotypes	**GG**	61 (50.83)	65 (54.17)	p=0.86, $\chi2$=0.29, $\chi2$ test
	GT	52 (43.33)	49 (40.83)	
	TT	7 (5.83)	6 (5.00)	
Alleles	**G**	174 (72.50)	179 (74.58)	p=0.68, Fisher's exact test
	T	66 (27.50)	61 (25.42)	
Carriers	**G**	113 (94.17)	114 (95.00)	p=1.00, Fisher's exact test
	TT	7 (5.83)	6 (5.00)	
	T	59 (49.17)	55 (45.83)	p=0.70, Fisher's exact test
	GG	61 (50.83)	65 (54.17)	

n = total number of observations in the data; NTRK2 = neurotrophic receptor tyrosine kinase 2 (tropomyosin receptor kinase B or TrkB).

5.3.5. Plasma BDNF concentration in subjects carrying different *NTRK2* rs1439050 genotypes and alleles

The distribution of rs1439050 polymorphic variants, located within the *NTRK2* gene encoding the TrkB receptor, was explored to determine their influence, if any, on the plasma concentration of unbound BDNF protein in the plasma. As presented in Table 13, there were no significant

differences in plasma BDNF levels of healthy subjects (p=0.42) or asthma patients (p=0.13) split into groups based on different *NTRK2* rs1439050 genotypes. There were also no significant differences in the plasma BDNF levels between healthy individuals (p=0.52) or asthma patients (p=0.23) carrying the G allele and TT genotype, or between healthy (p=0.36) or asthmatic carriers (p=0.21) of T allele and GG homozygous genotype (Table 13).

Table 13. Plasma BDNF concentration in healthy subjects and asthma patients carrying different *NTRK2* rs1439050 genotypes and alleles

***NTRK2* rs1439050 Polymorphism**		**Healthy Subjects**		**Asthma Patients**	
		Plasma BDNF Concentration (pg/mL), Median (IQR: 25%-75%)	**Statistical Analysis**	**Plasma BDNF Concentration (pg/mL), Median (IQR: 25%-75%)**	**Statistical Analysis**
Genotypes	**GG**	0.59 (0.49-0.94)	p=0.42, Kruskal-Wallis test	0.81 (0.59-1.15)	p=0.13, Kruskal-Wallis test
	GT	0.59 (0.39-0.84)		0.97 (0.63-1.37)	
	TT	0.65 (0.30-1.48)		0.51 (0.41-1.37)	
Carriers	**G**	0.59 (0.45-0.90)	p=0.52, U=336.50, Mann-Whitney test	0.91 (0.61-1.22)	p=0.23, U=241.50, Mann-Whitney test
	TT	0.65 (0.30-1.48)		0.51 (0.41-1.37)	
	T	0.60 (0.39-0.88)	p=0.36, U=1623.00, Mann-Whitney test	0.97 (0.58-1.37)	p=0.21, U=1550.00, Mann-Whitney test
	GG	0.59 (0.49-0.94)		0.81 (0.59-1.15)	

BDNF = brain-derived neurotrophic factor; IQR = interquartile range (25^{th} to 75^{th} percentile); NTRK2 = neurotrophic receptor tyrosine kinase 2 (tropomyosin receptor kinase B or TrkB).

5.3.6. Plasma BDNF concentration and asthma severity

The relationship between plasma BDNF concentration and asthma severity was also investigated. Figure 9 demonstrates that plasma BDNF levels did not differ significantly between the non-severe and severe asthma patients (p=0.71, U=1728.00, Mann-Whitney test).

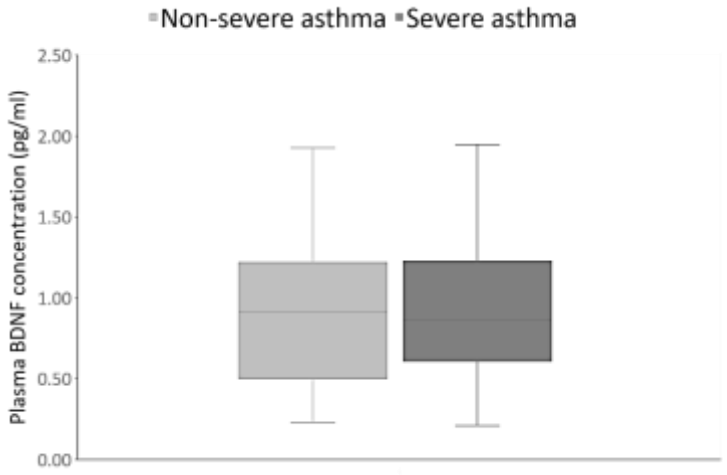

Figure 9. Plasma BDNF concentration (pg/mL) in non-severe asthma patients (n=59) compared to severe asthma patients (n=61). The central horizontal line inside each box and-whisker plot represents the median; the lower and upper ends of the boxes are the 25th and 75th percentiles, respectively; the error bars are the minimum and maximum values.

This finding was confirmed by further analysis with Kruskal-Wallis test followed by Dunn's multiple comparisons test. As shown in Table 14, plasma BDNF concentrations were significantly higher in both non-severe (p=0.005) and severe asthma patients (p=0.0004) compared to the control group.

Table 14. Plasma BDNF concentration in healthy subjects and asthma patients stratified according to severity into non-severe (mild-to-moderate) and severe asthma patients

Parameter	**Healthy Subjects (n=120)**	**Non-severe Asthma Patients (n=59)**	**Severe Asthma Patients (n=61)**	**Statistical Analysis**
Plasma BDNF Concentration (pmol/mL) Median (IQR: 25%-75%)	0.59 (0.45-0.92)	0.91 (0.50-1.22)	0.88 (0.61-1.29)	p<0.0001* Kruskal-Wallis test; p=0.005 non-severe asthma vs control; p=0.0004 severe asthma vs control, Dunn's multiple comparisons test

*statistically significant (p<0.01); BDNF = brain-derived neurotrophic factor; IQR = interquartile range (25th to 75th percentile).

5.3.7. *BDNF* Val66Met polymorphism (rs6265) and asthma severity

Non-severe and severe asthma patients did not significantly differ in their *BDNF* Val66Met genotype (p=0.13) or allele (p=0.11) frequencies (Table 15). As shown in Table 15, no significant differences based on asthma severity (non-severe versus severe) were observed in the distribution of the carriers of the GG genotype or the A allele (p=0.19), or in the frequencies of G allele or AA genotype carriers (p=0.24).

Table 15. Distribution of genotypes, alleles and carriers of *BDNF* Val66Met polymorphism in patients with non-severe and severe asthma

***BDNF* rs6265 Polymorphism**		**Non-Severe Asthma Patients, n (%)**	**Severe Asthma Patients, n (%)**	**Statistical Analysis**
Genotypes	**AA**	0 (0.00)	3 (4.92)	p=0.13,
	AG	19 (32.20)	24 (39.34)	$\chi 2$=4.04,
	GG	40 (67.80)	34 (55.74)	$\chi 2$ test
Alleles	**A**	19 (16.10)	30 (24.59)	p=0.11,
	G	99 (73.90)	92 (75.41)	Fisher's exact test
Carriers	**A**	19 (32.20)	27 (44.26)	p=0.19,
	GG	40 (67.80)	34 (55.74)	Fisher's exact test
	G	59 (100.00)	58 (95.08)	p=0.24,
	AA	0 (0.00)	3 (4.92)	Fisher's exact test

BDNF = brain-derived neurotrophic factor; n = total number of observations in the data.

5.3.8. *NTRK2* (*TrkB*) rs1439050 polymorphism and asthma severity

There were also no significant differences found in the *NTRK2* rs1439050 genotype (p=0.56) or allele (p=1.00) frequencies between the non-severe and severe asthma patients (Table 16). As demonstrated in Table 16, no significant differences between the non-severe and severe asthma patients were detected in the distribution of the carriers of the TT genotype or the G allele (p=0.43), or in the frequencies of T allele or GG genotype carriers (p=0.72).

Table 16. Distribution of genotypes, alleles and carriers of *NTRK2* rs1439050 polymorphism in non-severe (n=59) and severe (n=61) asthma patients

***NTRK2* rs1439050 Polymorphism**		**Non-Severe Asthma Patients, n (%)**	**Severe Asthma Patients, n (%)**	**Statistical Analysis**
Genotypes	**GG**	33 (55.93)	32 (52.46)	p=0.56,
	GT	22 (37.29)	27 (44.26)	χ^2=1.16,
	TT	4 (6.78)	2 (3.28)	χ^2 test
Alleles	**G**	88 (74.58)	91 (74.59)	p=1.00,
	T	30 (25.42)	31 (25.41)	Fisher's exact test
Carriers	**G**	55 (93.22)	59 (96.72)	p=0.43,
	TT	4 (6.78)	2 (3.28)	Fisher's exact test
	T	26 (44.07)	29 (47.54)	p=0.72,
	GG	33 (55.93)	32 (52.46)	Fisher's exact test

n = total number of observations in the data; NTRK2 = neurotrophic receptor tyrosine kinase 2 (tropomyosin receptor kinase B or TrkB).

5.3.9. The association of plasma BDNF concentrations with clinical characteristics of asthma patients

The analysis of the association between plasma BDNF concentrations and clinical characteristics of asthma patients revealed a positive correlation (p=0.023, r=0.21) between plasma BDNF concentration and duration of disease in asthma patients (Table 17), although this result was not significant after correcting for multiple testing. On the other hand, asthma patients with a history of pneumonia (0.69 pg/mL, IQR: 0.48-0.92) had nominally lower plasma BDNF concentrations (p=0.026) in comparison to asthma patients who had previously never developed pneumonia (0.95 pg/mL, IQR: 0.61-1.29). Plasma BDNF concentrations were nominally higher (p=0.025) in asthma patients with nasal polyps (1.12 pg/mL, IQR: 0.72-1.35) versus those without (0.81 pg/mL, IQR: 0.58-1.15). Furthermore, asthma patients with sensitivity to aspirin (1.22 pg/mL, IQR: 0.93-1.77) had significantly higher BDNF levels in plasma (p=0.009) than asthma patients lacking aspirin sensitivity (0.84 pg/mL, IQR: 0.57-1.18) (Table 17).

Table 17. The association of plasma BDNF concentration with clinical characteristics in asthma patients

Clinical Characteristics	Plasma BDNF Concentration (pg/mL)	Statistical test
Total Serum IgE (IU/mL)	*p*=0.50, r=-0.06	***Spearman correlation***
Blood eosinophils (×10^9/L)	*p*=0.69, r=0.04	
Blood neutrophils (×10^9/L)	*p*=0.92, r=-0.01	
FeNO (ppb)	*p*=0.98, r=-0.002	
FEV$_1$ (% of predicted value)	*p*=0.41, r=0.07	
FVC (% of predicted value)	*p*=0.65, r=-0.04	
PEF (% of predicted value)	*p*=0.58, r=-0.05	
DLCO (%)	*p*=0.055, r=-0.17	
Duration of disease (years)	*p*=0.023, r=0.21	
Comorbidities (n)	*p*=0.35, r=0.08	
Clinical characteristics	**Plasma BDNF Concentration (pg/mL)**	
Penicillin allergy	*p*=0.18, U=977.50	***Mann-Whitney test***
Nutritive allergy	*p*=0.10, U=458.50	
Animal dander / feather allergy	*p*=0.31, U=779.00	
Dust allergy	*p*=0.06, U=1408.00	
Pollen allergy	*p*=0.72, U=1709.00	
Fungal / mould allergy	*p*=0.28, U=390.50	
Early onset asthma (age <12 years)	*p*=0.12, U=817.00	
History of pneumonia	*p*=0.026, U=718.00	
Emergency intervention (ever)	*p*=0.53, U=1274.00	
Hospitalization for asthma (ever)	*p*=0.70, U=1724.00	
Nasal polyps	*p*=0.025, U=891.00	
Aspirin sensitivity	*p*=0.009*, U=314.00	
Allergen specific immunotherapy	*p*=0.99, U=787.00	
Oral corticosteroid therapy	*p*=0.58, U=1258.00	
Biological therapy	*p*=0.80, U=962.50	

*statistically significant (*p*<0.01); Mann-Whitney test.

BDNF = brain-derived neurotrophic factor; DLCO = diffusing capacity of lung for carbon monoxide; FeNO = fractional exhaled nitric oxide; FEV1 = forced expiratory volume in one second; FVC = forced vital capacity; IgE = immunoglobulin E; IQR = interquartile range (25th to 75th percentile); IU = international units; n = total number of observations in the data; PEF = peak expiratory flow; ppb = parts per billion.

5.3.10. Plasma BDNF concentration according to asthma phenotype

In addition, we investigated the plasma BDNF concentrations in various asthma phenotypes. As shown in Table 18, we found no differences in the plasma BDNF concentrations between asthma patients with T2-high and T2-low (*p*=0.43), non-allergic and allergic (*p*=0.41), or eosinophilic versus non-eosinophilic asthma (*p*=0.54) phenotypes. However, patients with

AERD demonstrated nominally higher plasma BDNF concentrations (p=0.017) than those without AERD (Table 18).

Table 18. Plasma BDNF concentration in patients with different asthma phenotypes

Asthma Phenotypes	Plasma BDNF Concentration (pg/mL), Median (IQR: 25%-75%)	Statistical Analysis
T2-high (n=94)	0.91 (0.60-1.30)	p=0.43, U=1097.00, Mann-Whitney test
T2-low (n=26)	0.82 (0.42-1.14)	
Non-allergic (n=42)	0.98 (0.60-1.29)	p=0.41, U=1487.00, Mann-Whitney test
Allergic (n=78)	0.82 (0.58-1.20)	
Non-eosinophilic (n=73)	0.92 (0.54-1.20)	p=0.54, U=1602.00, Mann-Whitney test
Eosinophilic (n=47)	0.88 (0.61-1.34)	
Non-AERD (n=111)	0.85 (0.58-1.18)	p=0.017, U=262.00, Mann-Whitney test
AERD (n=9)	1.22 (0.92-2.05)	

AERD = aspirin-exacerbated respiratory disease; BDNF = brain-derived neurotrophic factor; IQR = interquartile range (25^{th} to 75^{th} percentile); n = total number of observations in the data; T2 = type 2.

5.3.11. *BDNF* Val66Met polymorphism (rs6265) and asthma phenotype

As shown in Table 19, there were no significant differences in the distribution of the *BDNF* Val66Met genotypes (p=0.50), alleles (p=0.44), GG vs. A allele carriers (p=0.49), or AA vs. G carriers (p=1.00) between asthma patients with T2-high and T2-low phenotypes. In addition, the frequencies of *BDNF* Val66Met genotypes (p=0.27), alleles (p=0.18), GG vs. A allele carriers (p=0.24), and AA vs. G carriers (p=0.55) were not significantly different between non-allergic and allergic asthma patients (Table 19). Moreover, we observed no significant differences in the frequencies of *BDNF* Val66Met genotypes (p=0.60), alleles (p=0.62), GG vs. A allele carriers (p=0.71), or AA vs. G carriers (p=0.56), between patients with and without eosinophilic asthma. There were also no significant differences in the distribution of the *BDNF* Val66Met genotypes (p=0.86), alleles (p=1.00), GG vs. A allele carriers (p=1.00), or AA vs. G carriers (p=1.00), between asthma patients with and without AERD (Table 19).

Table 19. Distribution of genotypes, alleles and carriers of *BDNF* Val66Met polymorphism in patients with different asthma phenotypes

***BDNF* rs6265**		**T2-high, n (%)**	**T2-low, n (%)**	**Statistical Analysis**
Genotypes	**AA**	3 (3.19)	0 (0.00)	p=0.50, $\chi2$=1.37, $\chi2$ test
	AG	35 (37.23)	8 (30.77)	
	GG	56 (59.57)	18 (69.23)	
Alleles	**A**	41 (21.81)	8 (15.38)	p=0.44, Fisher's exact test
	G	147 (78.19)	44 (84.62)	
Carriers	**A**	38 (40.42)	8 (44.26)	p=0.49, Fisher's exact test
	GG	56 (59.58)	18 (55.74)	
	G	91(96.81)	26 (95.08)	p=1.00, Fisher's exact test
	AA	3 (3.19)	0 (0.00)	
***BDNF* rs6265**		**Non-allergic, n (%)**	**Allergic, n (%)**	**Statistical Analysis**
Genotypes	**AA**	0 (0.00)	3 (3.85)	p=0.27, $\chi2$=2.62, $\chi2$ test
	AG	13 (30.95)	30 (38.46)	
	GG	29 (69.05)	45 (57.69)	
Alleles	**A**	13 (15.48)	36 (23.08)	p=0.18, Fisher's exact test
	G	71 (84.52)	120 (76.92)	
Carriers	**A**	13 (30.95)	33 (42.31)	p=0.24, Fisher's exact test
	GG	29 (69.05)	45 (57.69)	
	G	44 (100.00)	75 (96.15)	p=0.55, Fisher's exact test
	AA	0 (0.00)	3 (3.85)	
***BDNF* rs6265**		**Non-eosinophilic, n (%)**	**Eosinophilic, n (%)**	**Statistical Analysis**
Genotypes	**AA**	1 (1.37)	2 (4.26)	p=0.60, $\chi2$=1.01, $\chi2$ test
	AG	26 (35.62)	17 (36.17)	
	GG	46 (63.01)	28 (59.57)	
Alleles	**A**	28 (19.18)	21 (22.34)	p=0.62, Fisher's exact test
	G	118 (80.82)	73 (77.66)	
Carriers	**A**	27 (36.99)	19 (40.43)	p=0.71, Fisher's exact test
	GG	46 (63.01)	28 (59.57)	
	G	72 (98.63)	45 (95.74)	p=0.56, Fisher's exact test
	AA	1 (1.37)	2 (4.26)	
***BDNF* rs6265**		**Non-AERD, n (%)**	**AERD, n (%)**	**Statistical Analysis**
Genotypes	**AA**	3 (2.70)	0 (0.00)	p=0.86, $\chi2$=0.30, $\chi2$ test
	AG	40 (36.04)	3 (33.33)	
	GG	68 (61.26)	6 (66.67)	
Alleles	**A**	46 (20.72)	3 (16.67)	p=1.00, Fisher's exact test
	G	176 (79.28)	15 (83.33)	
Carriers	**A**	43 (38.74)	3 (33.33)	p=1.00, Fisher's exact test
	GG	68 (61.26)	6 (66.67)	
	G	108 (97.30)	9 (100.00)	p=1.00, Fisher's exact test
	AA	3 (2.70)	0 (0.00)	

AERD = aspirin-exacerbated respiratory disease; BDNF = brain-derived neurotrophic factor; n = total number of observations in the data; T2 = type 2.

5.3.12. *NTRK2* (*TrkB*) rs1439050 polymorphism and asthma phenotype

The distribution of the *NTRK2* rs1439050 genotypes (p=0.14), alleles (p=0.07), GG vs. T allele carriers (p=0.55), and TT vs. G carriers (p=0.34) was not significantly different between asthma patients with T2-high and T2-low phenotypes (Table 20). Moreover, we observed no significant differences in the frequencies of *NTRK2* rs1439050 genotypes (p=0.09), alleles (p=0.06), GG vs. T allele carriers (p=0.13), or TT vs. G carriers (p=0.09) between non-allergic and allergic asthma patients. As demonstrated in Table 20, the frequencies of *NTRK2* rs1439050 genotypes (p=0.78), alleles (p=0.76), GG vs. T allele carriers (p=0.58), and TT vs. G carriers (p=1.00) were not significantly different between patients with and without eosinophilic asthma. In addition, asthma patients with and without AERD did not differ in their distribution of the *NTRK2* rs1439050 genotypes (p=0.77), alleles (p=1.00), GG vs. T allele carriers (p=1.00), or TT vs. G carriers (p=1.00) (Table 20).

Table 20. Distribution of genotypes, alleles and carriers of *NTRK2* rs1439050 polymorphism in patients with different asthma phenotypes

NTRK2 rs1439050		**T2-high, n (%)**	**T2-low, n (%)**	**Statistical Analysis**
Genotypes	**GG**	47 (50.00)	18 (69.23)	p=0.14,
	GT	41 (43.62)	8 (30.77)	$\chi2$=3.87,
	TT	6 (6.38)	0 (0.00)	$\chi2$ test
Alleles	**G**	135 (71.81)	44 (84.61)	p=0.07,
	T	53 (28.19)	8 (15.39)	Fisher's exact test
Carriers	**GG**	47 (50.00)	18 (69.23)	p=0.55,
	T	47 (50.00)	8 (30.77)	Fisher's exact test
	TT	6 (6.38)	0 (0.00)	p=0.34,
	G	88 (93.62)	26 (100.00)	Fisher's exact test
NTRK2 rs1439050		**Non-allergic, n (%)**	**Allergic, n (%)**	**Statistical Analysis**
Genotypes	**GG**	27 (64.29)	38 (48.72)	p=0.09,
	GT	15 (35.71)	34 (43.59)	$\chi2$=4.87,
	TT	0 (0.00)	6 (7.69)	$\chi2$ test
Alleles	**G**	69 (82.14)	110 (70.51)	p=0.06,
	T	15 (17.86)	46 (29.49)	Fisher's exact test
Carriers	**GG**	27 (64.29)	38 (48.72)	p=0.13,
	T	15 (35.71)	40 (51.28)	Fisher's exact test
	TT	0 (0.00)	6 (7.69)	p=0.09,
	G	42 (100.00)	72 (92.31)	Fisher's exact test
NTRK2 rs1439050		**Non-eosinophilic, n (%)**	**Eosinophilic, n (%)**	**Statistical Analysis**
Genotypes	**GG**	41 (56.16)	24 (51.06)	p=0.78,
	GT	28 (38.36)	21 (44.68)	$\chi2$=0.50,
	TT	4 (5.48)	2 (4.26)	$\chi2$ test
Alleles	**G**	110 (75.34)	69 (73.40)	p=0.76,
	T	36 (24.66)	25 (26.60)	Fisher's exact test
Carriers	**GG**	41 (56.16)	24 (51.06)	p=0.58,
	T	32 (43.84)	23 (48.94)	Fisher's exact test
	TT	4 (5.48)	2 (4.25)	p=1.00,
	G	69 (94.52)	45 (95.75)	Fisher's exact test
NTRK2 rs1439050		**Non-AERD, n (%)**	**AERD, n (%)**	**Statistical Analysis**
Genotypes	**GG**	60 (54.05)	5 (55.56)	p=0.77,
	GT	45 (40.54)	4 (44.44)	$\chi2$=0.52,
	TT	6 (5.41)	0 (0.00)	$\chi2$ test
Alleles	**G**	165 (74.32)	14 (77.78)	p=1.00,
	T	57 (25.68)	4 (22.22)	Fisher's exact test
Carriers	**GG**	60 (54.05)	5 (55.56)	p=1.00,
	T	51 (45.95)	4 (44.44)	Fisher's exact test
	TT	6 (5.40)	0 (0.00)	p=1.00,
	G	105 (94.60)	9 (100.00)	Fisher's exact test

AERD = aspirin-exacerbated respiratory disease; n = total number of observations in the data; NTRK2 = neurotrophic receptor tyrosine kinase 2 (tropomyosin receptor kinase B or TrkB); T2 = type 2.

6. DISCUSSION

There is emerging evidence indicating that peripheral 5-HT and BDNF are associated with asthma. The novelty of this study is that it is the first one to investigate the associations of platelet 5-HT concentration, platelet MAO-B activity, plasma BDNF concentration, and the SNPs of the *BDNF* (rs6265 [Val66Met]) and *NTRK2* (rs1439050) genes with adult asthma in an ethnically and racially homogeneous study population (i.e., Croatian Caucasians) of asthma patients and healthy controls.

The research findings are discussed in this section of the dissertation with respect to the general and specific aims of the study.

6.1. General characteristics of the study population

In our study, healthy individuals and asthma patients differed in their age, gender distributions and smoking status. The control subjects were younger than the asthma patients and composed largely of men, a finding that reflects the existing data on volunteer blood donors in Croatia, with a reported median age of 37 to 40 years and a male predominance of 83.9% [437-438]. The asthma group was older and consisted predominantly of females, which is similar to the literature in that after puberty, women continue to be affected by asthma more than men even after the age of 50 [22, 439]. As expected, the observed age difference between the asthma patients and the controls occurred by virtue of the significantly older individuals in the group of severe asthma patients, consistent with the data showing that asthma is more severe in older adults and in patients with longer asthma duration [440].

With respect to smoking prevalence, there were more active smokers among the healthy subjects than asthma patients in our study. This is expected given that asthma patients (30.83% ex-smokers in our study) are more likely in general to quit smoking because of more severe asthma symptoms, in addition to the obvious benefits of smoking cessation in improving lung function [441]. The observed 34.17% of smokers in the control group is consistent with the published data on smoking (37.45%) in the general population of Croatia [442]. On the other hand, the finding of 8.33% of active smokers in the asthma group is in line with the reports of smoking among asthma patients in Brazil and Spain, but not as high as recorded in many other countries [443].

Obesity is a growing global health crisis, with an ever-worsening epidemic of overweight and obese (BMI $\geq$ 30.0 kg/m^2) children and adults in Europe [444-445]. According to the latest

Eurostat data, 53% of the adult population living in the EU in 2019 was considered overweight (BMI ≥ 25.0 kg/m^2; 36% pre-obese and 17% obese) [446]. The highest proportion of overweight adults (65%) was recorded in Croatia and Malta [446]. Obesity is a common comorbidity in asthma, and may represent a unique asthma phenotype/endotype that is associated with more severe disease, unresponsive to conventional controller medications [447-449].

Adults with asthma and healthy subjects in our study had similar BMIs, and were mainly of normal weight (BMI 18.5-24.9 kg/m^2) or non-obese, but 18.33% of healthy individuals and 23.33% of asthma patients were classified as obese (BMI ≥ 30.0 kg/m^2). Nevertheless, the majority of individuals were considered overweight (BMI ≥ 25.0-29.9 kg/m^2; 45.00% of healthy subjects and 37.50% of asthmatics). Previous studies have shown that a higher BMI and body fat are associated with asthma, and obesity is now recognized as a major risk factor for adult asthma in all demographic groups [447, 449-450]. However, a more recent study on resting energy expenditure in asthma revealed no differences in the BMI of adult asthma patients and controls who were in the overweight/obese category based on mean BMI, but the authors point out that the asthma group consisted of only non-severe asthmatics [451]. Most recently, the findings of Zhang and co-authors (2022) suggest the importance of maintaining normal weight during adulthood to prevent asthma in later life [452].

6.2. Platelet 5-HT concentration and platelet MAO-B activity

6.2.1. Age, gender, smoking status, and body mass index (BMI) associations with 5-HT concentration in platelets

Blood platelet 5-HT concentration might serve as a suitable and easily accessible peripheral biomarker of asthma. A number of variables have been suggested to contribute to differences in normal (or reference) 5-HT values in control groups as well as patient groups, including age, gender and methodology (i.e., isolation procedure), among others, but not intraindividual differences over time [236]. In the limited number of published papers on asthma and 5-HT that have been mentioned previously in this dissertation, the parameters of age, gender, smoking, and BMI were neither specifically, nor secondarily, investigated in relation to blood 5-HT levels. Therefore, direct comparisons in this context to studies similar to ours were not possible here. However, indirect comparisons can be made to work in other research fields that have also analyzed these aforementioned parameters with respect to blood 5-HT.

Our finding of no significant correlation between platelet 5-HT concentration and age in adult asthma patients or healthy subjects seems to confirm some studies, but contrast others, albeit in different study populations outside of asthma. Contradictory results regarding the effect of age on platelet 5-HT levels have been reported in the literature by some studies within the context of their various research fields and objectives. Flachaire et al. (1990), for example, found age-related differences in platelet 5-HT concentrations in a healthy population, with elderly subjects having significantly lower levels compared to adults and children, though the samples sizes were rather limited [453]. In contrast, Kumar et al. (1998) reported a significant positive correlation between platelet 5-HT and age in a cohort of women [454]. Nonetheless, a recent study by Sagud et al. (2016) found no age-related associations with platelet 5-HT concentrations in either healthy subjects or depressed patients [432]. Nenadic-Sviglin et al. (2011) also showed no significant correlation between platelet 5-HT concentration and age in alcoholic patients or healthy subjects [455]. Thus, additional research in this area in the asthmatic population, with larger sample sizes, is clearly needed.

There is also no consensus regarding gender-related associations with platelet 5-HT in the literature. Concerning sex-related changes in platelet 5-HT levels, Sagud et al. (2016) reported no association [432]. Flachaire et al. (1990) also reported no significant gender-related differences in platelet 5-HT concentration in each age group (newborns, children, adults, and elderly) [453]. Therefore, our study showing no correlation between gender and platelet 5-HT concentration in asthma patients or healthy individuals is in line with these earlier studies, but in contradiction to others, such as the data from Pivac et al. (2004) that showed sex-related differences in platelet 5-HT concentrations (i.e., higher platelet 5-HT concentration in men than in women) in both healthy subjects and alcoholic patients [456]. Thus, further studies in this area in the asthmatic population, with larger sample sizes, are warranted.

Regarding smoking status, our finding that smokers and non-smokers in both the asthma and control groups had similar platelet 5-HT concentrations, with no statistically significant differences, aligns with the study by Sagud and colleagues (2016) showing no association between smoking and platelet 5-HT concentration in depressed patients or controls as well as with Pivac et al. (2004) demonstrating that smoking status has no effect on platelet 5-HT concentration in healthy individuals or alcoholic subjects [432, 456]. However, other studies have described that platelet 5-HT concentration is significantly affected by smoking in alcohol-dependent subjects [457-458]. Interestingly, Launay et al. (2009) found the platelet 5-

HT content to be similar in smokers and non-smokers, but lower in former smokers, despite the inhibition of MAO-B activity during smoking [459]. On the other hand, Padmavathi et al. (2015) reported decreased platelet 5-HT levels in smokers (in a group of healthy male volunteers) [291]. Therefore, more research in this area on a larger number of asthma cases and controls would help clarify these equivocal results.

From the literature, circulating 5-HT is suggested to be a key factor in the development of obesity [460]. The primary 5-HT receptor through which 5-HT modulates feeding and body weight is the 5-HT 2C receptor (5-HT_{2C} R), which has been a target of weight loss medications [461]. For example, the 5-HT_{2C} R agonist lorcaserin has been used for obesity treatment since 2012 [461]. The precise link between peripheral blood levels of 5-HT and obesity, however, is unclear, with some studies reporting elevated, and others reduced, peripheral 5-HT concentrations in the obese [e.g., 460, 462]. In our study, neither the asthma patients nor the healthy control subjects showed any BMI-related differences in platelet 5-HT concentration. This is in contrast to a study on whole blood 5-HT in healthy adult volunteers that demonstrated a significant negative correlation with BMI [460]. Moreover, Ritze et al. (2016) showed a reduction in serum 5-HT in obese compared with non-obese individuals [463], and Binetti et al. (2020) revealed decreased 5-HT levels in women with morbid obesity [464]. On the other hand, Young et al. (2018) reported higher plasma 5-HT levels in obese than in control subjects [462].

6.2.2. Age, gender, smoking status, and BMI associations with MAO-B activity in platelets

Platelet MAO-B activity also seems to be influenced by various factors, such as age, gender and smoking status [291, 465-467]. In earlier studies, as summarized in Berlin and Anthenelli's (2001) and Nicotra et al.'s (2004) reviews, MAO-B in the human brain appears to exhibit a positive correlation with age [e.g., 468-471], and the highest levels of MAO-B activity have been reported to occur in late adulthood [467]. However, studies examining the effects of age on MAO-B activity in thrombocytes have provided conflicting results [467]. While some have described an increase in platelet MAO-B activity with advancing age [e.g., 465], others have found no age-related differences [e.g., 472]. In line with the lack of effect of age on platelet MAO-B activity in the more recent studies by Pivac et al. (2006) in healthy Caucasian men from Croatia and Coccini et al. (2005) in healthy Caucasian men and women

in Italy, our study also showed no age-related changes in platelet MAO-B activity in men or women among both the asthma and healthy cohorts [466, 473].

MAO-B activity in thrombocytes seems to differ depending on the gender, as evidenced by our study showing that females in both groups (asthma and healthy) had increased platelet MAO-B activity compared to the males. This is consistent with the similar outputs of numerous earlier studies showing that enzyme activity was lower in men [e.g., 465, 472, 474], as reviewed in Berlin and Anthenelli (2001) [467]. More recent studies have produced similar results. For instance, in a study population of various ages and different ethnic backgrounds, Snell et al. (2002) found that females had significantly higher MAO-B activity compared with males, regardless of smoking status [475]. Coccini et al. (2005) also produced a similar trend, with MAO-B activity being significantly higher in healthy women than men [473]. Gender differences might be due to hormonal effects on MAO-B activity and this should be explored further.

It is now well established that smoking reduces MAO-B activity [e.g., 291], and this effect is suggested to be durable, with high MAO-B protein concentration persisting for years after quitting smoking due to increased MAO-B protein synthesis as a potential compensatory response for the decreased enzyme activity brought on by oxygen deprivation [281]. As expected, in our study, the decreased platelet MAO-B activity in cigarette smokers compared to non-smokers (former and never-smokers) in both asthmatics and healthy controls confirms the consistent findings of a number of earlier studies on brain and platelet MAO-B, as reviewed by Berlin and Anthenelli (2001) and Fowler et al. (2003) [290, 467]. In addition, Snell et al. (2002) revealed that platelet MAO-B activity was significantly reduced in smokers compared with non-smokers and ex-smokers in both males and females of different ethnic backgrounds [475]. The same association between enzyme activity of MAO-B and smoking status was later also reported in healthy male Croatian Caucasians [466], and more recently, by the same research team, in subjects with schizophrenia or conduct disorder [476] and post-traumatic stress disorder [477].

Obesity, a chronic metabolic disease associated with a state of increased oxidative stress, and thereby, generation of high amounts of reactive oxygen species (ROS) due to an excess of adipose tissue, has become a global pandemic in both children and adults [278, 478]. The importance of obesity in asthma has been discussed earlier. Despite this, the potential role of MAO as mitochondrial contributor to ROS production in obesity appears to have been

generally neglected over the years [278]. There have been a number of animal studies over the years showing an increased MAO activity in obese mice [479], dogs [480] and pigs [481]. Unfortunately, human studies are lacking and this observation has not yet been verified in humans [279]. With respect to the effects of BMI or obesity on MAO-B activity in thrombocytes, our study showed a negative correlation of platelet MAO-B activity with BMI in healthy subjects, but not asthma patients. Ehrlich et al. (2008) found a similar result in anorexic females, but not healthy control women, showing a significant negative correlation between BMI and platelet MAO-B activity, specifically, low enzyme activity in weight-recovered anorexic patients [482]. Other similar studies on anorexia have confirmed these findings, but given that there are no known studies explicitly focused on BMI and platelet MAO-B activity, we are unable to provide a comprehensive comparison with our results.

6.2.3. Platelet 5-HT concentration and platelet MAO-B activity in asthma patients and healthy control subjects

6.2.3.1. Platelet 5-HT concentration in asthma patients and healthy control subjects

Currently available data have suggested the important function of platelets and their release products, such as 5-HT, in various inflammatory diseases, including asthma [483-485]. Platelets have been found to actively participate in most of the main features of asthma [485]. In addition, it has been shown that 5-HT modulates activities of cells classically involved in asthma by regulating adhesion, migration and cytokine-chemokine production [379]. Research has increasingly implicated the serotonergic system in asthma. A number of studies have supported an association between 5-HT and asthma, but the exact role of 5-HT in asthma remains unclear. Most published studies to date have investigated free 5-HT levels in the serum or plasma of asthma patients [e.g. 259]. To date, despite the fact that platelets contain the majority of blood 5-HT, there is a scarcity of known published reports in the literature focused on clarifying the role of platelet 5-HT concentrations in asthma and its application to identifying novel asthma phenotypes [e.g., 268-269, 486].

We evaluated whether platelet 5-HT levels are associated with asthma and demonstrated that platelet 5-HT concentration in our study was significantly reduced in asthma patients compared to healthy controls. These findings corroborate those of earlier studies on 5-HT in thrombocytes. Malmgren et al. (1978), for example, reported that 5-HT accumulation in platelets from migraine and asthmatic patients, albeit acetylsalicylic acid (aspirin)-induced asthmatics, was substantially reduced in comparison to that in platelets from non-diseased

(i.e., healthy) individuals [486]. In a later study by the same research team, higher amounts of 5-HT were found in the serum and whole blood of asthmatic patients than normal (i.e., healthy) subjects [268]. Similarly, Matkar et al. (1999) observed lower levels of platelet biogenic amines (5-HT and histamine) in asthmatic patients compared to healthy subjects (aged 20-60 years) in a Punjabi study population [269]. Plausible explanations for the decreased concentrations of platelet 5-HT (or, increased levels of serum, plasma or whole blood concentrations of 5-HT) can be found in these earlier studies. Namely, platelets from patients with asthma have been shown to have a reduced capacity to accumulate 5-HT [486] and a disturbed active 5-HT uptake mechanism [487].

6.2.3.2. Platelet MAO-B activity in asthma patients and healthy control subjects

Although the two subtypes of MAOs, A and B, are located throughout the brain, only MAO-B exists in human platelets [488]. Despite differing in their preferred substrate specificity or binding affinity, MAO-A and MAO-B do not possess absolute substrate specificity, and both of them degrade dopamine, tryptamine and tyramine [195]. MAO-A catabolizes primarily 5-HT (with a 120-fold higher affinity than MAO-B), and, to a lesser degree, norepinephrine, whereas MAO-B preferentially degrades β-phenylethylamine and benzylamine [195]. In the absence of one of the MAO isoenzymes, the other can compensate, either partially or fully, by deaminating a certain amount of its nonpreferred biogenic amine substrates [195, 489-491]. Additionally, MAO-A and MAO-B can oxidize each other's preferred substrates when the substrate or enzyme concentration is changed [490, 492-493]. Specifically, it has been suggested that MAO-B might play a subsidiary role in the metabolism of catecholamines when MAO-A activity is deficient [491].

The biogenic amine 5-HT is mainly metabolized via its oxidative deamination [494]. During this process, compounds involved in oxidative stress, such as H_2O_2 and reactive aldehydes, are constantly generated as by-products [495]. MAO-related oxidative stress in brain diseases, cardiovascular and metabolic pathologies as well as cancer and immunologic diseases have been extensively studied [279, 496]. Asthma is a chronic inflammatory disease and it is believed that the enhanced oxidative stress observed in asthma subjects plays a critical role in its pathogenesis [496]. The specific function of MAO-generated oxidative stress and MAO-related inflammation in asthma, however, remains to be fully elucidated.

The association of platelet MAO-B activity with asthma, according to our extensive literature search and as far as we are aware, has yet to be investigated, and therefore, is currently

unknown. Research is needed to discover the contribution of MAO-B to asthma pathophysiology, if any. Platelet MAO-B activity was increased in our asthma patients compared to the healthy subjects. This finding is supported by Matkar et al. (1999) who demonstrated that plasma MAO enzymatic levels were significantly higher in asthma patients than healthy subjects (non-asthmatics) [269]. Moreover, in the work by Zarmouh et al. (2016), *Ferula assafoetida* resin (*aka* stinking assa; family Apiaceae) was found to be a highly potent and selective inhibitor of recombinant human MAO-B [497]. Interestingly, this oleo-gum-resin has been used traditionally for centuries around the world as a culinary spice and a phytomedicine, but in folklore medicine, it is mostly used to treat various diseases, including asthma, GI conditions and neuronal disorders [497-498]. If MAO-B indeed assumes the function of enzyme-catalyzed degradation of 5-HT in human thrombocytes in the absence of MAO-A, then an elevated platelet MAO-B activity in asthma patients could contribute to a decrease in concentration of platelet 5-HT, in comparison with healthy individuals, as observed in our study.

6.2.4. The association of platelet 5-HT concentration and platelet MAO-B activity with asthma severity

6.2.4.1. Platelet 5-HT concentration and asthma severity

We examined whether asthma severity is associated with platelet 5-HT concentration and determined that no such association exists given that our non-severe and severe asthma patients had similar values for 5-HT levels. However, when examining the relationship between the healthy controls and non-severe asthma patients, as well as the healthy controls and severe asthma patients, decreased 5-HT levels were discovered only in the severe asthma patients compared to the healthy subjects. Our findings were indirectly supported by an earlier study by Lechin et al. (1996) who focused on plasma (rather than platelet) 5-HT concentration, in addition to other neuroendocrine factors (norepinephrine, epinephrine, dopamine, and cortisol in plasma) in symptomatic versus asymptomatic asthma patients, ranging in age from 6 to 39 years (average age, 20.5 years) [259]. Lechin et al. (1996) established that increased levels of free 5-HT in plasma occurred during periods of exacerbations of asthma symptoms, with higher levels in symptomatic patients than asymptomatic patients [259]. In addition, there was a positive correlation of plasma 5-HT levels with clinical severity, but a negative one with pulmonary function, in symptomatic asthma patients [259]. In their study, free 5-HT was the only neuroendocrine factor that was

closely associated with these two parameters of asthma severity, which suggested that 5-HT might play an important role in the pathophysiology of acute asthma [259]. Their results support those of Malmgren et al. (1982) and Nemtsov et al. (1994) who had previously reported similar findings in asthma patients [259, 268, 499]. For instance, Nemtsov et al. (1994) recorded increased levels of free 5-HT in the plasma of symptomatic asthma patients [499], whereas in the study by Malmgren et al. (1982), higher amounts of 5-HT were found in the serum and whole blood of asthmatic patients than control (i.e., healthy) subjects, as mentioned earlier [268].

A plausible explanation for the reported differences in 5-HT levels in asthma patients versus healthy control subjects, or severe asthma patients versus control subjects, might be a defective uptake of 5-HT by platelets in asthma, that is, a severe impairment versus slight disturbance in platelet 5-HT uptake in symptomatic versus asymptomatic endogenous asthma patients, respectively, as demonstrated by Malmgren et al. (1982) [268]. Previous exposure to an increased concentration of free 5-HT in the plasma may contribute to the defect in platelet 5-HT uptake [268]. Another possible reason for the decreased platelet 5-HT levels, or increased plasma 5-HT levels, observed in symptomatic asthma patients could be an increased platelet aggregability that is always present in these asthma patients [259]. As reiterated earlier in this work, most of the 5-HT in the peripheral circulation is located in platelets, later to be released from their granules during platelet activation and aggregation. It has been shown that platelet aggregation increases during exacerbation periods in symptomatic asthma patients and decreases during periods of clinical improvement, and therefore, increased free 5-HT levels during asthma exacerbations may be secondary to this enhanced platelet aggregation [259]. In addition, free 5-HT levels have been shown to correlate positively with bronchoconstriction and clinical severity, whereas asthma exacerbations disappear immediately upon administration of tianeptine, a drug that enhances 5-HT uptake and serotonergic axons in the CNS [267].

Lechin and co-authors (2004) outlined some additional evidence for the role of peripheral and CNS mechanisms in the lungs that might contribute to altering 5-HT levels in the blood, thereby worsening asthma symptoms (i.e., increased clinical severity), and at the same time, explaining the therapeutic benefits of tianeptine in asthma. For instance, increased plasma 5-HT levels in asthmatics is not only triggered by platelet aggregation, but also nocturnal and/or diurnal hyperparasympathetic activity [267]. In addition, pulmonary neuroendocrine cells

located at parasympathetic terminals (i.e., presynaptic cells) release 5-HT, potentiating acetylcholine-induced contraction of bronchial muscle (mediated by 5-HT_3 and 5-HT_4 postsynaptic receptors on the bronchial muscle) [267]. Also, 5-HT released by medullary serotonergic axons stimulates the medullary vagal cardiorespiratory neurons, suggesting the involvement of 5-HT-mediated CNS mechanisms [267]. Moreover, drugs that interfere with 5-HT uptake (e.g., buspirone, with its main neuropharmacologic effect mediated via the 5-HT_{1A} receptors), by both platelets and 5-HT-terminals, can exacerbate existing asthma symptoms and trigger asthma attacks [267, 500].

6.2.4.2. Platelet MAO-B activity and asthma severity

Asthma severity was not associated with platelet MAO-B activity in our study. Non-severe and severe asthma patients had similar levels of platelet MAO-B activity. Furthermore, there was an increase in platelet MAO-B activity in the non-severe and severe asthma patients when each of them was compared to the healthy subjects. Unfortunately, no studies to date have been published on this topic in the literature; hence, comparisons could not be made with regards to our findings.

6.2.5. The association of platelet 5-HT concentration and platelet MAO-B activity with neutrophils in asthma

Platelets and neutrophils interact and modulate each other's functions during infection, inflammation and thrombosis [501]. There is accumulating experimental and clinical evidence for a role of platelet-neutrophil crosstalk in these clinical conditions [501]. Inflammatory responses have been shown to depend on this platelet-neutrophil interplay to help localize platelets to inflammatory sites, potentiate production of oxygen radicals and promote neutrophil activation [502]. In our study, blood neutrophil count was positively correlated with platelet MAO-B activity in asthma patients, though this was not a statistically significant finding after correcting for multiple testing. The interpretation for this potential correlation may lie in the close interconnection between neutrophils and platelets, as explained.

Blood neutrophil count, however, was not correlated with platelet 5-HT concentration in asthma patients in our study. From the literature, platelet 5-HT, along with other platelet-derived mediators, promotes the recruitment of neutrophils to sites of inflammation, such as in allergic asthma [503-505]. All the same, this neutrophil recruitment by platelet 5-HT might not necessarily be reflected in the blood neutrophil count in general. It is worth noting that neutrophil count in relation to platelet 5-HT concentration and platelet MAO-B activity in

healthy subjects was not examined in our study. Therefore, further studies may provide data to clarify and expand this important area of asthma research.

6.2.6. The association of platelet 5-HT concentration and platelet MAO-B activity with different asthma phenotypes

No differences in platelet 5-HT concentration between patients with various asthma phenotypes (T2-high and T2-low, non-allergic and allergic, eosinophilic and non-eosinophilic, AERD and non-AERD) were observed. Likewise, platelet MAO-B activity did not differ on the basis of the aforementioned asthma phenotypes. There are no known published studies to date on the association between platelet 5-HT levels or platelet MAO-B activity and asthma phenotype, apart from allergic asthma, thus, comparisons with the literature were very limited.

Although in our study there were no statistically significant differences in platelet 5-HT concentrations between allergic and non-allergic asthma patients, or between non-allergic asthma patients and healthy subjects, platelet 5-HT levels in patients with allergic asthma were nevertheless significantly lower than in healthy control subjects. At first glance, this may seem to conflict with very early studies that reported higher blood levels of 5-HT in patients with allergic asthma compared to healthy individuals [268, 506-507]. However, it should be noted that the 5-HT levels measured in these previous studies were derived from serum and whole blood samples, whereas the 5-HT concentrations in our study were measured in platelets. Therefore, an increased amount of free 5-HT in the blood may reflect a greater release of 5-HT into the circulation from the dense granules of platelets during platelet activation in allergic asthma patients, with a consequential decrease in the platelet 5-HT concentration in these patients compared to healthy subjects.

In line with this presumption is that the endogenous content of 5-HT in human thrombocytes, namely a decrease in platelet 5-HT concentration, has been used over the years in clinical research as a surrogate marker of platelet activation [236]. As described in a detailed review on the roles of blood platelets in the pathogenesis of allergic inflammation and bronchial asthma, platelets in subjects with asthma are more activated than those in healthy individuals [508]. Furthermore, as pointed out by Margraf and Zarbock (2019) in their review, platelets from allergic patients show an activated phenotype, as evidenced by studies showing increased plasma levels of platelet factor 4 (PF4; a chemokine also known as CXCL4) in asthma patients, increased platelet-eosinophil complex formation and 5-HT release, and

higher levels of soluble P-selectin (a platelet activation marker) [176, 509-512]. Interestingly, Dürk and colleagues (2013) showed that only platelet-derived 5-HT, and not mast cell-derived 5-HT, is a key factor in enhancing airway allergen inflammation, and that a lack of 5-HT (such as when TPH-1 is deficient or inhibited, and therefore, platelet sequestration of 5-HT is decreased) was associated with an impaired Th2-priming capacity of bone marrow dendritic cells (as a model of airway allergen inflammation), and thus, reduced allergen responses in the airways [176, 485].

In our study, platelet MAO-B activity was nominally higher in patients with allergic asthma than in those with non-allergic asthma. More specifically, asthma patients with pollen allergy had nominally higher platelet MAO-B activity than asthma patients without pollen allergy, but these differences were not statistically significant after correction for multiple testing. One interpretation of these results may be that asthma subjects with atopy likely have an increased inflammatory response. In a previous study using a murine model of asthma and ragweed pollen extract, it was concluded that increased oxidative stress plays a critical role in mounting an immune response [513]. Boldogh et al. (2005) discovered that removal of ROS generated by intrinsic pollen NADPH oxidase prevented an immune response and airway inflammation, demonstrating that the initiation and augmentation of antigen-induced allergic airway inflammation is signalled by ROS [513]. A more recent study by Cho and Moon (2010) used the mouse model of asthma and found that increased oxidative stress in the airways preceded the development of allergic inflammation, AHR and other pivotal features of asthma such as enhanced mucus secretion [496]. Therefore, it is strongly suggested that an increased level of ROS acts as a critical contributor to the induction of allergic airway inflammation [496]. Research is needed to determine whether increased platelet MAO-B activity might also contribute to increasing ROS in allergic asthma, in general, and pollen allergy, specifically.

If we presume that the platelet MAO-B enzyme is involved in platelet 5-HT degradation, the observed higher platelet MAO-B activity in our study of patients with allergic asthma is in accordance with the decreased platelet 5-HT levels in these individuals. Further studies in a larger population of asthma patients are needed, however, to confirm these results. In addition, it will be necessary to perform research that will help clarify the role of platelets and the extent of involvement of the platelet MAO-B enzyme in platelet 5-HT degradation in asthma.

6.3. Plasma BDNF concentration and the polymorphisms of *BDNF* and *NTRK2* (*TrkB*) genes

6.3.1. Age, gender, BMI, and smoking status associations with plasma BDNF concentration

Regarding BDNF concentration in plasma, our findings are in agreement with the results of a previous study by Sustar et al. (2019) showing no significant effects of age, gender, BMI, or smoking on plasma BDNF values [514]. On the other hand, the study of Pillai et al. (2012) found a negative correlation of plasma BDNF levels with age, as well as lower plasma BDNF levels in females than in males, possibly because of the weight differences; however, there was no correlation between BDNF plasma concentration and BMI [515]. In contrast to our data, Lommatzsch et al. (2005) reported a decrease in BDNF levels with increasing age or body weight, and there was a negative correlation of plasma BDNF values with BMI [516]. However, similar to our findings of nominally lower plasma BDNF levels in female versus male healthy subjects, women displayed lower platelet BDNF concentrations in comparison to men in the aforementioned study; although, when matched for weight, there were no significant gender differences regarding BDNF plasma levels [516]. Therefore, it is plausible that the nominally lower plasma BDNF levels seen in our female healthy subjects, showing no significant differences after correction for multiple testing, could be due to the lower weight of women than men in the control group.

Regarding the association between smoking and BDNF levels, there are discrepancies in the literature, demonstrating lower [517], higher [518] or unchanged [519] concentrations of BDNF in the serum or plasma of smokers compared to nonsmokers. Though these reports on circulating concentrations of BDNF are inconclusive, it is important to recognize that cigarette smoke, at least partly through oxidative stress, has been shown to increase the expression of BDNF and its receptors in ASM cells [520]. This smoke-induced enhancement of neurotrophin release and signalling may also occur in other cell types (e.g., epithelium and immune cells), thus contributing to variations in both circulating and local concentrations [520]. Given that BDNF itself mediates both short-term and long-term effects on ASM cells after cigarette smoke exposure [520], we surmise that the BDNF levels of the former smokers in the non-smoking cohorts of our study may have off-set any potential differences in the BDNF concentrations of the never-smokers in both the asthma and control groups.

6.3.2. Plasma BDNF concentration in healthy subjects and asthma patients

Our finding that asthma patients had significantly higher concentrations of plasma BDNF than healthy individuals is in contrast to earlier studies that reported no differences in the serum and sputum BDNF levels between healthy subjects and patients with asthma [410-411]. Nevertheless, our data are in line with the report on increased BDNF levels in the serum, platelets and plasma of asthma patients compared to the control group [344, 395]. It has also been shown that children with moderate and severe asthma had higher plasma BDNF levels than age-matched control subjects [405]. Moreover, the study of Freeman et al. (2017) demonstrated higher BDNF expression and secretion in ASM of asthma patients compared to patients without asthma [521].

6.3.3. Asthma severity and plasma BDNF concentration

A recent study, showing higher amounts of mature BDNF in the sputum of patients with severe asthma in comparison to healthy subjects, has suggested a possible association of BDNF expression with asthma severity [522]. However, our investigation found that while both the non-severe and severe asthma patients had significantly higher plasma BDNF concentrations than the control subjects, there was no difference in the plasma BDNF levels between these groups of asthma patients. Therefore, our findings imply that BDNF could serve as a potential biomarker of asthma, but not asthma severity. This is in contrast to the previously reported data suggesting BDNF as a potential biomarker for clinical severity of asthma in children [405]. These discrepancies might be explained by the fact that Müller et al. (2010) compared the plasma BDNF of pediatric patients categorized into three groups based on asthma severity (mild, moderate and severe) [405], whereas our study examined the plasma BDNF levels of non-severe (mild and moderate) versus severe asthma in adult patients.

On the other hand, the findings of our study are in line with the data of Szczepankiewicz et al. (2012), demonstrating similar serum BDNF levels in children with asthma of different severity, regardless of symptom activity [523]. Although differences in plasma BDNF concentration between non-severe and severe asthma patients were not observed in our study, there was a nominally positive correlation between plasma BDNF concentration and duration of asthma. The latter is usually also related to the severity of the disease. However, some studies have reported no correlation of serum BDNF levels with asthma duration [524].

6.3.4. Asthma phenotypes and plasma BDNF concentration

In our study, asthma patients with a history of pneumonia had nominally lower plasma BDNF concentrations in comparison to asthma patients who had never developed pneumonia. Lommatzsch and co-authors (2007), however, reported markedly reduced BDNF concentrations in the serum and platelets, but not plasma, of patients with acute bacterial lower respiratory tract infection, with normalization to control BDNF levels after antibiotic treatment [525]. Previous studies regarding the effect of viral infections on peripheral BDNF levels have been contradictory, with the most recent research by Azoulay et al. (2020) revealing lower serum BDNF levels in patients with severe or moderate severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection, followed by restoration of BDNF levels as patients recovered [526]. Further research is required to clarify the acute and long-term effects of respiratory tract infections on peripheral BDNF levels in the general population, and specifically, in asthma patients.

Interestingly, we observed the opposite trend of increased plasma BDNF levels in asthma patients with nasal polyps, as well as in those with aspirin sensitivity. As pertains to nasal polyps, Coffey et al. (2009) contrarily reported a decreased mean BDNF concentration, albeit in the sinus mucosa of patients with chronic rhinosinusitis compared to control subjects, and patients with nasal polyps had the most significant decrease [527]. On the other hand, our results are consistent with the findings of Jornot and colleagues (2007) in nasal polyps, showing that polyp epithelial cells expressed higher BDNF levels compared to turbinate-derived cells, and that BDNF secretion and production were markedly increased in response to pro-inflammatory cytokines [528]. As far as we are aware, there are currently no published data on BDNF levels in relation to aspirin sensitivity. More research is needed to define the role of BDNF in nasal polyps and aspirin sensitivity in asthma.

When patients were subdivided into those with or without AERD according to the presence or absence of the triad of clinical features (asthma, recurrent nasal polyps and sensitivity to aspirin or other NSAIDs), nominally higher plasma BDNF levels were observed in patients with AERD. To the best of our knowledge, there are no published data on blood BDNF levels in AERD patients. The importance of this asthma phenotype is underscored by the increased associated morbidity and costs, as well as its prevalence of 7.15% in asthma patients, as confirmed in our study (7.5%), and 14.89% in patients with severe asthma [529].

Plasma BDNF concentrations in our study did not differ significantly between the non-allergic and allergic asthma patients. This finding is in keeping with the study demonstrating no differences in the serum BDNF levels between patients with allergic asthma and healthy individuals [410], or between children with allergic asthma and those without an atopic background [523], as well as with the report of no association between atopy and BDNF concentrations in the serum of patients with chronic cough [411]. However, our results did not mirror earlier studies showing that patients with allergic asthma have higher BDNF levels compared to control subjects [344, 395]. Virchow et al. (1998) reported that BDNF is produced endobronchially following allergen provocation, suggesting a contribution to the pathogenesis of asthma [530]. In contrast to the study demonstrating lower amounts of BDNF in the eosinophil lysates of allergic asthma patients than control subjects [531], or the report on higher total BDNF in subjects with sputum eosinophilia [522], our study observed no differences in the plasma BDNF concentration between patients with and without eosinophilic asthma.

As far as we are aware, our work is the first to compare plasma BDNF levels between T2-low and T2-high asthma patients. Therefore, our results showing no differences could not be evaluated against the literature data.

6.3.5. The *BDNF* Val66Met polymorphism

The *BDNF* Val66Met polymorphism has previously been associated with asthma in children of Chinese Han [404, 414] and Slovak origin [403]. In addition, a recent meta-analysis has suggested that the G allele of *BDNF* Val66Met polymorphism is potentially associated with asthma risk in Caucasians [532]. However, in our study, no significant differences in the frequencies of *BDNF* Val66Met genotypes, alleles or carriers were found between the asthma patients and control group. Our findings are consistent with reports of no association of *BDNF* Val66Met polymorphism with the presence of asthma in children [533], as well as with asthma-related phenotypes in families with asthma [415], suggesting that this *BDNF* gene variation does not contribute significantly to asthma susceptibility or severity. However, the same research team later reported that *BDNF* Val66Met polymorphism in a haplotype (TTGC) with three other *BDNF* polymorphisms may contribute to asthma susceptibility, but does not influence asthma severity [534]. These findings are in agreement with our study, which found no significant differences in the frequencies of *BDNF* Val66Met genotypes, alleles or carriers between non-severe and severe asthma patients. In contrast, the study of

Zeilinger et al. (2009) suggested that *BDNF* Val66Met polymorphism contributes to the severe forms of asthma [402].

6.3.6. *BDNF* Val66Met polymorphism and plasma BDNF concentration

Regarding *BDNF* Val66Met polymorphism, it has been shown that the Met variant alters the intracellular trafficking and packaging of pro-BDNF, resulting in reduced secretion of the mature BDNF [354-355]. However, various studies reported contradictory findings about the possible influence of functional *BDNF* Val66Met polymorphism on BDNF levels, demonstrating that carriers of the Met allele have decreased [535], increased [536] or unchanged [537] serum BDNF levels. In our study, healthy individuals carrying the Met allele had significantly lower plasma BDNF levels in comparison to the carriers of the Val/Val genotype, supporting the findings that *BDNF* Val66Met polymorphism influences BDNF concentration in plasma. On the other hand, this association was not found in asthma patients, as no significant differences in plasma BDNF levels were observed when the patients were subdivided according to *BDNF* Val66Met genotypes and carriers. Our results in asthma patients are in agreement with the data showing that *BDNF* Val66Met variants do not affect the BDNF serum levels in children with asthma [416]. It is possible that in patients with asthma, due to the complex pathophysiology of this disease, other factors predominantly influence BDNF plasma levels, masking the effect of the functional *BDNF* Val66Met polymorphism.

6.3.7. *BDNF* Val66Met polymorphism and asthma phenotypes

In the present study, there were no differences in the distribution of *BDNF* Val66Met genotypes, alleles or carriers between the non-allergic and allergic asthma patients. This is in line with the reported lack of difference in the genotype distribution for clinical atopy between asthma patients and healthy subjects [533], and with no association of *BDNF* Val66Met polymorphism with asthma-related atopy [415] or asthma and any atopic disease in the cross-sectional study population [402]. In contrast, some reports have suggested an important contribution of *BDNF* variations in the predisposition and progression of allergic diseases [538-539].

To the best of our knowledge, the distribution of *BDNF* Val66Met variants among the other asthma phenotypes (T2-low vs. T2-high, eosinophilic vs. non-eosinophilic, and AERD vs. non-AERD) has not yet been investigated. Therefore, our results showing no differences could not be discussed here in relation to the available literature.

6.3.8. *NTRK2* rs1439050 polymorphism

Our study observed no differences in the distribution of *NTRK2* rs1439050 genotypes, alleles or carriers between healthy subjects and asthma patients, or between patients of different asthma severity or phenotypes. To the best of our knowledge, the association of *NTRK2* rs1439050 polymorphism with asthma, its severity and particular phenotypes has not been explored so far.

6.3.9. *NTRK2* rs1439050 polymorphism and plasma BDNF concentration

However, some studies have examined other polymorphic variants within genes encoding neurotrophic receptors, including the *TrkB* gene in asthma [401, 416]. Some of the studied polymorphisms represent functional variants that may affect TrkB receptor expression. Higher TrkB expression has been found in asthma patients compared to healthy control subjects [401, 521], and in bronchial eosinophils after segmental allergen provocation [540], but no differences in TrkB expression were observed between healthy subjects and patients with allergies in peripheral blood mononuclear cells [541]. Markedly increased mRNA levels of *TrkB* have also been detected in the inflamed lung tissue of a murine model of asthma [542]. In addition, cigarette smoke has been shown to be a potent inducer of TrkB expression and signalling in ASM [520], thus contributing to AHR in allergic asthma [400]. In addition to the TrkB receptor levels, polymorphic variants of neurotrophic receptor genes might also affect the concentrations of unbound/active BDNF protein. However, similar to the findings of Szczepankiewicz et al. (2013) [416], in the present study, plasma BDNF levels were not influenced by *NTRK2* rs1439050 polymorphism in healthy individuals or asthma patients.

6.4. Strengths and limitations of the study

This study has several strengths and weaknesses that have previously been addressed in our published paper related to the BDNF portion of this research. The most obvious limitation of the study is the methodological issue involving an unmatched study design, that is, asthma cases and control subjects were not balanced on age, gender or smoking status. However, there was equality in the sample sizes between the principal study groups (i.e., patients versus healthy individuals). Genetic polymorphism studies generally require very large sample sizes and this study included relatively small numbers, mainly due to quite restrictive exclusion criteria as well as financial constraints. Future studies should be conducted on much larger samples with a broader inclusion criteria to allow for the effects of comorbidities and medications, thereby, better reflecting the real-world data. Given that the role of anxiety and

depression in asthma is an ongoing area of research worldwide [e.g., 346, 543-548], it would be essential to include these patients in future studies. Another limitation of this study is that the level of physical activity and/or exercise regimen of the study subjects was not recorded. Previous studies have shown that BDNF is a potential mediator of exercise, and specifically, increased peripheral lactate levels following high intensity exercise are associated with increased peripheral BDNF concentrations [549]. Therefore, future research should take into account the amount and type of physical activity of the study population and the potential impact on plasma BDNF levels. Despite the aforementioned limitations, the study's main strength is that it was adequately powered with a sufficient number of participants to observe statistically significant associations and answer the research questions. Another strength of the study is that it examined an ethnically homogenous population, which is of particular importance for genetic studies, consisting of adult Caucasian Croatians for both the healthy controls and asthma patients. Furthermore, this study took into account both asthma severity and several asthma phenotypes that had not been covered sufficiently or with consistent results in the literature on serotonergic and neurotrophic systems, and therefore, should be an area of much greater focus in future investigations on asthma.

6.5. Summary

The results of this study, therefore, have provided evidence to confirm the research hypothesis that platelet 5-HT and plasma BDNF concentrations, and additionally platelet MAO-B activity, are statistically significantly altered in asthma patients compared to healthy control subjects. There were no differences in these three parameters, however, with respect to asthma severity or the various asthma phenotypes. Moreover, no association between platelet 5-HT levels, plasma BDNF concentration or platelet MAO-B activity was discovered in patients with allergic versus non-allergic asthma. The only exception was the discovery of significantly higher BDNF levels in the asthma patients with aspirin sensitivity and nominally higher levels in those with AERD (also known as Samter's Triad). There was also no association between the SNPs rs6265 (Val66Met) in the *BDNF* gene or rs1439050 in the *NTRK2* (*TrkB*) gene with asthma, or in particular, severe asthma or atopy. The genotype and allele frequencies of *BDNF* Val66Met and *NTRK2* rs1439050 polymorphisms did not show significant differences between asthma patients and healthy subjects or patients grouped according to severity or different asthma phenotypes. Plasma BDNF levels in the asthma patients were not influenced by the *BDNF* Val55Met genotype, allele or carrier distribution, in contrast to the healthy subjects. The *NTRK2* rs1439050 polymorphism did not affect the

levels of unbound BDNF protein in the plasma of the study population. These outcomes suggest that the aforementioned gene polymorphisms do not contribute to the risk of developing asthma, or specifically, severe asthma. Although *BDNF* Val66Met polymorphism influenced BDNF concentration in the plasma of healthy subjects, factors other than this gene polymorphism seem to influence plasma BDNF concentrations in asthma patients.

In conclusion, the findings of this study clearly indicate that asthma patients have significantly reduced platelet 5-HT levels, but increased platelet MAO-B activity and plasma BDNF levels, than healthy subjects. The reason for this is not entirely understood, but may suggest the role of a compensatory mechanism involving the activation of immune cells in asthma. Based on the results of this study, platelet 5-HT concentration, platelet MAO-B activity and plasma BDNF levels are not useful for establishing asthma severity or differentiating asthma phenotypes (i.e., allergic versus non-allergic asthma, T2-high versus T2-low asthma, non-eosinophilic versus eosinophilic asthma). Nevertheless, aspirin-sensitive asthma and AERD were found to be associated with significantly and nominally increased plasma BDNF levels, respectively, hence it is reasonable that BDNF could be used as a reliable measure to distinguish these groups of asthma patients.

The *BDNF* Val66Met genotype, allele and carrier distributions were not associated with differences in plasma BDNF concentration among asthma patients; though healthy subjects carrying the *BDNF* Val66Met GG genotype versus A allele carriers did have increased plasma BDNF concentrations. Therefore, plasma BDNF levels in asthma patients may be primarily related to the pathophysiology of the disease, but differences likely reflect a complex interplay between genetic predisposition and environmental factors.

7. CONCLUSIONS

A summary of the main research findings, as pertains to the hypothesis and objectives (general and specific) of this study, is presented here:

(1) Our study revealed decreased platelet 5-HT concentrations, increased platelet MAO-B activity and increased plasma BDNF concentrations in asthma patients in comparison to healthy control subjects. These results confirm our hypothesis that the concentrations of platelet 5-HT and plasma BDNF are significantly altered in asthmatic patients compared to healthy control subjects.

(2) We observed no significant differences in platelet 5-HT concentration, plasma BDNF concentration or platelet MAO-B activity between non-severe and severe asthma patients. These results suggest no association of platelet 5-HT concentration, plasma BDNF concentration or platelet MAO-B activity with asthma severity.

(3) Decreased platelet 5-HT concentrations were found in the severe asthma patients in comparison to healthy control subjects. These results confirm the hypothesis that platelet 5-HT concentrations are significantly altered in asthma patients compared to healthy control subjects. The effect is due mainly to the severe asthma patients since the non-severe asthma patients showed no significant differences in platelet 5-HT concentration compared to the healthy adults. On the other hand, platelet MAO-B activity was significantly higher in both non-severe and severe asthma patients in comparison to healthy control subjects.

(4) No significant differences in platelet 5-HT concentration, platelet MAO-B activity or plasma BDNF concentration were observed when patients were subdivided according to various asthma phenotypes (T2-high versus T2-low; allergic versus non-allergic; eosinophilic versus non-eosinophilic). These results suggest no association of platelet 5-HT concentration, platelet MAO-B activity or plasma BDNF concentration with these asthma phenotypes (T2-high versus T2-low; allergic versus non-allergic; eosinophilic versus non-eosinophilic).

(5) Our study showed higher plasma BDNF concentrations in asthma patients with nasal polyps than in those without, as well as in patients with sensitivity to aspirin than in asthma patients lacking aspirin sensitivity. Moreover, patients with AERD demonstrated nominally higher plasma BDNF concentrations than those without AERD. These results suggest an

association between plasma BDNF concentration and the combination of asthma, recurrent nasal polyps and sensitivity to aspirin.

(6) Asthma patients versus healthy control subjects, as well as asthma patients subdivided according to severity (severe vs non-severe) or phenotype (T2-high versus T2-low; allergic versus non-allergic; eosinophilic versus non-eosinophilic; AERD versus non-AERD), demonstrated no differences in the genotype or allele frequencies of *BDNF* rs6265 or *NTRK2* rs1439050 polymorphisms. These results do not support our hypothesis that the rs6265 (Val66Met) polymorphism in the *BDNF* gene is strongly associated with risk of severe asthma in Caucasian adults.

(7) We observed increased plasma BDNF concentrations in healthy subjects carrying the *BDNF* Val66Met GG genotype versus A allele carriers, supporting the findings that *BDNF* Val66Met polymorphism influences BDNF concentration in plasma. This result, however, was not obtained in the asthma patients. It is possible that in patients with asthma, due to the complex pathophysiology of this disease, other factors predominantly influence BDNF plasma levels, masking the effect of the functional *BDNF* Val66Met polymorphism. In addition, our findings demonstrated that *NTRK2* rs1439050 polymorphism did not affect plasma BDNF levels.

(8) There were no significant differences in plasma BDNF levels of healthy subjects or asthma patients split into groups based on different *NTRK2* rs1439050 genotypes, carrying the G allele and TT genotype, or T allele and GG homozygous genotype. These findings suggest that there was no influence of the *NTRK2* polymorphism on the plasma concentration of unbound BDNF protein in the plasma.

Taken together, our findings suggest that platelet 5-HT and plasma BDNF may be useful peripheral blood biomarkers for asthma in general, or in particular, asthma with aspirin sensitivity, but not asthma severity or the phenotypes examined here. However, further research is needed to better understand the role of peripheral 5-HT and BDNF levels, and MAO-B activity, as well as *BDNF* Val66Met and *NTRK2* (*TrkB*) gene polymorphisms in adult asthma. In addition, although interactions between 5-HT and BDNF systems in the CNS have been described as synergistic in nature and vital for normal neurodevelopment [550], their duality in the neuroinflammation of asthma remains to be further elucidated.

8. ABSTRACT

Association of platelet serotonin, plasma brain-derived neurotrophic factor (BDNF) and Val66Met BDNF gene polymorphism with asthma severity
(Katherina Bernadette Sreter, 2022)

Asthma is a common but complex chronic inflammatory disease of the respiratory airways. Serotonin (5-HT) and brain-derived neurotrophic factor (BDNF) are associated with regulation of lung function and immune responses; however, their exact involvement in the pathophysiology of asthma remains unclear. We investigated platelet 5-HT and plasma BDNF concentrations, platelet monoamine oxidase B (MAO-B) activity, and the distribution of genetic variants of *BDNF* rs6265 (Val66Met) and neurotrophic receptor tyrosine kinase 2 (*NTRK2*) rs1439050 polymorphisms in 120 asthma patients and 120 healthy subjects, and evaluated their relationship with asthma severity and phenotype. Platelet 5-HT concentrations were lower, whereas platelet MAO-B activity and plasma BDNF concentrations were higher in asthma patients. No differences were observed in these parameters between subjects with asthma subdivided according to asthma severity or phenotype. However, asthma patients with aspirin sensitivity and aspirin-exacerbated respiratory disease had higher plasma BDNF levels. The distribution of *BDNF* Val66Met and *NTRK2* rs1439050 genetic variants showed no differences between subjects with asthma and healthy individuals, or between patients with different asthma severity and phenotype. The *BDNF* Val66Met polymorphism, but not *NTRK2* rs1439050 polymorphism, influenced plasma BDNF levels in healthy subjects only. These results suggest that 5-HT and MAO-B in platelets as well as BDNF in plasma may serve as potential peripheral biomarkers for asthma but not asthma severity.

Keywords: asthma; plasma BDNF concentrations; platelet 5-HT levels; platelet MAO-B activity; *BDNF* and *NTRK2* polymorphisms; severity; phenotype

9. SAŽETAK

Povezanost trombocitnoga serotonina, moždanoga neurotrofnoga čimbenika (BDNF) u plazmi te Val66Met polimorfizma gena *BDNF* sa stupnjem težine astme
(Katherina Bernadette Sreter, 2022.)

Astma je česta ali kompleksna kronična upalna bolest dišnih putova. Serotonin (5-HT) i moždani neurotrofni čimbenik (BDNF) su povezani s regulacijom funkcije pluća i imunoloških odgovora; međutim, njihova točna uloga u patofiziologiji astme ostaje nerazjašnjena. Istražili smo koncentracije trombocitnog 5-HT-a i BDNF-a u plazmi, aktivnost trombocitne monoaminooksidaze B (MAO-B), i raspodjelu genskih varijanti *BDNF* rs6265 (Val66Met) polimorfizma i *NTRK2* (gen za neurotrofni receptor tirozin kinaza 2) rs1439050 polimorfizma u 120 pacijenata s astmom i 120 zdravih osoba, te istražili njihovu povezanost sa stupnjem težine i fenotipom astme. Koncentracije trombocitnog 5-HT-a bile su snižene, dok su aktivnost trombocitne MAO-B i koncentracije BDNF-a u plazmi bile povišene u pacijenata s astmom. Nisu opažene razlike u ovim parametrima između osoba oboljelih od astme podijeljenih prema težini bolesti ili fenotipu. Međutim, pacijenti s aspirin-osjetljivom astmom i aspirin-pogoršanom respiratornom bolešću imali su povišene razine BDNF-a u plazmi. Raspodjela genskih varijanti *BDNF* Val66Met i *NTRK2* rs1439050 polimorfizama nije pokazala razliku niti između pacijenata s astmom i zdravih osoba niti između oboljelih različitih stupnjeva težine i fenotipova astme. *BDNF* Val66Met polimorfizam, ali ne i *NTRK2* rs1439050 polimorfizam, utjecao je na razine BDNF-a u plazmi samo zdravih ispitanika. Ovi rezultati ukazuju da 5-HT i MAO-B u trombocitima, te BDNF u plazmi mogu poslužiti kao potencijalni periferni biomarkeri za astmu, ali ne i za težinu astme.

Ključne riječi: astma; koncentracija BDNF-a u plazmi; razina trombocitnog 5-HT-a; trombocitna MAO-B aktivnost; polimorfizmi *BDNF* i *NTRK2*; težina bolesti; fenotip

10. REFERENCES

1. Salter B, Lacy P, Mukherjee M. Biologics in asthma: a molecular perspective to precision medicine. Front Pharmacol 2022;12:793409.

2. Martinez FD. The state of asthma research: considerable advances, but still a long way to go. Am J Respir Crit Care Med 2019;199:397-9.

3. Chung KF. Personalised medicine in asthma: time for action: Number 1 in the Series "Personalised medicine in respiratory diseases" Edited by Renaud Louis and Nicolas Roche. Eur Respir Rev 2017;26:170064.

4. Pérez de Llano L, Dacal Rivas D, Blanco Cid N, Martin Robles I. Phenotype-guided asthma therapy: an alternative approach to guidelines. J Asthma Allergy 2021;14:207-17.

5. Padem N, Saltoun C. Classification of asthma. Allergy Asthma Proc 2019;40:385-8.

6. Barcik W, Boutin RCT, Sokolowska M, Finlay BB. The role of lung and gut microbiota in the pathology of asthma. Immunity 2020;52:241-55.

7. Peebles RS Jr, Aronica MA. Proinflammatory pathways in the pathogenesis of asthma. Clin Chest Med 2019;40:29-50.

8. Chung KF. Precision medicine in asthma: linking phenotypes to targeted treatments. Curr Opin Pulm Med 2018;24:4-10.

9. Global Initiative for Asthma. Global Strategy for Asthma Management and Prevention, 2022. Available from: https://ginasthma.org/gina-reports/ (accessed July 15, 2022).

10. Boulet LP, Reddel HK, Bateman E, Pedersen S, FitzGerald JM, O'Byrne PM. The Global Initiative for Asthma (GINA): 25 years later. Eur Respir J 2019;54:1900598.

11. Papi A, Saetta M, Fabbri L. Severe asthma: phenotyping to endotyping or vice versa? Eur Respir J 2017;49:1700053.

12. Global Asthma Network. The Global Asthma Report 2018. Available from: http://globalasthmareport.org/Global%20Asthma%20Report%202018.pdf (accessed May 30, 2021).

13. World Health Organization. 10 facts on asthma (updated August 2017). Available from: https://www.who.int/features/factfiles/asthma/en/ (accessed April 6, 2021).

14. Asher MI, García-Marcos L, Pearce NE, Strachan DP. Trends in worldwide asthma prevalence. Eur Respir J 2020;56:2002094.

15. GBD 2016 Disease and Injury Incidence and Prevalence Collaborators. Global, regional, and national incidence, prevalence, and years lived with disability for 328 diseases and injuries for 195 countries, 1990-2016: a systematic analysis for the Global Burden of Disease Study 2016. Lancet 2017;390:1211-59.

16. Eder W, Ege MJ, von Mutius E. The asthma epidemic. N Engl J Med 2006;355:2226-35.

17. Enilari O, Sinha S. The global impact of asthma in adult populations. Ann Glob Health 2019;85:2.

18. Mattiuzzi C, Lippi G. Worldwide asthma epidemiology: insights from the Global Health Data Exchange database. Int Forum Allergy Rhinol 2020;10:75-80.

19. Ellwood P, Ellwood E, Rutter C, Perez-Fernandez V, Morales E, García-Marcos L, et al., On behalf of the GAN Phase I Study Group. Global Asthma Network Phase I surveillance: geographical coverage and response rates. J Clin Med 2020;9:3688.

20. Dharmage SC, Perret JL, Custovic A. Epidemiology of asthma in children and adults. Front Pediatr 2019;7:246.

21. Asher MI, Montefort S, Björkstén B, Lai CK, Strachan DP, Weiland SK, et al.; ISAAC Phase Three Study Group. Worldwide time trends in the prevalence of symptoms of asthma, allergic rhinoconjunctivitis, and eczema in childhood: ISAAC Phases One and Three repeat multicountry cross-sectional surveys. Lancet 2006;368:733-43. Erratum in: Lancet 2007;370:1128.

22. Fuhlbrigge AL, Jackson B, Wright R. Gender and asthma. Immunol Allergy Clin North Am 2002;22:10.

23. Troisi RJ, Speizer FE, Willett WC, Trichopoulos D, Rosner B. Menopause, postmenopausal estrogen preparations, and the risk of adult-onset asthma. A prospective cohort study. Am J Respir Crit Care Med 1995;152:1183-8.

24. Fuseini H, Newcomb DC. Mechanisms driving gender differences in asthma. Curr Allergy Asthma Rep 2017;17:19.

25. European Commission, Eurostat. Online data for health problem: asthma. Last update: 17/12/2021. Available from: https://ec.europa.eu/eurostat/databrowser/view/HLTH_EHIS_CD1E__custom_13018 82/bookmark/table?lang=en&bookmarkId=82c8e263-760d-49fd-84a7-ddacddcea0c9 (accessed June 4, 2022).

26. European Commission, Eurostat. Finland: EU country with highest share of asthmatics. Last update: 17/12/2021. Available from: https://ec.europa.eu/eurostat/en/web/products-eurostat-news/-/edn-20210924-1 (accessed June 4, 2022).

27. Rezultati projekta Eurostat "Morbidity Statistics" – Podaci za hrvatsku, 2022 [Results from Eurostat "Morbidity Statistics" – Data for Croatia, 2022]. Available from: https://www.hzjz.hr/periodicne-publikacije/rezultati-projekta-eurostat-morbidity-statistics-podaci-za-hrvatsku/ (accessed July 15, 2022).

28. Stipić-Marković A, Pevec B, Pevec MR, Custović A. Prevalencija simptoma astme, alergijskog rinitisa i konjunktivitisa te atopijskog ekcema: ISAAC u populaciji skolske djece u Zagrebu [Prevalence of symptoms of asthma, allergic rhinitis, conjunctivitis and atopic eczema: ISAAC (International Study of Asthma and Allergies in Childhood) in a population of schoolchildren in Zagreb]. Acta Med Croatica 2003;57:281-5.

29. Banac S, Tomulić KL, Ahel V, Rozmanić V, Simundić N, Zubović S, et al. Prevalence of asthma and allergic diseases in Croatian children is increasing: survey study. Croat Med J 2004;45:721-6.

30. Munivrana H, Vorko-Jovic A, Munivrana S, Kursar M, Medlobi-Gluhak M, Vlahek P. The prevalence of allergic diseases among Croatian school children according to the ISAAC Phase One questionnaire. Med Sci Monit 2007;13:CR505-9.

31. Gudelj I, Mrkić Kobal I, Munivrana Škvorc H, Miše K, Vrbica Z, Plavec D, et al. Intraregional differences in asthma prevalence and risk factors for asthma among adolescents in Split-Dalmatia County, Croatia. Med Sci Monit 2012;18:PH43-50.

32. Banac S, Rožmanić V, Manestar K, Korotaj-Rožmanić Z, Lah-Tomulić K, Vidović I, et al. Rising trends in the prevalence of asthma and allergic diseases among school children in the north-west coastal part of Croatia. J Asthma 2013;50:810-4.

33. Kobal Mrkić I, Škvorc Munivrana H, Škvorc M, Munivrana Š. Time trends in asthma and atopic diseases in North-West part of Croatia – ISAAC Phase III (2013). Allergol Immunopathol (Madr) 2021;49:32-7.

34. Grle SP, Lampalo M, Cincar SŠ, Kardum LB, Gudelj I, Kasap EB, et al. Attitudes of Croatian pulmonologists concerning obstacles to earlier, more appropriate use of biologics in severe asthma: survey results. PLoS One 2021;16:e0253468.

35. Hekking PW, Wener RR, Amelink M, Zwinderman AH, Bouvy ML, Bel EH. The prevalence of severe refractory asthma. J Allergy Clin Immunol 2015;135:896-902.

36. Ebmeier S, Thayabaran D, Braithwaite I, Bénamara C, Weatherall M, Beasley R. Trends in international asthma mortality: analysis of data from the WHO Mortality Database from 46 countries (1993-2012). Lancet 2017;390:935-45.

37. Yaghoubi M, Adibi A, Safari A, FitzGerald JM, Sadatsafavi M. The projected economic and health burden of uncontrolled asthma in the United States. Am J Respir Crit Care Med 2019;200:1102-12.

38. Centers for Disease Control and Prevention. Data, statistics, and surveillance: asthma surveillance data (2019). Available from: https://www.cdc.gov/asthma/most_recent_national_asthma_data.htm (accessed April 10, 2021).

39. Butler CA, Heaney LG. Neurogenic inflammation and asthma. Inflamm Allergy Drug Targets 2007;6:127-32.

40. Nurmagambetov T, Kuwahara R, Garbe P. The economic burden of asthma in the United States, 2008-2013. Ann Am Thorac Soc 2018;15:348-56.

41. Gibson GJ, Loddenkemper R, Sibille Y, Lundbäck B, Fletcher M, eds. Lung health in Europe. Facts and figures. Sheffield, European Respiratory Society/European Lung Foundation, 2013. Available from: https://www.ers-education.org/lrmedia/2013/pdf/238593.pdf (accessed June 16, 2021).

42. Selroos O, Kupczyk M, Kuna P, Łacwik P, Bousquet J, Brennan D, et al. National and regional asthma programmes in Europe. Eur Respir Rev 2015;24:474-83. Erratum in: Eur Respir Rev 2019;28.

43. World Health Organization. European Health Information Gateway. SDR, Asthma, per 100 000 (updated November 18, 2021). Available from: https://gateway.euro.who.int/en/indicators/hfamdb_135-sdr-asthma-per-100-000/ (accessed July 15, 2022).

44. Mims JW. Asthma: definitions and pathophysiology. Int Forum Allergy Rhinol 2015;5:S2-6.

45. King GG, Farrow CE, Chapman DG. Dismantling the pathophysiology of asthma using imaging. Eur Respir Rev 2019;28:180111.

46. Pavord ID, Beasley R, Agusti A, Anderson GP, Bel E, Brusselle G, et al. After asthma: redefining airways diseases. Lancet 2018;391:350-400.

47. Lacwik P, Kupczyk M. Asthma phenotype overlap: more may be less. J Allergy Clin Immunol Pract 2021;9:363-4.

48. Hernandez-Pacheco N, Pino-Yanes M, Flores C. Genomic predictors of asthma phenotypes and treatment response. Front Pediatr 2019;7:6.

49. Ober C, Yao TC. The genetics of asthma and allergic disease: a 21st century perspective. Immunol Rev 2011;242:10-30.

50. Ullemar V, Magnusson PK, Lundholm C, Zettergren A, Melén E, Lichtenstein P, et al. Heritability and confirmation of genetic association studies for childhood asthma in twins. Allergy 2016;71:230-8.

51. Lim RH, Kobzik L, Dahl M. Risk for asthma in offspring of asthmatic mothers versus fathers: a meta-analysis. PLoS One 2010;5:e10134.

52. Skadhauge LR, Christensen K, Kyvik KO, Sigsgaard T. Genetic and environmental influence on asthma: a population-based study of 11,688 Danish twin pairs. Eur Respir J 1999;13:8-14.

53. Cookson WO, Sharp PA, Faux JA, Hopkin JM. Linkage between immunoglobulin E responses underlying asthma and rhinitis and chromosome 11q. Lancet 1989;1:1292-5.

54. Holloway JW, Yang IA, Holgate ST. Genetics of allergic disease. J Allergy Clin Immunol 2010;125:S81-94.

55. García-Sánchez A, Isidoro-García M, García-Solaesa V, Sanz C, Hernández-Hernández L, Padrón-Morales J, et al. Genome-wide association studies (GWAS) and their importance in asthma. Allergol Immunopathol (Madr) 2015;43:601-8.

56. Barnes KC. Genetic studies of the etiology of asthma. Proc Am Thorac Soc 2011;8:143-8.

57. Moffatt MF, Kabesch M, Liang L, Dixon AL, Strachan D, Heath S, et al. Genetic variants regulating ORMDL3 expression contribute to the risk of childhood asthma. Nature 2007;448:470-3.

58. Bunyavanich S, Schadt EE. Systems biology of asthma and allergic diseases: a multiscale approach. J Allergy Clin Immunol 2015;135:31-42.

59. Vicente CT, Revez JA, Ferreira MAR. Lessons from ten years of genome-wide association studies of asthma. Clin Transl Immunol 2017;6:e165.

60. Ntontsi P, Photiades A, Zervas E, Xanthou G, Samitas K. Genetics and epigenetics in asthma. Int J Mol Sci 2021;22:2412.

61. Russell RJ, Brightling C. Pathogenesis of asthma: implications for precision medicine. Clin Sci (Lond) 2017;131:1723-35.

62. Arora P, Ansari SH. Role of various mediators in inflammation of asthmatic airways. In: Pereira C, ed. Asthma – Biological Evidences [Internet]. London: IntechOpen; 2019. Available from: https://www.intechopen.com/chapters/66708 (accessed June 16, 2021).

63. Kaur R, Chupp G. Phenotypes and endotypes of adult asthma: moving toward precision medicine. J Allergy Clin Immunol 2019;144:1-12.

64. Woodruff PG, Modrek B, Choy DF, Jia G, Abbas AR, Ellwanger A, et al. T-helper type 2-driven inflammation defines major subphenotypes of asthma. Am J Respir Crit Care Med 2009;180:388-95. Erratum in: Am J Respir Crit Care Med 2009;180:796.

65. Kuo CS, Pavlidis S, Loza M, Baribaud F, Rowe A, Pandis I, et al.; U-BIOPRED Project Team‡. A transcriptome-driven analysis of epithelial brushings and bronchial biopsies to define asthma phenotypes in U-BIOPRED. Am J Respir Crit Care Med 2017;195:443-55.

66. Kuruvilla ME, Lee FE, Lee GB. Understanding asthma phenotypes, endotypes, and mechanisms of disease. Clin Rev Allergy Immunol 2019;56:219-33.

67. Caruso M, Morjaria J, Emma R, Amaradio MD, Polosa R. Biologic agents for severe asthma patients: clinical perspectives and implications. Intern Emerg Med 2018;13:155-76.

68. Fahy JV. Type 2 inflammation in asthma – present in most, absent in many. Nat Rev Immunol 2015;15:57-65.

69. Rupani H, Chauhan AJ. Measurement of FeNO in asthma: what the hospital doctor needs to know. Br J Hosp Med (Lond) 2019;80:99-104.

70. Brusselle G, Kips J, Joos G, Bluethmann H, Pauwels R. Allergen-induced airway inflammation and bronchial responsiveness in wild-type and interleukin-4-deficient mice. Am J Respir Cell Mol Biol 1995;12:254-9.

71. Grünig G, Warnock M, Wakil AE, Venkayya R, Brombacher F, Rennick DM, et al. Requirement for IL-13 independently of IL-4 in experimental asthma. Science 1998;282:2261-3.

72. Hogan SP, Koskinen A, Foster PS. Interleukin-5 and eosinophils induce airway damage and bronchial hyperreactivity during allergic airway inflammation in BALB/c mice. Immunol Cell Biol 1997;75:284-8.

73. Wills-Karp M, Luyimbazi J, Xu X, Schofield B, Neben TY, Karp CL, et al. Interleukin-13: central mediator of allergic asthma. Science 1998;282:2258-61.

74. Lambrecht BN, Hammad H, Fahy JV. The cytokines of asthma. Immunity 2019;50:975-91.

75. Barnes PJ. Targeting cytokines to treat asthma and chronic obstructive pulmonary disease. Nat Rev Immunol 2018;18:454-66.

76. Chang HS, Lee TH, Jun JA, Baek AR, Park JS, Koo SM, et al. Neutrophilic inflammation in asthma: mechanisms and therapeutic considerations. Expert Rev Respir Med 2017;11:29-40.

77. Ricciardolo FLM, Sorbello V, Folino A, Gallo F, Massaglia GM, Favatà G, et al. Identification of IL-17F/frequent exacerbator endotype in asthma. J Allergy Clin Immunol 2017;140:395-406.

78. Brightling CE, Bradding P, Symon FA, Holgate ST, Wardlaw AJ, Pavord ID. Mast-cell infiltration of airway smooth muscle in asthma. N Engl J Med 2002;346:1699-705.

79. Brightling CE, Ammit AJ, Kaur D, Black JL, Wardlaw AJ, Hughes JM, et al. The CXCL10/CXCR3 axis mediates human lung mast cell migration to asthmatic airway smooth muscle. Am J Respir Crit Care Med 2005;171:1103-8.

80. Brightling CE, Symon FA, Holgate ST, Wardlaw AJ, Pavord ID, Bradding P. Interleukin-4 and -13 expression is co-localized to mast cells within the airway smooth muscle in asthma. Clin Exp Allergy 2003;33:1711-6.

81. Sutcliffe A, Hollins F, Gomez E, Saunders R, Doe C, Cooke M, et al. Increased nicotinamide adenine dinucleotide phosphate oxidase 4 expression mediates intrinsic airway smooth muscle hypercontractility in asthma. Am J Respir Crit Care Med 2012;185:267-74.

82. Lloyd CM. IL-33 family members and asthma – bridging innate and adaptive immune responses. Curr Opin Immunol 2010;22:800-6.

83. Di Candia L, Gomez E, Venereau E, Chachi L, Kaur D, Bianchi ME, et al. HMGB1 is upregulated in the airways in asthma and potentiates airway smooth muscle contraction via TLR4. J Allergy Clin Immunol 2017;140:584-7.e8.

84. Berair R, Brightling CE. Asthma therapy and its effect on airway remodelling. Drugs 2014;74:1345-69.

85. Wardlaw AJ, Brightling C, Green R, Woltmann G, Pavord I. Eosinophils in asthma and other allergic diseases. Br Med Bull 2000;56:985-1003.

86. Jeffery PK. Remodeling in asthma and chronic obstructive lung disease. Am J Respir Crit Care Med 2001;164:S28-38.

87. Keglowich LF, Borger P. The three A's in asthma – airway smooth muscle, airway remodeling & angiogenesis. Open Respir Med J 2015;9:70-80.

88. Berair R, Hartley R, Mistry V, Sheshadri A, Gupta S, Singapuri A, et al. Associations in asthma between quantitative computed tomography and bronchial biopsy-derived airway remodelling. Eur Respir J 2017;49:1601507.

89. Stinson SE, Amrani Y, Brightling CE. D prostanoid receptor 2 (chemoattractant receptor-homologous molecule expressed on TH2 cells) protein expression in asthmatic patients and its effects on bronchial epithelial cells. J Allergy Clin Immunol 2015;135:395-406.

90. Frey A, Lunding LP, Ehlers JC, Weckmann M, Zissler UM, Wegmann M. More than just a barrier: the immune functions of the airway epithelium in asthma pathogenesis. Front Immunol 2020;11:761.

91. Nanda A, Wasan AN. Asthma in adults. Med Clin North Am 2020;104:95-108.

92. Sinyor B, Concepcion Perez L. Pathophysiology of asthma. [Updated 2021 May 9]. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing; 2021 Jan-. Available from: https://www.ncbi.nlm.nih.gov/books/NBK551579/ (accessed June 6, 2021).

93. Brussino L, Solidoro P, Rolla G. Is it severe asthma or asthma with severe comorbidities? J Asthma Allergy 2017;10:303-5.

94. Khalaf K, Paoletti G, Puggioni F, Racca F, De Luca F, Giorgis V, et al. Asthma from immune pathogenesis to precision medicine. Semin Immunol 2019;46:101294.

95. Busse PJ, McDonald VM, Wisnivesky JP, Gibson PG. Asthma across the ages: adults. J Allergy Clin Immunol Pract 2020;8:1828-38.

96. Fuchs O, Bahmer T, Rabe KF, von Mutius E. Asthma transition from childhood into adulthood. Lancet Respir Med 2017;5:224-34.

97. Löwhagen O. Diagnosis of asthma – new theories. J Asthma 2015;52:538-44.

98. Brigham EP, West NE. Diagnosis of asthma: diagnostic testing. Int Forum Allergy Rhinol 2015;5:S27-30.

99. Tarasidis GS, Wilson KF. Diagnosis of asthma: clinical assessment. Int Forum Allergy Rhinol 2015;5:S23-6.

100. McCormack MC, Enright PL. Making the diagnosis of asthma. Respir Care 2008;53:583-92.

101. Pellegrino R, Viegi G, Brusasco V, Crapo RO, Burgos F, Casaburi R, et al. Interpretative strategies for lung function tests. Eur Respir J 2005;26:948-68.

102. Coates AL, Wanger J, Cockcroft DW, Culver BH; Bronchoprovocation Testing Task Force: Kai-Håkon Carlsen, Diamant Z, et al. ERS technical standard on bronchial challenge testing: general considerations and performance of methacholine challenge tests. Eur Respir J 2017;49:1601526.

103. Davis BE, Blais CM, Cockcroft DW. Methacholine challenge testing: comparative pharmacology. J Asthma Allergy 2018;11:89-99.

104. Chibana K, Trudeau JB, Mustovich AT, Hu H, Zhao J, Balzar S, et al. IL-13 induced increases in nitrite levels are primarily driven by increases in inducible nitric oxide synthase as compared with effects on arginases in human primary bronchial epithelial cells. Clin Exp Allergy 2008;38:936-46. Erratum in: Clin Exp Allergy 2008;38:1409. Mustovitch, A T [corrected to Mustovich, A T].

105. Castillo JR, Peters SP, Busse WW. Asthma exacerbations: pathogenesis, prevention, and treatment. J Allergy Clin Immunol Pract 2017;5:918-27.

106. Bagnasco D, Ferrando M, Varricchi G, Passalacqua G, Canonica GW. A critical evaluation of anti-IL-13 and anti-IL-4 strategies in severe asthma. Int Arch Allergy Immunol 2016;170:122-31.

107. McGregor MC, Krings JG, Nair P, Castro M. Role of biologics in asthma. Am J Respir Crit Care Med 2019;199:433-45.

108. Pelaia C, Crimi C, Vatrella A, Tinello C, Terracciano R, Pelaia G. Molecular targets for biological therapies of severe asthma. Front Immunol 2020;11:603312.

109. Brusselle GG, Koppelman GH. Biologic therapies for severe asthma. N Engl J Med 2022;386:157-71.

110. Gruffydd-Jones K. Unmet needs in asthma. Ther Clin Risk Manag 2019;15:409-21.

111. Kistemaker LEM, Prakash YS. Airway innervation and plasticity in asthma. Physiology (Bethesda) 2019;34:283-98.

112. Kistemaker LE, Gosens R. Acetylcholine beyond bronchoconstriction: roles in inflammation and remodeling. Trends Pharmacol Sci 2015;36:164-71.

113. Kerstjens HA, Engel M, Dahl R, Paggiaro P, Beck E, Vandewalker M, et al. Tiotropium in asthma poorly controlled with standard combination therapy. N Engl J Med 2012;367:1198-207.

114. Kerstjens HA, Casale TB, Bleecker ER, Meltzer EO, Pizzichini E, Schmidt O, et al. Tiotropium or salmeterol as add-on therapy to inhaled corticosteroids for patients with moderate symptomatic asthma: two replicate, double-blind, placebo-controlled, parallel-group, active-comparator, randomised trials. Lancet Respir Med 2015;3:367-76.

115. Zampogna E, Zappa M, Spanevello A, Visca D. Pulmonary rehabilitation and asthma. Front Pharmacol 2020;11:542.

116. Cusack RP, Satia I, O'Byrne PM. Asthma maintenance and reliever therapy: should this be the standard of care? Ann Allergy Asthma Immunol 2020;125:150-5.

117. Kwah JH, Peters AT. Asthma in adults: principles of treatment. Allergy Asthma Proc 2019;40:396-402.

118. Bender BG, Pedan A, Varasteh LT. Adherence and persistence with fluticasone propionate/salmeterol combination therapy. J Allergy Clin Immunol 2006;118:899-904.

119. Cameron SJ, Cooper EJ, Crompton GK, Hoare MV, Grant IW. Substitution of beclomethasone aerosol for oral prednisolone in the treatment of chronic asthma. Br Med J 1973;4:205-7.

120. Pauwels RA, Pedersen S, Busse WW, Tan WC, Chen YZ, Ohlsson SV, et al.; START Investigators Group. Early intervention with budesonide in mild persistent asthma: a randomised, double-blind trial. Lancet 2003;361:1071-6.

121. John M, Lim S, Seybold J, Jose P, Robichaud A, O'Connor B, et al. Inhaled corticosteroids increase interleukin-10 but reduce macrophage inflammatory protein-1alpha, granulocyte-macrophage colony-stimulating factor, and interferon-gamma release from alveolar macrophages in asthma. Am J Respir Crit Care Med 1998;157:256-62.

122. Moore KW, de Waal Malefyt R, Coffman RL, O'Garra A. Interleukin-10 and the interleukin-10 receptor. Annu Rev Immunol 2001;19:683-765.

123. Morgan SJ, Deshpande DA, Tiegs BC, Misior AM, Yan H, Hershfeld AV, et al. β-agonist-mediated relaxation of airway smooth muscle is protein kinase A-dependent. J Biol Chem 2014;289:23065-74.

124. Johnson M. Beta2-adrenoceptors: mechanisms of action of beta2-agonists. Paediatr Respir Rev 2001;2:57-62.

125. D'Amato M, Vitale C, Molino A, Lanza M, D'Amato G. Anticholinergic drugs in asthma therapy. Curr Opin Pulm Med 2017;23:103-8.

126. Gosens R, Gross N. The mode of action of anticholinergics in asthma. Eur Respir J 2018;52:1701247.

127. Trinh HKT, Lee SH, Cao TBT, Park HS. Asthma pharmacotherapy: an update on leukotriene treatments. Expert Rev Respir Med 2019;13:1169-78.

128. Chauhan BF, Ducharme FM. Anti-leukotriene agents compared to inhaled corticosteroids in the management of recurrent and/or chronic asthma in adults and children. Cochrane Database Syst Rev 2012;2012:CD002314.

129. Morikawa M, Hagiwara Y, Gibo K, Goto T, Watase H, Hasegawa K; JEAN-3 Investigators. Methylxanthine use for acute asthma in the emergency department in Japan: a multicenter observational study. Acute Med Surg 2019;6:279-86.

130. Nair P, Milan SJ, Rowe BH. Addition of intravenous aminophylline to inhaled beta(2)-agonists in adults with acute asthma. Cochrane Database Syst Rev 2012;12:CD002742.

131. Tosca MA, Olcese R, Licari A, Ciprandi G. Allergen immunotherapy and asthma. Pediatr Allergy Immunol 2020;31:46-8.

132. Reddel HK, FitzGerald JM, Bateman ED, Bacharier LB, Becker A, Brusselle G, et al. GINA 2019: a fundamental change in asthma management: treatment of asthma with short-acting bronchodilators alone is no longer recommended for adults and adolescents. Eur Respir J 2019;53:1901046.

133. Global Initiative for Asthma. Global Strategy for Asthma Management and Prevention, 2019. Available from: https://ginasthma.org/archived-reports/ (accessed July 9, 2022).

134. Schuers M, Chapron A, Guihard H, Bouchez T, Darmon D. Impact of non-drug therapies on asthma control: a systematic review of the literature. Eur J Gen Pract 2019;25:65-76.

135. Ledford DK, Lockey RF. Asthma and comorbidities. Curr Opin Allergy Clin Immunol 2013;13:78-86.

136. Wenzel SE. Asthma: defining of the persistent adult phenotypes. Lancet 2006;368:804-13.

137. Wenzel SE, Schwartz LB, Langmack EL, Halliday JL, Trudeau JB, Gibbs RL, et al. Evidence that severe asthma can be divided pathologically into two inflammatory subtypes with distinct physiologic and clinical characteristics. Am J Respir Crit Care Med 1999;160:1001-8.

138. Miranda C, Busacker A, Balzar S, Trudeau J, Wenzel SE. Distinguishing severe asthma phenotypes: role of age at onset and eosinophilic inflammation. J Allergy Clin Immunol 2004;113:101-8.

139. Kupczyk M, Dahlén B, Sterk PJ, Nizankowska-Mogilnicka E, Papi A, Bel EH, et al.; BIOAIR investigators. Stability of phenotypes defined by physiological variables and biomarkers in adults with asthma. Allergy 2014;69:1198-204.

140. Moore WC, Meyers DA, Wenzel SE, Teague WG, Li H, Li X, et al.; National Heart, Lung, and Blood Institute's Severe Asthma Research Program. Identification of asthma phenotypes using cluster analysis in the Severe Asthma Research Program. Am J Respir Crit Care Med 2010;181:315-23.

141. Shaw DE, Sousa AR, Fowler SJ, Fleming LJ, Roberts G, Corfield J, et al.; U-BIOPRED Study Group. Clinical and inflammatory characteristics of the European U-BIOPRED adult severe asthma cohort. Eur Respir J 2015;46:1308-21. Erratum in: Eur Respir J 2017;49.

142. Loza MJ, Djukanovic R, Chung KF, Horowitz D, Ma K, Branigan P, et al.; ADEPT (Airways Disease Endotyping for Personalized Therapeutics) and U-BIOPRED (Unbiased Biomarkers for the Prediction of Respiratory Disease Outcome Consortium) investigators. Validated and longitudinally stable asthma phenotypes based on cluster analysis of the ADEPT study. Respir Res 2016;17:165.

143. McDonald VM, Fingleton J, Agusti A, Hiles SA, Clark VL, Holland AE, et al.; participants of the Treatable Traits Down Under International Workshop; Treatable Traits Down Under International Workshop participants. Treatable traits: a new paradigm for 21st century management of chronic airway diseases: Treatable Traits Down Under International Workshop report. Eur Respir J 2019;53:1802058.

144. Pembrey L, Barreto ML, Douwes J, Cooper P, Henderson J, Mpairwe H, et al. Understanding asthma phenotypes: the World Asthma Phenotypes (WASP) international collaboration. ERJ Open Res 2018;4:00013-2018.

145. Gülşen A. Asthma phenotypes and current biological treatments. In: Chapoval SP, ed. Recent advances in asthma research and treatments [Internet]. London: IntechOpen; 2021. Available from: https://www.intechopen.com/chapters/76328 (accessed June 17, 2021).

146. Ray A, Camiolo M, Fitzpatrick A, Gauthier M, Wenzel SE. Are we meeting the promise of endotypes and precision medicine in asthma? Physiol Rev 2020;100:983-1017.

147. Robinson D, Humbert M, Buhl R, Cruz AA, Inoue H, Korom S, et al. Revisiting type 2-high and type 2-low airway inflammation in asthma: current knowledge and therapeutic implications. Clin Exp Allergy 2017;47:161-75.

148. Simpson JL, Scott R, Boyle MJ, Gibson PG. Inflammatory subtypes in asthma: assessment and identification using induced sputum. Respirology 2006;11:54-61.

149. Nau F Jr, Miller J, Saravia J, Ahlert T, Yu B, Happel KI, et al. Serotonin 5-HT_2 receptor activation prevents allergic asthma in a mouse model. Am J Physiol Lung Cell Mol Physiol 2015;308:L191-8.

150. Erspamer V, Vialli M. Ricerche sul secreto delle cellule enterocromaffini [Research on the secretion of enterochromaffin cells]. Boll d Soc Med-chir Pavia 1937;51:357-63.

151. Rapport MM, Green AA, Page IH. Partial purification of the vasoconstrictor in beef serum. J Biol Chem 1948;174:735-41.

152. Rapport MM, Green AA, Page IH. Crystalline serotonin. Science 1948;108:329-30.

153. Rapport MM. Serum vasoconstrictor (serotonin). V. The presence of creatinine in the complex. A proposed structure of the vasoconstrictor principle. J Biol Chem 1949;180:961-9.

154. Erspamer V, Asero B. Identification of enteramine, the specific hormone of the enterochromaffin cell system, as 5-hydroxytryptamine. Nature 1952;169:800-1.

155. Schoenichen C, Bode C, Duerschmied D. Role of platelet serotonin in innate immune cell recruitment. Front Biosci (Landmark Ed) 2019;24:514-26.

156. Twarog BM, Page IH. Serotonin content of some mammalian tissues and urine and a method for its determination. Am J Physiol 1953;175:157-61.

157. Whitaker-Azmitia PM. The discovery of serotonin and its role in neuroscience. Neuropsychopharmacol 1999;21:S2-8.

158. Berger M, Gray JA, Roth BL. The expanded biology of serotonin. Annu Rev Med 2009;60:355-66.

159. Charnay Y, Léger L. Brain serotonergic circuitries. Dialogues Clin Neurosci 2010;12:471-87.

160. Berumen LC, Rodríguez A, Miledi R, García-Alcocer G. Serotonin receptors in hippocampus. ScientificWorldJournal 2012;2012:823493.

161. Yabut JM, Crane JD, Green AE, Keating DJ, Khan WI, Steinberg GR. Emerging roles for serotonin in regulating metabolism: new implications for an ancient molecule. Endocr Rev 2019;40:1092-107.

162. Kanova M, Kohout P. Serotonin – its synthesis and roles in the healthy and the critically ill. Int J Mol Sci 2021;22:4837.

163. Lv J, Liu F. The role of serotonin beyond the central nervous system during embryogenesis. Front Cell Neurosci 2017;11:74.

164. Diksic M, Young SN. Study of the brain serotonergic system with labeled alpha-methyl-L-tryptophan. J Neurochem 2001;78:1185-200.

165. De Ponti F. Pharmacology of serotonin: what a clinician should know. Gut 2004;53:1520-35.

166. Gershon MD, Tack J. The serotonin signaling system: from basic understanding to drug development for functional GI disorders. Gastroenterology 2007;132:397-414.

167. Cataldo LR, Suazo J, Olmos P, Bravo C, Galgani JE, Fex M, et al. Platelet serotonin levels are associated with plasma soluble leptin receptor concentrations in normoglycemic women. J Diabetes Res 2019;2019:2714049.

168. Spohn SN, Mawe GM. Non-conventional features of peripheral serotonin signalling – the gut and beyond. Nat Rev Gastroenterol Hepatol 2017;14:412-20.

169. Veenstra-VanderWeele J, Anderson GM, Cook EH Jr. Pharmacogenetics and the serotonin system: initial studies and future directions. Eur J Pharmacol 2000;410:165-81.

170. Lau WK, Chan-Yeung MM, Yip BH, Cheung AH, Ip MS, Mak JC; COPD Study Group of the Hong Kong Thoracic Society. The role of circulating serotonin in the development of chronic obstructive pulmonary disease. PLoS One 2012;7:e31617.

171. Kim TH, An SH, Cha JY, Shin EK, Lee JY, Yoon SH, et al. Association of 5-hydroxytryptamine (serotonin) receptor 4 (5-HTR4) gene polymorphisms with asthma. Respirology 2011;16:630-8.

172. Walther DJ, Peter JU, Winter S, Höltje M, Paulmann N, Grohmann M, et al. Serotonylation of small GTPases is a signal transduction pathway that triggers platelet alpha-granule release. Cell 2003;115:851-62.

173. Lam DD, Garfield AS, Marston OJ, Shaw J, Heisler LK. Brain serotonin system in the coordination of food intake and body weight. Pharmacol Biochem Behav 2010;97:84-91.

174. Namkung J, Kim H, Park S. Peripheral serotonin: a new player in systemic energy homeostasis. Mol Cells 2015;38:1023-8.

175. Ahern GP. 5-HT and the immune system. Curr Opin Pharmacol 2011;11:29-33.

176. Dürk T, Duerschmied D, Müller T, Grimm M, Reuter S, Vieira RP, et al. Production of serotonin by tryptophan hydroxylase 1 and release via platelets contribute to allergic airway inflammation. Am J Respir Crit Care Med 2013;187:476-85.

177. Sari Y, Johnson VR, Weedman JM. Role of the serotonergic system in alcohol dependence: from animal models to clinics. Prog Mol Biol Transl Sci 2011;98:401-43.

178. Chabbi-Achengli Y, Coman T, Collet C, Callebert J, Corcelli M, Lin H, et al. Serotonin is involved in autoimmune arthritis through Th17 immunity and bone resorption. Am J Pathol 2016;186:927-37.

179. Gilliam FG, Hecimovic H, Gentry MS. Serotonergic therapy in epilepsy. Curr Opin Neurol 2021;34:206-12.

180. Sourbron J, Lagae L. Serotonin receptors in epilepsy: novel treatment targets? Epilepsia Open 2022;7:231-46.

181. Cazzola I, Matera MG. 5-HT modifiers as a potential treatment of asthma. Trends Pharmacol Sci 2000;21:13-6.

182. Ménard G, Turmel V, Bissonnette EY. Serotonin modulates the cytokine network in the lung: involvement of prostaglandin E2. Clin Exp Immunol 2007;150:340-8.

183. Canal CE. Serotonergic psychedelics: experimental approaches for assessing mechanisms of action. Handb Exp Pharmacol 2018;252:227-60.

184. Cunningham KA, Howell LL, Anastasio NC. Serotonin neurobiology in cocaine use disorder. In: Müller CP, Cunningham KA, eds. Handbook of the behavioral neurobiology of serotonin. London, United Kingdom: Academic Press; 2020. p. 745-802.

185. Dahlstroem A, Fuxe K. Evidence for the existence of monoamine-containing neurons in the central nervous system. I. Demonstration of monoamines in the cell bodies of brain stem neurons. Acta Physiol Scand Suppl 1964;232:1-55.

186. Best J, Nijhout HF, Reed M. Serotonin synthesis, release and reuptake in terminals: a mathematical model. Theor Biol Med Model 2010;7:34.

187. Celada P, Puig MV, Artigas F. Serotonin modulation of cortical neurons and networks. Front Integr Neurosci 2013;7:25.

188. Morrissey JJ, Walker MN, Lovenberg W. The absence of tryptophan hydroxylase activity in blood platelets. Proc Soc Exp Biol Med 1977;154:496-9.

189. Pardridge WM. The role of blood-brain barrier transport of tryptophan and other neutral amino acids in the regulation of substrate-limited pathways of brain amino acid metabolism. J Neural Transm Suppl 1979;15:43-54.

190. Richard DM, Dawes MA, Mathias CW, Acheson A, Hill-Kapturczak N, Dougherty DM. L-tryptophan: basic metabolic functions, behavioral research and therapeutic indications. Int J Tryptophan Res 2009;2:45-60.

191. Ślifirski G, Król M, Turło J. 5-HT receptors and the development of new antidepressants. Int J Mol Sci 2021;22:9015.

192. Ichiyama A, Nakamura S, Nishizuka Y, Hayaishi O. Enzymic studies on the biosynthesis of serotonin in mammalian brain. J Biol Chem 1970;245:1699-709.

193. Höltje M, Winter S, Walther D, Pahner I, Hörtnagl H, Ottersen OP, et al. The vesicular monoamine content regulates VMAT2 activity through Galphaq in mouse platelets. Evidence for autoregulation of vesicular transmitter uptake. J Biol Chem 2003;278:15850-8.

194. Mercado CP, Kilic F. Molecular mechanisms of SERT in platelets: regulation of plasma serotonin levels. Mol Interv 2010;10:231-41.

195. Bortolato M, Shih JC. Behavioral outcomes of monoamine oxidase deficiency: preclinical and clinical evidence. Int Rev Neurobiol 2011;100:13-42.

196. Badawy AA. Tryptophan metabolism in alcoholism. Nutr Res Rev 2002;15:123-52.

197. Beck O, Helander A. 5-hydroxytryptophol as a marker for recent alcohol intake. Addiction 2003;98:63-72.

198. Fishman AP, Pietra GG. Handling of bioactive materials by the lung (second of two parts). N Engl J Med 1974;291:953-9.

199. Dawson CA, Linehan JH, Rickaby DA, Bronikowski TA. Kinetics of serotonin uptake in the intact lung. Ann Biomed Eng 1987;15:217-27.

200. Sandler M, Reveley MA, Glover V. Human platelet monoamine oxidase activity in health and disease: a review. J Clin Pathol 1981;34:292-302.

201. Liu T, Borjigin J. N-acetyltransferase is not the rate-limiting enzyme of melatonin synthesis at night. J Pineal Res 2005;39:91-6.

202. Tordjman S, Chokron S, Delorme R, Charrier A, Bellissant E, Jaafari N, et al. Melatonin: pharmacology, functions and therapeutic benefits. Curr Neuropharmacol 2017;15:434-43.

203. Hannon J, Hoyer D. Molecular biology of 5-HT receptors. Behav Brain Res 2008;195:198-213.

204. Paderina DZ, Boiko AS, Pozhidaev IV, Bocharova AV, Mednova IA, Fedorenko OY, et al. Genetic polymorphisms of 5-HT receptors and antipsychotic-induced metabolic dysfunction in patients with schizophrenia. J Pers Med 2021;11:181.

205. Mlinar B, Montalbano A, Baccini G, Tatini F, Berlinguer Palmini R, Corradetti R. Nonexocytotic serotonin release tonically suppresses serotonergic neuron activity. J Gen Physiol 2015;145:225-51.

206. Quentin E, Belmer A, Maroteaux L. Somato-dendritic regulation of raphe serotonin neurons; a key to antidepressant action. Front Neurosci 2018;12:982.

207. Piñeyro G, Castanon N, Hen R, Blier P. Regulation of [3H]5-HT release in raphe, frontal cortex and hippocampus of 5-HT1B knock-out mice. Neuroreport 1995;7:353-9.

208. Vanhatalo S, Soinila S. The concept of chemical neurotransmission – variations on the theme. Ann Med 1998;30:151-8.

209. Murphy DL, Lerner A, Rudnick G, Lesch KP. Serotonin transporter: gene, genetic disorders, and pharmacogenetics. Mol Interv 2004;4:109-23.

210. Zhou FC, Tao-Cheng JH, Segu L, Patel T, Wang Y. Serotonin transporters are located on the axons beyond the synaptic junctions: anatomical and functional evidence. Brain Res 1998;805:241-54.

211. Bauer M, Bschor T, Pfennig A, Whybrow PC, Angst J, Versiani M, et al.; WFSBP Task Force on Unipolar Depressive Disorders. World Federation of Societies of Biological Psychiatry (WFSBP) guidelines for biological treatment of unipolar depressive disorders in primary care. World J Biol Psychiatry 2007;8:67-104.

212. Chen X, Margolis KJ, Gershon MD, Schwartz GJ, Sze JY. Reduced serotonin reuptake transporter (SERT) function causes insulin resistance and hepatic steatosis independent of food intake. PLoS One 2012;7:e32511.

213. Luo L, Zhang J, Lee J, Tao A. Platelets, not an insignificant player in development of allergic asthma. Cells 2021;10:2038.

214. Maclean JA, Schoenwaelder SM. Serotonin in platelets. In: Pilowsky PM, ed. Serotonin: the mediator that spans evolution. London: Elsevier; 2019. p. 239-70.

215. Segal DM, Taurog JD, Metzger H. Dimeric immunoglobulin E serves as a unit signal for mast cell degranulation. Proc Natl Acad Sci USA 1977;74:2993-7.

216. Janković BD. Neuroimmunomodulation: facts and dilemmas. Immunol Lett 1989;21:101-18.

217. Karege F, Widmer J, Bovier P, Gaillard JM. Platelet serotonin and plasma tryptophan in depressed patients: effect of drug treatment and clinical outcome. Neuropsychopharmacology 1994;10:207-14.

218. Rendu F, Brohard-Bohn B. The platelet release reaction: granules' constituents, secretion and functions. Platelets 2001;12:261-73.

219. Rand R, Reid G. Source of 'serotonin' in serum. Nature 1951;4270:385.

220. Zucker MB, Friedman BK, Rapport MM. Identification and quantitative determination of serotonin (5-hydroxytryptamine) in blood platelets. Proc Soc Exp Biol Med 1954;85:282-5.

221. Mauler M, Bode C, Duerschmied D. Platelet serotonin modulates immune functions. Hämostaseologie 2016;36:11-6.

222. Li N, Wallén NH, Ladjevardi M, Hjemdahl P. Effects of serotonin on platelet activation in whole blood. Blood Coagul Fibrinolysis 1997;8:517-23.

223. Ebermeyer T, Cognasse F, Berthelot P, Mismetti P, Garraud O, Hamzeh-Cognasse H. Platelet innate immune receptors and TLRs: a double-edged sword. Int J Mol Sci 2021;22:7894.

224. Spigset O, Mjorndal T. The effect of fluvoxamine on serum prolactin and serum sodium concentrations: relation to platelet 5-HT2A receptor status. J Clin Psychopharmacol 1997;17:292-7.

225. Mück-Seler D, Pivac N. Serotonin: an overview. Period Biol 2011;113:29-41.

226. Audhya T, Adams JB, Johansen L. Correlation of serotonin levels in CSF, platelets, plasma, and urine. Biochim Biophys Acta 2012;1820:1496-501.

227. Hilaire G, Voituron N, Menuet C, Ichiyama RM, Subramanian HH, Dutschmann M. The role of serotonin in respiratory function and dysfunction. Respir Physiol Neurobiol 2010;174:76-88.

228. Momi S, Pichford S, Gresele P, Page CP. Platelets and airway diseases. In: Gresele P, Kleiman N, Lopez J, Page C, eds. Platelets in thrombotic and non-thrombotic disorders. Springer: Cham; 2017. p. 1149-68.

229. Yamamoto Y, Hasegawa H, Inoue F, Ikeda K, Ichiyama A. Serotonin in the lung. Demonstration of a close correlation to blood platelet. Agents Actions 1986;18:351-8.

230. Cazzola M, Matera MG, D'Amato G, Rossi F. Effects of serotonin on airways: recent developments. Allergy 1995;50:1-10.

231. Lechin F, van der Dijs B, Lechin AE. Severe asthma and plasma serotonin. Allergy 2002;57:258-9.

232. Lechin F, Lechin AE, van der Dijs B. Tianeptine, plasma serotonin, and pulmonary hypertension. Lancet 2003;361:87.

233. Chebbo M, Duez C, Alessi MC, Chanez P, Gras D. Platelets: a potential role in chronic respiratory diseases? Eur Respir Rev 2021;30:210062.

234. Ulrich S, Huber LC, Fischler M, Treder U, Maggiorini M, Eberli FR, et al. Platelet serotonin content and transpulmonary platelet serotonin gradient in patients with pulmonary hypertension. Respiration 2011;81:211-6.

235. Udenfriend S, Weissbach H. Turnover of 5-hydroxytryptamine (serotonin) in tissues. Proc Soc Exp Biol Med 1958;97:748-51.

236. Hervig TA, Farstad M, Vollset SE. Endogenous serotonin in human blood platelets: factors that may influence reference values. Platelets 1996;7:47-52.

237. Jernej B, Banović M, Cicin-Sain L, Hranilović D, Balija M, Oresković D, et al. Physiological characteristics of platelet/circulatory serotonin: study on a large human population. Psychiatry Res 2000;94:153-62.

238. Fernandez-Rodriguez S, Broadley KJ, Ford WR, Kidd EJ. Increased muscarinic receptor activity of airway smooth muscle isolated from a mouse model of allergic asthma. Pulm Pharmacol Ther 2010;23:300-7.

239. Segura P, Vargas MH, Córdoba-Rodríguez G, Chávez J, Arreola JL, Campos-Bedolla P, et al. Role of 5-HT2A, 5-HT4 and 5-HT7 receptors in the antigen-induced airway hyperresponsiveness in guinea-pigs. Clin Exp Allergy 2010;40:327-38.

240. Arreola-Ramírez JL, Vargas MH, Manjarrez-Gutiérrez G, Alquicira J, Gutiérrez J, Córdoba G, et al. Modifications of plasma 5-HT concentrations during the allergic bronchoconstriction in guinea pigs. Exp Lung Res 2013;39:269-74.

241. Pavón-Romero GF, Serrano-Pérez NH, García-Sánchez L, Ramírez-Jiménez F, Terán LM. Neuroimmune pathophysiology in asthma. Front Cell Dev Biol 2021;9:663535.

242. Adner M, Rose AC, Zhang Y, Swärd K, Benson M, Uddman R, et al. An assay to evaluate the long-term effects of inflammatory mediators on murine airway smooth muscle: evidence that TNFalpha up-regulates 5-HT(2A)-mediated contraction. Br J Pharmacol 2002;137:971-82.

243. Nagatomo T, Rashid M, Abul Muntasir H, Komiyama T. Functions of 5-HT2A receptor and its antagonists in the cardiovascular system. Pharmacol Ther 2004;104:59-81.

244. Nichols DE, Nichols CD. Serotonin receptors. Chem Rev 2008;108:1614-41.

245. Stefulj J, Jernej B, Cicin-Sain L, Rinner I, Schauenstein K. mRNA expression of serotonin receptors in cells of the immune tissues of the rat. Brain Behav Immun 2000;14:219-24.

246. León-Ponte M, Ahern GP, O'Connell PJ. Serotonin provides an accessory signal to enhance T-cell activation by signaling through the 5-HT7 receptor. Blood 2007;109:3139-46.

247. Inoue M, Okazaki T, Kitazono T, Mizushima M, Omata M, Ozaki S. Regulation of antigen-specific CTL and Th1 cell activation through 5-hydroxytryptamine 2A receptor. Int Immunopharmacol 2011;11:67-73.

248. Mikulski Z, Zaslona Z, Cakarova L, Hartmann P, Wilhelm J, Tecott LH, et al. Serotonin activates murine alveolar macrophages through 5-HT2C receptors. Am J Physiol Lung Cell Mol Physiol 2010;299:L272-80.

249. Kang BN, Ha SG, Bahaie NS, Hosseinkhani MR, Ge XN, Blumenthal MN, et al. Regulation of serotonin-induced trafficking and migration of eosinophils. PLoS One 2013;8:e54840.

250. Campos-Bedolla P, Vargas MH, Segura P, Carbajal V, Calixto E, Figueroa A, et al. Airway smooth muscle relaxation induced by 5-HT(2A) receptors: role of Na(+)/K(+)-ATPase pump and Ca(2+)-activated K(+) channels. Life Sci 2008;83:438-46.

251. Mao HQ, Morimoto K, Shirakawa T, Hopkin JM, Hashimoto T, Furuyama J, et al. Association between serotonin type 2 receptor (HTR2) and bronchial asthma in humans. J Med Genet 1996;33:525.

252. Pauwels RA, Van der Straeten ME. An animal model for adenosine-induced bronchoconstriction. Am Rev Respir Dis 1987;136:374-8.

253. Manzini S, Ballati D. 2-chloroadenosine induction of vagally-mediated and anaesthetized guinea pigs. Br J Pharmacol 1990;100:251-6.

254. Cazzola M, Matera MG, Santangelo G, Assogna G, D'Amato G, Rossi F, et al. Effect of the selective 5-HT2 antagonist ketanserin on adenosine-induced bronchoconstriction in asthmatic subjects. Immunopharmacology 1992;23:21-8.

255. 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.

256. Flanagan TW, Sebastian MN, Battaglia DM, Foster TP, Cormier SA, Nichols CD. 5-HT_2 receptor activation alleviates airway inflammation and structural remodeling in a chronic mouse asthma model. Life Sci 2019;236:116790.

257. Nau F Jr, Yu B, Martin D, Nichols CD. Serotonin 5-HT_{2A} receptor activation blocks TNF-α mediated inflammation in vivo. PLoS One 2013;8:e75426.

258. Lechin AE, Varon J, van der Dijs B, Lechin F. Plasma catecholamines and indolamines during attack and remission on severe bronchial asthma: possible role of stress [abstract]. Am J Respir Crit Care Med 1994;149:A778.

259. 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.

260. Lechin F. Asthma, asthma medication and autonomic nervous system dysfunction. Clin Physiol 2001;21:723.

261. Pretorius, E. Asthma medication and the role of serotonin in the development of cognitive and psychological difficulties. Early Child Dev Care 2005;175:139-51.

262. Lechin F, van der Dijs B, Orozco B, Jara H, Rada I, Lechin ME, et al. Neuropharmacologic treatment of bronchial asthma with the antidepressant tianeptine: a double-blind, crossover placebo-controlled study. Clin Pharmacol Ther 1998;64:223-32.

263. Lechin F, van der Dijs B, Orozco B, Jara H, Rada I, Lechin ME, et al. The serotonin uptake-enhancing drug tianeptine suppresses asthmatic symptoms in children: a double-blind, crossover, placebo-controlled study. J Clin Pharmacol 1998;38:918-25.

264. Mück-Seler D, Pivac N, Šagud M, Jakovljević M, Mihaljević-Peles A. The effects of paroxetine and tianeptine on peripheral biochemical markers in major depression. Prog Neuropsychopharmacol Biol Psychiatry 2002;26:1235-43.

265. Lechin F, van der Dijs B, Jara H, Orozco B, Baez S, Benaim M, et al. Effects of buspirone on plasma neurotransmitters in healthy subjects. J Neural Transm (Vienna) 1998;105:561-73.

266. Lechin F. Central and plasma 5-HT, vagal tone and airways. Trends Pharmacol Sci 2000;21:425.

267. Lechin F, van der Dijs B, Lechin AE. Treatment of bronchial asthma with tianeptine. Methods Find Exp Clin Pharmacol 2004;26:697-701.

268. Malmgren R, Grubbström J, Olsson P, Theorell H, Tornling G, Unge G. Defective serotonin (5-HT) transport mechanism in platelets from patients with endogenous and allergic asthma. Allergy 1982;37:29-39.

269. Matkar NM, Rupwate RU, Desai NK, Kamat SR. Comparative study of platelet histamine and serotonin with their corresponding plasma oxidases in asthmatics with normals. J Assoc Physicians India 1999;47:878-82.

270. Ramsay RR. Monoamine oxidases: the biochemistry of the proteins as targets in medicinal chemistry and drug discovery. Curr Top Med Chem 2012;12:2189-209.

271. Saccone NL, Rice JP, Rochberg N, Williams JT, Goate A, Reich T, et al. Linkage for platelet monoamine oxidase (MAO) activity: results from a replication sample. Alcohol Clin Exp Res 2002;26:603-9.

272. Hare ML. Tyramine oxidase: a new enzyme system in liver. Biochem J 1928;22:968-79.

273. Schnaitman C, Erwin VG, Greenawalt JW. The submitochondrial localization of monoamine oxidase. An enzymatic marker for the outer membrane of rat liver mitochondria. J Cell Biol 1967;32:719-35.

274. Lee HW, Choi H, Nam SJ, Fenical W, Kim H. Potent inhibition of monoamine oxidase B by a piloquinone from marine-derived *Streptomyces* sp. CNQ-027. J Microbiol Biotechnol 2017;27:785-90.

275. Bach AW, Lan NC, Johnson DL, Abell CW, Bembenek ME, Kwan SW, et al. cDNA cloning of human liver monoamine oxidase A and B: molecular basis of differences in enzymatic properties. Proc Natl Acad Sci USA 1988;85:4934-8.

276. Kaludercic N, Carpi A, Nagayama T, Sivakumaran V, Zhu G, Lai EW, et al. Monoamine oxidase B prompts mitochondrial and cardiac dysfunction in pressure overloaded hearts. Antioxid Redox Signal 2014;20:267-80.

277. Kaludercic N, Mialet-Perez J, Paolocci N, Parini A, Di Lisa F. Monoamine oxidases as sources of oxidants in the heart. J Mol Cell Cardiol 2014;73:34-42.

278. Sturza A, Popoiu CM, Ionică M, Duicu OM, Olariu S, Muntean DM, et al. Monoamine oxidase-related vascular oxidative stress in diseases associated with inflammatory burden. Oxid Med Cell Longev 2019;2019:8954201.

279. Sommer N, Schulz R. Mitochondrial monoamine oxidase: another player in pulmonary hypertension? Am J Respir Cell Mol Biol 2021;64:277-8.

280. Lan NC, Heinzmann C, Gal A, Klisak I, Orth U, Lai E, et al. Human monoamine oxidase A and B genes map to Xp 11.23 and are deleted in a patient with Norrie disease. Genomics 1989;4:552-9.

281. Rendu F, Peoc'h K, Berlin I, Thomas D, Launay JM. Smoking related diseases: the central role of monoamine oxidase. Int J Environ Res Public Health 2011;8:136-47.

282. Ostadkarampour M, Putnins EE. Monoamine oxidase inhibitors: a review of their anti-inflammatory therapeutic potential and mechanisms of action. Front Pharmacol 2021;12:676239.

283. Edmondson DE, Mattevi A, Binda C, Li M, Hubalek F. Structure and mechanism of monoamine oxidase. Curr Med Chem 2004;11:1983-93.

284. Chen K, Wu HF, Shih JC. The deduced amino acid sequences of human platelet and frontal cortex monoamine oxidase B are identical. J Neurochem 1993;61:187-90.

285. Donnelly CH, Murphy DL. Substrate and inhibitor-related characteristics of human platelet monoamine oxidase. Biochem Pharmacol 1977;26:853-8.

286. Coccini T, Castoldi AF, Gandini C, Randine G, Vittadini G, Baiardi P, et al. Platelet monoamine oxidase B activity as a state marker for alcoholism: trend over time during withdrawal and influence of smoking and gender. Alcohol Alcohol 2002;37:566-72.

287. Wyatt RJ, Murphy DL, Belmaker R, Cohen S, Donnelly CH, Pollin W. Reduced monoamine oxidase activity in platelets: a possible genetic marker for vulnerability to schizophrenia. Science 1973;179:916-8.

288. Fowler CJ, Tipton KF, MacKay AVP, Youdim MBH. Human platelet monoamine oxidase: a useful enzyme in the study of psychiatric disorders? Neuroscience 1982;7:1577-94.

289. Whitfield JB, Pang D, Bucholz KK, Madden PA, Heath AC, Statham DJ, et al. Monoamine oxidase: associations with alcohol dependence, smoking and other measures of psychopathology. Psychol Med 2000;30:443-54.

290. Fowler JS, Logan J, Wang GJ, Volkow ND. Monoamine oxidase and cigarette smoking. Neurotoxicology 2003;24:75-82.

291. Padmavathi P, Reddy VD, Swarnalatha K, Hymavathi R, Varadacharyulu NCh. Influence of altered hormonal status on platelet 5-HT and MAO-B activity in cigarette smokers. Indian J Clin Biochem 2015;30:204-9.

292. Ruchkin VV, Koposov RA, af Klinteberg B, Oreland L, Grigorenko EL. Platelet MAO-B, personality, and psychopathology. J Abnorm Psychol 2005;114:477-82.

293. Rommelspacher H, May T, Dufeu P, Schmidt LG. Longitudinal observations of monoamine oxidase B in alcoholics: differentiation of marker characteristics. Alcohol Clin Exp Res 1994;18:1322-9.

294. Soyka M, Bondy B, Benda E, Preuss U, Hegerl U, Möller H. Platelet monoamine oxidase activity in alcoholics with and without a family history of alcoholism. Eur Addict Res 2000;6:57-63.

295. Muck-Seler D, Presecki P, Mimica N, Mustapic M, Pivac N, Babic A, et al. Platelet serotonin concentration and monoamine oxidase type B activity in female patients in early, middle and late phase of Alzheimer's disease. Prog Neuropsychopharmacol Biol Psychiatry 2009;33:1226-31.

296. Youdim MB. Platelet monoamine oxidase B: use and misuse. Experientia 1988;44:137-41.

297. Ramsay RR. Molecular aspects of monoamine oxidase B. Prog Neuropsychopharmacol Biol Psychiatry 2016;69:81-9.

298. Gealageas R, Devineau A, So PPL, Kim CMJ, Surendradoss J, Buchwalder C, et al. Development of novel monoamine oxidase-B (MAO-B) inhibitors with reduced blood-brain barrier permeability for the potential management of noncentral nervous system (CNS) diseases. J Med Chem 2018;61:7043-64.

299. Carradori S, Secci D, Petzer JP. MAO inhibitors and their wider applications: a patent review. Expert Opin Ther Pat 2018;28:211-26.

300. Deshwal S, Di Sante M, Di Lisa F, Kaludercic N. Emerging role of monoamine oxidase as a therapeutic target for cardiovascular disease. Curr Opin Pharmacol 2017;33:64-9.

301. Shih JC. Monoamine oxidase isoenzymes: genes, functions and targets for behavior and cancer therapy. J Neural Transm (Vienna) 2018;125:1553-66.

302. Vitiello L, Marabita M, Sorato E, Nogara L, Forestan G, Mouly V, et al. Drug repurposing for Duchenne muscular dystrophy: the monoamine oxidase B inhibitor safinamide ameliorates the pathological phenotype in *mdx* mice and in myogenic cultures from DMD patients. Front Physiol 2018;9:1087.

303. Lin A, Song C, Kenis G, Bosmans E, De Jongh R, Scharpé S, et al. The in vitro immunosuppressive effects of moclobemide in healthy volunteers. J Affect Disord 2000;58:69-74.

304. Bielecka AM, Paul-Samojedny M, Obuchowicz E. Moclobemide exerts anti-inflammatory effect in lipopolysaccharide-activated primary mixed glial cell culture. Naunyn Schmiedebergs Arch Pharmacol 2010;382:409-17.

305. Cui Y, Liu KW, Liang Y, Ip MS, Mak JC. Inhibition of monoamine oxidase-B by selegiline reduces cigarette smoke-induced oxidative stress and inflammation in airway epithelial cells. Toxicol Lett 2017;268:44-50.

306. Ekuni D, Firth JD, Nayer T, Tomofuji T, Sanbe T, Irie K, et al. Lipopolysaccharide-induced epithelial monoamine oxidase mediates alveolar bone loss in a rat chronic wound model. Am J Pathol 2009;175:1398-409.

307. Putnins EE, Goebeler V, Ostadkarampour M. Monoamine oxidase-B inhibitor reduction in pro-inflammatory cytokines mediated by inhibition of cAMP-PKA/EPAC signaling. Front Pharmacol 2021;12:741460.

308. Deshwal S, Forkink M, Hu CH, Buonincontri G, Antonucci S, Di Sante M, et al. Monoamine oxidase-dependent endoplasmic reticulum-mitochondria dysfunction and mast cell degranulation lead to adverse cardiac remodeling in diabetes. Cell Death Differ 2018;25:1671-85.

309. Lieb J. Remission of rheumatoid arthritis and other disorders of immunity in patients taking monoamine oxidase inhibitors. Int J Immunopharmacol 1983;5:353-7.

310. Kast RE. Crohn's disease remission with phenelzine treatment. Gastroenterology 1998;115:1034-5.

311. Barnes PJ. Inflammatory mechanisms in patients with chronic obstructive pulmonary disease. J Allergy Clin Immunol 2016;138:16-27.

312. Elisia I, Lam V, Cho B, Hay M, Li MY, Yeung M, et al. The effect of smoking on chronic inflammation, immune function and blood cell composition. Sci Rep 2020;10:19480.

313. Cui Y, Liu KWK, Ip MSM, Liang Y, Mak JCW. Protective effect of selegiline on cigarette smoke-induced oxidative stress and inflammation in rat lungs *in vivo*. Ann Transl Med 2020;8:1418.

314. Nocker RE, Schoonbrood DF, van de Graaf EA, Hack CE, Lutter R, Jansen HM, et al. Interleukin-8 in airway inflammation in patients with asthma and chronic obstructive pulmonary disease. Int Arch Allergy Immunol 1996;109:183-91.

315. Pease JE, Sabroe I. The role of interleukin-8 and its receptors in inflammatory lung disease: implications for therapy. Am J Respir Med 2002;1:19-25.

316. Mathov E. Acción perjudicial, agravante, de un inhibidor de la monamine oxidasa (iproniazida) en al asma grave irreductible [Harmful aggravating action of an inhibitor of monoamine oxidase (iproniazid) in severe unremitting asthma]. Rev argent Alergia 1959;6:187.

317. Mathov E. The risks of monoamine oxidase inhibitors in the treatment of bronchial asthma. J Allergy 1963;34:483-8.

318. Cortés JL, Radillo SA, Villagran J. Inhibidores de la monamine oxidase y reacciones alergicas [Inhibitors of monoamine oxidase and allergic reactions]. Rev argent Alergia 1961;8:89.

319. Keefe KM, Sheikh IS, Smith GM. Targeting neurotrophins to specific populations of neurons: NGF, BDNF, and NT-3 and their relevance for treatment of spinal cord injury. Int J Mol Sci 2017;18:548.

320. Notaras M, van den Buuse M. Brain-derived neurotrophic factor (BDNF): novel insights into regulation and genetic variation. Neuroscientist 2019;25:434-54.

321. Bathina S, Das UN. Brain-derived neurotrophic factor and its clinical implications. Arch Med Sci 2015;11:1164-78.

322. Maisonpierre PC, Le Beau MM, Espinosa R 3rd, Ip NY, Belluscio L, de la Monte SM, et al. Human and rat brain-derived neurotrophic factor and neurotrophin-3: gene structures, distributions, and chromosomal localizations. Genomics 1991;10:558-68.

323. Barde YA, Edgar D, Thoenen H. Purification of a new neurotrophic factor from mammalian brain. EMBO J 1982;1:549-53.

324. Hempstead BL. Brain-derived neurotrophic factor: three ligands, many actions. Trans Am Clin Climatol Assoc 2015;126:9-19.

325. Miranda M, Morici JF, Zanoni MB, Bekinschtein P. Brain-derived neurotrophic factor: a key molecule for memory in the healthy and the pathological brain. Front Cell Neurosci 2019;13:363.

326. Fernandes BS, Berk M, Turck CW, Steiner J, Gonçalves CA. Decreased peripheral brain-derived neurotrophic factor levels are a biomarker of disease activity in major psychiatric disorders: a comparative meta-analysis. Mol Psychiatry 2014;19:750-1.

327. Fernandes BS, Molendijk ML, Köhler CA, Soares JC, Leite CM, Machado-Vieira R, et al. Peripheral brain-derived neurotrophic factor (BDNF) as a biomarker in bipolar disorder: a meta-analysis of 52 studies. BMC Med 2015;13:289.

328. Karege F, Perret G, Bondolfi G, Schwald M, Bertschy G, Aubry JM. Decreased serum brain-derived neurotrophic factor levels in major depressed patients. Psychiatry Res 2002;109:143-8.

329. Karege F, Bondolfi G, Gervasoni N, Schwald M, Aubry JM, Bertschy G. Low brain-derived neurotrophic factor (BDNF) levels in serum of depressed patients probably results from lowered platelet BDNF release unrelated to platelet reactivity. Biol Psychiatry 2005;57:1068-72.

330. Sen S, Duman R, Sanacora G. Serum brain-derived neurotrophic factor, depression, and antidepressant medications: meta-analyses and implications. Biol Psychiatry 2008;64:527-32.

331. Shimizu E, Hashimoto K, Okamura N, Koike K, Komatsu N, Kumakiri C, et al. Alterations of serum levels of brain-derived neurotrophic factor (BDNF) in depressed patients with or without antidepressants. Biol Psychiatry 2003;54:70-5.

332. Green MJ, Matheson SL, Shepherd A, Weickert CS, Carr VJ. Brain-derived neurotrophic factor levels in schizophrenia: a systematic review with meta-analysis. Mol Psychiatry 2011;16:960-72.

333. Weickert CS, Lee CH, Lenroot RK, Bruggemann J, Galletly C, Liu D, et al. Increased plasma brain-derived neurotrophic factor (BDNF) levels in females with schizophrenia. Schizophr Res 2019;209:212-7.

334. Singh J, Verma R, Raghav R, Sarkar S, Sood M, Jain R. Brain-derived neurotrophic factor (BDNF) levels in first-episode schizophrenia and healthy controls: a comparative study. Asian J Psychiatr 2020;54:102370.

335. Jiang L, Zhang H, Wang C, Ming F, Shi X, Yang M. Serum level of brain-derived neurotrophic factor in Parkinson's disease: a meta-analysis. Prog Neuropsychopharmacol Biol Psychiatry 2019;88:168-74.

336. Karimi N, Ashourizadeh H, Akbarzadeh Pasha B, Haghshomar M, Jouzdani T, Shobeiri P, et al. Blood levels of brain-derived neurotrophic factor (BDNF) in people with multiple sclerosis (MS): a systematic review and meta-analysis. Mult Scler Relat Disord 2022;65:103984.

337. Naegelin Y, Saeuberli K, Schaedelin S, Dingsdale H, Magon S, Baranzini S, et al. Levels of brain-derived neurotrophic factor in patients with multiple sclerosis. Ann Clin Transl Neurol 2020;7:2251-61.

338. Comini-Frota ER, Rodrigues DH, Miranda EC, Brum DG, Kaimen-Maciel DR, Donadi EA, et al. Serum levels of brain-derived neurotrophic factor correlate with the number of T2 MRI lesions in multiple sclerosis. Braz J Med Biol Res 2012;45:68-71.

339. Faria MC, Gonçalves GS, Rocha NP, Moraes EN, Bicalho MA, Gualberto Cintra MT, et al. Increased plasma levels of BDNF and inflammatory markers in Alzheimer's disease. J Psychiatr Res 2014;53:166-72.

340. Ng TKS, Ho CSH, Tam WWS, Kua EH, Ho RC. Decreased serum brain-derived neurotrophic factor (BDNF) levels in patients with Alzheimer's disease (AD): a systematic review and meta-analysis. Int J Mol Sci 2019;20:257.

341. Kim BY, Lee SH, Graham PL, Angelucci F, Lucia A, Pareja-Galeano H, et al. Peripheral brain-derived neurotrophic factor levels in Alzheimer's disease and mild cognitive impairment: a comprehensive systematic review and meta-analysis. Mol Neurobiol 2017;54:7297-311.

342. Gutierrez A, Corey-Bloom J, Thomas EA, Desplats P. Evaluation of biochemical and epigenetic measures of peripheral brain-derived neurotrophic factor (BDNF) as a biomarker in Huntington's disease patients. Front Mol Neurosci 2020;12:335.

343. Prakash YS, Martin RJ. Brain-derived neurotrophic factor in the airways. Pharmacol Ther 2014;143:74-86.

344. Lommatzsch M, Schloetcke K, Klotz J, Schuhbaeck K, Zingler D, Zingler C, et al. Brain-derived neurotrophic factor in platelets and airflow limitation in asthma. Am J Respir Crit Care Med 2005;171:115-20.

345. Fujimura H, Altar CA, Chen R, Nakamura T, Nakahashi T, Kambayashi J, et al. Brain-derived neurotrophic factor is stored in human platelets and released by agonist stimulation. Thromb Haemost 2002;87:728-34.

346. Yang Y, Zhao M, Zhang Y, Shen X, Yuan Y. Correlation of 5-HTT, BDNF and NPSR1 gene polymorphisms with anxiety and depression in asthmatic patients. Int J Mol Med 2016;38:65-74.

347. Braun A, Lommatzsch M, Lewin GR, Virchow JC, Renz H. Neurotrophins: a link between airway inflammation and airway smooth muscle contractility in asthma? Int Arch Allergy Immunol 1999;118:163-5.

348. Gejl AK, Enevold C, Bugge A, Andersen MS, Nielsen CH, Andersen LB. Associations between serum and plasma brain-derived neurotrophic factor and influence of storage time and centrifugation strategy. Sci Rep 2019;9:9655.

349. Le Blanc J, Fleury S, Boukhatem I, Bélanger JC, Welman M, Lordkipanidzé M. Platelets selectively regulate the release of BDNF, but not that of its precursor protein, proBDNF. Front Immunol 2020;11:575607.

350. Naegelin Y, Dingsdale H, Säuberli K, Schädelin S, Kappos L, Barde YA. Measuring and validating the levels of brain-derived neurotrophic factor in human serum. eNeuro 2018;5:ENEURO.0419-17.2018.

351. Hanson IM, Seawright A, van Heyningen V. The human BDNF gene maps between FSHB and HVBS1 at the boundary of 11p13-p14. Genomics 1992;13:1331-3.

352. Pruunsild P, Kazantseva A, Aid T, Palm K, Timmusk T. Dissecting the human BDNF locus: bidirectional transcription, complex splicing, and multiple promoters. Genomics 2007;90:397-406.

353. Sheikh HI, Hayden EP, Kryski KR, Smith HJ, Singh SM. Genotyping the BDNF rs6265 (val66met) polymorphism by one-step amplified refractory mutation system PCR. Psychiatr Genet 2010;20:109-12.

354. Egan MF, Kojima M, Callicott JH, Goldberg TE, Kolachana BS, Bertolino A, et al. The BDNF val66met polymorphism affects activity-dependent secretion of BDNF and human memory and hippocampal function. Cell 2003;112:257-69.

355. Chen ZY, Patel PD, Sant G, Meng CX, Teng KK, Hempstead BL, et al. Variant brain-derived neurotrophic factor (BDNF) (Met66) alters the intracellular trafficking and activity-dependent secretion of wild-type BDNF in neurosecretory cells and cortical neurons. J Neurosci 2004;24:4401-11.

356. Notaras M, Hill R, van den Buuse M. A role for the BDNF gene Val66Met polymorphism in schizophrenia? A comprehensive review. Neurosci Biobehav Rev 2015;51:15-30.

357. Chiaruttini C, Vicario A, Li Z, Baj G, Braiuca P, Wu Y, et al. Dendritic trafficking of BDNF mRNA is mediated by translin and blocked by the G196A (Val66Met) mutation. Proc Natl Acad Sci USA 2009;106:16481-6.

358. Hariri AR, Goldberg TE, Mattay VS, Kolachana BS, Callicott JH, Egan MF, et al. Brain-derived neurotrophic factor val66met polymorphism affects human memory-related hippocampal activity and predicts memory performance. J Neurosci 2003;23:6690-4.

359. Pezawas L, Verchinski BA, Mattay VS, Callicott JH, Kolachana BS, Straub RE, et al. The brain-derived neurotrophic factor val66met polymorphism and variation in human cortical morphology. J Neurosci 2004;24:10099-102.

360. Bath KG, Lee FS. Variant BDNF (Val66Met) impact on brain structure and function. Cogn Affect Behav Neurosci 2006;6:79-85.

361. Chen ZY, Jing D, Bath KG, Ieraci A, Khan T, Siao CJ, et al. Genetic variant BDNF (Val66Met) polymorphism alters anxiety-related behavior. Science 2006;314:140-3.

362. Klein R, Nanduri V, Jing SA, Lamballe F, Tapley P, Bryant S, et al. The trkB tyrosine protein kinase is a receptor for brain-derived neurotrophic factor and neurotrophin-3. Cell 1991;66:395-403.

363. Soppet D, Escandon E, Maragos J, Middlemas DS, Reid SW, Blair J, et al. The neurotrophic factors brain-derived neurotrophic factor and neurotrophin-3 are ligands for the trkB tyrosine kinase receptor. Cell 1991;65:895-903.

364. Nakagawara A, Liu XG, Ikegaki N, White PS, Yamashiro DJ, Nycum LM, et al. Cloning and chromosomal localization of the human TRK-B tyrosine kinase receptor gene (NTRK2). Genomics 1995;25:538-46.

365. Luberg K, Wong J, Weickert CS, Timmusk T. Human TrkB gene: novel alternative transcripts, protein isoforms and expression pattern in the prefrontal cerebral cortex during postnatal development. J Neurochem 2010;113:952-64.

366. Klein R, Parada LF, Coulier F, Barbacid M. TrkB, a novel tyrosine protein kinase receptor expressed during mouse neural development. EMBO J 1989;8:3701-9.

367. Klein R, Conway D, Parada LF, Barbacid M. The trkB tyrosine protein kinase gene codes for a second neurogenic receptor that lacks the catalytic kinase domain. Cell 1990;61:647-56.

368. Middlemas DS, Lindberg RA, Hunter T. TrkB, a neural receptor protein-tyrosine kinase: evidence for a full-length and two truncated receptors. Mol Cell Biol 1991;11:143-53.

369. Gupta VK, You Y, Gupta VB, Klistorner A, Graham SL. TrkB receptor signalling: implications in neurodegenerative, psychiatric and proliferative disorders. Int J Mol Sci 2013;14:10122-42.

370. Segal RA. Selectivity in neurotrophin signaling: theme and variations. Annu Rev Neurosci 2003;26:299-330.

371. Chao MV. Neurotrophins and their receptors: a convergence point for many signalling pathways. Nat Rev Neurosci 2003;4:299-309.

372. Minichiello L. TrkB signalling pathways in LTP and learning. Nat Rev Neurosci 2009;10:850-60.

373. Numakawa T, Suzuki S, Kumamaru E, Adachi N, Richards M, Kunugi H. BDNF function and intracellular signaling in neurons. Histol Histopathol 2010;25:237-58.

374. Park H, Poo MM. Neurotrophin regulation of neural circuit development and function. Nat Rev Neurosci 2013;14:7-23.

375. Renz H. Neurotrophins in bronchial asthma. Respir Res 2001;2:265-8.

376. Manti S, Brown P, Perez MK, Piedimonte G. The role of neurotrophins in inflammation and allergy. Vitam Horm 2017;104:313-41.

377. Nockher WA, Renz H. Neurotrophins and asthma: novel insight into neuroimmune interaction. J Allergy Clin Immunol 2006;117:67-71.

378. Nassenstein C, Kerzel S, Braun A. Neurotrophins and neurotrophin receptors in allergic asthma. Prog Brain Res 2004;146:347-67.

379. Braun A, Lommatzsch M, Neuhaus-Steinmetz U, Quarcoo D, Glaab T, McGregor GP, et al. Brain-derived neurotrophic factor (BDNF) contributes to neuronal dysfunction in a model of allergic airway inflammation. Br J Pharmacol 2004;141:431-40.

380. Prakash Y, Thompson MA, Meuchel L, Pabelick CM, Mantilla CB, Zaidi S, et al. Neurotrophins in lung health and disease. Expert Rev Respir Med 2010;4:395-411.

381. Braun A, Nockher WA, Renz H. Control of nerve growth and plasticity. Curr Opin Pharmacol 2002;2:229-34.

382. Barnes PJ. Neurogenic inflammation in airways. Int Arch Allergy Appl Immunol 1991;94:303-9.

383. Barnes PJ. Neurogenic inflammation and asthma. J Asthma 1992;29:165-80.

384. Barnes PJ. Neurogenic inflammation in the airways. Respir Physiol 2001;125:145-54.

385. Joos GF, De Swert KO, Schelfhout V, Pauwels RA. The role of neural inflammation in asthma and chronic obstructive pulmonary disease. Ann N Y Acad Sci 2003;992:218-30.

386. Lomia M, Tchelidze T, Pruidze M. Bronchial asthma as neurogenic paroxysmal inflammatory disease: a randomized trial with carbamazepine. Respir Med 2006;100:1988-96.

387. Walter MJ, Holtzman MJ. A centennial history of research on asthma pathogenesis. Am J Respir Cell Mol Biol 2005;32:483-9.

388. Arráez-Aybar LA, Navia-Álvarez P, Fuentes-Redondo T, Bueno-López JL. Thomas Willis, a pioneer in translational research in anatomy (on the 350th anniversary of Cerebri anatome). J Anat 2015;226:289-300.

389. Reed CE. The pathogenesis of asthma. Med Clin North Am 1974;58:55-63.

390. Audrit KJ, Delventhal L, Aydin Ö, Nassenstein C. The nervous system of airways and its remodeling in inflammatory lung diseases. Cell Tissue Res 2017;367:571-90.

391. Raji MA. The nervous system as potential targets for asthma treatments: lessons learned from a centennial history of asthma research. Am J Respir Cell Mol Biol 2005;33:211.

392. Jain S, Jain KC. Effect of phenytoin sodium in the management of poorly controlled bronchial asthma at a rural health center in Phalodi, Rajasthan, India. J Asthma 1991;28:201-11.

393. Satia I, Nagashima A, Usmani OS. Exploring the role of nerves in asthma; insights from the study of cough. Biochem Pharmacol 2020;179:113901.

394. Drake MG, Cook M, Fryer AD, Jacoby DB, Scott GD. Airway sensory nerve plasticity in asthma and chronic cough. Front Physiol 2021;12:720538.

395. Noga O, Hanf G, Schäper C, O'Connor A, Kunkel G. The influence of inhalative corticosteroids on circulating nerve growth factor, brain-derived neurotrophic factor and neurotrophin-3 in allergic asthmatics. Clin Exp Allergy 2001;31:1906-12.

396. Lommatzsch M, Lindner Y, Edner A, Bratke K, Kuepper M, Virchow JC. Adverse effects of salmeterol in asthma: a neuronal perspective. Thorax 2009;64:763-9.

397. Gosens R, Zaagsma J, Meurs H, Halayko AJ. Muscarinic receptor signaling in the pathophysiology of asthma and COPD. Respir Res 2006;7:73.

398. Prakash YS, Thompson MA, Pabelick CM. Brain-derived neurotrophic factor in TNF-alpha modulation of Ca2+ in human airway smooth muscle. Am J Respir Cell Mol Biol 2009;41:603-11.

399. Aravamudan B, Thompson MA, Pabelick CM, Prakash YS. Mechanisms of BDNF regulation in asthmatic airway smooth muscle. Am J Physiol Lung Cell Mol Physiol 2016;311:L270-9.

400. Britt RD Jr, Thompson MA, Wicher SA, Manlove LJ, Roesler A, Fang YH, et al. Smooth muscle brain-derived neurotrophic factor contributes to airway hyperreactivity in a mouse model of allergic asthma. FASEB J 2019;33:3024-34.

401. Dragunas G, Woest ME, Nijboer S, Bos ST, van Asselt J, de Groot AP, et al. Cholinergic neuroplasticity in asthma driven by TrkB signaling. FASEB J 2020;34:7703-17.

402. Zeilinger S, Pinto LA, Nockher WA, Depner M, Klopp N, Illig T, et al. The effect of BDNF gene variants on asthma in German children. Allergy 2009;64:1790-4.

403. Jesenak M, Babusikova E, Evinova A, Banovcin P, Dobrota D. Val66Met polymorphism in the BDNF gene in children with bronchial asthma. Pediatr Pulmonol 2015;50:631-7.

404. Yinli C, Jie H, Li Z, Jun G, Peiling L, Weihong Y. Association between brain-derived neurothropic factor variants and asthma in Chinese Han children. Acta Paediatr 2013;102:e247-50.

405. Müller GC, Pitrez PM, Teixeira AL, Pires PS, Jones MH, Stein RT, et al. Plasma brain-derived neurotrophic factor levels are associated with clinical severity in school age children with asthma. Clin Exp Allergy 2010;40:1755-9.

406. Ricci A, Felici L, Mariotta S, Mannino F, Schmid G, Terzano C, et al. Neurotrophin and neurotrophin receptor protein expression in the human lung. Am J Respir Cell Mol Biol 2004;30:12-9.

407. Braun A, Lommatzsch M, Renz H. The role of neurotrophins in allergic bronchial asthma. Clin Exp Allergy 2000;30:178-86.

408. Wetmore C, Olson L. Neuronal and nonneuronal expression of neurotrophins and their receptors in sensory and sympathetic ganglia suggest new intercellular trophic interactions. J Comp Neurol 1995;353:143-59.

409. Brigadski T, Leßmann V. The physiology of regulated BDNF release. Cell Tissue Res 2020;382:15-45.

410. Joachim RA, Noga O, Sagach V, Hanf G, Fliege H, Kocalevent RD, et al. Correlation between immune and neuronal parameters and stress perception in allergic asthmatics. Clin Exp Allergy 2008;38:283-90.

411. Koskela HO, Purokivi MK, Romppanen J. Neurotrophins in chronic cough: association with asthma but not with cough severity. Clin Respir J 2010;4:45-50.

412. Baj G, Carlino D, Gardossi L, Tongiorgi E. Toward a unified biological hypothesis for the BDNF Val66Met-associated memory deficits in humans: a model of impaired dendritic mRNA trafficking. Front Neurosci 2013;7:188.

413. Voegeli G, Ramoz N, Shekhtman T, Courtet P, Gorwood P, Kelsoe JR. Neurotrophin genes and antidepressant-worsening suicidal ideation: a prospective case-control study. Int J Neuropsychopharmacol 2016;19:pyw059.

414. Wang JY, Wang AL, Han W, Mu ZL. Association between a functional single nucleotide polymorphism in the brain-derived neurotrophic factor gene and risk of child asthma. Genet Mol Res 2015;14:16233-40.

415. Szczepankiewicz A, Rose-Zerilli MJ, Barton SJ, Holgate ST, Holloway JW. Association analysis of brain-derived neurotrophic factor gene polymorphisms in asthmatic families. Int Arch Allergy Immunol 2009;149:343-9.

416. Szczepankiewicz A, Rachel M, Sobkowiak P, Kycler Z, Wojsyk-Banaszak I, Schöneich N, et al. Neurotrophin serum concentrations and polymorphisms of neurotrophins and their receptors in children with asthma. Respir Med 2013;107:30-6.

417. Global Initiative for Asthma. Global Strategy for Asthma Management and Prevention 2014. Available from: https://ginasthma.org/archived-reports/ (accessed May 30, 2021).

418. Global Initiative for Asthma. Global Strategy for Asthma Management and Prevention 2017. Available from: https://ginasthma.org/archived-reports/ (accessed May 30, 2021).

419. World Medical Association. Declaration of Helsinki – ethical principles for medical research involving human subjects, October 2013. Available from: https://www.wma.net/policies-post/wma-declaration-of-helsinki-ethical-principles-for-medical-research-involving-human-subjects/ (accessed February 4, 2020).

420. Quanjer PH, Tammeling GJ, Cotes JE, Pedersen OF, Peslin R, Yernault JC. Lung volumes and forced ventilatory flows. Report Working Party Standardization of Lung Function Tests, European Community for Steel and Coal. Official Statement of the European Respiratory Society. Eur Respir J 1993;16:5-40.

421. Cotes JE, Chinn DJ, Quanjer PH, Roca J, Yernault JC. Standardization of the measurement of transfer factor (diffusing capacity). Report Working Party Standardization of Lung Function Tests, European Community for Steel and Coal. Official Statement of the European Respiratory Society. Eur Resp J 1993;16:41-52.

422. Eiringhaus K, Renz H, Matricardi P, Skevaki C. Component-resolved diagnosis in allergic rhinitis and asthma. J Appl Lab Med 2019;3:883-98.

423. Buhl R, Humbert M, Bjermer L, Chanez P, Heaney LG, Pavord I, et al.; Expert group of the European Consensus Meeting for Severe Eosinophilic Asthma. Severe eosinophilic asthma: a roadmap to consensus. Eur Respir J 2017;49:1700634.

424. Wenzel SE. Asthma phenotypes: the evolution from clinical to molecular approaches. Nat Med 2012;18:716-25.

425. Pavord I, Bahmer T, Braido F, Cosío BG, Humbert M, Idzko M, et al. Severe T2-high asthma in the biologics era: European experts' opinion. Eur Respir Rev 2019;28:190054.

426. Fitzpatrick AM, Chipps BE, Holguin F, Woodruff PG. T2-"low" asthma: overview and management strategies. J Allergy Clin Immunol Pract 2020;8:452-63.

427. Kim SD, Cho KS. Samter's triad: state of the art. Clin Exp Otorhinolaryngol 2018;11:71-80.

428. Pivac N, Mück-Seler D, Barisić I, Jakovljević M, Puretić Z. Platelet serotonin concentration in dialysis patients with somatic symptoms of depression. Life Sci 2001;68:2423-33.

429. Pivac N, Nedic G, Mustapic M, Babic A, Stipcevic T, Borovecki F, et al. The lack of genotype-phenotype relationship between platelet serotonin concentration and serotonin transporter gene promoter polymorphism in healthy subjects. Neurosci Lett 2009;462:45-8.

430. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem 1951;193:265-75.

431. Krajl M. A rapid microfluorimetric determination of monoamine oxidase. Biochem Pharmacol 1965;14:1684-6.

432. Sagud M, Nikolac Perkovic M, Vuksan-Cusa B, Maravic A, Svob Strac D, Mihaljevic Peles A, et al. A prospective, longitudinal study of platelet serotonin and plasma brain-derived neurotrophic factor concentrations in major depression: effects of vortioxetine treatment. Psychopharmacology (Berl) 2016;233:3259-67.

433. R&D Systems, Inc. Quantikine® Colorimetric Sandwich ELISA Kit Assay Principle. Available online: https://www.rndsystems.com/products/quantikine-colorimetric-sandwich-elisa-assay-principle (accessed April 20, 2021).

434. Miller SA, Dykes DD, Polesky HF. A simple salting out procedure for extracting DNA from human nucleated cells. Nucleic Acids Res 1988;16:1215.

435. TaqMan® SNP genotyping technology. Available online: https://www.bionumerics.com/applications/taqman-based-snp-genotyping (accessed April 20, 2021).

436. Thermo Fisher Scientific Inc. Interpreting scatterplots in genotyping experiments. Available online: https://www.thermofisher.com/hr/en/home/life-science/pcr/real-time-pcr/real-time-pcr-learning-center/genotyping-analysis-real-time-pcr-information/interpreting-scatter-plots-genotyping-experiments.html (accessed April 20, 2021).

437. World Health Organization - Regional Office for Europe. Blood services in south-eastern Europe 2007. Available from: https://www.euro.who.int/__data/assets/pdf_file/0014/90401/E90300.pdf (accessed April 25, 2021).

438. Vuk T, Ljubičić J, Gulan Harcet J, Očić T, Jukić I. Post-donation information management – contribution to the safety of transfusion treatment. Transfus Clin Biol 2019;26:353-4.

439. Zein JG, Denson JL, Wechsler ME. Asthma over the adult life course: gender and hormonal influences. Clin Chest Med 2019;40:149-61.

440. Zein JG, Dweik RA, Comhair SA, Bleecker ER, Moore WC, Peters SP, et al.; Severe Asthma Research Program. Asthma is more severe in older adults. PLoS One 2015;10:e0133490.

441. Chatkin JM, Dullius CR. The management of asthmatic smokers. Asthma Res Pract 2016;2:10.

442. Glavak Tkalić R, Miletić GM, Sakoman S. Prevalence of substance use among the general population: situation in Croatia and comparison with other European countries. Druš Istraž 2013;22:557-78.

443. Katsaounou P, Ioannou M, Hyland ME, Odemyr M, Spranger O, Lindberg A, et al. Smoking asthmatics, a neglected large phenotype of asthmatic patients. Open J Asthma 2019;3:1-8.

444. Hruby A, Hu FB. The epidemiology of obesity: a big picture. Pharmacoeconomics 2015;33:673-89.

445. World Health Organization (WHO). New WHO report: Europe can reverse its obesity "epidemic". Copenhagen, Denmark, 2022. Available from: https://www.who.int/europe/news/item/03-05-2022-new-who-report--europe-can-reverse-its-obesity--epidemic (accessed July 15, 2022).

446. Eurostat. Over half of adults in the EU are overweight. July 21, 2021. Available from: https://ec.europa.eu/eurostat/web/products-eurostat-news/-/ddn-20210721-2 (accessed June 4, 2022).

447. Dixon AE, Holguin F, Sood A, Salome CM, Pratley RE, Beuther DA, et al.; American Thoracic Society Ad Hoc Subcommittee on Obesity and Lung Disease. An official American Thoracic Society Workshop report: obesity and asthma. Proc Am Thorac Soc 2010;7:325-35.

448. Peters U, Dixon AE, Forno E. Obesity and asthma. J Allergy Clin Immunol 2018;141:1169-79.

449. Miethe S, Karsonova A, Karaulov A, Renz H. Obesity and asthma. J Allergy Clin Immunol 2020;146:685-93.

450. Beuther DA, Sutherland ER. Overweight, obesity, and incident asthma: a meta-analysis of prospective epidemiologic studies. Am J Respir Crit Care Med 2007;175:661-6.

451. Mey JT, Matuska B, Peterson L, Wyszynski P, Koo M, Sharp J, et al. Resting energy expenditure is elevated in asthma. Nutrients 2021;13:1065.

452. Zhang W, Du J, Wang S, Ma H. Association of weight change patterns across adulthood with incident asthma: a retrospective cohort study. Sci Rep 2022;12:9756.

453. Flachaire E, Beney C, Berthier A, Salandre J, Quincy C, Renaud B. Determination of reference values for serotonin concentration in platelets of healthy newborns, children, adults, and elderly subjects by HPLC with electrochemical detection. Clin Chem 1990; 36:2117-20.

454. Kumar AM, Weiss S, Fernandez JB, Cruess D, Eisdorfer C. Peripheral serotonin levels in women: role of aging and ethnicity. Gerontology 1998;44:211-6.

455. Nenadic-Sviglin K, Nedic G, Nikolac M, Kozaric-Kovacic D, Stipcevic T, Muck Seler D, et al. Suicide attempt, smoking, comorbid depression, and platelet serotonin in alcohol dependence. Alcohol 2011;45:209-16.

456. Pivac N, Mück-Seler D, Mustapić M, Nenadić-Sviglin K, Kozarić-Kovacić D. Platelet serotonin concentration in alcoholic subjects. Life Sci 2004;76:521-31.

457. Svob Strac D, Nedic Erjavec G, Nikolac Perkovic M, Nenadic-Sviglin K, Konjevod M, Grubor M, et al. The association between *HTR1B* gene rs13212041 polymorphism and onset of alcohol abuse. Neuropsychiatr Dis Treat 2019;15:339-47.

458. Nedic Erjavec G, Bektic Hodzic J, Repovecki S, Nikolac Perkovic M, Uzun S, Kozumplik O, et al. Alcohol-related phenotypes and platelet serotonin concentration. Alcohol 2021;97:41-9.

459. Launay JM, Del Pino M, Chironi G, Callebert J, Peoc'h K, Mégnien JL, et al. Smoking induces long-lasting effects through a monoamine-oxidase epigenetic regulation. PLoS One 2009;4:e7959.

460. Hodge S, Bunting BP, Carr E, Strain JJ, Stewart-Knox BJ. Obesity, whole blood serotonin and sex differences in healthy volunteers. Obes Facts 2012;5:399-407.

461. Georgescu T, Lyons D, Heisler LK. Role of serotonin in body weight, insulin secretion and glycaemic control. J Neuroendocrinol 2021;33:e12960.

462. Young RL, Lumsden AL, Martin AM, Schober G, Pezos N, Thazhath SS, et al. Augmented capacity for peripheral serotonin release in human obesity. Int J Obes (Lond) 2018;42:1880-89.

463. Ritze Y, Schollenberger A, Hamze Sinno M, Bühler N, Böhle M, Bárdos G, et al. Gastric ghrelin, GOAT, leptin, and leptinR expression as well as peripheral serotonin are dysregulated in humans with obesity. Neurogastroenterol Motil 2016;28:806-15.

464. Binetti J, Bertran L, Riesco D, Aguilar C, Martínez S, Sabench F, et al. Deregulated serotonin pathway in women with morbid obesity and NAFLD. Life (Basel) 2020;10:245.

465. Bridge TP, Soldo BJ, Phelps BH, Wise CD, Francak MJ, Wyatt RJ. Platelet monoamine oxidase activity: demographic characteristics contribute to enzyme activity variability. J Gerontol 1985;40:23-8.

466. Pivac N, Knezevic J, Mustapic M, Dezeljin M, Muck-Seler D, Kozaric-Kovacic D, et al. The lack of association between monoamine oxidase (MAO) intron 13 polymorphism and platelet MAO-B activity among men. Life Sci 2006;79:45-9.

467. Berlin I, Anthenelli RM. Monoamine oxidases and tobacco smoking. Int J Neuropsychopharmacol 2001;4:33-42.

468. Nicotra A, Pierucci F, Parvez H, Senatori O. Monoamine oxidase expression during development and aging. Neurotoxicology 2004;25:155-65.

469. Fowler CJ, Wiberg A, Oreland L, Marcusson J, Winblad B. The effect of age on the activity and molecular properties of human brain monoamine oxidase. J Neural Transm 1980;49:1-20.

470. Kornhuber J, Konradi C, Mack-Burkhardt F, Riederer P, Heinsen H, Beckmann H. Ontogenesis of monoamine oxidase-A and -B in the human brain frontal cortex. Brain Res 1989;499:81-6.

471. Volchegorskii IA, Shemyakov SE, Turygin VV, Malinovskaya NV. Comparative analysis of age-related changes in activities of monoamine oxidase-B and antioxidant defense enzymes in various structures of human brain. Bull Exp Biol Med 2001;132:760-2.

472. Anthenelli RM, Tipp J, Li TK, Magnes L, Schuckit MA, Rice J, et al. Platelet monoamine oxidase activity in subgroups of alcoholics and controls: results from the Collaborative Study on the Genetics of Alcoholism. Alcohol Clin Exp Res 1998;22:598-604.

473. Coccini T, Randine G, Castoldi AF, Balloni L, Baiardi P, Manzo L. Lymphocyte muscarinic receptors and platelet monoamine oxidase-B as biomarkers of CNS function: effects of age and gender in healthy humans. Environ Toxicolo Pharmacol 2005;19:715-20.

474. Saccone NL, Rice JP, Rochberg N, Goate A, Reich T, Shears S, et al. Genome screen for platelet monoamine oxidase (MAO) activity. Am J Med Genet 1999;88:517-21.

475. Snell LD, Glanz J, Tabakoff B; WHO/ISBRA Study on State and Trait Markers ofAlcohol Use and Dependence Investigators. Relationships between effects of smoking, gender, and alcohol dependence on platelet monoamine oxidase-B: activity, affinity labeling, and protein measurements. Alcohol Clin Exp Res 2002;26:1105-13.

476. Nikolac Perkovic M, Svob Strac D, Nedic Erjavec G, Uzun S, Podobnik J, Kozumplik O, et al. Monoamine oxidase and agitation in psychiatric patients. Prog Neuropsychopharmacol Biol Psychiatry 2016;69:131-46.

477. Svob Strac D, Kovacic Petrovic Z, Nikolac Perkovic M, Umolac D, Nedic Erjavec G, Pivac N. Platelet monoamine oxidase type B, MAOB intron 13 and MAOA-uVNTR polymorphism and symptoms of post-traumatic stress disorder. Stress 2016;19:362-73.

478. McMurray F, Patten DA, Harper ME. Reactive oxygen species and oxidative stress in obesity-recent findings and empirical approaches. Obesity (Silver Spring) 2016;24:2301-10.

479. Tong JH, D'Iorio A, Kandaswami C. On the characteristics of mitochondrial monoamine oxidase in pancreas and adipose tissues from genetically obese mice. Can J Biochem 1979;57:197-200.

480. Wanecq E, Bour S, Verwaerde P, Smih F, Valet P, Carpéné C. Increased monoamine oxidase and semicarbazide-sensitive amine oxidase activities in white adipose tissue of obese dogs fed a high-fat diet. J Physiol Biochem 2006;62:113-23.

481. Carter K, Nelson M, Robidoux J, Lerman L, Anderson E. Kinetics of neurotransmitter metabolism by monoamine oxidase in porcine heart differs by location and is increased with obesity/metabolic syndrome [abstract]. FASEB J 2017;31:S883.16.

482. Ehrlich S, Franke L, Schott R, Salbach-Andrae H, Pfeiffer E, Lehmkuhl U, et al. Platelet monoamine oxidase activity in underweight and weight-recovered females with anorexia nervosa. Pharmacopsychiatry 2008;41:226-31.

483. Arreola R, Becerril-Villanueva E, Cruz-Fuentes C, Velasco-Velázquez MA, Garcés-Alvarez ME, Hurtado-Alvarado G, et al. Immunomodulatory effects mediated by serotonin. J Immunol Res 2015;2015:354957.

484. Kornerup KN, Page CP. The role of platelets in the pathophysiology of asthma. Platelets 2007;18:319-28.

485. Idzko M, Pitchford S, Page C. Role of platelets in allergic airway inflammation. J Allergy Clin Immunol 2015;135:1416-23.

486. Malmgren R, Olsson P, Tornling G, Unge G. Acetylsalicyclic asthma and migraine – a defect in serotonin (5-HT) uptake in platelets. Thromb Res 1978;13:1137-9.

487. Malmgren R, Olsson P, Tornling G, Unge G. The 5-hydroxytryptamine take-up mechanism in normal platelets and platelets from migraine and asthmatic patients. Thromb Res 1980;18:733-41.

488. Young WF Jr, Laws ER Jr, Sharbrough FW, Weinshilboum RM. Human monoamine oxidase. Lack of brain and platelet correlation. Arch Gen Psychiatry 1986;43:604-9.

489. Chen K, Holschneider DP, Wu W, Rebrin I, Shih JC. A spontaneous point mutation produces monoamine oxidase A/B knock-out mice with greatly elevated monoamines and anxiety-like behavior. J Biol Chem 2004;279:39645-52.

490. Cases O, Seif I, Grimsby J, Gaspar P, Chen K, Pournin S, et al. Aggressive behavior and altered amounts of brain serotonin and norepinephrine in mice lacking MAOA. Science 1995;268:1763-6.

491. Lenders JW, Eisenhofer G, Abeling NG, Berger W, Murphy DL, Konings CH, et al. Specific genetic deficiencies of the A and B isoenzymes of monoamine oxidase are characterized by distinct neurochemical and clinical phenotypes. J Clin Invest 1996;97:1010-9.

492. Shih JC, Chen K, Ridd MJ. Monoamine oxidase: from genes to behavior. Annu Rev Neurosci 1999;22:197-217.

493. Hsu MC, Shih JC. Photoaffinity labeling of human placental monoamine oxidase-A by 4-fluoro-3-nitrophenyl azide. Mol Pharmacol 1988;33:237-41.

494. Prah A, Purg M, Stare J, Vianello R, Mavri J. How monoamine oxidase A decomposes serotonin: an empirical valence bond simulation of the reactive step. J Phys Chem B 2020;124:8259-65.

495. Umbarkar P, Singh S, Arkat S, Bodhankar SL, Lohidasan S, Sitasawad SL. Monoamine oxidase-A is an important source of oxidative stress and promotes cardiac dysfunction, apoptosis, and fibrosis in diabetic cardiomyopathy. Free Radic Biol Med 2015;87:263-73.

496. Cho YS, Moon HB. The role of oxidative stress in the pathogenesis of asthma. Allergy Asthma Immunol Res 2010;2:183-7.

497. Zarmouh NO, Messeha SS, Elshami FM, Soliman KF. Natural products screening for the identification of selective monoamine oxidase-B inhibitors. European J Med Plants 2016;15:14802.

498. Iranshahy M, Iranshahi M. Traditional uses, phytochemistry and pharmacology of asafoetida (Ferula assa-foetida oleo-gum-resin) – a review. J Ethnopharmacol 2011;134:1-10.

499. Nemtsov V, Kachanova T, Fedoseev G, Zhikharev SS. Serotonin level and vascular disturbances in bronchial asthma [abstract]. Chest 1994;106:S146.

500. Jann MW. Buspirone: an update on a unique anxiolytic agent. Pharmacotherapy 1988;8:100-16.

501. Lisman T. Platelet-neutrophil interactions as drivers of inflammatory and thrombotic disease. Cell Tissue Res 2018;371:567-76.

502. Page C, Pitchford S. Neutrophil and platelet complexes and their relevance to neutrophil recruitment and activation. Int Immunopharmacol 2013;17:1176-84.

503. Duerschmied D, Suidan GL, Demers M, Herr N, Carbo C, Brill A, et al. Platelet serotonin promotes the recruitment of neutrophils to sites of acute inflammation in mice. Blood 2013;121:1008-15.

504. Cloutier N, Allaeys I, Marcoux G, Machlus KR, Mailhot B, Zufferey A, et al. Platelets release pathogenic serotonin and return to circulation after immune complex-mediated sequestration. Proc Natl Acad Sci USA 2018;115:E1550-59.

505. Yue M, Hu M, Fu F, Ruan H, Wu C. Emerging roles of platelets in allergic asthma. Front Immunol 2022;13:846055.

506. Bakulin MP, Ioffe EI. Soderzhanie biologicheski aktivnykh veshchestv (gistamina i serotonina) u bol'nykh bronkhial'noĭ astmoĭ [Contents of biologically active substances (histamine and serotonin) in patients with bronchial asthma]. Ter Arkh 1979;51:45-9.

507. MacHaffie RA, Menebroker LR, Mahler DJ, Barak AJ. Studies in allergy: II. Serum serotonin levels in nonallergic, pretreatment, and posttreatment allergic human beings and in normal and sensitized guinea pigs. J Allergy 1960;31:106-10.

508. Takeda T, Morita H, Saito H, Matsumoto K, Matsuda A. Recent advances in understanding the roles of blood platelets in the pathogenesis of allergic inflammation and bronchial asthma. Allergol Int 2018;67:326-33.

509. Margraf A, Zarbock A. Platelets in inflammation and resolution. J Immunol 2019;203:2357-67.

510. Kowal K, Pampuch A, Kowal-Bielecka O, DuBuske LM, Bodzenta-Łukaszyk A. Platelet activation in allergic asthma patients during allergen challenge with Dermatophagoides pteronyssinus. Clin Exp Allergy 2006;36:426-32.

511. Pitchford SC, Yano H, Lever R, Riffo-Vasquez Y, Ciferri S, Rose MJ, et al. Platelets are essential for leukocyte recruitment in allergic inflammation. J Allergy Clin Immunol 2003;112:109-18.

512. Johansson MW, Han ST, Gunderson KA, Busse WW, Jarjour NN, Mosher DF. Platelet activation, P-selectin, and eosinophil β1-integrin activation in asthma. Am J Respir Crit Care Med 2012;185:498-507.

513. Boldogh I, Bacsi A, Choudhury BK, Dharajiya N, Alam R, Hazra TK, et al. ROS generated by pollen NADPH oxidase provide a signal that augments antigen-induced allergic airway inflammation. J Clin Invest 2005;115:2169-79.

514. Sustar A, Perkovic MN, Erjavec GN, Strac DS, Pivac N. Association between reduced brain-derived neurotrophic factor concentration & coronary heart disease. Indian J Med Res 2019;150:43-9.

515. Pillai A, Bruno D, Sarreal AS, Hernando RT, Saint-Louis LA, Nierenberg J, et al. Plasma BDNF levels vary in relation to body weight in females. PLoS One 2012;7:e39358.

516. Lommatzsch M, Zingler D, Schuhbaeck K, Schloetcke K, Zingler C, Schuff-Werner P, et al. The impact of age, weight and gender on BDNF levels in human platelets and plasma. Neurobiol Aging 2005;26:115-23.

517. Bhang SY, Choi SW, Ahn JH. Changes in plasma brain-derived neurotrophic factor levels in smokers after smoking cessation. Neurosci Lett 2010;468:7-11.

518. Jamal M, Van der Does W, Elzinga BM, Molendijk ML, Penninx BW. Association between smoking, nicotine dependence, and BDNF Val66Met polymorphism with BDNF concentrations in serum. Nicotine Tob Res 2015;17:323-9.

519. Xia H, Du X, Yin G, Zhang Y, Li X, Cai J, et al. Effects of smoking on cognition and BDNF levels in a male Chinese population: relationship with BDNF Val66Met polymorphism. Sci Rep 2019;9:217.

520. Sathish V, Vanoosten SK, Miller BS, Aravamudan B, Thompson MA, Pabelick CM, et al. Brain-derived neurotrophic factor in cigarette smoke-induced airway hyperreactivity. Am J Respir Cell Mol Biol 2013;48:431-8.

521. Freeman MR, Sathish V, Manlove L, Wang S, Britt RD Jr, Thompson MA, et al. Brain-derived neurotrophic factor and airway fibrosis in asthma. Am J Physiol Lung Cell Mol Physiol 2017;313:L360-70.

522. Watanabe T, Fajt ML, Trudeau JB, Voraphani N, Hu H, Zhou X, et al. Brain-derived neurotrophic factor expression in asthma. Association with severity and type 2 inflammatory processes. Am J Respir Cell Mol Biol 2015;53:844-52.

523. Szczepankiewicz A, Rachel M, Sobkowiak P, Kycler Z, Wojsyk-Banaszak I, Schöneich N, et al. Serum neurotrophin-3 and neurotrophin-4 levels are associated with asthma severity in children. Eur Respir J 2012;39:1035-7.

524. Salama AA, Mostafa GA, Abd Al-Aziz MM, Ibrahim MN. Brain-derived neurotrophic factor in asthmatic children. Egypt J Pediatr Allergy Immunol 2003;1:102-9.

525. Lommatzsch M, Niewerth A, Klotz J, Schulte-Herbrüggen O, Zingler C, Schuff-Werner P, et al. Platelet and plasma BDNF in lower respiratory tract infections of the adult. Respir Med 2007;101:1493-9.

526. Azoulay D, Shehadeh M, Chepa S, Shaoul E, Baroum M, Horowitz NA, et al. Recovery from SARS-CoV-2 infection is associated with serum BDNF restoration. J Infect 2020;81:e79-81.

527. Coffey CS, Mulligan RM, Schlosser RJ. Mucosal expression of nerve growth factor and brain-derived neurotrophic factor in chronic rhinosinusitis. Am J Rhinol Allergy 2009;23:571-4.

528. Jornot L, Grouzmann E, Lacroix JS, Rochat T. BDNF and DPP-IV in polyps and middle turbinates epithelial cells. Rhinology 2007;45:129-33.

529. Rajan JP, Wineinger NE, Stevenson DD, White AA. Prevalence of aspirin-exacerbated respiratory disease among asthmatic patients: a meta-analysis of the literature. J Allergy Clin Immunol 2015;135:676-81.e1.

530. Virchow JC, Julius P, Lommatzsch M, Luttmann W, Renz H, Braun A. Neurotrophins are increased in bronchoalveolar lavage fluid after segmental allergen provocation. Am J Respir Crit Care Med 1998:158;2002-5.

531. Noga O, Englmann C, Hanf G, Grützkau A, Seybold J, Kunkel G. The production, storage and release of the neurotrophins nerve growth factor, brain-derived neurotrophic factor and neurotrophin-3 by human peripheral eosinophils in allergics and non-allergics. Clin Exp Allergy 2003;33:649-54.

532. Xie X, Zhu Y, Zhang J, Zhai C, Feng W, Pan Y, et al. Association between Val66Met polymorphisms in brain-derived neurotrophic factor gene and asthma risk: a meta-analysis. Inflamm Res 2015;64:875-83.

533. Szczepankiewicz A, Breborowicz A, Skibińska M, Wiłkość M, Tomaszewska M, Hauser J. Association analysis of brain-derived neurotrophic factor gene polymorphisms in asthmatic children. Pediatr Allergy Immunol 2007;18:293-7.

534. Szczepankiewicz A, Bręborowicz A, Sobkowiak P, Popiel A. Association of BDNF gene polymorphism with asthma in polish children. World Allergy Organ J 2010;3:235-8.

535. Bhang S, Ahn JH, Choi SW. Brain-derived neurotrophic factor and serotonin transporter gene-linked promoter region genes alter serum levels of brain-derived neurotrophic factor in humans. J Affect Disord 2011;128:299-304.

536. Lang UE, Hellweg R, Sander T, Gallinat J. The Met allele of the BDNF Val66Met polymorphism is associated with increased BDNF serum concentrations. Mol Psychiatry 2009;14:120-2.

537. Terracciano A, Piras MG, Lobina M, Mulas A, Meirelles O, Sutin AR, et al. Genetics of serum BDNF: meta-analysis of the Val66Met and genome-wide association study. World J Biol Psychiatry 2013;14:583-9.

538. Andiappan AK, Parate PN, Anantharaman R, Suri BK, Wang de Y, Chew FT. Genetic variation in BDNF is associated with allergic asthma and allergic rhinitis in an ethnic Chinese population in Singapore. Cytokine 2011;56:218-23.

539. Jin P, Andiappan AK, Quek JM, Lee B, Au B, Sio YY, et al. A functional brain-derived neurotrophic factor (BDNF) gene variant increases the risk of moderate-to-severe allergic rhinitis. J Allergy Clin Immunol 2015;135:1486-93.e8.

540. Nassenstein C, Braun A, Erpenbeck VJ, Lommatzsch M, Schmidt S, Krug N, et al. The neurotrophins nerve growth factor, brain-derived neurotrophic factor, neurotrophin-3, and neurotrophin-4 are survival and activation factors for eosinophils in patients with allergic bronchial asthma. J Exp Med 2003;198:455-67.

541. Nassenstein C, Möhring UH, Luttmann W, Virchow JC Jr, Braun A. Differential expression of the neurotrophin receptors p75NTR, TrkA, TrkB and TrkC in human peripheral blood mononuclear cells. Exp Toxicol Pathol 2006;57:55-63.

542. Nassenstein C, Dawbarn D, Pollock K, Allen SJ, Erpenbeck VJ, Spies E, et al. Pulmonary distribution, regulation, and functional role of Trk receptors in a murine model of asthma. J Allergy Clin Immunol 2006;118:597-605.

543. Goodwin RD, Jacobi F, Thefeld W. Mental disorders and asthma in the community. Arch Gen Psychiatry 2003;60:1125-30.

544. Di Marco F, Santus P, Centanni S. Anxiety and depression in asthma. Curr Opin Pulm Med 2011;17:39-44.

545. Wong KO, Hunter Rowe B, Douwes J, Senthilselvan A. Asthma and wheezing are associated with depression and anxiety in adults: an analysis from 54 countries. Pulm Med 2013;2013:929028.

546. Gerald JK, Moreno FA. Asthma and depression: it's complicated. J Allergy Clin Immunol Pract 2016;4:74-5.

547. Szilasi ME, Pak K, Kardos L, Varga VE, Seres I, Mikaczo A, et al. The alteration of irisin-brain-derived neurotrophic factor axis parallels severity of distress disorder in bronchial asthma patients. Front Neurosci 2017;11:653.

548. Cooley C, Park Y, Ajilore O, Leow A, Nyenhuis SM. Impact of interventions targeting anxiety and depression in adults with asthma. J Asthma 2022;59:273-87.

549. Müller P, Duderstadt Y, Lessmann V, Müller NG. Lactate and BDNF: key mediators of exercise induced neuroplasticity? J Clin Med 2020;9:1136.

550. Homberg JR, Molteni R, Calabrese F, Riva MA. The serotonin-BDNF duo: developmental implications for the vulnerability to psychopathology. Neurosci Biobehav Rev 2014;43:35-47.

11. CURRICULUM VITAE

Dr. Katherina Bernadette Sreter earned her medical degree from the University of Zagreb, School of Medicine (Medical Studies in English Programme). Before starting medical school, Dr. Sreter obtained a Bachelor of Science (Biology) and Bachelor of Arts (Psychology) degree at the University of Ottawa, Canada. After completing her one-year mandatory rotating internship, Dr. Sreter worked as a general practitioner in Bol, Island of Brač, and Zagreb, Croatia. During her residency training in pulmonology, Dr. Sreter attended a month-long clinical observership in lung cancer at the Princess Margaret Cancer Centre and Toronto General Hospital in Toronto, Canada, in 2016. Upon finishing her residency in 2018, Dr. Sreter underwent a year-long Clinical Research Fellowship (2019-2020) at the Lung Unit (Thoracic Medical Oncology) of the Royal Marsden Hospital in London, United Kingdom. Dr. Sreter is a co-author of a book chapter, and has published several articles and conference abstracts in peer-reviewed journals. She has actively participated in various congresses, conferences, workshops, and research seminars, both nationally and internationally. She has served as a sub-investigator on multiple clinical trials in asthma and thoracic oncology. Dr. Sreter is currently a Consultant Respiratory Medicine Specialist at the University Hospital Centre “Sestre Milosrdnice” in Zagreb, Croatia.