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Identification of underlying molecular mechanisms of obesity-associated asthma

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„Alles wissenschaftliche Arbeiten ist nichts anderes, als immer neuen Stoff in allgemeine Gesetze zu bringen“.

Wilhelm von Humboldt

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1. Abbreviations

Abbreviation	Explanation
GINA	Global Initiative for Asthma
PEF	peak expiratory flow rate
FVC	forced vital capacity
FEV_1	forced expiratory volume in the first second
ISAAC	Asthma and Allergies in Children
ECRHS	European Community Respiratory Health Survey
ATS	American Thoracic Society
GWASs	Genome-wide association studies
ORMDL3	ORMDL sphingolipid biosynthesis regulator 3
IL33	interleukin 33
HLA-DR/DQ	major histocompatibility complex, class II-DR/DQ
TSLP	thymic stromal lymphopoietin
IgE	immunoglobulin E
HDM	house dust mite
RV	Rhinovirus
CCL20	chemokine (C-C motif) ligand 20
ILCs	innate lymphoid cells
AHR	airway hyper-responsiveness
DCs	Airway dendritic cells
APCs	antigen-presenting cells
cDCs	Conventional dendritic cells
pDCs	Plasmacytoid dendritic cells
Tc	T cytotoxic cells
Th	T helper cells
IFNy	interferon gamma
PCs	plasma cells
B mem	memory B cells
GM-CSF	granulocyte-macrophage colony-stimulating factor
TNF-α	tumor necrosis factor α
TGF-β	transforming growth factor-β

CCL11	chemokines such as eotaxins-1
ROS	highly reactive oxygen species
NETs	neutrophil extracellular traps
FFAs	free fatty acids
BMI	Body mass index
EVs	Extracellular vesicles
nt	nucleotides
miRNAs	microRNAs
mRNAs	messenger RNAs
DNMT1	DNA Methyltransferase 1
ICS	inhaled corticosteroids
LABA	long-acting beta-agonist
NF-κB	nuclear factor-κB
AP1	activator protein 1
ADRB2	β2-adrenoceptor
SABA	short-acting β2-adrenergic receptor agonists
LTRA	leukotriene receptor antagonists
cysLTs	cysteinyl-leukotrienes
DPBS	Dulbecco's phosphate buffered saline
BD	Becton-Dickinson
ChIP-Seq	chromatin immunoprecipitation sequencing
RNA-Seq	RNA sequencing
RT	room temperature
NTA	Nanoparticle tracking analysis
PCA	Principal Component Analyses
FDR	false discovery rate
DE	differentially expressed
DEGs	differentially expressed genes
GO-BP	Gene Ontology Biological processes
RPS27	Ribosomal Protein S27
TSS	Transcription Start Site
kb	kilo base
FeNO	fractional exhaled nitric oxide

CRP	C-reactive protein
GATA3	GATA Binding Protein 3
Ctrl/controls	Healthy controls
FC	Fold Change
DDB1	DNA binding protein 1
SMC3	structural maintenance of chromosomes 3
TMEM88	transmembrane protein 88
DDIT3	DNA damage inducible transcript 3
HNRNPA2B1	heterogeneous nuclear ribonucleoprotein A2/B1
GPCR	G-coupled protein receptor
IFITM3	interferon-induced transmembrane protein 3
GBP3	guanylate binding protein 3
CXCL-3	C-X-C Motif Chemokine Ligand 3
TUBA8	tubulin alpha 8
TUBB1	tubulin beta 1 class VI
EMT	epithelial-to-mesenchymal transition
ISGs	IFN stimulated genes
EIF2AK2	eukaryotic translation initiation factor 2 alpha kinase 2
IFIT3	protein with tetratricopeptide repeats 3
CNOT1	CCR4-NOT transcription complex subunit 1
CNOT6L	CCR4-NOT transcription complex subunit 6 like
SOPs	standard operating procedures
SOCS3	suppressor of cytokine signaling 3
JAK-STAT	Janus kinase signal transducer and activator of transcription
TLR1	Toll-like receptor 1
RTIs	respiratory tract infections
TUBB2A	tubulin beta 2A class IIa
TUBB2B	tubulin beta 2B class IIb
PF4	platelet factor 4
PPBP	pro-platelet basic protein
T regs	CD4+ T regulatory cells
HFD	High fat diet

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3. Introduction:

3.1 Asthma definition and attacks

According to the latest update of Global Initiative for Asthma (GINA, 2021) “Asthma is a heterogeneous disease, usually characterized by chronic airway inflammation. It is 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” (Global Initiative for Asthma - GINA 2021).

Asthma attacks occur when the symptoms become worse suddenly, which can be severe and life threatening if not treated immediately. Many triggers may lead to acute asthma exacerbations including respiratory tract infections including viruses, exposure to an allergen such as animal dander, exposure to an irritant such as cigarette smoke, or sometimes hard physical exercise (Global Initiative for Asthma - GINA 2021; Bush 2019).

3.2 Asthma diagnosis

Diagnosis of asthma comprises two basic components: a history of clinical symptoms and physical as well as clinical testing. Clinical suspicion of asthma should appear in the presence of symptoms mentioned above. Usually questionnaires of family history, life style, recurrence of symptoms are given to the patient as a first clinical evaluation (Tarasidis and Wilson 2015; Tay et al. 2018; Brigham and West 2015).

Airway obstruction and the degree of the obstruction are the mainstay of asthma diagnosis. They are commonly examined by a spirometry with a bronchodilator reversibility test. Lung function parameters which can be measured by spirometry include peak expiratory flow rate (PEF), forced vital capacity (FVC), and forced expiratory volume in the first second (FEV_1). A reduction in FEV_1/FVC ratio below 0.90 or below the lowest 5% of the reference population indicates obstruction (Global Initiative for Asthma - GINA 2021). It must be taken into consideration that the patients are not on any type of asthma treatment because this could lead to a reduction in the

sensitivity and specificity of diagnostic tests. A summary of standard asthma diagnostic tests is shown in Table 1 (Brigham and West 2015).

Table 1: Available diagnostic testing for asthma (Brigham and West, Int Forum Allergy Rhinol 2015)

Confirmation of airway obstruction
Spirometry
Demonstration of variability in airway obstruction
Basic measurements
Peak expiratory flow (repeated measures)
Spirometry (repeated measures)
Assessment of a response to treatment
Spirometry pre-bronchodilator and post-bronchodilator
Spirometry post-initiation of a controller therapy
Bronchial provocation
Nonpharmacologic: exercise or eucapnic voluntary ventilation
Pharmacologic: hypertonic saline, mannitol, or methacholine
Other supportive tests
Allergy testing
Fractional exhaled nitric oxide

3.3 Asthma epidemiology

An approximate number of 300 million people worldwide are affected by asthma (Nanda and Wasan 2020). The prevalence of asthma is increasing rapidly in the industrial, westernized areas like United States, Europe, and Australia (Figure 1) (Stern et al. 2020). However, the prevalence gap is closing slowly between the modern and developing countries as the latter have started to adapt to new modern lifestyles (Croisant 2014).

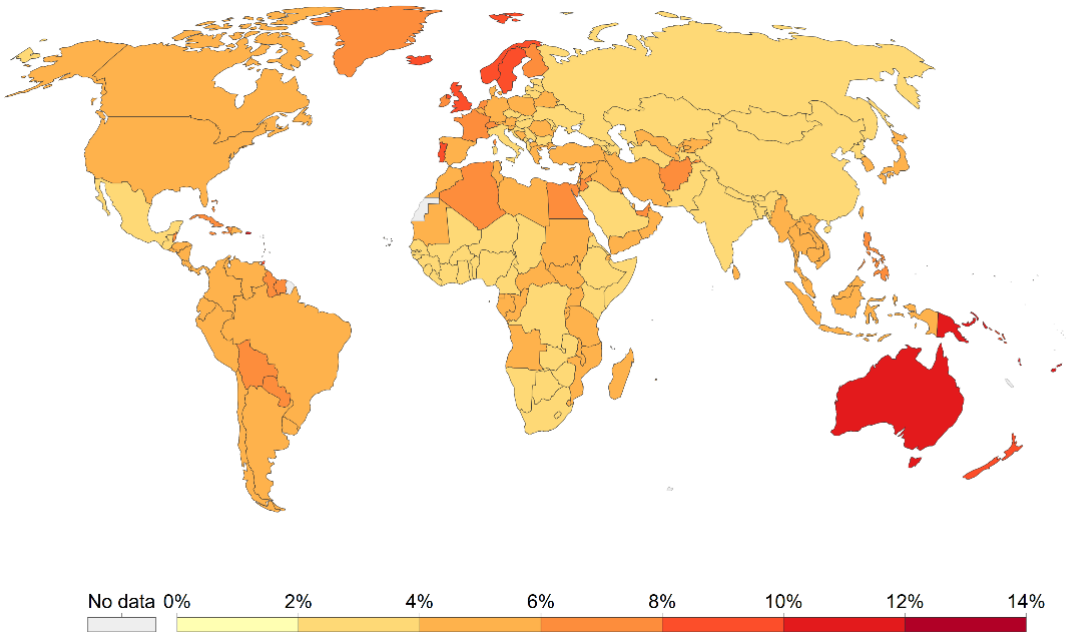


Figure 1: Worldwide asthma prevalence in 2017 (IHME, Global Burden of Disease)

Many different factors like age, race, gender, socioeconomic status, and others play a crucial role in the prevalence of asthma. Overall, there is an increasing prevalence trend in children compared to adults (Loftus and Wise 2016). It has been also reported that whereas during childhood males has a higher risk of developing asthma than females, the opposite trend is observed in the adulthood. In the United States there is a lower prevalence rate in the non-hispanic white population compared to the black population (Trivedi and Denton 2019).

The International Study of Asthma and Allergies in Children (ISAAC) and the European Community Respiratory Health Survey (ECRHS) are among the most popular initiatives that have been established to study prevalence of asthma. The ISAAC study conducted a questionnaire survey among the school children. It came to a conclusion that environmental factors can be responsible for wide variations in asthma prevalence. On the contrary, the ECRHS study targeted the adults. In both ISAAC and ECRHS results, English-speaking areas showed a high prevalence of asthma exacerbations (Stern et al. 2020).

The economic burden deriving from health care associated with asthma is huge. According to the 2018 press releases of the American Thoracic Society (ATS), the United States is spending more than 80 billion dollars annually on asthma management. An estimated number of 185 children and 3262 adults died from asthma

in 2007, while, asthma deaths in 2010 were 3404 (Asthma Costs the U.S. Economy More than $80 Billion Per Year 2021; Loftus and Wise 2015).

3.4 Asthma causes, triggers, and risk factors

The complex heterogeneous background of asthma is caused by multiple factors. Environmental and genetic factors as well as their mutual interaction contribute to the development of asthma (Figure 2) (Mims 2015).

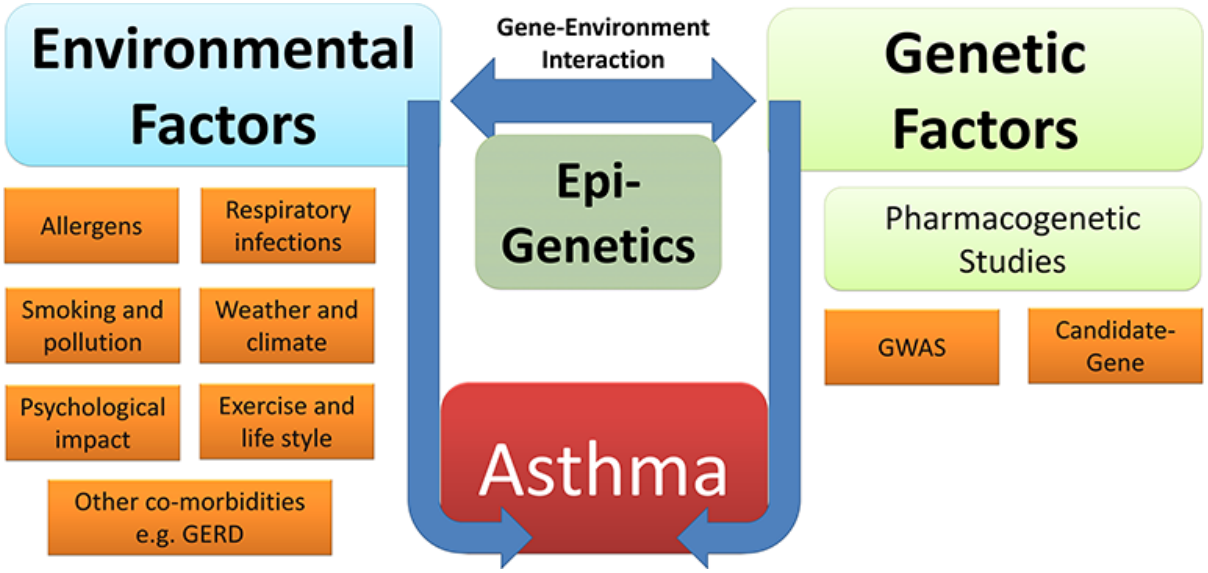


Figure 2: Asthma causes and risk factors (Youssef *et al*, intechopen.com 2016); GWAS: genome-wide association study; GERD: Gastroesophageal reflux disease

3.4.1 Genetic perturbations in asthma

Asthma is not a monogenic disease, yet a variety of genes have been identified to be associated with asthma prevalence (Thomsen 2015). Therefore, Genetic factors contribute strongly to asthma disease. Family and twin studies showed that 60–70% of asthma cases are related to genetic factors. Those genetic factors can be in a shape of genetic variants or aberrant gene expression patterns, the latter of which can be inherited from parents to offspring through complex epigenetic mechanisms (Thomsen

2014). Overall, the heritability estimates are in the range of 35-95% for asthma (Ober and Yao 2011).

Genome-wide association studies (GWASs) are one of the biggest genetic platforms that inspected the genetic perturbations of asthma and other diseases. Over 140 susceptibility loci have been detected by GWAS to contribute to asthma pathogenesis (Han et al. 2020). ORMDL sphingolipid biosynthesis regulator 3 (*ORMDL3*) gene in the 17q21 locus is the first and most widely replicated gene associated with childhood asthma discovered by GWAS (Moffatt et al. 2007).

Other loci were identified to be associated with asthma; these include interleukin 33 (*IL33*; on 9p24), major histocompatibility complex, class II-DR/DQ (*HLA-DR*/*DQ;* on 6p21), *IL1RL1*/*IL18R1* (on 2q12), thymic stromal lymphopoietin (*TSLP;* on 5q22), and *IL13* (on 5q31) (Torgerson et al. 2011; Gudbjartsson et al. 2009). Yet 5q31-33 loci are one of the main susceptibility loci for asthma and high immunoglobulin E (IgE) levels. This region includes a set of cytokine genes, such as those encoding IL-3, IL-4, IL-5, IL-9, IL-13 and other important proteins (Renauld 2001).

3.4.2 Environmental triggers in asthma

Besides genetic factors, environmental triggers can lead to asthma development and progression, which can be divided in two categories. Firstly, allergic triggers including allergens like house dust mite (HDM) and pollens. Secondly, non-allergic triggers including smoking, air pollution, and physical exercise (Gautier and Charpin 2017).

Allergen exposure is the main factor that is considered to be the most common cause of asthmatic exacerbations in adults and children (Baxi and Phipatanakul 2010). HDM as an indoor allergen has increased the risk of asthma in children that have exposed to it (Celedón et al. 2007).

Viruses or respiratory viral infections are environmental risk factors which can worsen the clinical symptoms or even associate with asthma development e.g. Rhinovirus (RV), the common cold virus, is one of the most relevant viruses contributing to asthma exacerbations and causing wheezing in children (Jartti et al. 2020; Chau-Etchepare et al. 2019).

Smoking and air pollution affect lung function directly and might cause several diseases including asthma. According to Polosa and Thomson *et al*, asthmatics who smoke are likely to experience a higher rate of asthma exacerbations than asthmatics who do not smoke (Polosa and Thomson 2013).

3.5 Asthma cellular inflammation

Inflammation of lower lung airways in asthma, caused by the above-mentioned risk factors, engages a variety of cell types, which represent both innate and adaptive immune systems like epithelial cells, macrophages, dendritic cells, eosinophils, neutrophils, mast cells, T and B lymphocytes and others (European Medical Journal 2018).

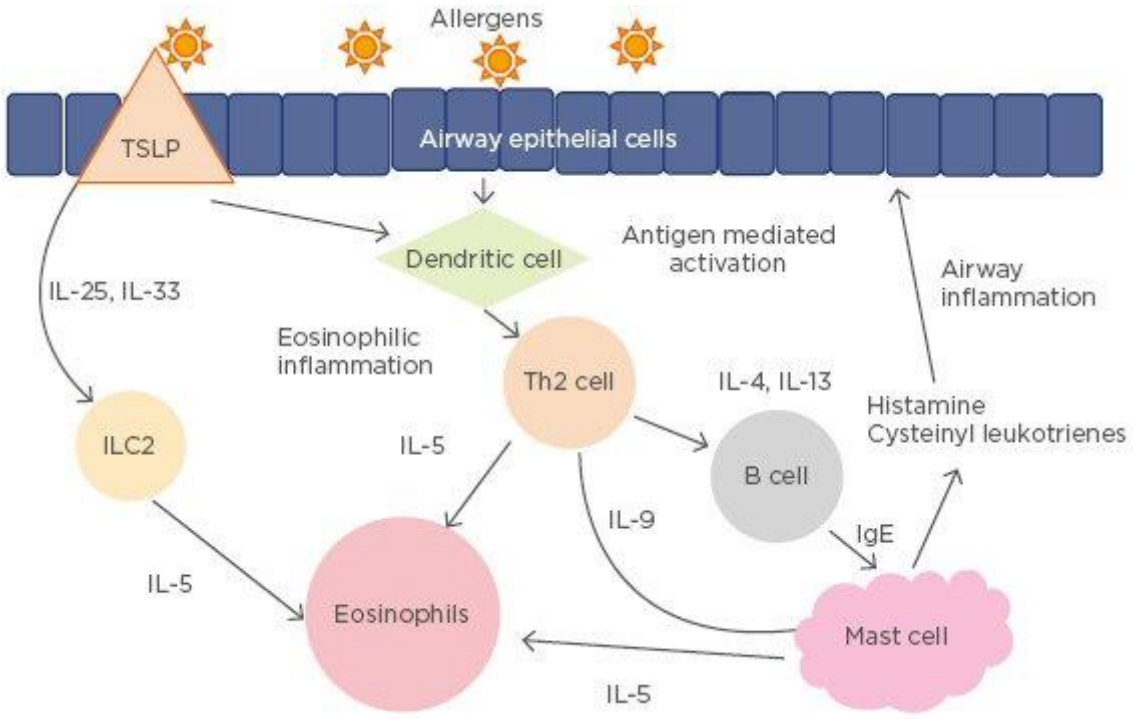


Figure 3: Multiple cell types playing a key role in asthma pathogenesis (EMJ. 2018;3[4]:111-121.)

3.5.1 Epithelial cells

Airway epithelium is a barrier interface between the external environment and the internal cellular composition, which makes it provide the first line of defense against allergens and pathogens. Disruption of airway epithelium facilitates easy access of

inhaled environmental particles and soluble compounds into the airway wall to interact with immune and inflammatory cells. Abnormal communication between the disrupted and stressed airway epithelial cells promotes airway remodeling responses and makes the individual prone to develop chronic inflammation such as asthma (Wang et al. 2008; Hellings and Steelant 2020).

In the pathogenesis of asthma on the epithelium level, secretion of chemokine (C-C motif) ligand 20 (CCL20), IL25, IL33 and TSLP is substantially increased following stress by allergens and other pathogenic substances. Those cytokines activate other immune cells such dendritic cells and innate lymphoid cells (ILCs). This could lead eventually to infiltration of many inflammatory cells into the airway submucosa, which makes also the basis of airway hyper-responsiveness (AHR) and airway remodeling (Liu et al. 2018; Gon and Hashimoto 2018).

There are several types of airway epithelial cells. *Ciliated epithelial cells* comprise a large number of cilia, which are capable of clearing mucus and attached environmental particles from the airway epithelium. Reduction in their clearance function leads to accumulation of mucus and asthma symptoms (Munkholm and Mortensen 2014). M*ucus-secreting goblet cells* secret mucus as an innate immune response to the environmental particles, which act as a trap and prevent them from passing through. *Airway basal cells* act as stem like cells to generate other airway epithelial cells and maintain the cellular homeostasis. Moreover, they mediate innate immune response by secreting pro inflammatory cytokine like IL-6 and IL-8 (Heijink et al. 2020; Hellings and Steelant 2020).

3.5.2 Airway dendritic cells

Airway dendritic cells (DCs) are the most efficient antigen-presenting cells (APCs) in the innate immune system. They act as a scaffold between the innate and adaptive immune systems (Morianos and Semitekolou 2020). There are two main types of airway dendritic cells:

Conventional dendritic cells (cDCs) play a crucial role in allergen sensitization in asthma through activating different adaptive immune cells. They have been reported

to initiate allergic airway inflammation in the lungs of mice after HDM sensitization *in vivo* (Plantinga et al. 2013).

Plasmacytoid DCs (pDCs) in turn can secret enormous amounts of type 1 interferons in response to viral infections (Peters et al. 2019). They can also induce regulatory T cells in order to tolerate sensitization of harmless inhaled particles. That can in turn prevent undesired allergic or asthmatic reactions (Kassner et al. 2010; Kool et al. 2009).

3.5.3 T and B lymphocytes

T lymphocytes play a central role in the adaptive immune system by receiving signals (peptides) from antigen-presenting cells (e.g. dendritic cells) and generating downstream signals (Holgate 2012). They are subdivided into two types: *T cytotoxic cells* (Tc, CD8+*)* and *T helper cells* (Th, CD4+).

The main task of CD8+ T cells is to eradicate virus-infected cells. They can be further differentiated into two subsets: *Tc1* and *Tc2.* Both subsets contribute to airway inflammation and development of asthma by secreting various types of inflammatory cytokines including interferon gamma (IFNy), IL-12 and IL-4 (Betts and Kemeny 2009).

The role of CD4+ T cells in allergic inflammation and asthma pathogenesis have been extensively studied. CD4+ T cells own unique plasticity to differentiate into a variety of subsets including Th1, Th2, Th9, Th17, Tregs and others (Figure 4) (Wambre et al. 2012).

As shown in Figure 4, the subsets of CD4+ T cells are related to different types of airway inflammation associated with asthma. For example, in type-2 allergic asthma (Th2-driven asthma), IL4 stimulates B cells to differentiate into IgE-producing plasma cells. Subsequently, secreted IgE molecules bind to their receptors on basophils and mast cells, which promotes, upon binding of the allergen, their maturation and production of histamine and proteases that can eventually damage the epithelial barrier and cause AHR (Ling and Luster 2016; Larché et al. 2003). IL5 mediates eosinophil infiltration, while IL13 triggers secretion of mucus by goblet cells and development of AHR (Wambre et al. 2012).

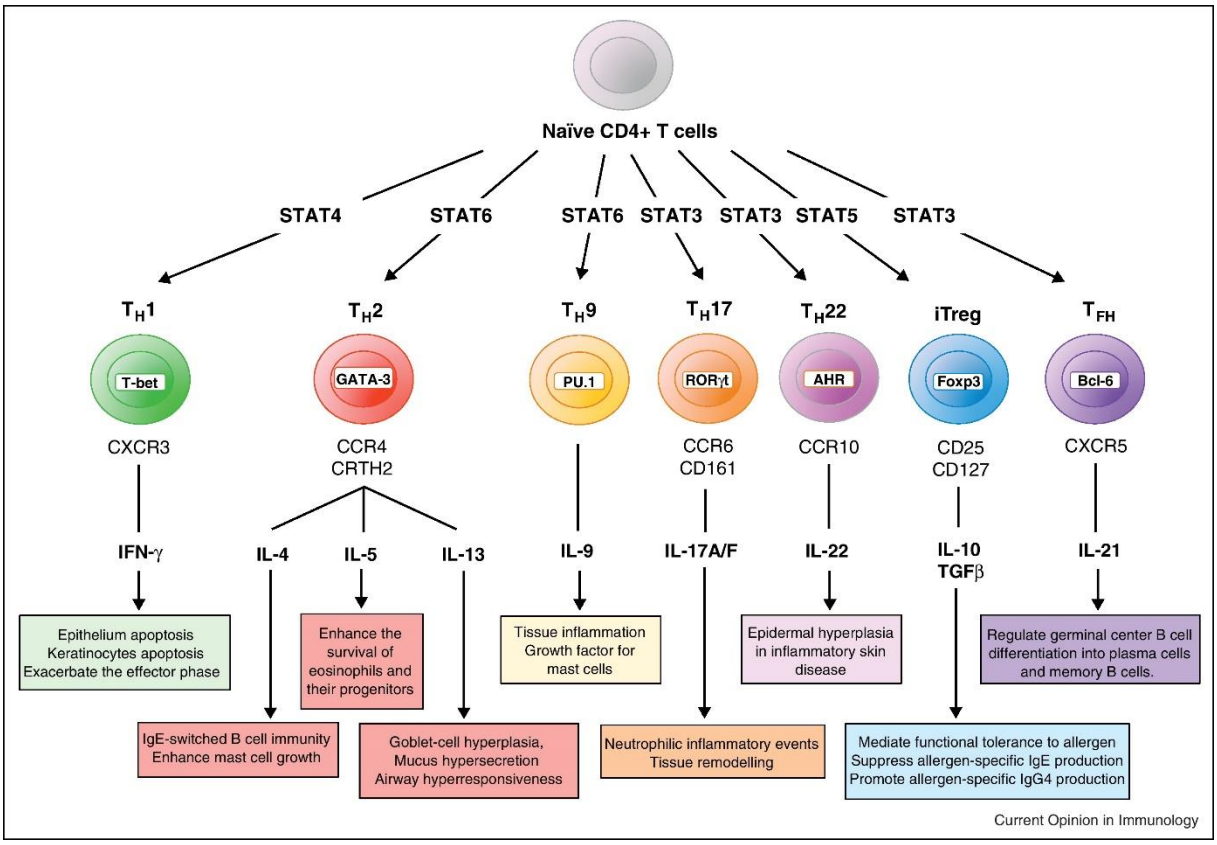


Figure 4: CD4+ T cell subsets and their role in allergic inflammation (Wambre *et al*, Curr Opin Immunol 2012)

On the other hand, Th1 cells have been found to be the major producers of IFNy protein that mediates the immune response against viruses and bacteria. Type-1 allergic responses in conjunction with type-2 allergic responses may even act synergistically to induce airway inflammation and AHR (Hansen et al. 1999; Ford et al. 2001). Importantly, Th1 and Th17 cells are thought to be major drivers of airway inflammation in non-type-2 forms of asthma, which it is often associated with neutrophilic airway inflammation (Lambrecht et al. 2019).

B lymphocytes are the major effector cells in humoral immunity (Inoue et al. 2018). They respond upon antigen encounter and cytokines of T lymphocytes by developing into IgM-, IgG-, IgA-, or IgE-secreting *plasma cells* (PCs) or *memory B* (B mem) cells, which in turn contribute to a variety of disease states including asthma and allergy (Cyster and Allen 2019). As previously stated IgE molecules play a crucial role in type-

2 asthma by binding to basophils and mast cells and causing AHR (Potaczek et al. 2017).

3.5.4 Eosinophils

The major role of eosinophils has been linked to host defense against parasitic infections by the release of cytotoxic cellular contents (Kariyawasam and Robinson 2007). Eosinophils responses are mainly triggered by IL-5, a cytokine released from different types of immune cells mainly including CD4 Th2 cells and ILC2s (Gurram and Zhu 2019). Increased numbers of eosinophils in the peripheral blood have been associated with eosinophilic disorders such as eosinophilic type of asthma (Matucci et al. 2018). Accumulation of eosinophils in the airway bronchi leads to epithelial cells damage through degranulation and release of toxic mediators such as eosinophil-derived neurotoxin, eosinophil cationic protein, and eosinophil peroxidase. They also produce a number of regulatory and pro-inflammatory cytokines such as IL-3, IL-4, IL-6, granulocyte-macrophage colony-stimulating factor (GM-CSF), tumor necrosis factor α (TNF-α), transforming growth factor-β (TGF-β) and chemokines such as eotaxins-1 (CCL11) and RANTES (CCL5). Those inflammatory mediators released by eosinophils contribute to airway remodeling in both allergic and non-allergic asthma (Kita 2011), thus eosinophils play a key role in asthma pathogenesis. Eosinophilic asthma has been considered as one of the most severe types of asthma. Airway eosinophilia can be detected in > 50% of asthmatic patients (Pelaia et al. 2017).

3.5.5 Neutrophils

Neutrophils are the most predominant phagocytes in the peripheral blood. They are trained to fight infectious pathogens efficiently and rapidly (Lehrer et al. 1988; Wang and Arase 2014). The defense mechanisms used by neutrophils to recognize and phagocytose microbes are the generation and release of destructive molecules including proteases, highly reactive oxygen species (ROS) and neutrophil extracellular traps (NETs). They also secret a variety of signaling proteins, including cytokines,

chemotactic molecules, and other mediators that are involved in their effector functions (Mayadas et al. 2014; Cheng and Palaniyar 2013).

Harmful effects of neutrophils can be generated through a leak of their previously-mentioned secretions from living and dying neutrophils during inflammation. This could damage adjacent normal tissue cells and cause airway inflammation. Persistent neutrophilic inflammation could lead to acute lung injury that can progress to persistent inflammation, which occurs in chronic lung diseases such as asthma (Ravi et al. 2019; Snelgrove et al. 2018).

3.6 Asthma phenotypes and endotypes

Heterogeneity in clinical features, inflammatory patterns and responses to therapy has made it very clear that asthma is not a single disease, but rather a complex disorder or even a syndrome. While phenotyping refers to grouping individuals with similar observable clinical features, endophenotyping or ‘endotyping’ assembles individuals on the basis of underlying molecular mechanisms or therapy responses (Anderson 2008; Lötvall et al. 2011).

Historically, asthma has been subphenotyped based on disease severity (mild, moderate and severe), IgE levels (atopic and non-atopic), eosinophils counts (eosinophilic and non-eosinophilic) and others (Wenzel 2012). Recently two classifications or categories of asthma have been widely approved based on many studies (Koczulla et al. 2017; Potaczek et al. 2020). As shown in Figure 5, under the umbrellas of **type 2-driven asthma** and **non-type 2-driven asthma** there are a variety of asthma phenotypes based on disease severity, age of onset, and clinical and molecular characteristics.

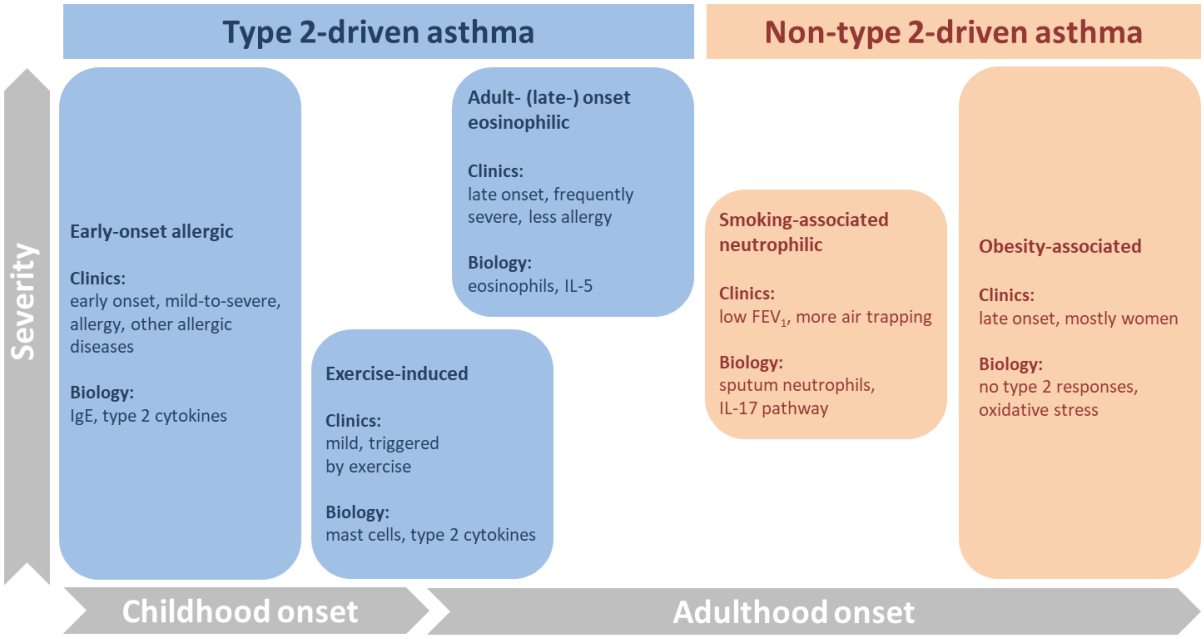


Figure 5: Examples of well-established type 2-driven and non-type 2-driven asthma phenotypes. Abbreviations: IgE, immunoglobulin E; IL, interleukin; FEV_1, forced expiratory volume in 1 s. (Potaczek *et al*, Cell Signal 2020)

3.6.1 Type 2-driven asthma

Of the most commonly accepted phenotypes, this classification of asthma includes *early-onset allergic* asthma that occurs mainly in children with mild to severe phenotype, *exercise-induced* asthma that is triggered after hard physical exercise in both children and adults with mild phenotype, and *late onset eosinophilic* asthma, which is the most severe phenotype in adults (Figure 5) (Desai and Oppenheimer 2016; Kuruvilla et al. 2019).

The underlying molecular mechanisms of type 2-driven asthma are generally summarized in Figure 6. In brief, cytokines of epithelial cells including IL-25, IL-33, and TSLP activate the airway DCs to present their cargo of allergens or viruses to different immune cells including ILC2s and CD4+ T cells. The latter produce a decent amount of inflammatory cytokines like IL-4, IL-5, IL-9, and IL13 that stimulate further cells, including mucus-secreting goblet cells, mast cells, basophils, and eosinophils and this way cause asthma inflammation and exacerbations (Fahy 2015).

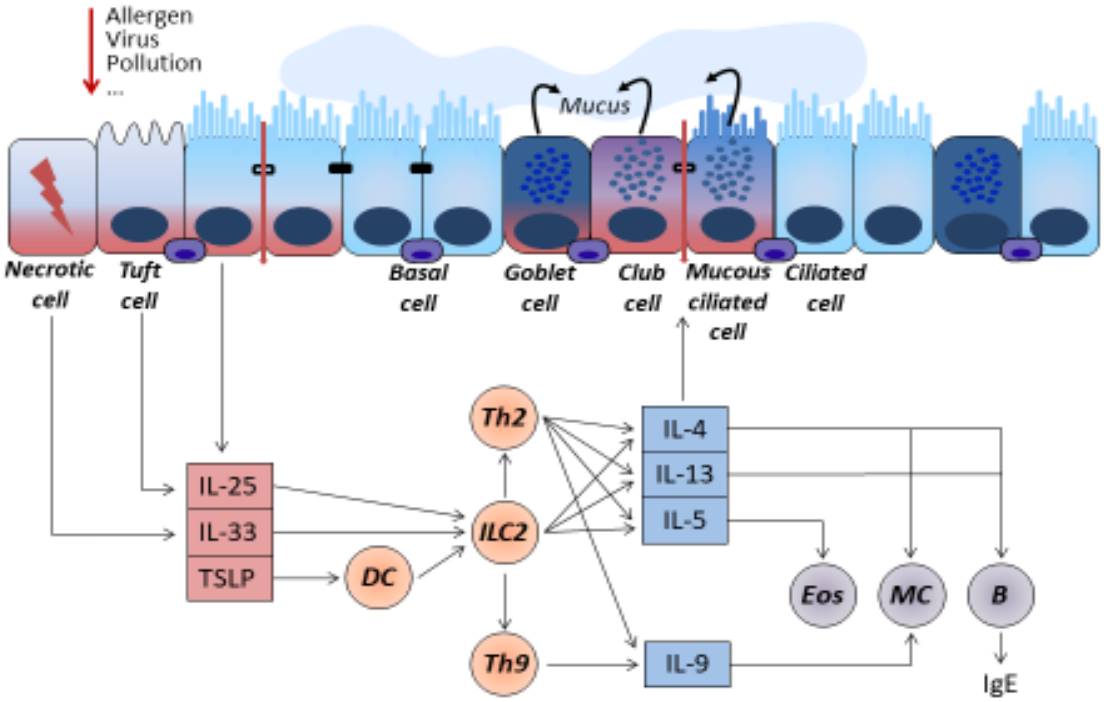


Figure 6: Molecular mechanisms of Type 2-driven asthma (Potaczek *et al*, Cell Signal. 2020)

3.6.2 Non-type 2-driven asthma

Multiple clinical forms of asthma are classified under the non-type 2-driven asthma phenotype including, of the widely recognized, well-established phenotypes, *smoking-associated neutrophilic* asthma and *obesity-associated* asthma. Both asthma phenotypes occur mostly in adults with mild to severe disease (Potaczek et al. 2020).

A summary of the underlying molecular mechanisms of non-type 2-driven asthma is shown in Figure 7. Neutrophils are the major infiltrating and inflammatory cell type that contributes to this asthma category. Maturation and activation of neutrophils are triggered by Th17 secreted cytokines like IL-17A, IL17F, IL-21, and IL-22 (Duvall et al. 2019). Additionally, adipose tissue mediators like free fatty acids (FFAs) also recruit neutrophils. Migration of accumulated neutrophils to the airway epithelium facilitates ultimately airway neutrophilia and causes asthma exacerbations (Watanabe et al. 2019).

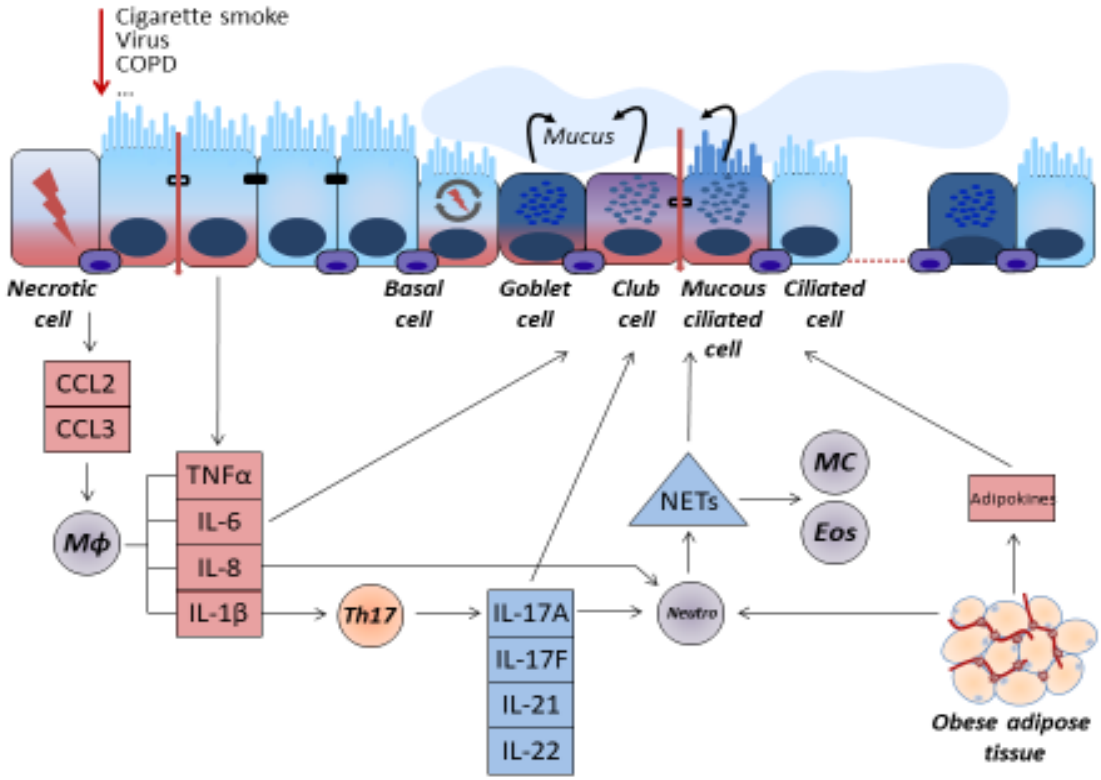


Figure 7: Molecular mechanisms of non-Type 2-driven asthma (Potaczek *et al*, Cell Signal. 2020)

3.7 Association of asthma with obesity

Obesity is a complex immunometabolic disease affecting more than 500 million adults worldwide (Tajima and Pawankar 2019; Carpaij and van den Berge 2018). Causes of obesity are various including sedentary life style, poor-quality diet, genetic susceptibility, and others. Moreover, obesity is considered as a risk factor for many other diseases such as cardiovascular disorders, diabetes, and hypertension (Miethe et al. 2018).

There is an accumulating evidence of an association between increase in body mass index (BMI) and the chance of developing asthma. In addition, different studies have demonstrated that being overweight (BMI 25-29.9) or obese (BMI ≥30) is associated with an increased risk for developing asthma exacerbations in adults (Bédard et al. 2017; Beuther and Sutherland 2007; Burgess et al. 2017). Besides, obese asthmatics show only a limited response to classical asthma treatments and are in need of higher treatment doses (Mohanan et al. 2014).

The mechanism of how obesity affects asthma development and clinical course is not fully understood. Nevertheless, adipocytes of obese individuals secret a huge load of pro-inflammatory cytokines such as TNF-a and IL-6, inflammatory adipokines including adiponectin and leptin and other mediators such as FFAs (Carpaij and van den Berge 2018). The latter has been shown to recruit neutrophils to the airway epithelium and cause neutrophilic airway inflammation in asthmatic patients (Watanabe et al. 2019).

3.8 Role of extracellular vesicles (EVs) and their associated microRNAs (miRNAs) in asthma

Extracellular vesicles (EVs) are circulating vesicles secreted by most cell types and found predominately in the body fluids. They are coated with a phospholipid bilayer and surface marker proteins (Ref). There are three major types of extracellular vesicles based on their size, formation process, or their molecular load (Mohan et al. 2020):

1. Exosomes in size from 30-150 nm in diameter.
2. Macrovesicles in size from 100-1000 nm in diameter.
3. Apoptotic bodies in size >1000 nm in diameter.

Extracellular vesicles are loaded with a cargo of proteins, RNAs (tRNA, rRNA and miRNAs), oligo DNAs, and lipids (Figure 8), which are used for cell-cell communications, in which they shuttle their cargo from parent to recipient cells (Fujita et al. 2014). Many studies have investigated the role of extracellular vesicles and their associated microRNAs (miRNAs) in various dieses including asthma (Bartel et al. 2020; Bahmer et al. 2021; Zhao et al. 2019b).

MiRNAs are small types of non-coding RNAs with a length range around 22 nucleotides (nt). They act as suppressors of messenger RNAs (mRNAs) translation by cleaving mRNAs and blocking their translation into functional proteins (Ha and Kim 2014; Hudder and Novak 2008). The EV associated miRNAs have been found to play a key role in asthma pathogenesis. Dysregulated expression of EV miR-34a, miR-92b, and miR-210 in nasal lavages of asthmatic children correlated positively with their lung function measurements (Bartel et al. 2020). Serum exosomal miR-125b has been shown to present at lower levels in asthma patients compared to healthy controls. Moreover, the expression levels of this miRNA correlated positively with asthma

severity degree in those patients (Zhao et al. 2019a). Both human and mice investigations have found that serum exosomal miR-126 was up-regulated in asthmatic patients compared to healthy controls, which was additionally proved in the lung tissues of asthma model mice. The target DNA Methyltransferase 1 (*DNMT1*) gene mRNA was found to be down-regulated in the same tissue. This indicated a potential role of miR-126 in asthma pathogenesis (Zhao et al. 2019b).

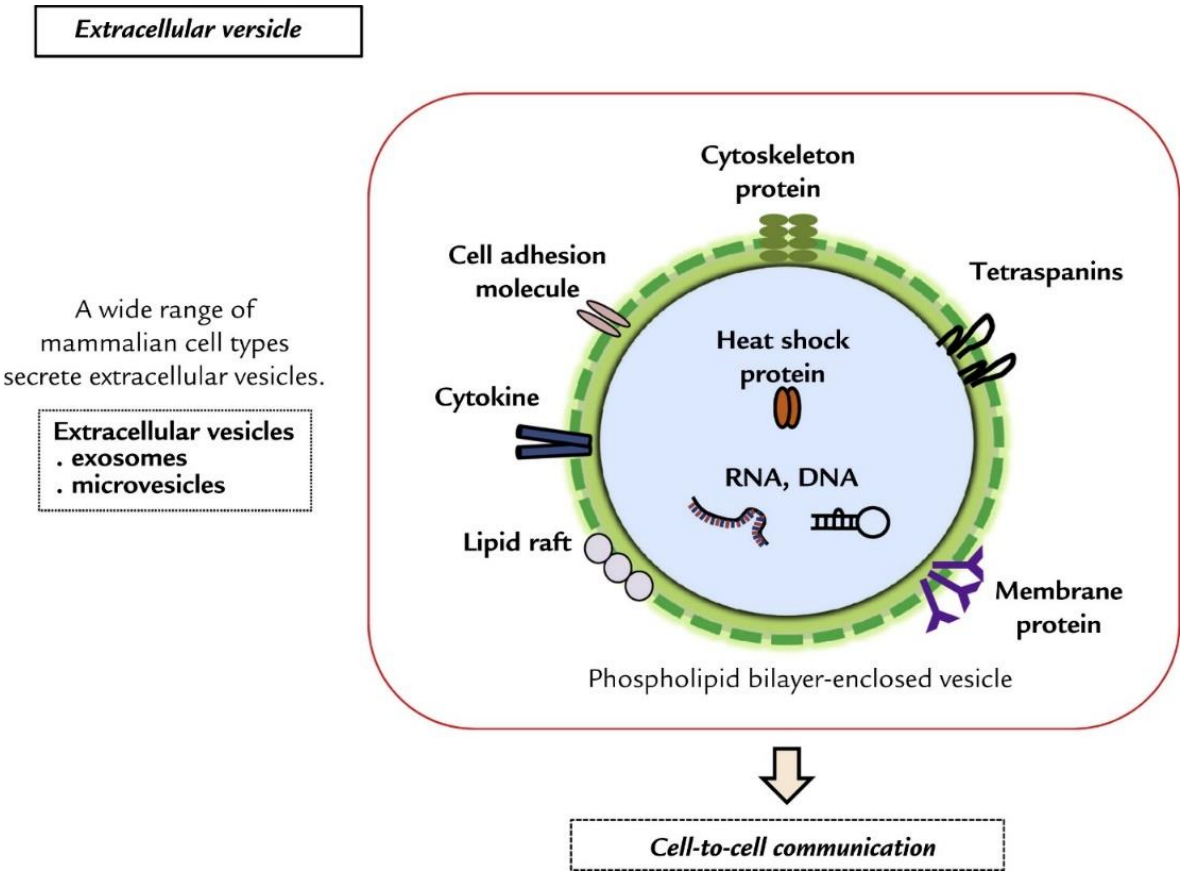


Figure 8: Molecular content and surface components of extracellular vesicles (Fujita *et al*, Clin Ther 2014)

3.9 Asthma treatment

Asthma is a heterogeneous disease with multiple clinical phenotypes and molecular endotypes that makes it vary in severity and response to treatment. The old paradigm of “one dose fits all” has gradually vanished after the tremendous advancement in the medical and biological fields. The primary treatment of asthma begins with prevention aiming to avoid risk factors such allergens and smoking, yet intervention with pharmacological drugs is still required (Larenas-Linnemann 2009). The main asthma

treatments are composed of controller therapy such as inhaled corticosteroids (ICS) and reliever therapy like β2-agonists. According to the GINA guidelines, increasing amount of drug doses and a supplement of additional drugs is recommended based on the severity of the asthma exacerbations, starting with a low dose of ICS for mild to moderate asthma and a high dose of ICS with a long-acting beta-agonist (LABA) for severe asthma (Figure 9) (Global Initiative for Asthma – GINA 2019). This is a standard treatment only, yet there are several other alternative medical and biological therapeutic options available for asthma treatment.

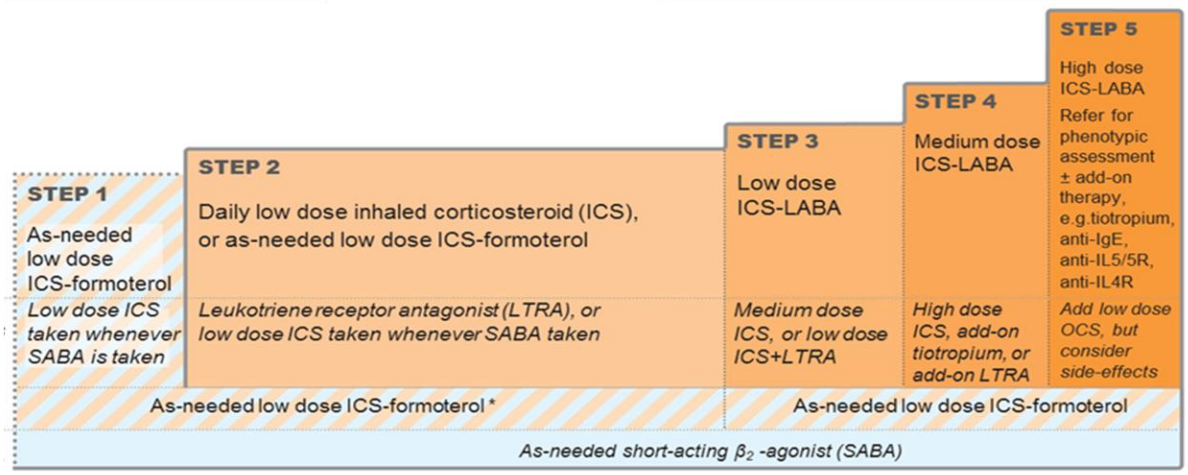


Figure 9: GINA 2019 asthma treatment guidelines.

3.9.1 ICS

ICS are the first line and most effective anti-inflammatory asthma treatment. The main target of ICS biological mechanism is suppressing the type 2-driven asthma inflammation, in which ICS impair the expression of various inflammatory cytokines and chemokines (Barnes et al. 1998). Corticosteroid molecules diffuse through cell plasma membrane to attach to their cognate intracellular receptors in the cytoplasm. This binding effect is then transmitted to the nucleus, where it negatively influences the gene expression of different type 2 inflammation-associated transcription factors including nuclear factor-κB (NF-κB) and activator protein 1 (AP1) (Barnes and Adcock 2003). Unfortunately, ICS have been found to show a variety of side effects such as metabolic disturbances, adrenal insufficiency, skin bruising and others (Kelly and

Nelson 2003; Dahl 2006). Examples of ICS medications are fluticasone and budesonide.

3.9.2 β2-agonists

β2-agonists are bronchodilator drugs that act upon β2-adrenoceptor (ADRB2) to mediate relaxation of airway smooth muscles and assure more airflow through the respiratory tract during acute asthma exacerbations (Page and Spina 2006).

There are two main types of β2-agonist drugs. Firstly, short-acting β2-adrenergic receptor agonists (SABA) that are given to asthmatic patients during acute asthma attacks, including salbutamol and terbutaline (Expert Panel Report 3 (EPR-3): Guidelines for the Diagnosis and Management of Asthma-Summary Report 2007 2007). Secondly, LABA such as formoterol and salmeterol, are regularly given to asthmatic patients to reduce the occasional need of SABA drugs (Mysore and Ruffin 2011). A combination of ICS and β2-agonists have been associated with a lower risk of asthma exacerbations (Sobieraj et al. 2018; Ismaila et al. 2014).

3.9.3 Leukotriene receptor antagonists (LTRA)

Leukotriene receptor antagonist (LTRA) drugs, including montelukast and zafirlukast, are meant to act against potent bronchoconstrictors such as cysteinyl-leukotrienes (cysLTs: LTC_4, LTD_4 and LTE_4) (Bjermer and Diamant 2002). LTRA have also been utilized as monotherapy for persistent asthma (Kemp 2003). Moreover, LTRA have shown superiority over ICS in reducing asthma exacerbations (Sin et al. 2004).

3.9.4 Biologic treatment for asthma

Treating asthma with biologics has increased rapidly in recent years. Biologics are naturally antibody-derived substances that are able to highly specific target a potential mediator associated with a specific disease mechanism (Eisenberg 2012).

Some of those biologic treatments have been recently approved in the asthma therapy field:

Anti-immunoglobulin E (IgE) drugs such as omalizumab which binds to high-affinity and low-affinity receptor binding sites on IgE molecules, preventing mast cell/basophil degranulation; therefore reducing asthma exacerbations (Menzella et al. 2015).

Anti-interleukin 5 (IL-5) drugs such as mepolizumab deigned against IL-5 protein that stimulates production of longer survival of eosinophils. It has been shown that mepolizumab reduces asthma exacerbations in eosinophilic asthmatics (Pavord et al. 2012).

Anti-interleukin 4/13 (IL-4/13) drugs such as Dupilumab that binds IL-4Rα and and prevents binding of both IL-4 and IL-13 to the IL-4 receptor alpha, thus, Dupilumab has exhibited strong clinical efficacy against type-2 driven asthma (Le Floc'h et al. 2020; Pepper et al. 2017).

Anti-interleukin 2 receptors (IL-2Rs) drugs such as daclizumab that acts against interleukin 2 activity and T cell activation. Added to ICS, daclizumab was able to further improve lung function and asthma control in adults with moderate to severe persistent asthma (Busse et al. 2008).

3.10 Hypotheses and aims

Nowadays, there is an unmet medical need for personalized treatment based on a precise identification of the disease phenotype. As shown in the previous chapter, most of the current asthma treatment regimens are meant to treat asthma in general, not its particular, diverse phenotypes. But applying a combination of conventional therapies for one severe or mild asthmatic patient is in fact nothing more than a clinical trial that might simply fail anytime. Therefore, a mechanistic comprehension of the molecular endotype underlying the disease in specific subgroups of asthmatic patients that share similar clinical characteristics is urgently required. This will make it possible to design and match specific therapies or therapeutic regimens targeting specific and well understood asthma endotypes. Such progress would be especially important in the case of obesity-associated asthma lacking so far any specific treatment approaches,

even though the prevalence of asthma, obesity, and the obesity-associated asthma is constantly increasing.

Therefore, the hypotheses of this work are that in vivo peripheral blood CD4+ T cells activation patterns and plasma exosomal microRNAs expression profiles in obese asthmatics can translate the clinical phenotypes into a set of molecular mechanisms, this will generate molecular phenotypes that can more precisely define underlying endotypes, i.e. underlying differential pathomechanisms which could lead to stratified theraputic concepts in line with personalized medicine strategies.

The aim of this work was to decipher the underlying molecular mechanism of the low type-2 obesity-related asthma phenotype using modern genome wide techniques. Therefore, the objectives were as follows: (1) to investigate the impact of timing of blood samples processing on transcriptomic and epigenomic profiles of blood CD4+ T cells in order to define optimal experimental setup before processing precious biomaterial obtained from the patients, (2) to utilize peripheral blood CD4+ T cells as endo-phenotyping model for obese asthmatics and study their activation patterns which can be translated into a set of molecular mechanisms, (3) to discover the biological pathways associated with the activation states of CD4+ T cells, (4) to underpin the role of the EV miRNAs in low type 2 obesity-related asthma and define their target genes and pathways in the CD4+ T cells and (5) to link the previous molecular findings with the clinical measurements and investigate their potential correlation insights.

4. Material and methods

4.1 Material tables

Table 2: Blood collection and CD4+ T cells isolation.

Product	**Company**
S-Monovette K3 EDTA collection tube	Sarstedt, Nümbrecht, Germany
S-Monovette Lithium-Heparin collection tube	Sarstedt, Nümbrecht, Germany
Biocoll-Separating solution	Biochrom, Berlin, Germany
StraightFrom™ Whole Blood CD4 MicroBeads	Miltenyi Biotec, Bergisch Gladbach, Germany
Whole Blood Column kit	Miltenyi Biotec, Bergisch Gladbach, Germany
Magnetic QuadroMACS Separator	Miltenyi Biotec, Bergisch Gladbach, Germany
MACS MultiStand	Miltenyi Biotec, Bergisch Gladbach, Germany
Dulbecco's phosphate buffered saline (DPBS)	Merck, Darmstadt, Germany
Fetal bovine serum	Merck, Darmstadt, Germany
Cell Counter CASY	OLS OMNI Life Science, Bremen, Germany
CD4 (RPA-T4) APC-H7 antibody	Becton-Dickinson (BD), Franklin Lakes, New Jersey, USA
CD8 (RPA-T8) FITC antibody	BD, Franklin Lakes, New Jersey, USA
CD19 (HIB19) PercP Cy5.5 antibody	BioLegend, San Diego, CA, USA
CD3 (UCHT1) PECy7 antibody	Thermo Fisher Scientific, Waltham, MA, USA
FACSAria III	BD, Franklin Lakes, New Jersey, USA

Table 3: RNA extraction, chromatin immunoprecipitation (ChIP) analysis, library preparation and sequencing.

Product	Comapny
RNeasy Mini Kit	Qiagen, Hilden, Germany
RNase-Free DNase Set	Qiagen, Hilden, Germany
2-Mercaptoethanol	Carl Roth, Karlsruhe, Germany
Agilent High Sensitivity DNA Kit	Agilent Technologies, Santa Clara, CA, USA
Agilent RNA 6000 Pico Kit	Agilent Technologies, Santa Clara, CA, USA
Agilent Bioanalyzer 2100	Agilent Technologies, Santa Clara, CA, USA
37% formaldehyde solution	Merck, Dermstadt, Germany
Glycine	Carl Roth, Karlsruhe, Germany
True MicroChIP kit	Diagenode, Liège, Belgium
Bioruptor® Pico	Diagenode, Liège, Belgium
1.5 ml Bioruptor® Pico Microtubes with Caps	Diagenode, Liège, Belgium
H3K27ac polyclonal rabbit antibody	Diagenode, Liège, Belgium
IgG rabbit antibody as an isotype control	Diagenode, Liège, Belgium
MicroChIP DiaPure columns	Diagenode, Liège, Belgium
Library Preparation Kit v3 with dual indexes	Diagenode, Liège, Belgium
QuantSeq 3' mRNA-Seq Library Prep Kit REV for Illumina	Lexogen, Vienna, Austria
MJ Mini Personal thermal cycler	Bio-Rad, Hercules, CA, USA
Ampure XP Beads	Beckmann Coulter, Pasadena CA, USA
Qubit dsDNA HS Assay Kit	Thermo Fisher Scientific, Waltham, MA, USA
Qubit RNA HS Assay Kit	Thermo Fisher Scientific, Waltham, MA, USA

Qubit 3 Fluorometer	Thermo Fisher Scientific, Waltham, MA, USA
High Output Kit v2.5	Illumnia, San Diego, CA, USA
Illumina NextSeq550 platform	Illumnia, San Diego, CA, USA
BGISEQ-500 platform	BGI, Shenzhen, China
GraphPad Prism 7 software	GraphPad, San Diego, CA, USA

Table 4: Plasma EVs extraction, EVs RNA extraction, library preparation and sequencing.

Product	**Comapny**
BD 303172 Plastipak™ Sterile Luer Slip 1ml Syringes	Becton-Dickinson (BD), New Jersey, United States
Millex-AA Syringe Filter Unit, 0.80 µm	Sigma-Aldrich/Merck, Darmstadt, Germany
exoRNeasy Midi	Qiagen, Hilden, Germany
Buffer XE	Qiagen, Hilden, Germany
Qubit microRNA Assay Kit	Qiagen, Hilden, Germany
QIAseq miRNA Library Kit	Qiagen, Hilden, Germany
LightCycler 480	Roche, Basel, Switzerland
Freedom EVO150 liquid handling platform	Tecan, Männedorf, Switzerland
1X EvaGreen® Dye	Biotium, Hayward, CA, USA
LabChip GX	PerkinElmer, Waltham, MA, USA
KAPA Library Quantification Kit	Roche, Basel, Switzerland
Illumina HiSeq 4000 platfrom	Illumnia, San Diego, CA, USA
NTA instrument and ZetaVIEW software	Particle Metrix, Ammersee, Germany

4.2 Buffers

MACS buffer

Dulbecco's phosphate buffered saline + 0.5% Fetal bovine serum

1% 2-Mercaptoethanol in RLT buffer for RNA extraction

500 µL 2-Mercaptoethanol + 50 mL RLT buffer

TE buffer PH 8.0

200 of EDTA (0.5 M, pH 8.0) + 1000 µl of Tris-Cl (1 M, pH 8.0) + H_2O to 100 mL

4.3 Methods

4.3.1 Establishment of optimal conditions for blood CD4+ T cells processing

4.3.1.1 Cell sorting for blood CD4+ T cells

Venous blood was collected using S-Monovette Lithium-Heparin collection tubes from three non-atopic (healthy) and three atopic volunteers from two centers; Pediatric Allergology, Department of Pediatrics, Dr. von Hauner Children's Hospital, University Hospital, LMU Munich and Department of Pediatric Pneumology, Allergology and Neonatology, Hannover Medical School. 10 ml blood from each volunteer were processed immediately and additional 10 ml were left at room temperature (RT) for 24 hours for delayed processing. Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized blood by Biocoll density-gradient centrifugation and stained with fluorescent-labeled antibodies: CD8 (RPA-T8) FITC, CD4 (RPA-T4) APC-H7, CD19 (HIB19) PercP-Cy5.5 and CD3 (UCHT1) PECy7. CD3+CD4+CD8-CD19- T-cells were sorted by fluorescent activated cell sorting (FACS) using FACSAria III. Debris and doublets were excluded from sorting by gating for intact cells using forward and side scatters followed by gating for lymphocytes and surface stained CD3+, CD4+, and CD19+ cells based on multi-parameter staining panel. Finally, CD4+CD3+ T cells were sorted. After sorting, cells were pelleted and re-suspended in RLT-Buffer with 1% 2-mercaptoenthanol for transcriptome analysis and immediately transferred to -80 °C for storage. Cells for chromatin immunoprecipitation (ChIP) analysis were cross-linked with 1% formaldehyde (final concentration) by adding 27 µL of 37% formaldehyde to 1 mL of cell suspension and incubated for 8 min at RT. The cross-linking reaction was quenched with 125 mM Glycine (final concentration) by adding 115 µL of 125 mM Glycine to the previous reaction volume and incubated for 5 minutes at RT. Subsequently the cross-linked cells were pelleted at 300 g for 10 minutes at 4 °C, the supernatant was discarded and the cells were rinsed with 1 mL DPBS containing protease inhibitor mix (1 µL of protease inhibitor was added per 200 µL of DPBS,

calculated accordingly for the rest of the steps). The cells were pelleted again at 300xg for 10 m at 4 °C and supernatant was discarded. The cell pellet was snap frozen in liquid nitrogen and stored at -80 °C.

4.3.1.2 RNA extraction

RNA was extracted from 500,000 blood derived CD4+ T cells utilizing the RNeasy Mini Kit. According to the manufacturer's protocol, lysate samples were thawed down at 37 °C, 350 µL of 70% ethanol was added to each sample and well re-suspended. Samples were transferred to RNeasy Mini spin tubes and centrifuged for 15 sec at 8,000xg; flow-through was discarded. RNeasy columns were washed with 350 µL of RW1 buffer and centrifuged at the same previous setting.

For DNase digestion, the RNase-Free DNase Set was used. 10 µL DNase I stock solution were added to 70 µL Buffer RDD and mixed gently. DNase I incubation mix (80 µL) was added directly to the RNeasy column membrane, which was placed on benchtop for 15 min at RT. 350 µL of Buffer RW1 were added to RNeasy column and centrifuged for 15 sec at 8,000xg, flow-through was discarded. 500 µL of Buffer RPE were added to the RNeasy spin column and centrifuged for 15 min at 8,000xg; flow-through was discarded; this step was repeated. RNeasy spin columns were placed in a new 2 ml collection tube and centrifuged at full speed for 1 min to dry the membrane. Afterwards the RNeasy spin columns were placed in a new 1.5 mL collection tube, 30 µL RNase-free water were added directly to the spin column membrane, and the columns in the tubes were centrifuged for 1 min at 8,000xg to elute the RNA. Quality of the extracted RNA was assessed by Agilent RNA 6000 Pico Kit and RNA concentration was measured by Qubit RNA HS Assay Kit.

4.3.1.3 RNA library preparation and sequencing

Library preparation was conducted using the QuantSeq 3' mRNA-Seq Library Prep Kit REV for Illumina according to the manufacturer's protocol. To synthesize the first cDNA strand, 5 µL of FS1 Buffer was added to 5 µL of RNA samples (50 ng of RNA). This mix was incubated 3 min at 85 °C, then cooled down to 42 °C. Afterwards FS2/E1

mastermix was prepared by adding 9.5 µL of FS2 buffer to 0.5 µL E1 and pre-warmed 3 min at 42 °C. 10 µL FS2/E1 mix were added to the FS1/RNA mix while keeping the sample at 42 °C in the thermocycler and incubated 15 min at 42 °C. To remove the residual RNA, 5 µL RS were added to the reaction tube, mixed well, incubated 10 min at 95 °C, and then cooled down to 25 °C.

To synthesize the second cDNA strand, 10 µL SS1 were added to the reaction tube, mixed well, Incubated 1 min at 98 °C, slowly ramped down to 25 °C (0.5 °C/sec), and incubated 30 min at 25 °C. 5 µL SS2/E2 mix were added to the reaction tube, mixed well, and incubated 15 min at 25 °C.

To purify the cDNA, 16 µL PB were added to the reaction tube, mixed well, incubated 5 min at RT. Mix was placed on magnet for 5 min, and supernatant was discarded. 40 µL EB were added, tubes were removed from magnet, mixed well, and incubated 2 min at RT. 56 µL PS were added, mixed well, incubated for 5 min at RT and placed on magnet for 5 min. Supernatant was discarded, beads were washed twice with 120 µL 80% EtOH for 30 sec, and air dried for 5 min. 20 µL EB were added, tubes were removed from magnet, mixed well, and incubated 2 min at RT. Tubes were placed on magnet for 5 min. Afterwards, 17 µL of the supernatant were transferred into a fresh PCR plate.

To amplify the cDNA library, a mastermix with 7 µL PCR and 1 µL E3 per reaction was prepared, and mixed well. 8 µL PCR/E3 mastermix and 5 µL i7 Primer were added to 17 µL of each purified cDNA library. The reaction tubes were placed in the thermocycler for the following program: 30 sec at 98 °C, 15X (10 sec at 98 °C, 20 sec at 65 °C, 30 sec at 72 °C), 1 min at 72 °C and 10 min at 10 °C.

To purify the amplified libraries, 30 µL PB were added to each library tube, mixed well, and incubated 5 min at RT. Library tubes were placed on magnet for 5 min and then the supernatants were discarded. 30 µL EB were added, tubes were removed from magnet, mixed well and incubated 2 min at RT. 30 µL PS were added, mixed well, incubated 5 min at RT. The tubes were placed on magnet 5 min, and supernatant was discarded. Beads were washed twice with 120 µL 80 % EtOH for 30 sec, and air dried for 5 min. 20 µL EB were added, tubes removed from magnet, mixed well and incubated 2 min at RT. Tubes were placed on magnet for 5 min, 15 µL of the supernatant transferred into a fresh PCR plate. Sequencing was performed on an Illumina NextSeq550 platform, High Output Kit v2.5, 50 bases single-end reads

according to the manufacturer's instructions, both library preparation and sequencing were performed at the Genomics Core Facility of the Medical Faculty of the Philipps University of Marburg.

4.3.1.4 ChIP analysis

ChIP experiments were performed using the True MicroChIP kit. According to the manufacturer´s protocol, for each 10,000 to 100,000 cells, the cell pellet was thawed down and re-suspended in 25 µL of Lysis Buffer tL1 containing protease inhibitor mix and incubated for 5 min on ice. Then 75 µl of HBSS containing protease inhibitor mix were added, and afterwards the chromatin lysate was sheared using the Bioruptor® Pico sonication device combined with the Bioruptor® Water cooler for 13 cycles using a 30" [ON] 30" [OFF] settings. Shearing was conducted in 1.5 ml Bioruptor® Pico Microtubes with Caps.

The sheared chromatin was centrifuged at 14,000xg for 10 min at 4C° to get rid of the cell debris. The chromatin was transferred to low retention and DNase free tubes and diluted with 100 µL of ChIP Buffer tC1 containing protease inhibitor mix, 20 µL of the chromatin solution were set aside at 4 C° as an input control, and the remaining chromatin was incubated overnight on a rotator at 40 rpm at 4 °C for immunoselection in the presence of the following amounts of antibodies: 0.75 µg of H3K4me3 polyclonal rabbit antibody, 1 µg of H3K27me3 polyclonal rabbit antibody, 0.75 µg of H3K27ac polyclonal rabbit antibody, and 1 µg IgG rabbit antibody as an isotype control.

In the next morning, 10 µl of the Protein-A coated magnetic beads were used for each IP reaction. They were first washed twice with 1 mL of Beads Wash Buffer tBW1 and added to each chromatin IP reaction tube, and then tubes were incubated on a rotator for 2 hours at 4 °C.

The reaction IP tubes were placed on a magnetic rack for 1 min and the supernatants were discarded. Beads were re-suspended with 100 µL of Wash Buffer tW1 and rotated at 40 rpm for 4 min at 4 °C. The previous washing step was repeated with Wash Buffers tW2, tW3 and tW4.

After removing the last wash buffer, beads were re-suspended with 200 µL of Elution Buffer tE1 and rotated at 40 rpm for 30 min at RT, 180 µL of Elution Buffer tE1 were added also to the input control tubes.

The IP reaction tubes were placed on the magnetic rack for 1 min and the supernatants were transferred to new tubes. Then 8 µL of Elution Buffer tE2 were added to both IP reaction and input control tubes and incubated overnight at 65 °C in a water bath to perform the de-cross-linking reaction.

In the next morning and after the de-cross-linking reaction was done, the immunoprecipitated DNA was purified using the Microchip Diapure columns kit by adding 1 mL of ChIP DNA binding buffer to each DNA containing tube and 600 µL of the DNA solution was run on the spin columns provided with collection tubes twice and centrifuged using the same setting for the whole purification steps at 1,200xg for 30 sec at RT. The spin columns were washed twice with 200 µL of DNA wash buffer. After washing, the spin columns were placed on new collections tubes and DNA was eluted with 30 µL of DNA elution buffer and stored at -80 °C until sequencing. Concentration of eluted DNA concentration was measured by Qubit dsDNA HS Assay Kit.

4.3.1.5 ChIP Library preparation and sequencing

ChIP libraries were prepared using the MicroPlex Library Preparation Kit v3 with dual indexes according to the manufacturer's protocol. To prepare the DNA template, 2 µL Dual Template Preparation Buffer and 1 µL Dual Template Preparation Enzyme were mixed and added to 10 µL ChIP DNA (0.5 ng of DNA). Reaction tubes were placed in the thermocycler for 25 min at 22 °C, for 20 min at 55°C and cooled down to 4 °C.

To synthesize the libraries, 1 µL Dual Library Synthesis Buffer and 1 µL Dual Library Synthesis Enzyme were mixed and added to the reaction tubes which were placed in the thermocycler for 40 min at 22 °C and cooled down to 4 °C.

To amplify the libraries, 25 µL Dual Library Amplification Buffer, 1 µL Dual Library Amplification Enzyme and 4 µL Nuclease-Free Water were mixed well and added to each reaction tube, 5 µL of a unique Dual Indexing Reagent were also added to each reaction tube. Reaction tubes were placed on the thermocycler for the following program: 3 min at 72 °C, 2 min at 85 °C, 2 min at 98 °C, 4X (20 sec at 98 °C, 20 sec

at 67 °C, and 40 sec at 72 °C), 12X (20 sec at 98 °C and 50 sec at 72 °C) and cooled down 4 °C.

To purify the libraries, 50 µL AMPure XP beads were added to each reaction/library tube, mixed well and incubated for 5 min at RT. Tubes were placed on magnet for 5 min and supernatants were discarded. Beads were washed twice with 150 µL of 80% EtOH without disturbing the bead pellet, each time for 30 sec. Supernatants were discarded and beads were air dried for 5 min. DNA was eluted from beads by re-suspending the beads in 20 µL of 1x Low TE buffer, pH 8. Tubes were placed on magnet for 2 min and the supernatant containing the DNA was transferred to a new tube. DNA quality and concentration were assessed by Agilent High Sensitivity DNA Kit and Qubit dsDNA HS Assay Kit, respectively.

Library sequencing was performed as in point 2.3.1.3.

4.3.1.6 Bioinformatic analysis of RNA-Seq and ChIP-Seq

Subsequent RNA-Seq and ChIP-Seq bioinformatic workflows were performed using Galaxy platform (Jalili et al. 2020). Starting with RNA-Seq, quality of reads was assessed by *FastQC v0.72*. Afterwards reads were aligned to the human reference genome (*Homo sapiens* hg38 full) with *RNA STAR v2.7.8a*. Mapped reads were counted with *featureConts v2.0.1*, followed by exon *DESeq2 v2.11.40.6* analysis to determine the differentially expressed genes. *Enrichr*, *BioPlanet* and Gene Ontology databases were utilized to perform functional pathways enrichment and biological processes analyses, respectively (Chen et al. 2013; Kuleshov et al. 2016; Xie et al. 2021). ChIP-Seq reads were aligned to the same human reference genome using *Bowtie2 v2.4.2*. *Deeptools* were utilized to generate enriched score genomic heatmaps and overlapped genes profiles. Differential whole gene analysis was conducted by *DESeq2*. Values of heat maps were calculated as Z-scores of normalized gene expression counts.

4.3.2 Blood CD4+ T cells transcriptome methods of the obesity-associated asthma study

4.3.2.1 CD4+ T cell isolation from peripheral blood

9 mL peripheral venous blood were drawn from the antecubital vein of each donor in S-Monovette K3 EDTA collection tubes. The whole blood was transferred into a 50 mL “Falcon” tube. 450 µL of StraightFrom™ Whole Blood CD4 MicroBeads, human were added, mixed well, and incubated for 15 min at 4 °C. 25 mL of MACS buffer were added, suspension was centrifuged at 1,500 rpm for 10 min at RT without a brake. Supernatant was discarded till about 10 mL level (the same volume of the supernatant as blood cell pellet was always kept), blood was re-suspended and mixed well. Whole Blood Column was placed on the magnetic MACS MultiStand and QuadroMACS Separator, and washed with 3 mL MACS buffer. The blood suspension was added to the column in portions of 3 mL till the whole blood was through. Column was then washed twice, each time with 3 mL MACS buffer. 4 mL elution buffer were added on the column, immediately flushed with the provided plunger into new 15 mL tube, and centrifuged at 1,500 rpm for 5 min at RT. Supernatant was discarded and the pellet was re-suspended in 2 mL MACS buffer. 5 µL of the cell suspension was added into CASY-ton cup and cells were counted using the Casy counter.

4.3.2.2 RNA Extraction

See point 4.3.1.2

4.3.2.3 BGI Library preparation and RNA-Sequencing

RNA library preparation and sequencing were performed by BGI Genome Sequencing Service in Shenzen, China. Briefly, mRNA molecules were purified from total RNA using oligo (dT)-attached magnetic beads. Further mRNA molecules were fragmented into small pieces. Afterwards first-strand cDNA was generated using random hexamer-

primed reverse transcription, followed by a second-strand cDNA synthesis. The synthesized cDNA was subjected to end-repair and 3’ adenylation. Adapters were ligated to the ends of the 3’ adenylated cDNA fragments. The cDNA fragments were subsequently amplified with primers towards adapters from previous step. PCR products were purified with Ampure XP Beads and dissolved in elution buffer solution. Library quality was validated on the Agilent Technologies 2100 bioanalyzer. Double-stranded PCR products were heat denatured and circularized by the splint oligo sequence. The single strand circular DNA (ssCir DNA) were formatted as the final library. The library was amplified with phi29 to make DNA nanoball (DNB) which had more than 300 copies of one molecule. The DNBs were load into the patterned nanoarray and paired-end 100 bases reads were generated.

4.3.2.4 Bioinformatic analysis of the CD4 + T cells mRNA-Seq

The bioinformatic analysis was partly done by Ho-Ryo Chung, Clemens Thölken and Leigh Marsh. Sequenced reads were mapped with Salmon v1.3.0 (Srivastava et al. 2020) in quantification mode against the reference human transcriptome of cDNA and ncRNA sequences (GRCh38) with the respective genome background as decoys. Resulting read count estimates were rounded to integers and differential gene expression analysis was performed with DEseq2 (Love et al. 2014). iDEP v.0.91 was used to create a correlation matrix of the matched samples (Ge et al. 2018). Functional pathway and gene ontology analyses were conducted using Enrichr Bioplanet and GO Biological Process. Pathway network analyses were built with R package (ggraph v2.0.5). Gene network analysis was performed by STRING PPI v.11.0 using default settings (Szklarczyk et al. 2019). Gene networks were further processed by Cytoscape v.23 add-ins MCODE and Clustermaker (Shannon et al. 2003). Correlation plots of clinical and molecular parameters were generated by R package (Corrplot v.0.84) implemented with default Pearson correlation.

4.3.2.5 Statistical analysis

Unpaired t test applying a significance threshold of $P < 0.05$ was performed for comparisons of numerical data by using GraphPad Prism 7 software (GraphPad, USA).

4.3.3 Plasma EV miRNA methods of obesity-associated asthma study

4.3.3.1 Small RNA/miRNA extraction from plasma EVs

EVs were isolated using the exoRNeasy Midi kit, according to the manufacturer's instructions. 1 mL of each Plasma sample was thawed at 37 °C and pre-filtered with a syringe Millex-AA Syringe Filter 0.8 µm. 1 mL of Buffer XBP was added to each plasma sample and mixed with plasma well immediately by gently inverting the tube five times. Plasma sample/Buffer XBP mix was added to the exoEasy spin column, centrifuged for 1 min at 500 x g, and then flow-through was discarded. 3.5 ml Buffer XWP were added and tube was centrifuged for 1 min at 5,000 x g to wash the column and remove residual buffer, flow-through was discarded together with the collection tube. The spin column was transferred to a fresh collection tube.

For total RNA or miRNA isolation, 700 µL QIAzol were added to the membrane and centrifuged at 5,000 x g for 5 min to collect the lysate and transfer completely to a 2 mL tube. The lysate tube was vortexed briefly and incubated for 5 min at RT. After incubation, 90 µL chloroform were added and tube was shaken vigorously for 15 sec. Tube was incubated for 3 min at RT and centrifuged at 12,000 x g for 15 min at 4 °C. ~ 400 µL of the upper aqueous phase were transferred to a new collection tube. 800 µL (2 volumes) of 100% EtOH and mixed thoroughly by pipetting. 700 µL sample were added into an RNeasy MinElute spin column and centrifuged at 8000 x g for 15 sec at RT; flow-through was discarded. The previous step was repeated using the remainder of the sample. Afterwards 700 µL Buffer RWT were added to the RNeasy MinElute spin column and centrifuged at 8,000 x g for 15 sec; flow-through was discarded. 500 µL Buffer RPE was added onto the RNeasy MinElute spin column and centrifuged for 15 sec at ≥8000 x g, flow-through was discarded. The previous step was repeated but centrifuged for 2 min at ≥8000 x g. RNeasy MinElute spin column was placed in a new 2 mL collection tube, the lid of the spin column was opened and the tube was

centrifuged at full speed for 5 min to dry the membrane, flow-through and the collection tube were discarded. RNeasy MinElute spin column was placed in a new 1.5 mL collection tube, 14 µL RNase-free water were added directly to the center of the spin column membrane, incubated for 1 min at RT, and then centrifuged for 1 min at full speed to elute the RNA.

4.3.3.2 Nanoparticle tracking analysis (NTA)

NTA analysis was performed at Institute for Tumor Immunology, Medical Faculty, Philipps University of Marburg, ZetaVIEW software startup was performed, then NTA instrument was rinsed using 5 mL of filtered water to get rid of the remaining particles existed from the previous session. Auto alignment was done by injecting 2 mL of polystyrene particles into the NTA instrument, which was rinsed again afterwards. The optimal particle per frame value ranges from 140 to 200 particles/frame. Therefore, plasma EV samples were diluted 1:500 in pre-filtered PBS, 1 mL of each diluted EV sample was injected into the instrument which was rinsed after each measurement.

The manufacturer's default software settings for EVs, liposomes or nanospheres were selected accordingly. For each measurement, two video cycles were recorded by scanning 11 cell positions and capturing 30 frames per position with the following settings: Focus: autofocus; Camera sensitivity for all samples: 75; Shutter: 70; Scattering Intensity: detected automatically; Cell temperature: 25°C; pH: 7.0. After capture, the videos were analyzed by the in-built ZetaView Software 8.05.05 SP2 with specific analysis parameters: Max size: 1000; Min size: 5; Min brightness: 25. Hardware: embedded laser: 40 mW at 488 nm; camera: CMOS.

4.3.3.3 Small RNA sequencing using the QIAseq miRNA Library Kit

Small library preparation was conducted in the Laboratory for Epigenetics and Environment, Centre National De Recherche En Génomique Humaine, CEA - Institut De Biologie François Jacob, Evry, France (Jorg Tost's Lab).10 ng of EV miRNA were used to prepare the Qiaseq miRNA library on a Freedom EVO150 liquid handling

platform. Therefore, QIAseq miRNA NGS 3' Adapter was diluted 1:5 in Nuclease-free water. 1 µL QIAseq miRNA NGS 3' Adapter, 1 µL QIAseq miRNA NGS RI, 1 µL QIAseq miRNA NGS 3' Ligase, 2 µL QIAseq miRNA NGS 3' Buffer, and 10 µL 2x miRNA Ligation Activator were mixed well and added to 5 µL of EV miRNA sample. The mix was incubated for 60 min at 28 °C and 20 min at 65 °C, then it was kept for 5 min at 4 °C.

QIAseq miRNA NGS 5' Adapter was diluted 1:2.5 in Nuclease-free water. 20 µL Nuclease-free water, 2 µL QIAseq miRNA NGS 5' Buffer, 1 µL QIAseq miRNA NGS RI, 1 µL QIAseq miRNA NGS 5' Ligase and 1 µL QIAseq miRNA NGS 5' Adapter were mix well and added to the 20 µL of the previous mix. The new mix was incubated for 30 min at 28 °C and 20 min at 65 °C, then it was kept for 5 min at 4 °C.

To perform reverse transcription, 2 µL QIAseq miRNA NGS was added to the previous mix and incubated 2 min at 75 °C, 2 min at 70 °C, 2 min at 65 °C, 2 min at 60 °C, 2 min at 55 °C, 5 min at 37 °C, 5 min at 25 °C and 5 min at 4 °C.

QIAseq miRNA NGS RT Primer was diluted 1:5 in Nuclease-free water. 2 µL QIAseq miRNA NGS RT Primer, 2 µL Nuclease-free water, 12 µL QIAseq miRNA NGS RT Buffer, 1 µL QIAseq miRNA NGS RI, 1 µL QIAseq miRNA NGS RT Enzyme were mixed well and added to previous mix. The new mix was incubated for 60 min at 50 °C, 15 min at 70 °C and 5 min at 4 °C.

To wash the magnetic beads, 400 µL of QIAseq Beads were added to a 2 mL microfuge tube and briefly centrifuged and immediately separated on a magnet stand. Beads were carefully separated and the supernatant was discarded. The tube was removed from the magnet stand. 150 µL QIAseq miRNA NGS Bead Binding Buffer were carefully pipetted onto the beads and thoroughly vortexed. The tube was briefly centrifuged and beads were immediately separated on a magnetic stand. Supernatant was carefully removed and discarded. Beads were removed from magnet and resuspended in 400 µL of the QIAseq miRNA NGS Bead Binding Buffer.

To clean up prepared libraries, the tubes/plates containing the cDNA reactions were centrifuged. 143 µL of washed beads were added to each tube containing the cDNA reaction, vortexed for 3 sec, centrifuged briefly again and incubated for 5 min at RT. Tubes were placed on a magnetic stand for ~4 min until beads have fully migrated and supernatant was discarded. 200 µL of 80% ethanol were added and immediately

removed, this step was repeated. Tube lids were kept open and beads were air dried at RT for 10 min. DNA was eluted by adding 17 µL of Nuclease-free water to the tubes, closing the tubes and then removing them from the magnetic stand. Beads were carefully pipetted up and down until all the beads are thoroughly resuspended, briefly centrifuged, and then incubated for 2 min at RT. Beads tubes were placed on magnet for 2 min and supernatant containing the clean cDNA was transferred.

To amplify the cDNA, 8 µL QIAseq miRNA NGS Library Buffer, 1.5 µL HotStarTaq® DNA Polymerase, and 15.5 µL Nuclease-free water were mixed well and added to 15 µL of the clean cDNA. The mix was incubated 15 min at 95 °C, 15X (15 sec at 95 °C, 30 sec at 60 °C, 15 sec at 72 °C), 2 min at 72 °C and cooled down to 4 °C. Amplified cDNA was cleaned up as before and stored -20 °C until further use.

Library peak distribution was controlled and the average size was calculated using LabChip GX. The peak of the miRNA-sized library is approximately 180 bp, while 188 bp peak corresponds to piRNA. Molarity of the miRNA library was determined by qPCR method using KAPA Library Quantification Kit following the manufacturer's recommendations. Paired-end miRNA sequencing was performed in 12-plex on a HiSeq 4000 with sequencing read length of 2x75 cycles.

4.3.3.4 Bioinformatic analysis of EV small RNA-Seq

The bioinformatics analysis was partly conducted in Jorg Tost's lab, Clemens Thölken, and Leigh Marsh. A modified version of the pipeline provided by Qiagen for use with the QiaSeq protocol was used to process raw data (fastq files –> counts table). Raw reads were firstly trimmed for adapters' sequences using *Cutadapt 1.18 (Martin 2011)*(Ref). Reads were mapped using *bowtie (V1.1.2)* (Langmead et al. 2009) against mature and stem-loop miRNA databases (miRbase V22) as well as a proprietary in-house developed database, which takes natural variation of small RNA sequences into account. The aligned reads were counted by *SAM tools 0.1.19* (Li et al. 2009). The obtained read counts were passed for post-processing analysis, including counts normalization and outliers search.

The expected peak size of mapped reads of a size peaking at 22-23 nt was verified. Counts normalization to TPM (Transcripts Per Million) was performed during the post-

processing. A threshold of TPM >10 was applied to filter out lowly expressed miRNAs and then assess if there are any significant differences in the overall distribution of particular samples using Principal Component Analyses (PCA) and normalized count statistics. The filtered count file was used for the differential analysis using *Deseq2 (V1.6.3)* and in-house R scripts were used to interpret and evaluate the result. R scripts were used for graphical representations such as the PCA plot. *TAM 2* was used to predict the miRNA functions and clusters using a cut-off false discovery rate (FDR) < 0.05 and fold change (FC) ≥ 1.5 (Li et al. 2018). miRTargetLink 2.0 was used to predict miRNA gene targets (Kern et al. 2021).

5. Results

5.1 Establishment of suitable conditions for blood CD4+ T cells processing for transcriptomics and epigenomics studies

5.1.1 Study design

For this study, three atopic patients and three healthy donors were recruited at Pediatric Allergology, Department of Pediatrics, Dr. von Hauner Children's Hospital, University Hospital, LMU Munich and Department of Pediatric Pneumology, Allergology and Neonatology, Hannover Medical School. The study was approved by the University of Lübeck ethics committee (Alliance study approval No. 12-215) and all participants gave written informed consent.

Two blood samples were drawn from each of the six donors (12 blood samples in total). CD4+ T cells were isolated from the first blood within 2 hours (immediately) or from the second blood sample after 24 hours (delayed). The isolated CD4+ T cells were divided in two portions. The first cells portion was directly lysed in RLT buffer supplemented with 1% 2-Mercaptoethanol for downstream RNA extraction and RNA-Seq analysis, while the second cells portion was subjected to cross-linking with 1% formaldehyde for subsequent ChIP-Seq analysis, afterwards profound bioinformatics analysis was conducted (Figure 10).

5.1.2 RNA-Seq analysis detected hundreds of differentially expressed genes (DEGs) in immediately processed CD4+ T cells

After performing RNA-Seq and bioinformatic analyses for the immediately processed CD4+ T cells from peripheral blood of the atopic patients and healthy donors, there were 672 up-regulated genes and 631 down-regulated genes in CD4+ T cells obtained from atopic patients (A0) compared to those from healthy donors (H0); with a significance threshold at FDR<0.1 (Figure 11. A).

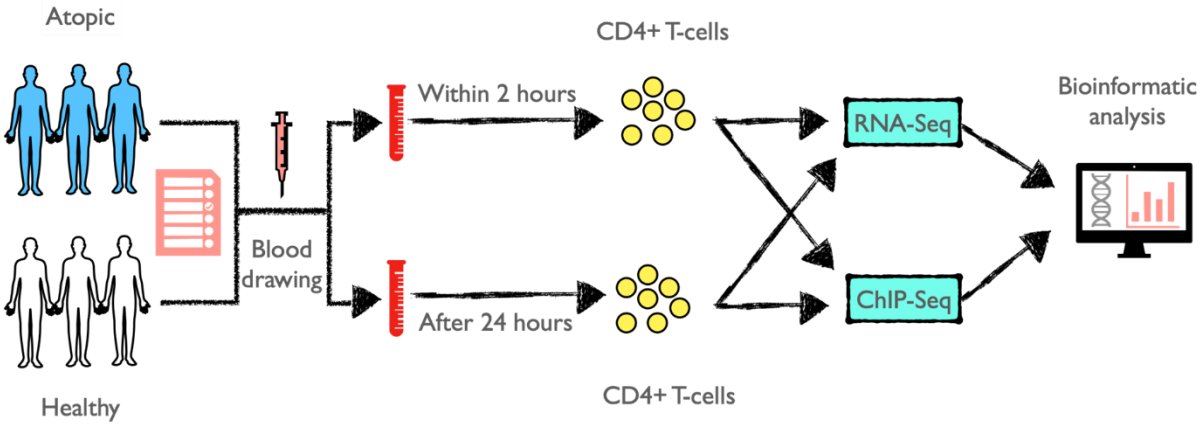


Figure 10: Study design to investigate the time-dependent effects of immediate and delayed CD4+ T cells processing on the transcriptomic and epigenomic profiles.

Gene Ontology Biological processes (GO-BP) that associated with the top 10 up- and down-regulated genes included: *Response to interleukin-4, Positive regulation of immune system, and Regulation of signal transduction by p53 class mediator* (Figure 11. B). By conducting a functional biological pathway analysis for the DEGs that comprise FC≥1.5 and FDR<0.1, atopy-related biological pathways were among the affected pathways by the up-regulated genes including *Interleukin-9 regulation of target genes*, *interleukin-6 signaling pathway*, and *T helper cell surface molecules* (Figure 12. A). The enriched genes of these pathways are shown in Figure 12. B.

5.1.3 Transcriptomic profiles of delayed processed CD4+ T were altered dramatically in atopic donors

In contrast to the comparison of immediately processed CD4+ T cells, differential analysis of delayed processed CD4+ T cells of atopic (A24) versus healthy (H24) donors revealed only 3 up-regulated genes (Ribosomal Protein S27: *RPS27, RPL41,* and *RPL13*) at FDR<0.1. (Figure 13. A). To investigate the reason for the gene expression impairment, a direct comparison between the delayed and immediate CD4+ T cells processing for atopic and healthy donors was conducted. Only slight changes in gene expression by 19 DEGs were detected in delayed versus immediately processed CD4+ T cells of healthy donors (Figure 13. B).

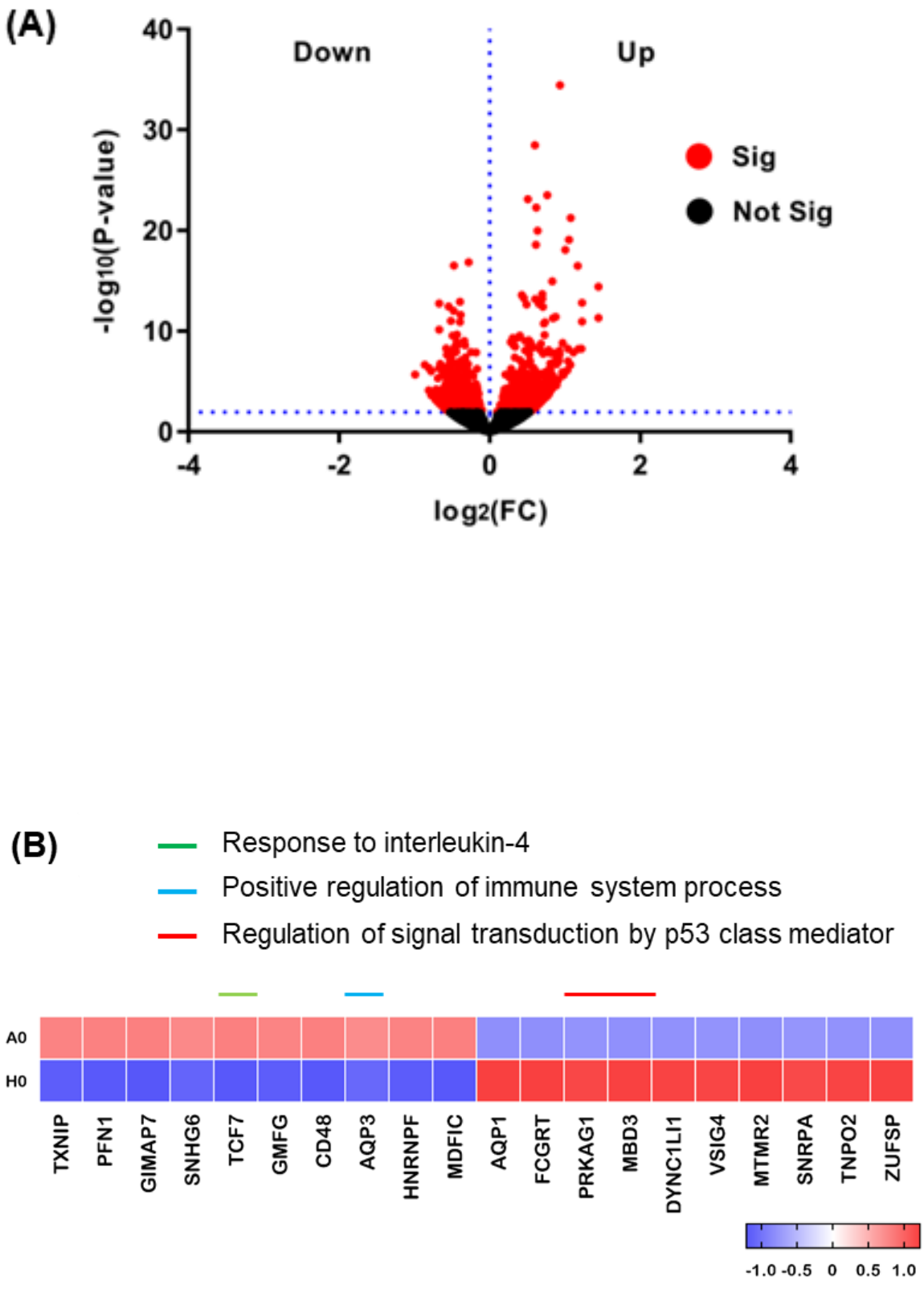
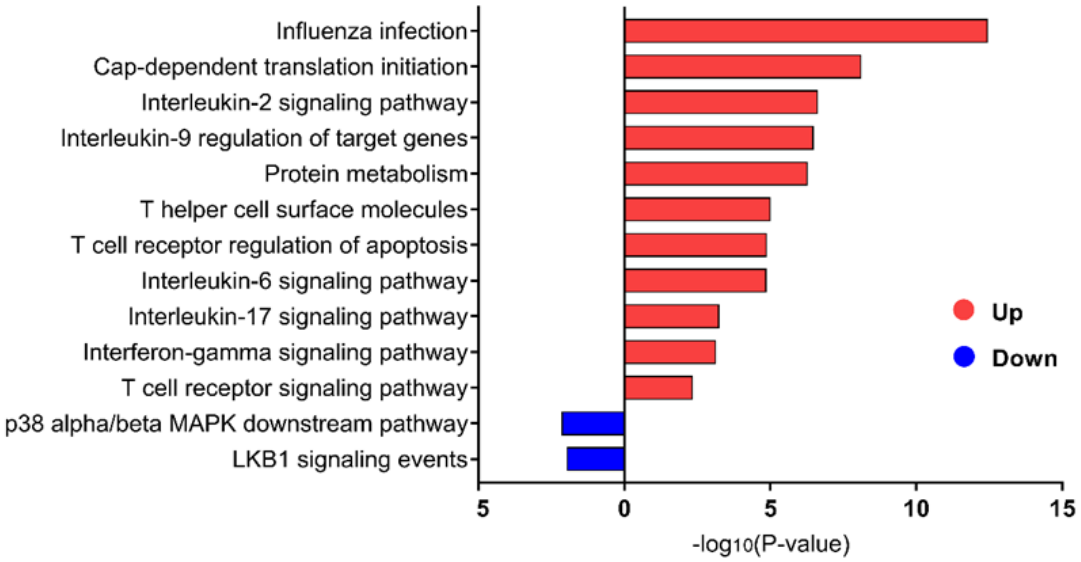


Figure 11: **RNA-Seq analysis of immediately processed CD4+ T cells of atopic and healthy individuals**. **(A)** Volcano plot showing the significant up- and down-regulated genes (red) at FDR <0.1 and **(B)** Top 10 significant up- and down-regulated genes and their associated biological processes,

(A)



(B)

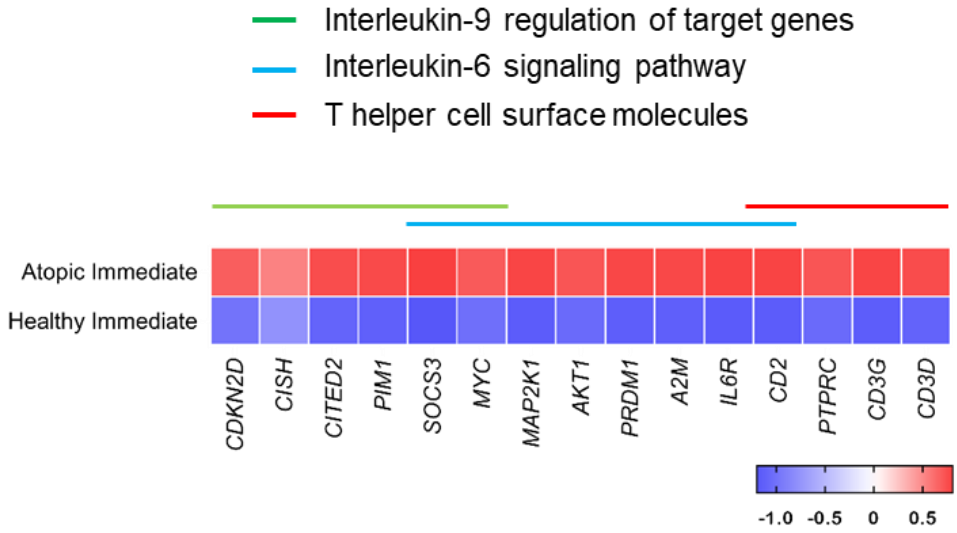


Figure 12: **Functional Biological pathway analysis of immediately processed CD4+ T cells of atopic and healthy donors**. **(A)** Biological pathways affected by the dysregulated genes at FC ≥1.5 and FDR <0.1 and **(B)** three selected atopy related pathways with their associated genes.

Surprisingly, a drastic change in gene expression by 320 DEGs was observed in delayed versus immediately processed CD4+ T cells of atopic donors (Figure 13. C).

By performing a biological pathway analysis for the DEGs that comprise FC≥1.5 and FDR<0.1 in delayed versus immediate (atopic) comparison, most of the previous atopy-related molecular pathways were downregulated, while some RNA degradation-related pathways were up-regulated (Figure 14. A). The enriched genes of 3 selected pathways are shown in Figure 14. B.

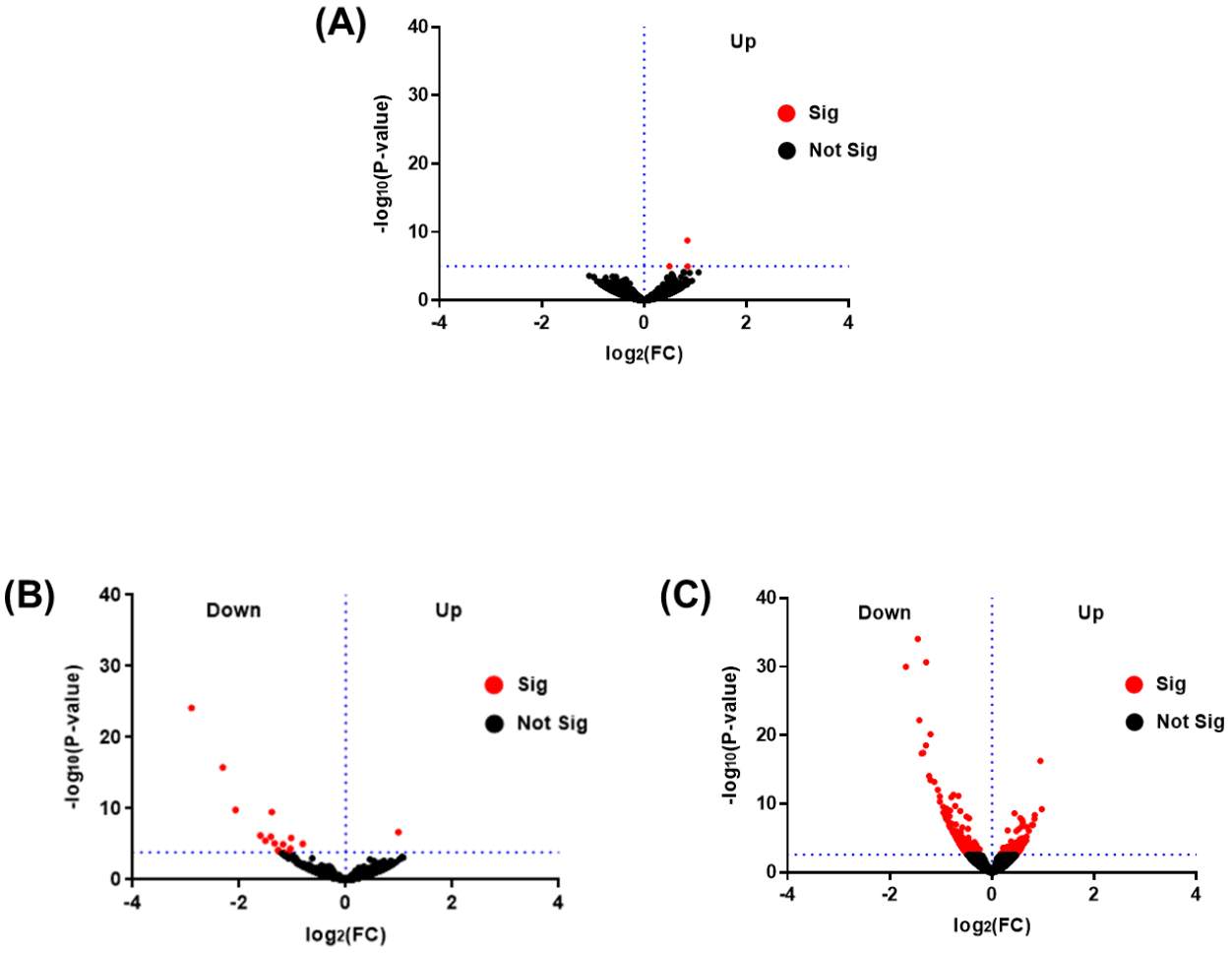
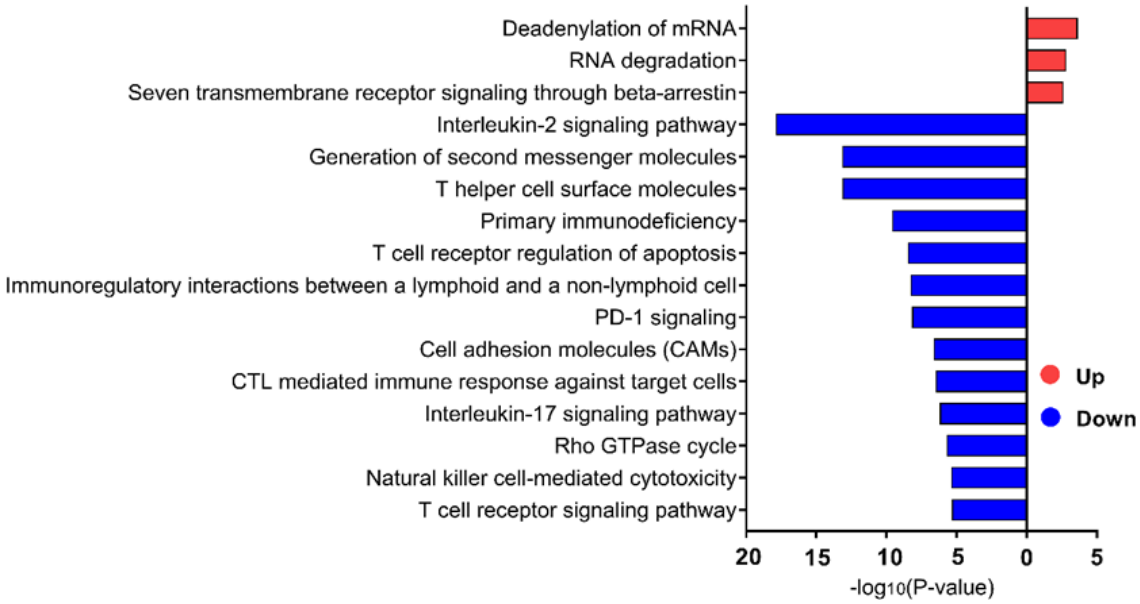


Figure 13: **RNA-Seq analysis of delayed processed CD4+ T cells of atopic and healthy donors.** Volcano plot showing the significant up- and down-regulated genes (red) at FDR <0.1 of **(A)** atopic versus healthy (delayed CD4+ processing), **(B)** delayed versus Immediate CD4+ T cells processing (healthy donors) and **(C)** delayed versus Immediate CD4+ T cells processing (atopic donors).

5.1.4 Epigenetic profiles of immediate and delayed processed CD4+ T cells of atopic and healthy donors

To validate the transcriptomic results of the delayed versus immediate CD4+ T cells processing on the epigenetic level, acetylation of H3K27 was investigated as a histone modification that is associated with an active transcription machinery (Paauw et al. 2018). By plotting genomic heat maps centered at the Transcription Start Site (TSS) region extended to 3 kilo base (kb) up- and down- stream the TSS. The results of the H3K27ac assessment were concordant with the transcriptomic profiles, i.e. there was a large depletion in the acetylation signal of the delayed processed CD4+ T cells of atopic donors compared to immediately processed CD4+ T of the same donors (Figure 15. A), whereas the H3K27ac signal remained largely unaffected by the delayed CD4+ T cells processing in healthy donors (Figure 15. B). Combined coverage plots were created to compare the two H3K37ac coverage signals of the delayed and immediate conditions (Figure 15. C, D). Similar to the previous results, there was a huge reduction in the H3K27ac coverage of delayed processed CD4+ T cells of atopic donors compared to immediately processed CD4+ T of the same donors (Figure 15. C), while a very slight reduction in the H3K27ac coverage was observed in the delayed versus immediate CD4+ T cell processing of healthy donors (Figure 15. D). By performing differential analysis of the whole H3K27 acetylated gene sets for delayed versus immediate CD4+ T cells processing of atopic and healthy donors, volcano plots showed that a huge number of H3K27 acetylated genes in CD4+ T cells were down-regulated and less were up-regulated after storing blood samples of atopic patients overnight at FDR <0.1 (Figure 16. A), in contrast, there was no difference at all in the acetylated genes between the delayed and immediately processed CD4+ T cells of healthy donors (Figure 16. B). *Interleukine 2 signaling* and *T cell receptor regulation of apoptosis pathways* were downregulated in both transcriptomic and H3K27 acetylation profiles of delayed processed CD4+ T cells in atopic patients (Table 5). The top 30 significant affected pathways by the downregulated genes are listed in Table 5.

(A)



(B)

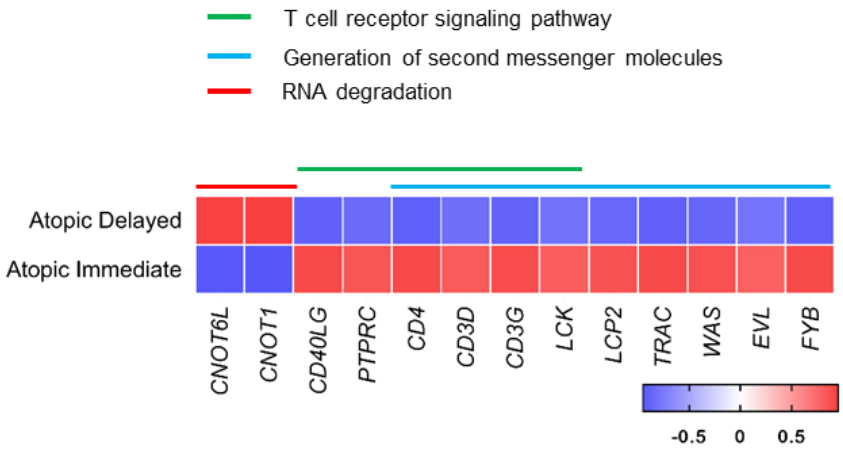


Figure 14: **Functional Biological pathway analysis of immediately versus delayed processed CD4+ T cells of atopic individuals**. **(A)** Biological pathways affected by the dysregulated genes at FC ≥1.5 and FDR <0.1 and **(B)** three selected pathways with their associated genes.

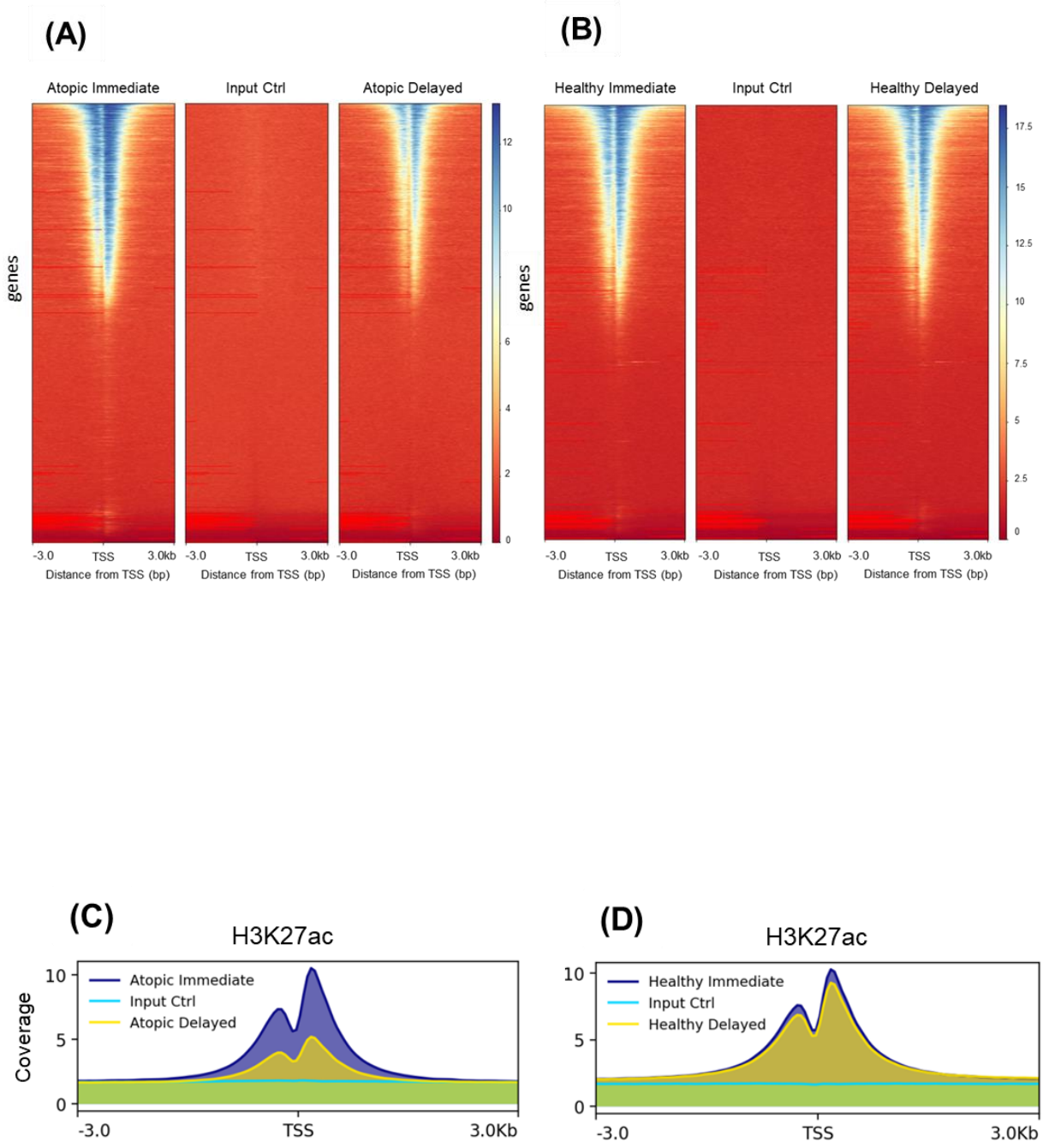


Figure 15: **H3K27ac ChIP-Seq analysis of CD4+ T cells verifying the findings delayed blood cells processing effects on the transcriptomic profile**. Genomic heat maps showing the H3K27ac signals of 3kb around TSS for delayed versus immediately processed CD4+ T cells of **(A)** atopic patients and **(B)** healthy donors. Density Plots depicting the combined H3K27ac coverage for delayed versus immediately processed CD4+ T cells of **(C)** atopic patients and **(D)** healthy donors.

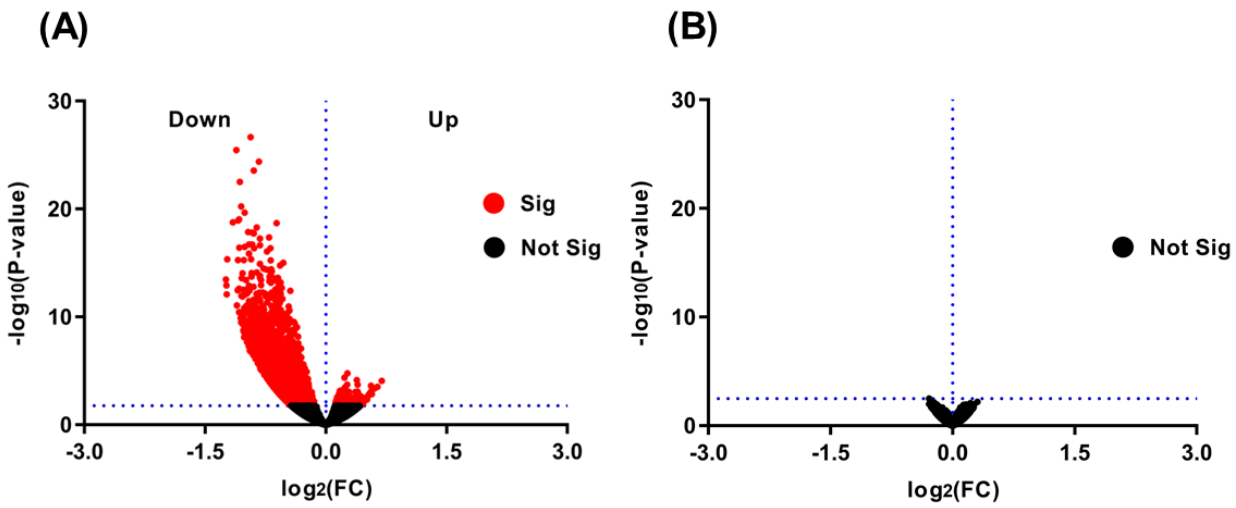


Figure 16: **H3K27ac ChIP-Seq analysis of CD4+ T cells verifying the findings of delayed blood cells processing effects on the transcriptomic profile**. Volcano plots depicting genes associated with differential H3K27 acetylation identified by ChIP-Seq analysis (significance cut-off at FDR<0.1) for delayed versus immediately processed CD4+ T cells of **(A)** atopic patients and **(B)** healthy donors.

Table 5: H3K27ac ChIP-Seq significant pathways affected by down-regulated genes of Delayed vs. Immediate (Atopic).

Term	**P-value**	**Adjusted P-value**
Translation	3.36E-20	3.04E-17
Influenza viral RNA transcription and replication	6.97E-20	3.16E-17
Cytoplasmic ribosomal proteins	1.98E-19	6.00E-17
Systemic lupus erythematosus	5.34E-19	1.21E-16
Influenza infection	6.30E-17	1.14E-14
Packaging of telomere ends	3.26E-13	4.93E-11
RNA polymerase I promoter opening	4.53E-13	5.87E-11
Cap-dependent translation initiation	4.55E-12	5.16E-10
T cell receptor regulation of apoptosis	4.31E-11	4.34E-09
Activation of mRNA upon binding of the cap-binding complex and eIFs, and subsequent binding to 43S	6.91E-11	6.27E-09

Meiotic recombination	1.91E-10	1.57E-08
Protein metabolism	6.45E-10	4.87E-08
Amyloids	1.79E-09	1.25E-07
Telomere maintenance	2.18E-09	1.41E-07
Gene expression	2.99E-09	1.81E-07
Meiotic synapsis	6.98E-09	3.76E-07
RNA polymerase I transcription	7.46E-09	3.76E-07
Meiosis	7.46E-09	3.76E-07
Deposition of new CENP-A-containing nucleosomes at the centromere	2.34E-08	1.12E-06
Transcription	2.49E-08	1.13E-06
RNA polymerase I, RNA polymerase III, and mitochondrial transcription	4.82E-06	2.04E-04
Type II interferon signaling (interferon-gamma)	4.95E-06	2.04E-04
Chromosome maintenance	5.32E-06	2.10E-04
Interleukin-2 signaling pathway	1.52E-05	5.73E-04
Messenger RNA splicing: major pathway	1.95E-05	7.09E-04
Diurnally regulated genes with circadian orthologs	3.06E-05	0.001069
Respiratory electron transport, ATP biosynthesis by chemiosmotic coupling, and heat production by uncoupling proteins	1.18E-04	0.003957
p75 neurotrophin receptor signaling via NF-kB	7.34E-04	0.023787
Clathrin derived vesicle budding	8.94E-04	0.027101
mRNA stability regulation by proteins that bind AU-rich elements	8.96E-04	0.027101
mRNA stability regulation by proteins that bind AU-rich elements	0.00126	0.036878

5.2 Transcriptomic analysis of blood CD4+ T cells of low type-2 obese and non-obese asthmatics

5.2.1 Study population and design

One hundred forty-four asthmatic patients were recruited at the Department of Medicine, Pulmonary and Critical Care Medicine, University Medical Centre Giessen and Marburg. All patients were diagnosed with asthma according to current clinical guideline diagnostics. Sub-groups of patients were selected based on their BMI, blood eosinophil counts, and blood total serum IgE levels. To selectively focus on type-2 low asthmatics, only patients with low blood eosinophil counts (<300 eos/µL) and fractional exhaled nitric oxide (FeNO<25) according to GINA guidelines (Global Initiative for Asthma - GINA 2021), as well as serum total IgE levels (<300 kU/L) were included in this study. Based on the selected criteria, patients that were included in this study were: 10 obese, non-atopic asthmatic adults with high BMI (36.67±6.9), 10 non-obese, non-atopic asthmatic adults with normal BMI (23.88±2.73), and 10 healthy controls with normal BMI (23.6±3.7). Detailed patients' characteristics are shown in Table 6. Clinical parameters including C-reactive protein (CRP), blood cell counts, serum IgE levels, and lung function parameters were measured as part of routine clinical assessment. Healthy controls (Ctrl/Control) were recruited at the Comprehensive Biobank Marburg, Medical Faculty of the Philipps University Marburg. The study was approved by the local ethics committee (approval No. 155/08/; 202/12) and all participants gave written informed consent.

Table 6: Patient characteristics. Data are expressed as mean ± SD. BMI = body mass index; FEV_1 = forced expiratory volume in the first second; FVC = forced vital capacity.

	Obese asthmatics	**Non-obese asthmatics**	**P-value**
N	10	10	1
Age [years]	46.30 ± 10.75	39.30 ± 13.94	0.23
Male [%]	30	10	0.29
BMI [kg/m^2]	36.67 ± 6.90	23.88 ± 2.73	<0.0001
FeNO [ppb]	15.72 ± 7.93	16.78 ± 11.64	0.81
FEV_1 [l]	2.89 ± 0.80	2.73 ± 1.10	0.58
FVC [l]	3.79 ± 1.00	3.50 ± 1.00	0.52

5.2.2 Comparison of clinical parameters between type-2 low obese and non-obese asthmatics

By comparing the low type-2 obese and non-obese asthmatics using a set of different clinical and laboratory parameters, including inflammation markers such as CRP and FeNO, lung function parameters such as FEV_1 and FVC, and age of onset, apart from BMI, there was no significant difference between the two asthma groups (Table 6, Figure 17). In other words, the measured clinical and laboratory parameters were not able to distinguish between the two asthma groups. However, the next aim was to find out whether a difference between the low type-2 obese and non-obese asthmatics could be detected at the level of the transcriptomic profile of the peripheral blood CD4+ T cells of those patients and gain a mechanistic understanding of the underlying mechanism of the low type-2 obese asthma phenotype.

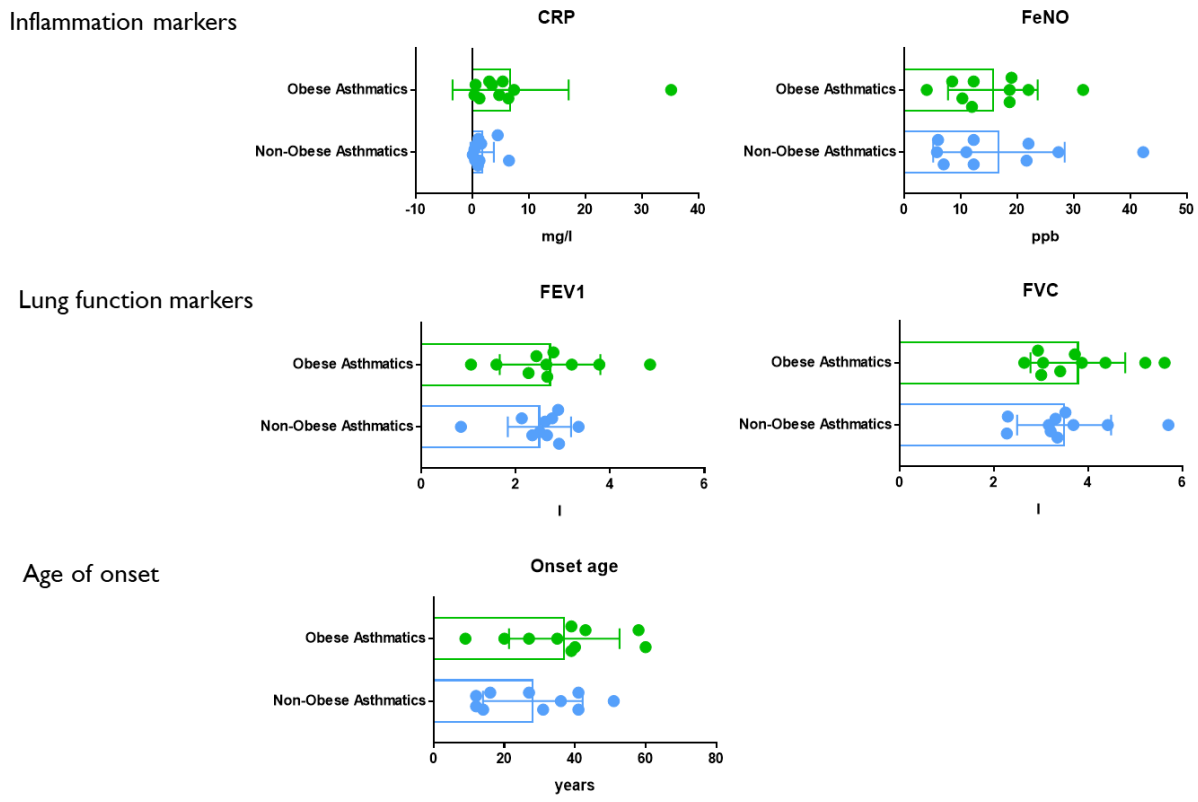


Figure 17: A comparison between low type-2 obese asthmatics and non-obese asthmatics using clinical and laboratory parameters.

5.2.3 Transcriptomic analysis of low type-2 obese asthmatics

After performing RNA-Seq and bioinformatic analyses for the three study groups, a list of Th2 related genes were checked in order to make sure that low type-2 asthma phenotype has been correctly chosen based on the previous clinical criteria. None of the Th2 related genes showed significant differences for both asthma groups compared to healthy controls (Radens et al. 2020). Interestingly, the GATA Binding Protein 3 (*GATA3*), the master transcription factor for Th2 differentiation and activation, was even significantly lower expressed in CD4+ T cells in both asthma groups compared to controls (Table 7).

Table 7: Th2 related genes in both asthma groups compared to healthy controls with their associated Fold Change (FC) and FDR values.

	Obese asthmatics vs. Ctrl		**Non-obese asthmatics vs. Ctrl**	
Th2 genes	**Log2(FC)**	**FDR**	**Log2(FC)**	**FDR**
GATA3	-0.489	0.001	-0.446	0.004
TNFSF11	-0.026	0.981	0.316	0.753
IL17RB	-0.062	0.909	0.083	0.909
AKAP12	1.031	0.368	0.916	0.511
HPGDS	0.033	0.981	0.331	0.812
LRRC32	-0.591	0.222	-0.616	0.258
PTGDR2	0.160	0.726	0.598	0.114
BACE2	0.048	0.962	0.306	0.768
NRIP3	-0.124	0.824	0.058	0.943
CHDH	-0.396	0.442	0.106	0.902
NCS1	-0.593	0.750	0.293	0.909
PKP2	0.250	0.619	0.723	0.094

To get a full overview of the transcriptomic profiles of the two asthma groups, a correlation matrix was created based on 75% of total expressed genes as a default option in IDEP v0.92 bioinformatic tool. The low type-2 obese asthmatics showed more clustering variability compared to the other two groups, while non-obese asthmatics

represented rather the most homogenous population (Figure 18. A). Afterwards, pairwise comparisons of the three study groups were performed at FDR <0.1, and up-regulation of 647 and 386 genes was observed for obese asthmatics versus controls and non-obese asthmatics versus controls, respectively, 195 genes were shared between the two comparisons (Figure 18. B, left). The pairwise comparisons showed also down-regulation of 657 and 483 genes for obese asthmatics versus controls and non-obese asthmatics versus controls, respectively; 287 genes were shared between the two comparisons (Figure 18. B, right). Interestingly, there was a difference of 141 up-regulated genes and 142 down-regulated genes by comparing obese asthmatics to non-obese asthmatics (Figure 18. B).

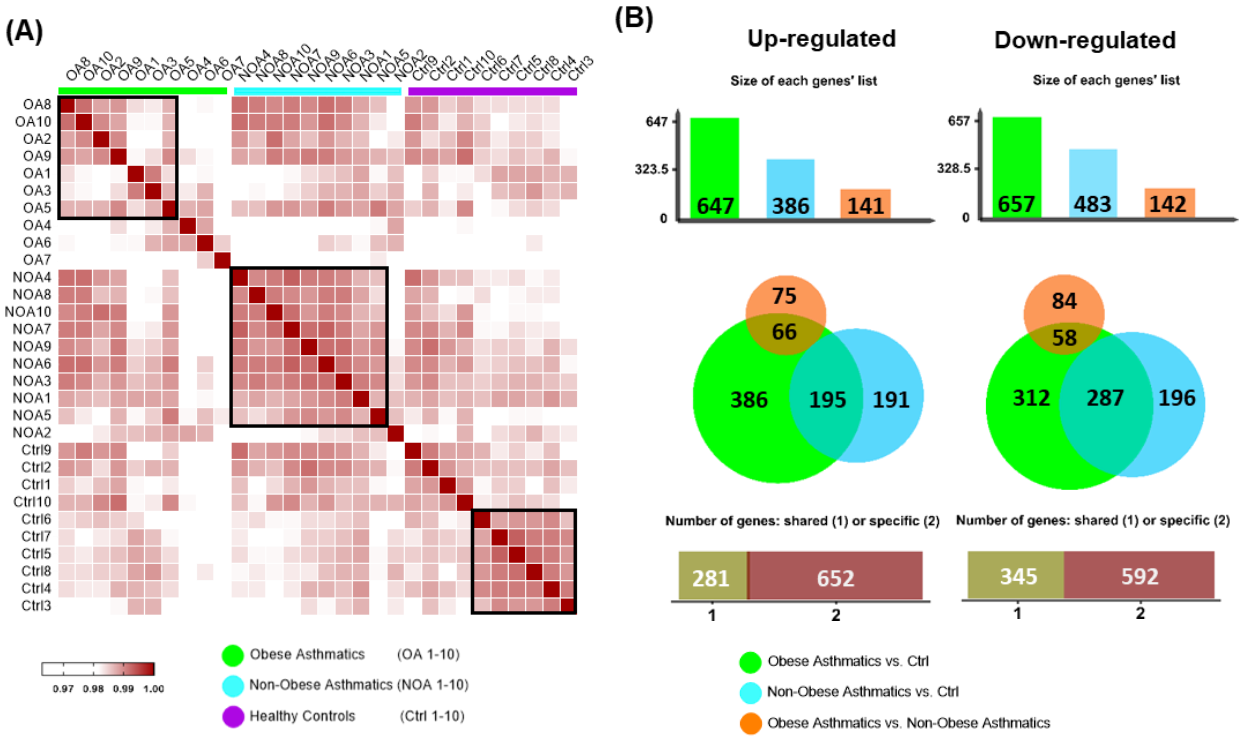


Figure 18: **Transcriptomic analysis of CD4+ T cells for low type-2 obese asthmatics and non-obese asthmatics. (A)** Correlation matrix of 75% of expressed genes of the three study groups, **(B)** Venn diagrams showing a pairwise comparison of the three study groups at FDR <0.1.

To separately investigate each of the three comparisons, for each of them a volcano plot was created (Figure 19. A-C, up) as well as a heat map was plotted to depict the top 10 significantly up- and down-regulated genes alongside with their associated significant biological processes (BP, GO terms) for at least two genes in each comparison (two genes make a significant BP at FDR<0.05) (Figures 19. A-C, down).

The results showed that the *positive regulation of viral release from host cell* biological process was enriched in the obese asthmatics versus controls represented by the up-regulated damage specific DNA binding protein 1 (*DDB1*) and structural maintenance of chromosomes 3 (*SMC3*) genes (Figure 19. A, down).

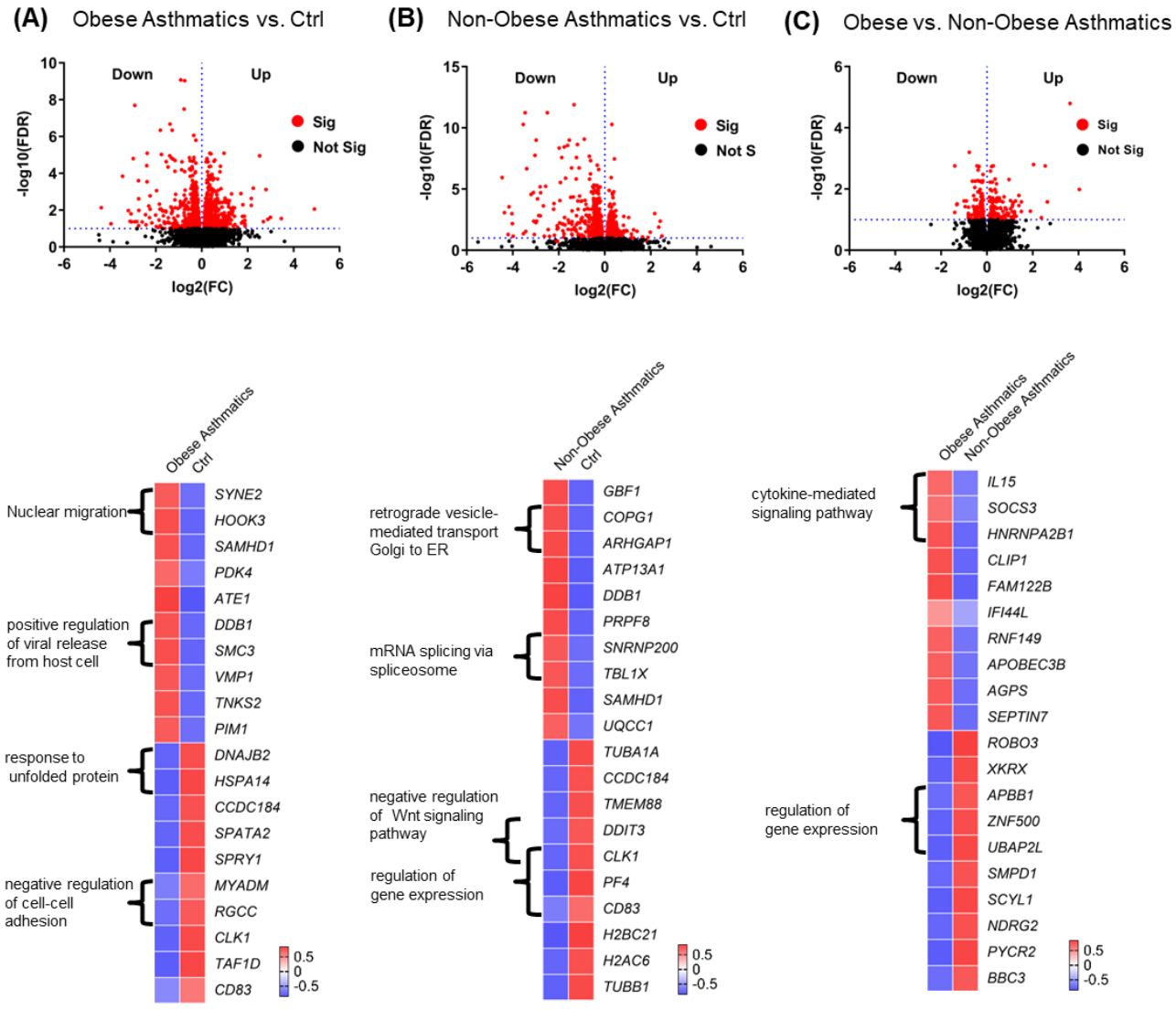


Figure 19: **Separate pairwise comparisons of CD4+ T cells transcriptome for low type-2 obese asthmatics and non-obese asthmatics**. **(A-C, up)** volcano plots showing the differentially regulated genes at FDR<0.1, **(A-C, down)** volcano plots depicting the top significant 10 up- and downregulated genes and their associated significant biological processes (BP).

The *negative Regulation of Wnt signaling* pathway was enriched in non-obese asthmatics compared to controls represented by the down-regulated transmembrane protein 88 (*TMEM88*) and DNA damage inducible transcript 3 (*DDIT3*) genes (Figure 19. B, down). In the comparison of obese asthmatics versus non-obese asthmatics, *IL15*, *SOCS3*, and heterogeneous nuclear ribonucleoprotein A2/B1 (*HNRNPA2B1*)

genes contributed to *cytokine-mediated signaling* pathway (Figure 19. C, down). Further biological processes of the 10 up- and down-regulated genes are shown in (Figure 19. A-C, down).

5.2.4 Functional biological pathway analysis of low type-2 obese asthmatics

Functional biological pathway analysis was performed utilizing the DEGs that comprised FC≥1.5 and FDR<0.1 for the three comparisons. A set of shared biological pathways with different significance levels have been identified when comparing differential gene expression of low type-2 obese asthmatics versus controls and low type-2 non-obese asthmatics versus controls. *T cell receptor regulation of apoptosis* was the most significant pathway in obese asthmatic, while *Interleuin-1 regulation of extracellular matrix* was the most significant molecular pathway for non-obese asthmatics (Figure 20. A). To better understand the interconnection between the top 15 significant pathways, genes that are shared among the pathways have been identified (Figure 20. B). Based on the shared genes, a correlation matrix have been created to figure out potential pathway clusters (Figure 21. And a topological pathway network have been built (Figure 21. B). Three clusters of pathways have been detected including *cytokine and chemokine signaling* pathways in one cluster which have the biggest number of shared genes (size of circle), while *T cell receptor regulation of apoptosis* was the major pathway in the second cluster and had direct connection to the *EGFR1 pathway* in the third cluster (Figure 21. B). Similar pathway analyses ware applied to the other two comparisons, i.e. obese asthmatics versus controls and obese asthmatics versus non-obese asthmatics (Figure 22. A-D). Surprisingly, *interferon signaling* and *viral infection pathways* were overrepresented in the analyses (Figure 22, A). Accordingly, the pathway network analysis showed two pathway clusters centralized by the *interferon signaling pathway* (Figure 22. D).

Figure 20: **Functional biological pathway analysis of low type-2 obese and non-obese asthmatics**. **(A)** Top 15 significant pathways shared between low type-2 obese versus controls and non-obese asthmatics versus controls **(B)** overview of shared/mutual genes among the 15 biological pathways.

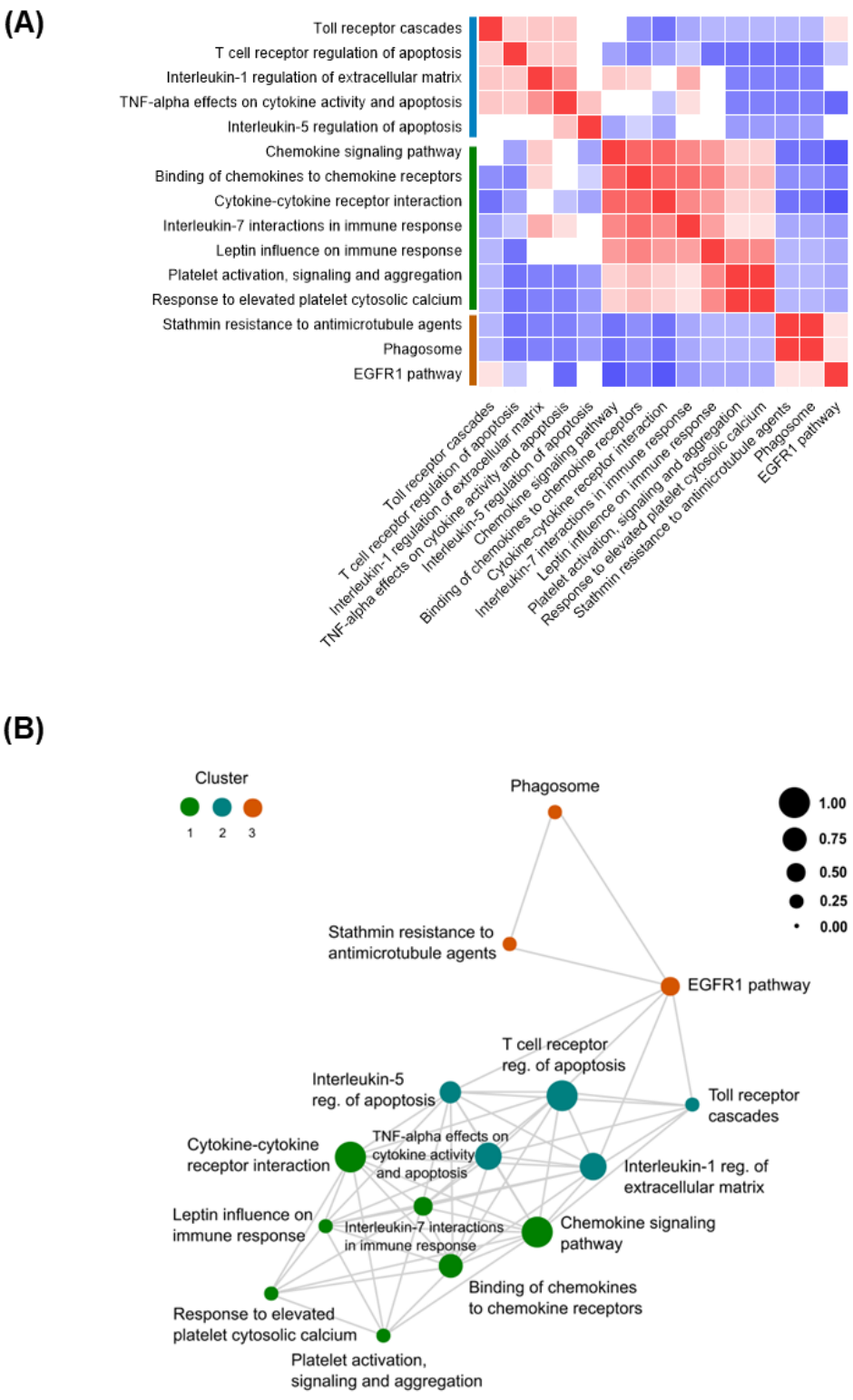


Figure 21: **Clustering and topological analysis of the enriched biological pathways of low type-2 asthma**. **(A)** Correlation matrix analysis based on the mutual genes, **(B)** topological pathway analysis depicting the proximity between the pathways (line length), the number of mutual genes in each pathway (size of circle; 1 is the highest and the rest is proportional to the highest), and pathway clusters (color of the circle).

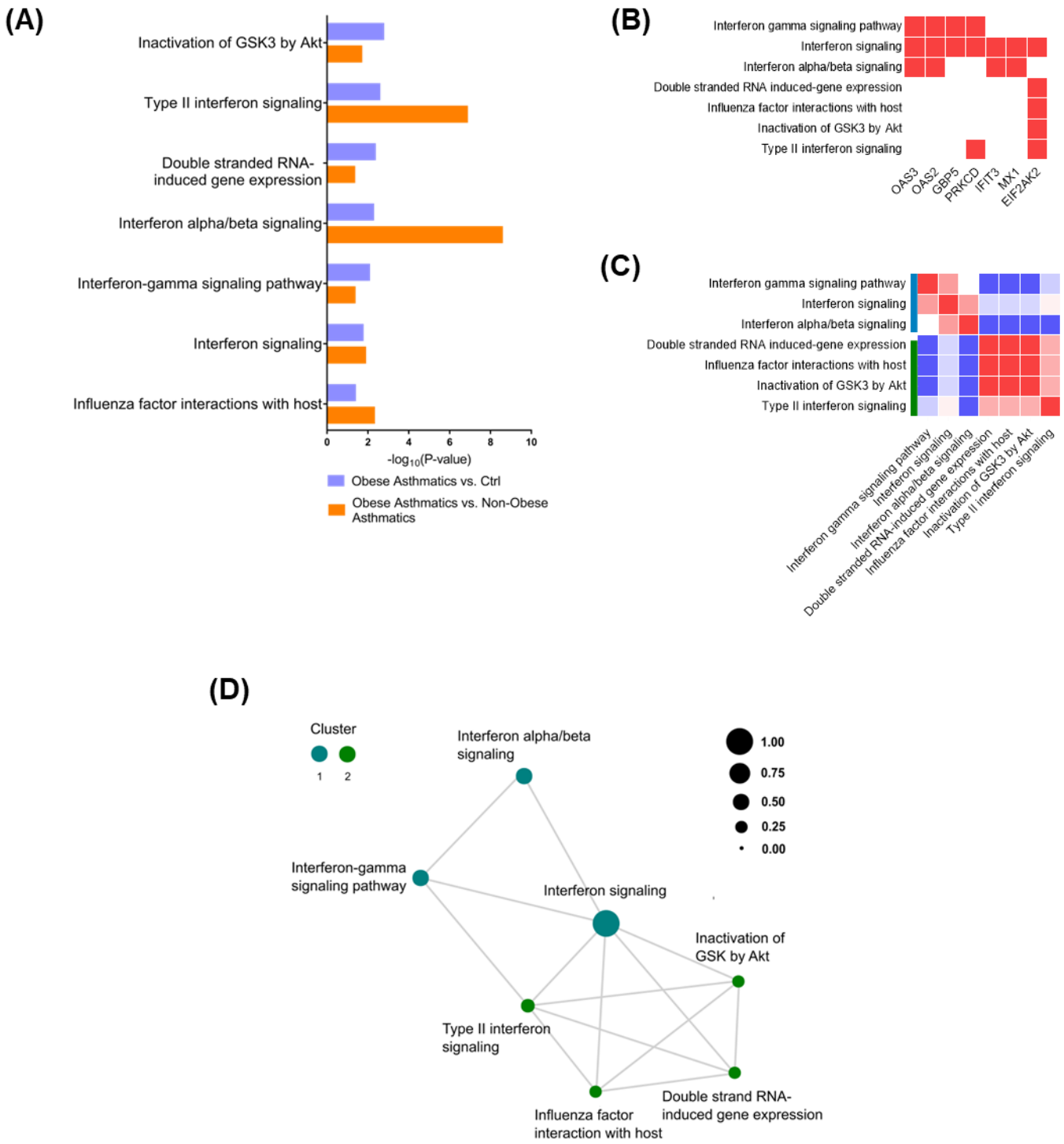


Figure 22: **Clustering and topological analysis of the enriched biological pathways of low type-2 obese asthma**. **(A)** Biological pathways that are shared between obese asthma versus controls and obese-asthma vs. non-obese asthma comparisons **(B)** shared/mutual genes among the 7 pathways **(C)** correlation matrix analysis based on the mutual genes **(D)** topological pathway analysis depicting the proximity between the pathway (line length), the number of mutual genes in each pathway (size of circle), and pathway clusters (color of the circle).

5.2.5 Common and specific biological pathways of low type-2 asthma and obesity asthma phenotypes

To narrow down the common and specific biological pathways, genes that have FC ≥1.5 and FDR <0.1 were first fed into STRING PPI to create huge protein/gene clusters and, afterwards, the gene clusters were subdivided into smaller clusters based on their topological connection using MCODE and Clustermaker add-ins of Cytoscape. As a final step, only genes clusters, that have shared pathways between each two comparisons, have been chosen. As a result, *G-coupled protein receptor (GPCR)* and *gap junction pathways* were shared between obese asthmatics versus controls and non-obese asthmatics versus controls (Figure 23). On the other side, *the interferon signaling pathways* was again shared between obese asthmatics versus controls and obese asthmatics versus non-obese asthmatics (Figure 24). Differential expression of the whole genes in the networks are shown in Figure 25.

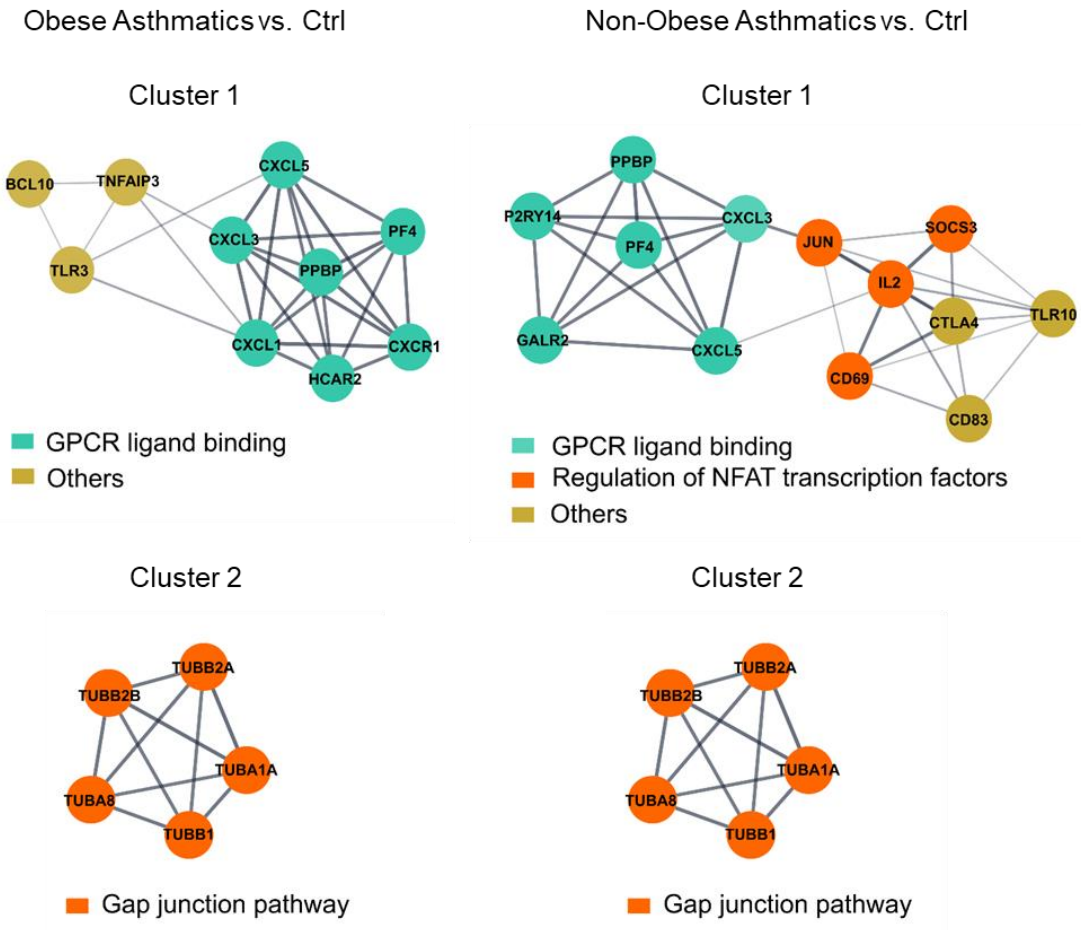


Figure 23: **Gene network and pathway analyses of low type-2 asthma**. Shared pathways and their associated genes between the two comparisons; obese asthmatics vs. Controls and non-obese asthmatics vs. Controls.

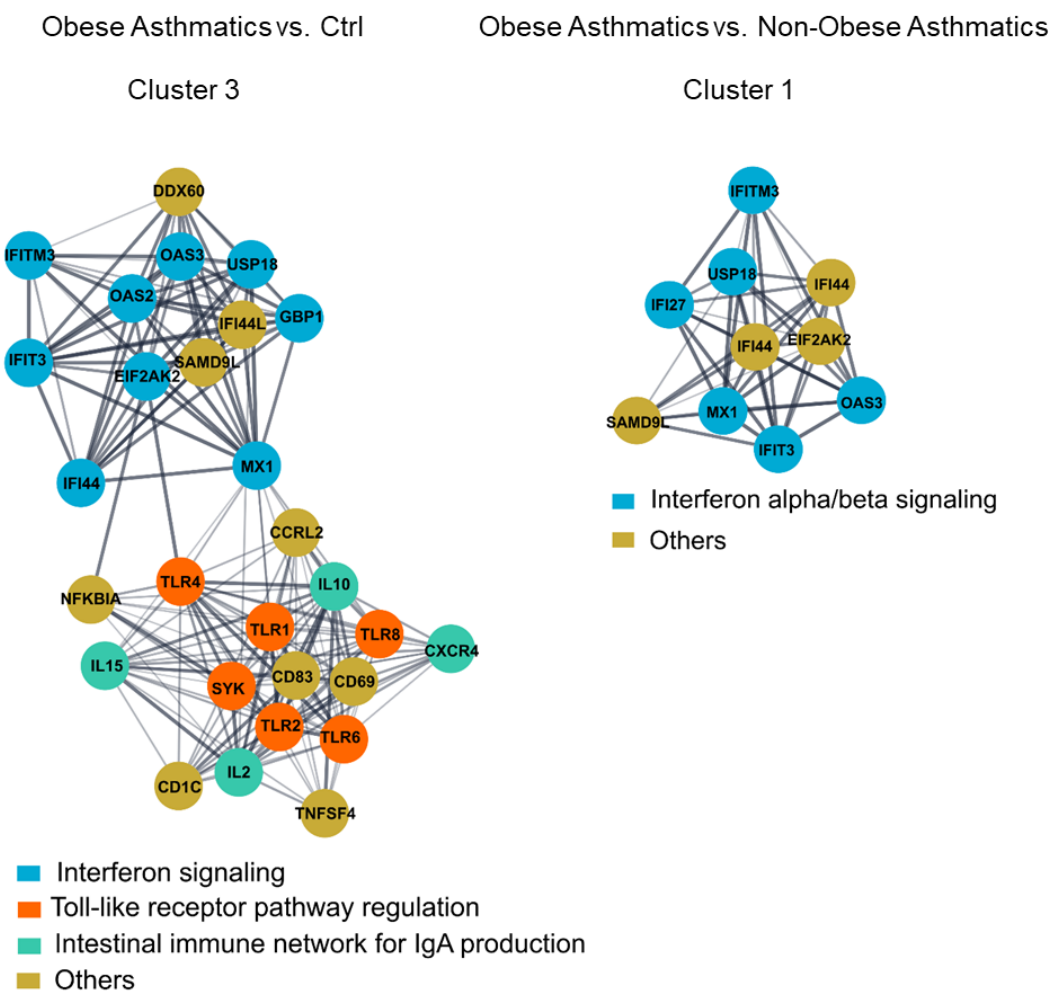


Figure 24: **Gene network and pathway analyses of low type-2 obese asthma**: Shared pathways and their associated genes between the two comparisons; obese asthmatics vs. controls and obese asthmatics vs. non-obese asthmatics.

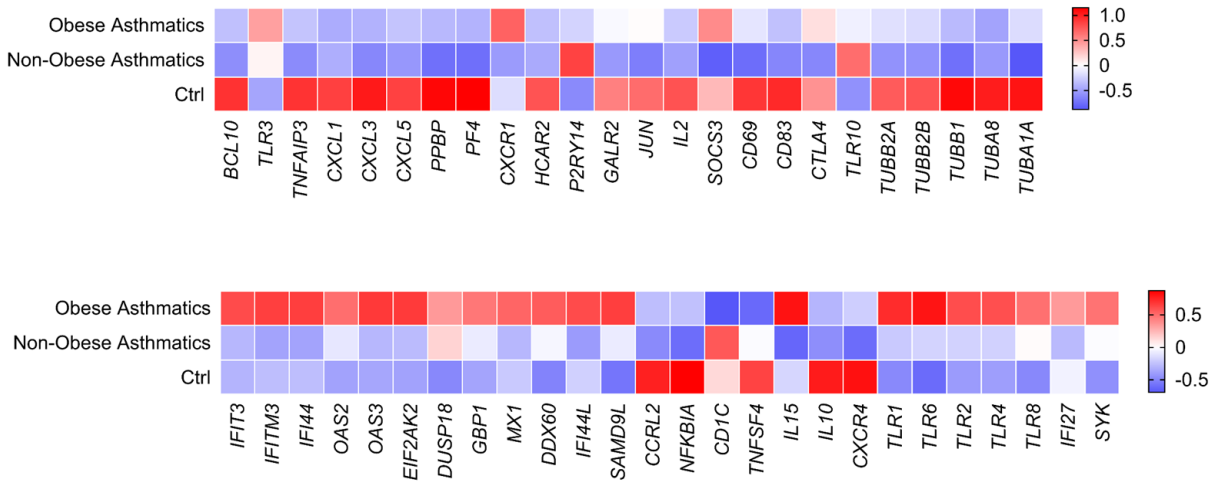


Figure 25: Differential expression of all genes appearing in the gene networks of figure 23 and 24.

5.2.6 Correlation between expressions of the candidate pathway enriched genes and clinical parameters

As shown in the figure 17 and table 6, a set of clinical and laboratory parameters was unable to differentiate between the two low type-2 asthma groups. Nevertheless, correlation between these parameters and expression levels of enriched genes within the three candidate pathways was conducted. The majority of interferon signaling pathway enriched genes correlated positively with the lung function parameters, the strongest correlation has been observed for interferon-induced transmembrane protein 3 (*IFITM3*) and guanylate binding protein 3 (*GBP3*) genes (Figure 26). In contrast, the airway inflammation marker (FeNO) showed a negative correlation with all interferon signaling pathway enriched genes. A similar correlation pattern has been observed in obese asthmatics between the expression levels of the GPCR ligand binding enriched genes and lung function parameters, except for C-X-C Motif Chemokine Ligand 3 (*CXCL-3*) that showed an opposite correlation pattern (Figure 27. A, left). On the contrary, the previous correlation pattern was not found in the case of non-obese asthmatics (Figure 27. A, right). In non-obese asthmatics, a negative correlation has been observed between expression levels of gap junction pathway enriched genes and lung function parameters, except for tubulin alpha 8 gene (*TUBA8*) that showed an opposite correlation (Figure 27. B, right). Similar to the previous *TUBA8* correlation pattern, tubulin beta 1 class VI (*TUBB1*) correlated positively with lung function parameters and negatively with FeNO in low type-2 obese asthmatics (Figure 27. B, left).

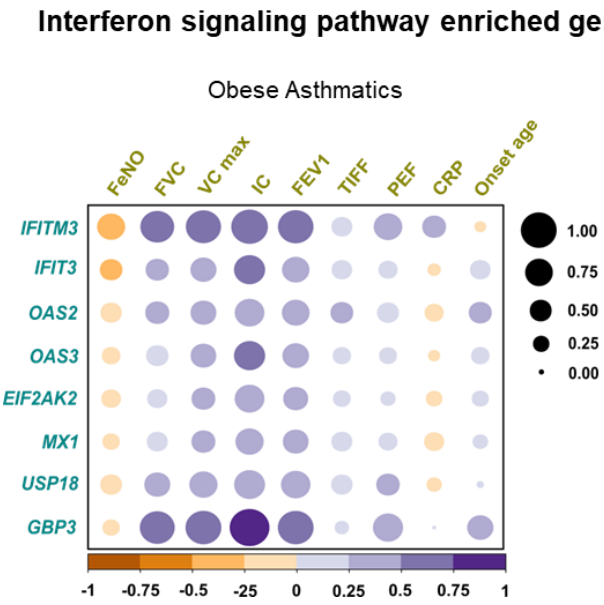


Figure 26: Pearson correlation between clinical and laboratory parameters and expression levels of interferon signaling pathway enriched genes in low-type 2 obese asthmatics.

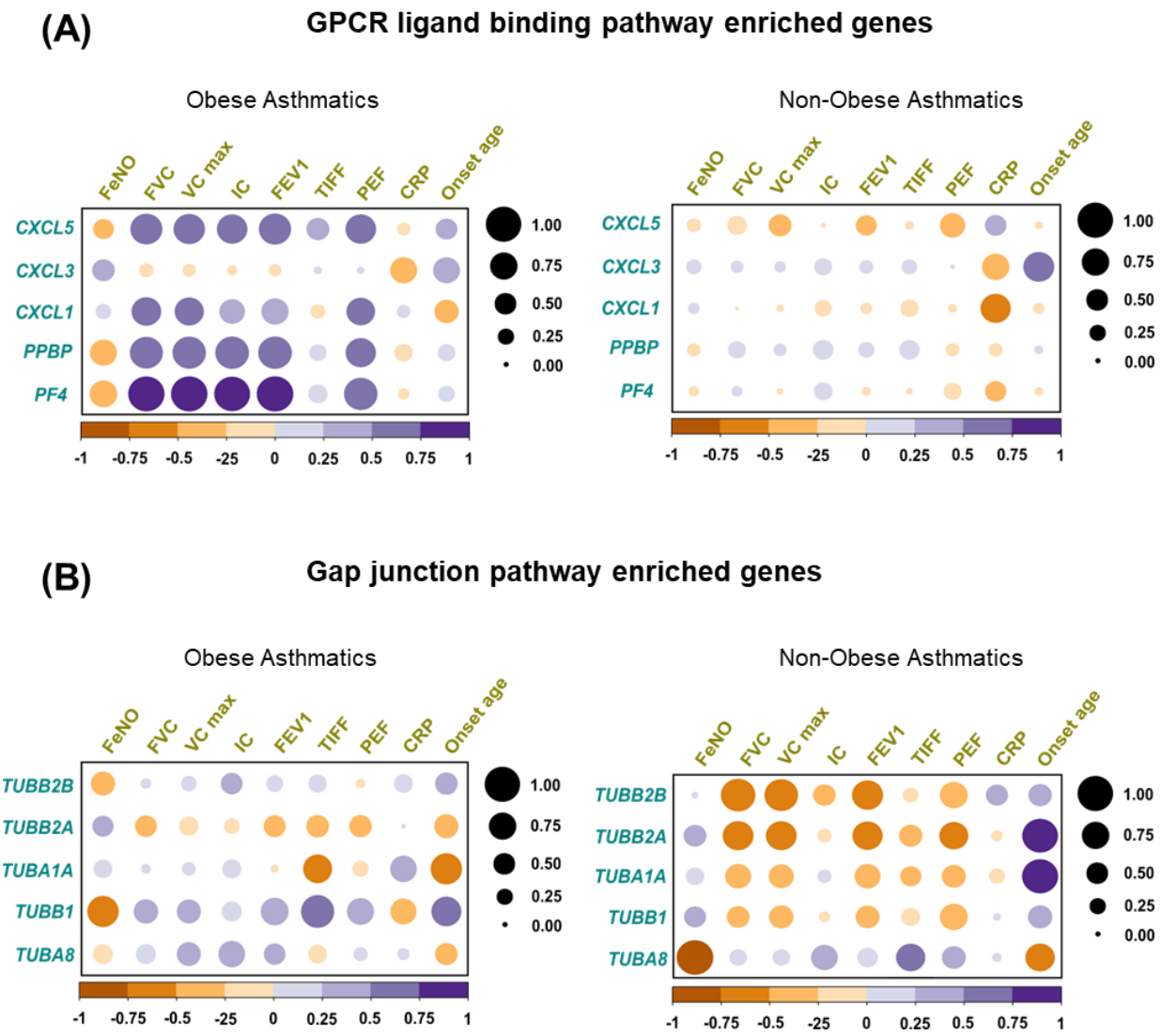


Figure 27: **Pearson correlation between clinical parameters and gene expression levels of two biological pathways of low type-2 asthma**. **(A)** Correlation between clinical parameters and GPCR ligand pathway binding enriched genes in obese asthmatics and non-obese asthmatics and **(B)** Correlation between clinical parameters and gap junction pathway enriched genes in obese asthmatics and non-obese asthmatics.

5.3 Plasma EV miRNA-Seq analysis of low type-2 obese asthmatics and non-obese asthmatics

5.3.1 Study design and EVs characterization

Plasma was collected from the same blood samples as in the previous transcriptomic study. Following isolation of EVs from 1 mL of plasma of ten obese asthmatics, ten non-obese asthmatics and ten healthy controls, EV size and concentration were measured by means of NTA. The NTA analysis showed similar size distributions of

EVs/particles in samples of the three study groups. The EVs mean size was as follows: 159.2 nm, 168.0 nm and 174.5 nm for obese asthmatics, non-obese asthmatics and healthy controls, respectively (Figure 28. A-C).

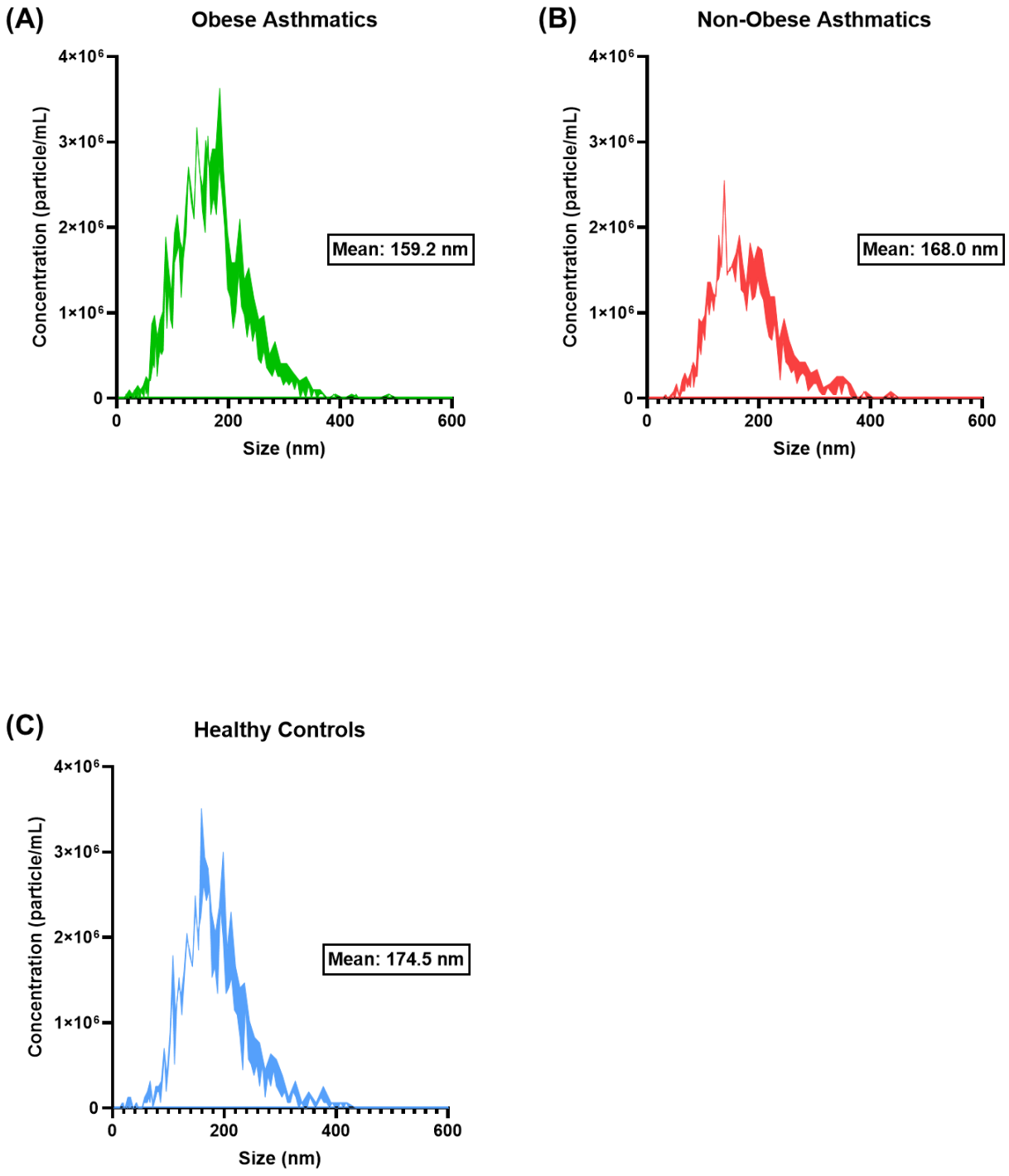


Figure 28: **Nanoparticle tracking analysis (NTA) of the isolated EVs.** EVs/particle size distribution and concentrations for **(A)** obese asthmatics, **(B)** non-obese asthmatics and **(C)** healthy controls.

5.3.2 EV miRNA sequencing of low type-2 obese asthmatics and non-obese asthmatics

Following total RNA extraction from EVs, small RNA sequencing and bioinformatic analyses, RNA biotypes were evaluated. Their distribution was shown to vary between the three study groups. Focusing only on miRNAs, there were 63.9%, 44.2% and 53.2% of total sequencing reads associated with miRNAs in obese asthmatics, non-obese asthmatics, and healthy controls, respectively (Figure 29). By creating a PCA plot to figure out the distribution and clustering of the 3 study groups, there was an obvious separation between the 3 groups based on the most variable 500 miRNAs (Figure 30). Afterwards, a pairwise comparison was performed for the 3 groups and volcano plots were created using a significance threshold at FDR<0.05. For obese asthmatics versus controls, a total number of 706 differentially regulated miRNAs were detected (349 up and 357 down; Figure 31. A), for non-obese asthmatics versus controls, a total number of 636 differentially regulated miRNAs were detected (332 up and 304 down; Figure 31. B), and for obese asthmatics versus non-obese asthmatics), a total number of 679 differentially regulated miRNAs were detected (338 up and 341 down; Figure 31. C).

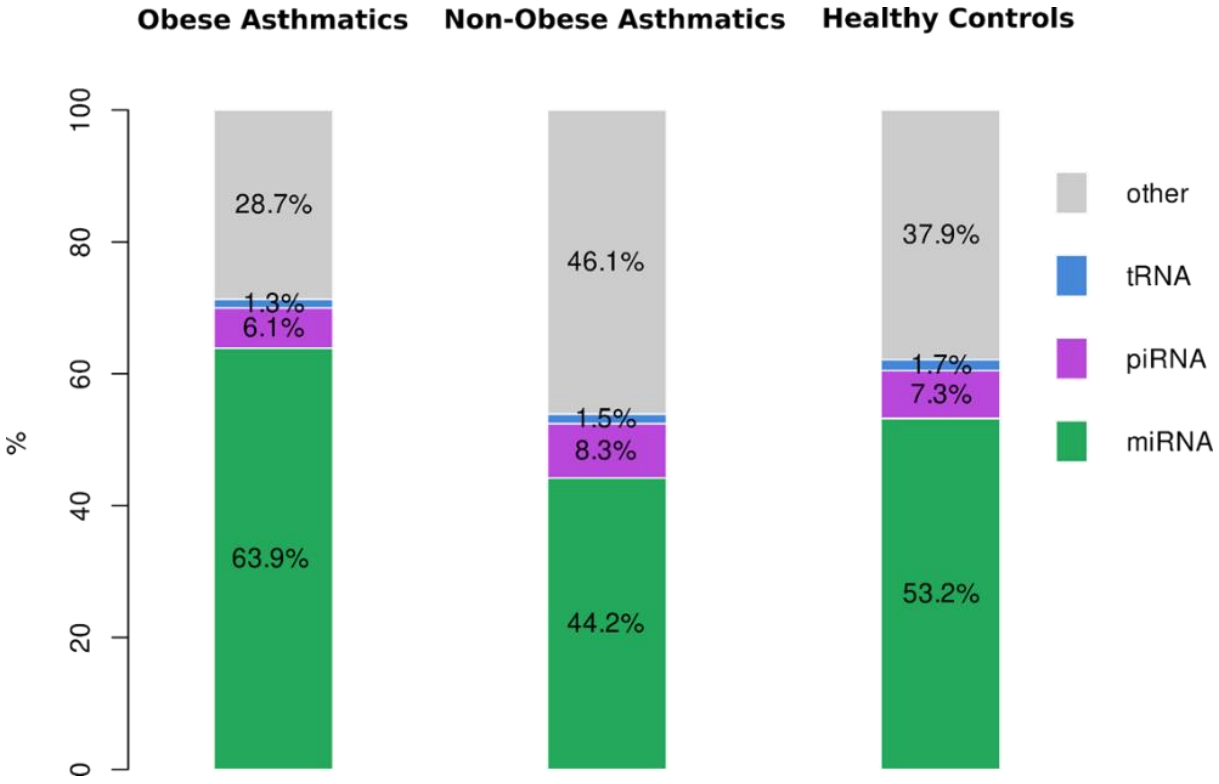


Figure 29: Distribution of small RNA biotypes in obese asthmatics, non-obese asthmatics, and healthy controls.

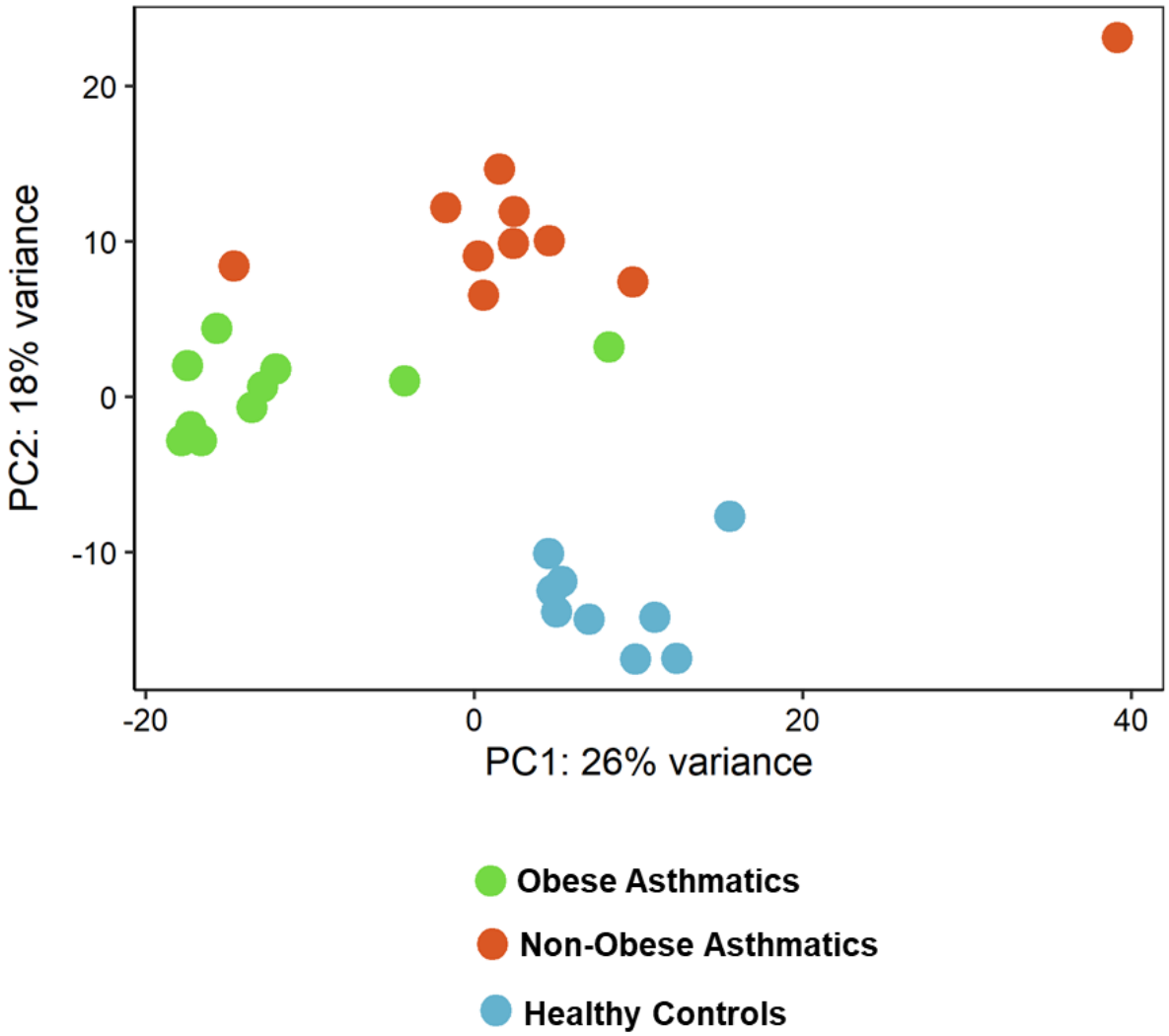


Figure 30: Principle component analysis (PCA) for the most variable 500 miRNAs in the above listed three study groups.

5.3.3 Functions and clusters of the differentially regulated miRNAs in low type-2 obese asthmatics and non-obese asthmatics

By performing functional analysis on the differentially regulated miRNAs of the three previous comparisons using TAM 2.0 tool at FC≥1.5 and FDR<0.05, a set of various functions were associated with the differentially regulated miRNAs including *inflammation*, *adipocyte differentiation*, and *epithelial-to-mesenchymal transition*

(EMT) in obese asthmatics versus controls comparison (Figure 32. A). Similar functions were detected in the obese asthmatics versus non-obese asthmatics comparison with additional unique functions such as *latent virus replication* (Figure 32. C). Functions like *neutrophil differentiation* and *vascular smooth muscle differentiation* were associated with the differentially regulated miRNAs in the non-obese asthmatics versus controls comparison (Figure 32. B).

A “miRNA cluster” is a group of miRNAs that are transcribed from adjacent genes, which acts together to regulate specific cellular processes (Lai and Vera 2013). By conducting miRNA cluster analyses, there were a variety of miRNA clusters playing different biological roles in each of the three comparisons. Starting with obese asthmatics versus controls, two miRNA clusters were enriched, namely miR-2392 and miR-106b clusters, which were associated with biological processes such as *regulation of interferon-gamma mediated signaling pathway* and *viral process*, respectively (Figure 33. A).

These results are concordant with the CD4+ T cells transcriptomic results of the same subjects. In non-obese asthmatics versus controls, miR-514a-3, miR-642b and miR-3619 clusters were enriched and associated with *vesicle-mediated transport*, *cytokine-mediated signaling pathway* and *cell junction assembly*, respectively (Figure 33. B). miR-6749, miR-3619 and miR-424 clusters were associated with *viral entry into host cell*, *tight junction assembly* and *positive regulation of type I interferon production*, respectively in obese asthmatics versus non-obese asthmatics (Figure 33. C).

5.3.4 Detecting the DE miRNA gene targets in the CD4+ T cells

To link the solid findings of the transcriptomic study of the CD4+ T cells of obese asthmatics with the DE miRNAs of plasma EVs of the same study subjects, 15 down-regulated miRNAs targeting 6 of the 8 candidate IFN stimulated genes (ISGs) in CD4+ T cells have been detected (Figure 34). Among the down-regulated miRNAs were miR-665, miR-4419b, miR-4769-3p, miR-6893-5p and miR-4743-3p which target the ISG: eukaryotic translation initiation factor 2 alpha kinase 2 (*EIF2AK2*). Other down-regulated miRNAs were miR-6721-5p, miR-1207-5p, miR-6132 and miR-4700-3p

targeting the ISG (*IFTIM3*), the rest of the miRNAs and their putative ISG targets are shown in Figure 34.

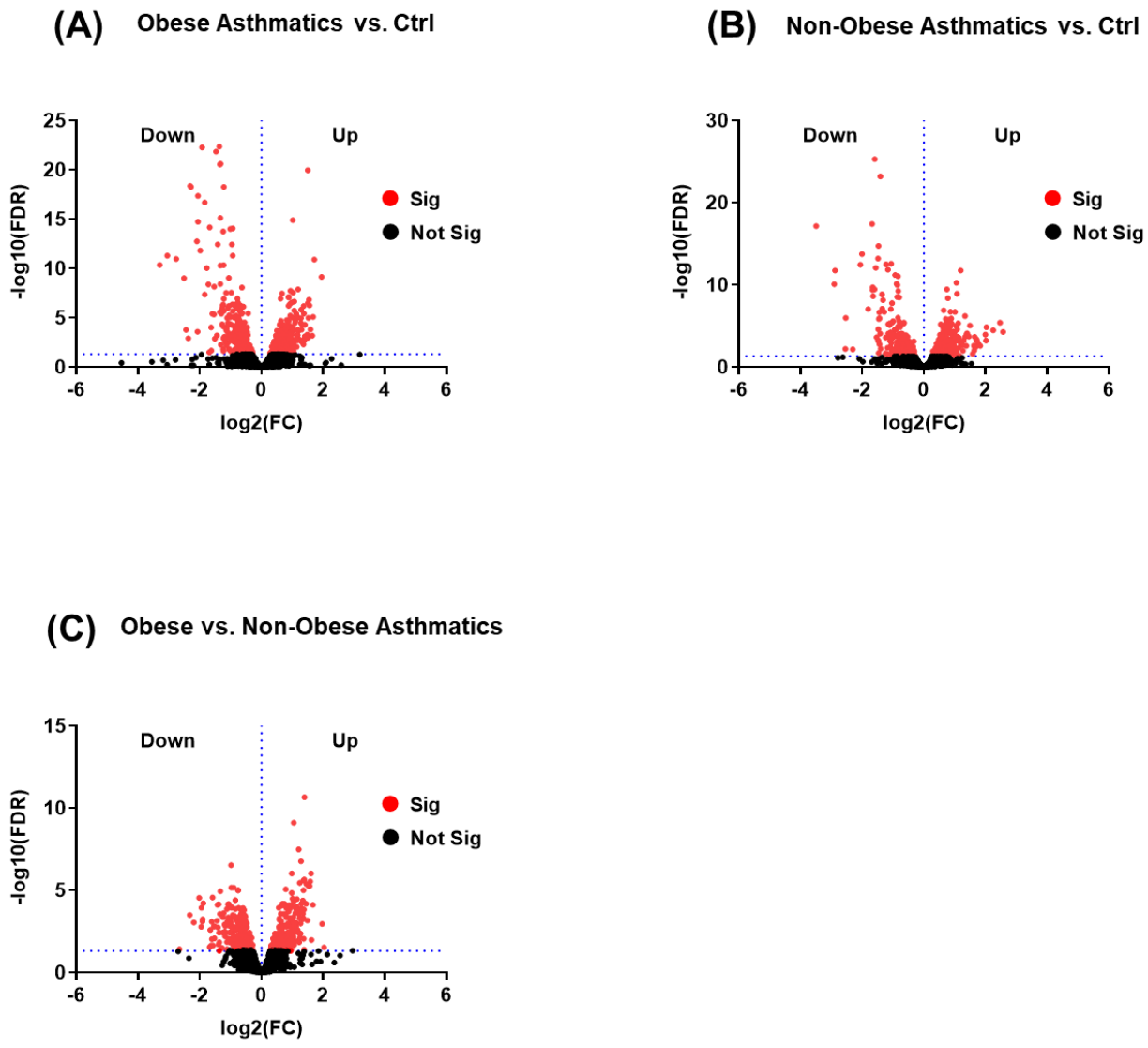


Figure 31: **Differentially regulated miRNAs in obese and non-obese asthmatics**. Volcano plots depicting the Differentially regulated miRNAs at FDR <0.05 for **(A)** obese asthmatics versus controls, **(B)** non-obese asthmatics versus controls and **(C)** obese asthmatics versus non-obese asthmatics.

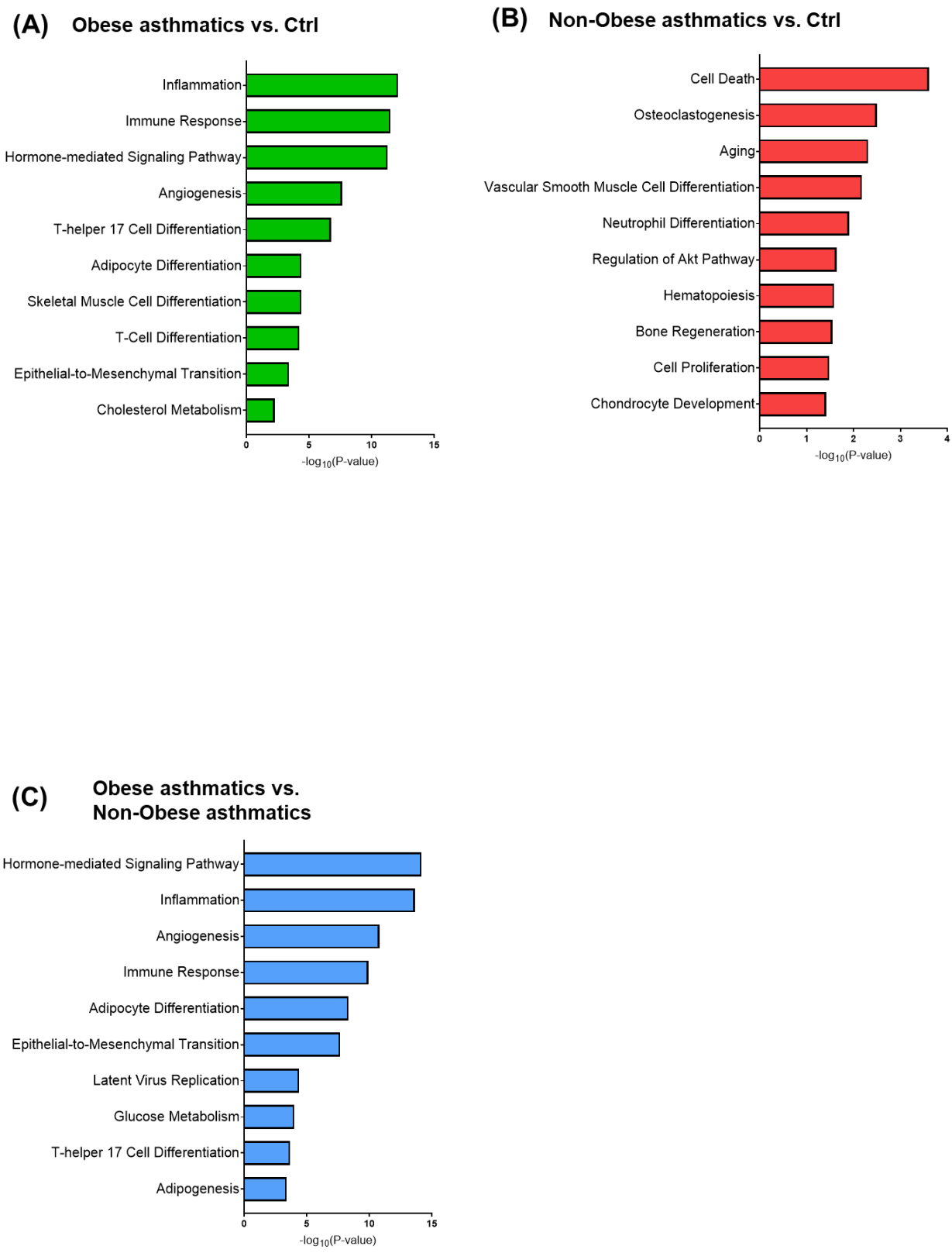


Figure 32: **Functions of DE miRNAs of the three comparisons at FC >1.5 and FDR <0.05**, for **(A)** obese asthmatics vs. Controls, **(B)** non-obese asthmatics vs. Controls and **(C)** obese asthmatics vs. non-obese asthmatics comparisons.

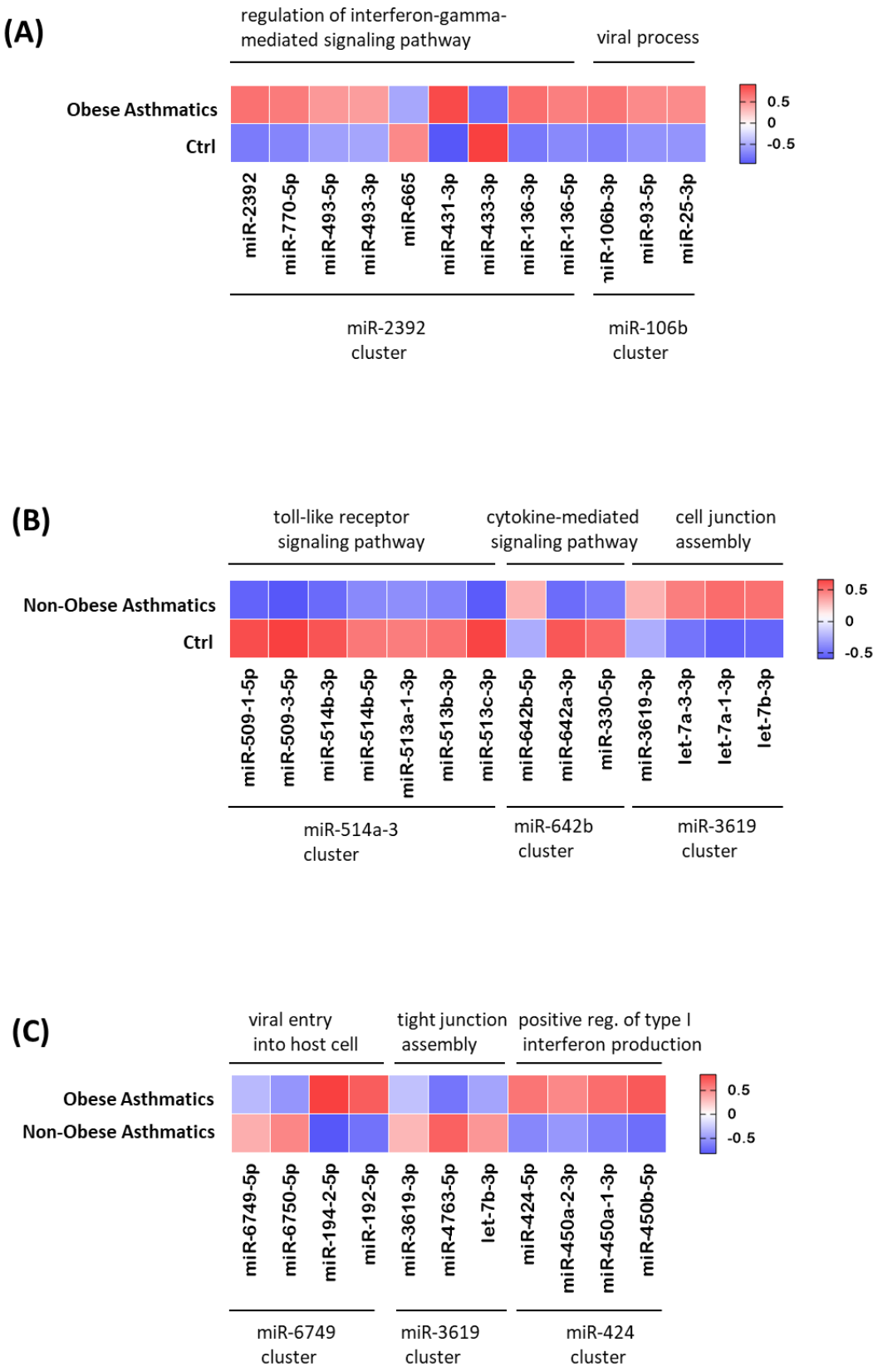


Figure 33: **miRNA clusters and their associated biological processes (BP:GO terms)**, for **(A)** obese asthmatics vs. Controls, **(B)** non-obese asthmatics vs. Controls, and **(C)** obese asthmatics vs. non-obese asthmatics comparisons.

In contrast, 15 up-regulated miRNAs of plasma EVs were identified to target seven candidate ISGs in CD4+ T cells in obese asthmatics (Figure 35). miR-20b-5p, miR-17-5p, miR-93-5p, miR-20a-5p were found to target the ISG (*GBP3*). Whereas, miR-92b-3p, miR-99a-5p, miR-146a-5p and miR-202-5p were found to target interferon induced protein with tetratricopeptide repeats 3 (*IFIT3*), the latter two miRNAs can target also *IFITM3* and *EIF2AK2*, respectively. The rest of the miRNAs and their ISG targets are shown in Figure 35.

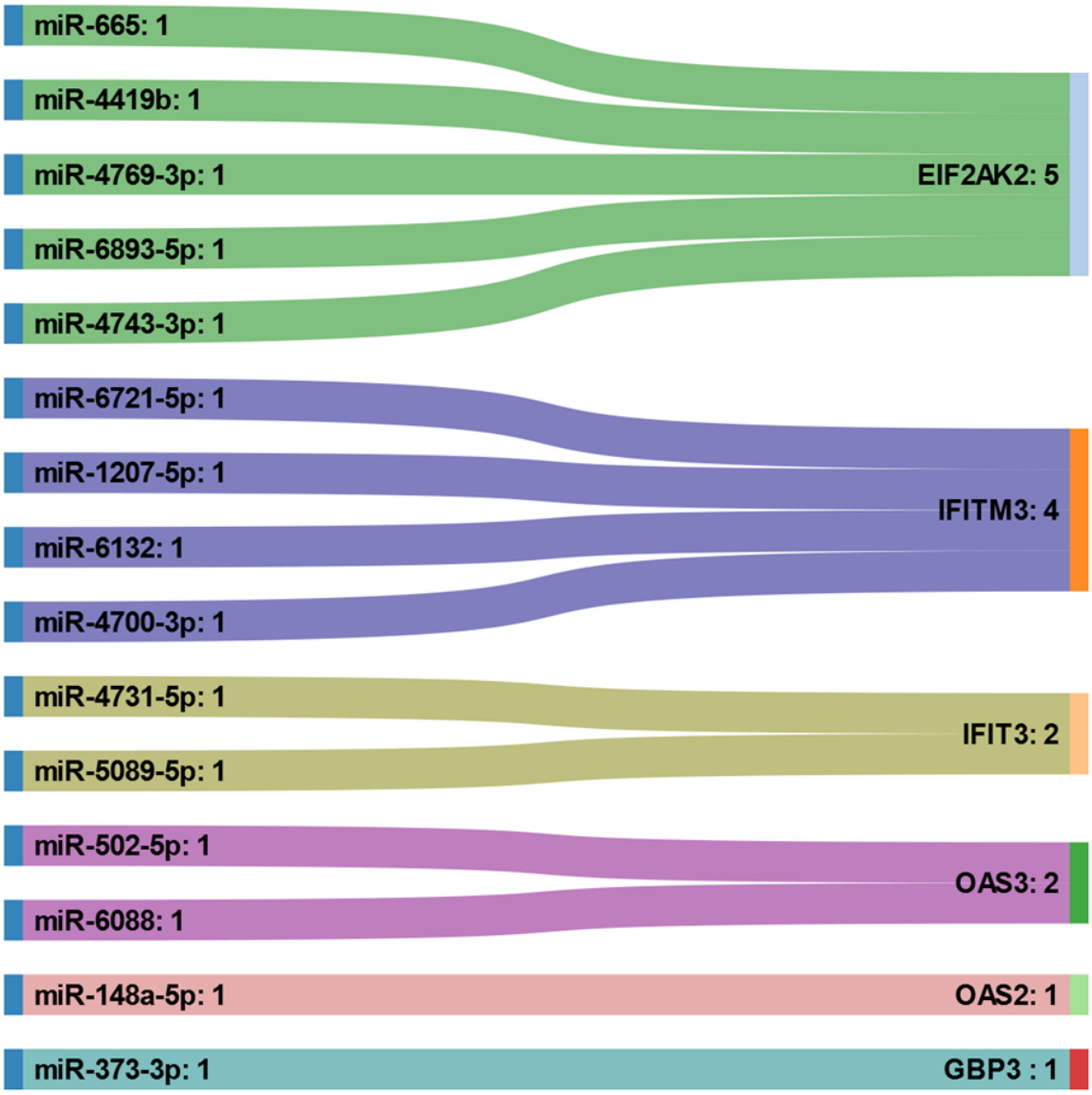


Figure 34: **Down-regulated EVs miRNAs potentially targeting the ISGs of the blood CD4+ T cells in obese asthmatics versus controls.** Sanky diagrams showing 15 down-regulated plasma EV miRNAs and their six target candidate ISGs in blood CD4+ T cells.

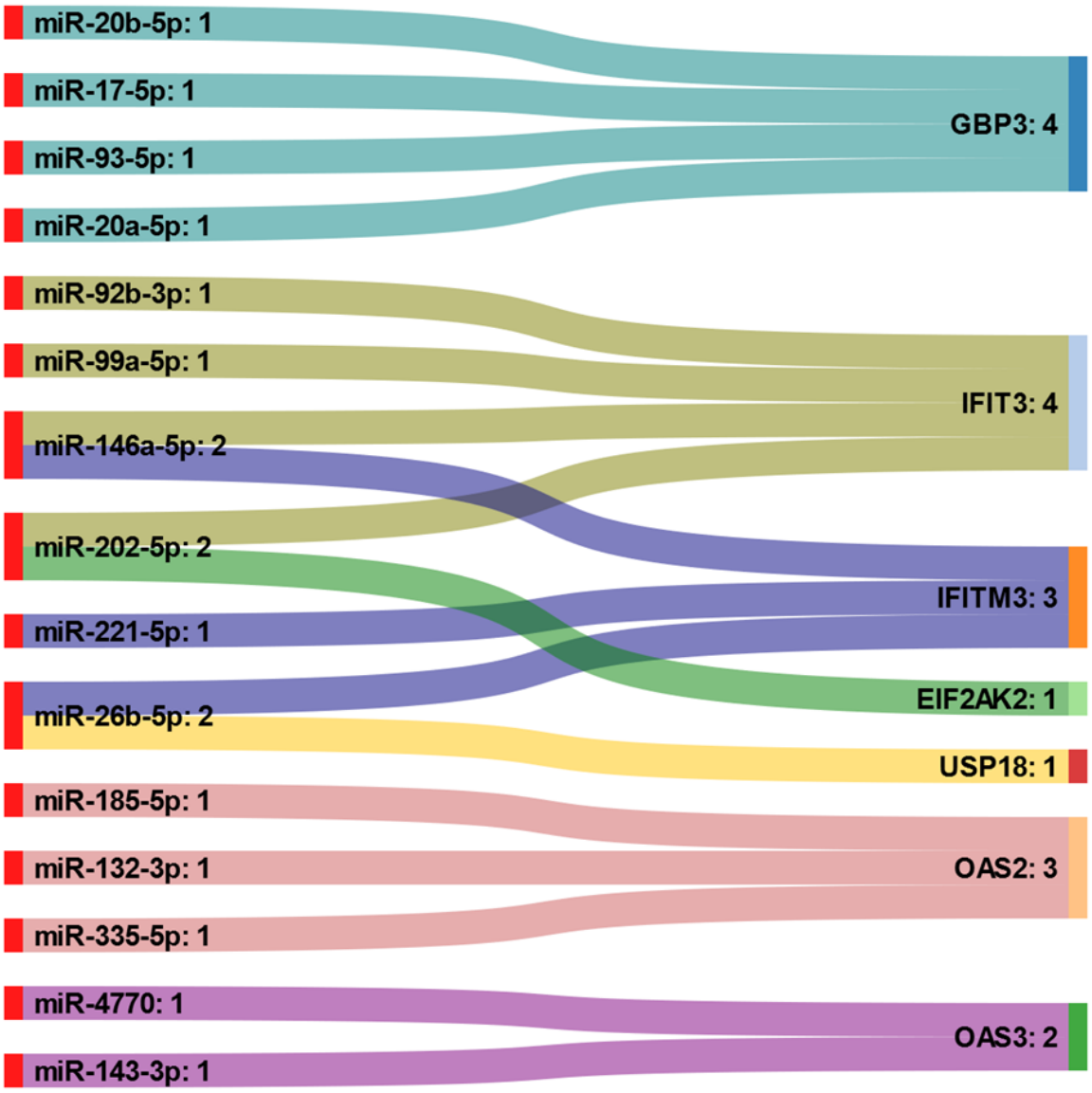


Figure 35: **Up-regulated EVs miRNAs potentially targeting the ISGs of the blood CD4+ T cells in obese asthmatics versus Controls.** Sanky diagrams showing 15 up-regulated plasma EV miRNAs and their 7 target candidate ISGs in blood CD4+ T cells.

6. Discussion

Asthma is a chronic inflammatory disease of the lung airways affecting more than 300 million people worldwide (Nanda and Wasan 2020). Due to its heterogeneity, asthma has been underdiagnosed in too many cases (Kaur and Chupp 2019). Moreover, conventional asthma therapies have frequently failed to treat asthmatic patients, reduce their asthma exacerbations or ease their disturbing symptoms (DiMango et al. 2018). In the last few decades, several studies have focused on the heterogeneous pathogenesis of asthma and described it as a disorder with various clinical phenotypes including eosinophilic asthma, neutrophilic asthma, obesity-associated asthma and others (Wenzel 2012; Kaur and Chupp 2019). Therefore, it is now mandatory to unscramble the specific underlying molecular mechanisms for each of these phenotypes (Schoettler and Strek 2020; Hekking and Bel 2014). Much efforts have been elaborated in designing biologics that target molecules associated with type-2 driven asthma such as omalizumab (anti-IgE) and mepolizumab (anti-IL-5) (Akar-Ghibril et al. 2020; Chung 2018), yet less work has been invested in designing similar treatments targeting molecules of the other (non-type-2) asthma phenotypes such as obesity-associated asthma. This is due to the lack of the mechanistic understanding of their underlying molecular mechanisms driven by a plenty of immune cells, their inflammatory mediators and their secreted EVs into the body fluids such as the blood.

Peripheral blood is a valuable source of molecular information that can be obtained through several multi-omics techniques (Su et al. 2020). The minimally invasive sampling of peripheral blood has made it an appealing target for identification of biomarkers and monitoring therapy responses in the field of allergies and atopy (Jiang et al. 2019; Gallant and Ellis 2018). *Ex vivo* handling of blood samples prior to specific cell population isolation is a big challenge in gaining robust and reproducible results. One of the critical points in blood sampling and processing is the time-dependent effects accompanying the delayed cells processing after *ex vivo* storage of blood (Baechler et al. 2004; Dvinge et al. 2014). Therefore, initially a study was conducted to investigate such effects on both transcriptomic and epigenetic levels of blood CD4+ T cells from atopic and healthy donors. CD4+ T cells were included here due to the fact they will be investigated in more detail in the upcoming study representing a cell type that plays a central role in adaptive immune system and comprises a unique plasticity to differentiate into several T helper (Th) cell subsets such as Th1, 2, and 17 which

have been associated with many different diseases including atopy and asthma (Potaczek et al. 2017).

The RNA-Seq results of this pilot study showed that immediate processing of blood CD4+ T cells generated hundreds of DEGs in atopic patients compared to healthy donors at FDR <0.1. Well-known atopy-related biological pathways including IL-2 and IL-6 signaling pathways were enriched for these DEGs. On the other side, delayed (~24 hours) CD4+ T cells processing generated only 3 DEGs: Ribosomal Protein S27 (RPS27), RPL41, and RPL13. While the transcriptomic profiles of blood CD4+ T cells in atopic donors were harshly affected by the prolonged *ex vivo* storage, the CD4+ T cells of healthy donors remained almost intact. By comparing the transcriptomic profiles of the delayed and immediately processed blood CD4+ T cells from atopic donors, most of the atopy-related pathways were down-regulated. Interestingly, RNA degradation genes such as CCR4-NOT transcription complex subunit 1 (*CNOT1*) and CCR4-NOT transcription complex subunit 6 like (*CNOT6L*) were up-regulated during the *ex vivo* blood storage. The sudden depletion of the atopy-related RNA transcripts could be interpreted that the absence of the external stimuli in CD4+ T cells has facilitated specific RNA degradation gene products to compromise the atopy related gene RNA transcripts (Shirai et al. 2014). Similar results have been also observed in a recent study by Massoni-Badosa et al. They utilized single-cell transcriptomic analysis on peripheral blood derived PBMC, in which T cells have changed their gene expression significantly after 8 hours of incubation at room temperature (Massoni-Badosa et al. 2020).

H3K27ac ChIP-Seq analysis of CD4+ T cells has additionally been implemented to validate the transcriptomic results on the epigenetic level due to the strong association between the histone modification H3K27ac and gene expression (Paauw et al. 2018). The results of the ChIP-Seq analysis were concordant with those of the transcriptomic analysis of CD4+ T cells of both atopic and healthy donors. Similarly, atopy-related pathways such as *IL2 signaling* and *T -cell receptor signaling pathways* were down-regulated alongside with other gene expression pathways in the delayed versus immediate blood CD4+ T cells processing of atopic patients.

The results raise the question about the ambiguous role of the RNA degradation genes in switching the gene expression profile of CD4+ T cells between the disease (atopy) and health conditions. Moreover, establishing new methods and finalizing standard

operating procedures (SOPs) based on healthy donor samples seems to be not sufficient for planning bigger studies. Therefore, integration of patients' samples in preliminary studies is mandatory in order to gain realistic results and pave the way for well-designed clinical and molecular studies.

After establishing optimal experimental conditions concerning the timing of CD4+ T cells processing from human peripheral blood, the next step was to explore CD4+ T cells signatures and understand their associated molecular pathways in the obesity-associated asthma phenotype, which can contribute to disentangle the complex pathogenesis of this asthma phenotype. To fulfill this purpose, 10 low type-2 obese asthmatics and 10 low type-2 non-obese asthmatics were recruited alongside with 10 non-obese healthy controls. These 20 asthmatic patients were chosen from one hundred forty-four asthmatics based on preselected criteria including blood eosinophils count<300 cell/µL, FeNO<25 ppb and serum total IgE levels< 300 kU/L, while both asthmatic groups differed significantly in their BMI levels. Such criteria were meant to identify molecular markers that might help defining asthma phenotypes/endotypes in patients that are similar for classical asthma biomarkers and clinical features.

The biostatistical analyses of the laboratory and clinical measurements including lung function parameters, CRP and age of onset did not reveal any significant difference between the two asthma groups. On the other side, the genome-wide gene expression analysis revealed hundreds of differentially expressed genes in CD4+ T cells between those obese asthmatics and non-obese asthmatics. *IL15* and suppressor of cytokine signaling 3 (*SOCS3*) were among the top 10 significant upregulated genes in obese asthmatics compared to non-obese asthmatics. These two genes are considered as obesity-associated marker genes (Pedroso et al. 2019; Barra et al. 2010; Duan et al. 2017). Moreover, there was a weak clustering and a high variability between the low type-2 obese asthmatic individuals in gene expression of CD4+ T cells compared to low type-2 non-obese asthmatics. This observation could be interpretated that low type-2 obesity-associated asthma might be composed of several asthma subtypes due to variable immunopathogenic effects of different adipose tissue-associated mediators, which in turn modulate inflammatory processes of asthma (Cho et al. 2014; Endo et al. 2015; Papatriantafyllou 2013).

IFN signaling pathway is one of the major pathways in the immune system to fight against pathogens. The route of *IFN signaling pathway* begins with binding IFN

proteins to their receptors on the cell surface, followed by the initiation of Janus kinase signal transducer and activator of transcription (JAK-STAT) signaling pathways (Schneider et al. 2014). These signaling pathways lead eventually to induction of expression of many ISGs (Schoggins 2019). ISGs like *EIF2AK2*, *IFIT3*, *IFITM3* and others were found to be differentially expressed in obese asthmatics compared to both healthy controls and non-obese asthmatics. This could also indicate that CD4+ T cells in obese asthmatics have been driven in a Th1 polarization manner. This observation could be supported by the negative correlation between the interferon signaling genes and the airway inflammation biomarker “FeNO”, which is considered to be a type-2 inflammation marker (Korn et al. 2020).

The close topological proximity between *interferon signaling* and *viral infection pathways* (e.g. *influenza factor interaction with host*) in obese asthmatics could give a hint of viral infection traces in obese asthmatic individuals. This observation was supported by the elevated expression of Toll-like receptor pathway regulation genes such as Toll-like receptor 1 (*TLR1*), *TLR-2*, *TLR-3*, *TLR-4*, *TLR-6*, and *TLR-8* in obese asthmatics compared to healthy controls since some of these TLRs play an essential role in recognizing pathogenic nucleic acids and induce IFNs production as a defense mechanism against viral infections (Carty and Bowie 2010; Zakeri and Russo 2018). Tang *et al* stated that obesity could facilitate respiratory tract infections (RTIs) and could lead ultimately to asthma exacerbations in adults (Tang et al. 2019). Concordant results have been reported about the association between obesity and viral infection by Maccioni *et al* (Maccioni et al. 2018). Remarkably, this association was also investigated during the COVID-19 pandemic. Various studies have stated that obesity can complicate the outcomes of the SARS-CoV-2 viral infection (Gao et al. 2021; Yates et al. 2021).

Enrichment of a leptin-related pathway (leptin influence on immune response) in low type-2 non-obese asthmatics albeit with slightly lower significance has raised the question about the prominent role of the leptin hormone in low type-2 asthma phenotype in general. Zhang *et al* reported that higher levels of circulating leptin correlated positively with asthma exacerbations (Zhang et al. 2017). This could be additionally noted from the close proximity between the leptin influence on immune response pathway and other asthma related pathways like cytokine-cytokine receptor interaction.

Modulated regulation of *GPCR ligand binding pathway* associated genes such as those encoding C-X-C motif chemokine ligand 1 (*CXCL1*), *CXCL3*, *CXCL5*, platelet factor 4 (*PF4*), and pro-platelet basic protein (*PPBP*) has been observed in both low type-2 obese asthmatics and non-obese asthmatics compared to healthy controls. The activation of the *GPCR signaling pathway* upon ligand binding mediates a wide range of extra- and intra-cellular responses, this has shed light upon this pathway as a potential target for therapy in various diseases including asthma (Wendell et al. 2020; Deshpande and Penn 2006). Almost one third of marketed drugs target GPCRs directly or indirectly (Hauser et al. 2017). The unique robust correlation between the five above-mentioned genes and most of the lung function parameters specifically in obese asthmatics indicates the key role of the GPCR signaling pathway in the obesity associated asthma phenotype.

Several tubulin genes including those encoding tubulin alpha 1a (*TUBA1A*), *TUBA8*, *TUBB1*, tubulin beta 2A class IIa (*TUBB2A*) and tubulin beta 2B class IIb (*TUBB2B*) are fundamental components of the *gap junction pathway*. This pathway represents another route of cell-cell communication by building up hemi-channels between two adjacent cells and exchanging signaling molecules (Giepmans et al. 2001; Elgueta et al. 2009). *In vitro* CD4+ T cells have been shown to communicate with macrophages through gap junctions as a part of antigene cross presentation process (Bermudez-Fajardo et al. 2007). Additionally, the interaction between CD4+ T regulatory cells (T regs) and DCs through gap junctions have augmented the supersessive immune effects of T regs and hampered the immune response of the CD8+ T cells (Ring et al. 2010). On the other hand, blocking the gap junctions of the T regs has abrogated their suppressive characteristics (Bopp et al. 2007). This might also explain the persistent inflammatory response in the low type-2 asthma phenotype.

After interrogating the role of *IFN signaling pathway* in peripheral blood derived CD4+ T cells of low type-2 obese asthmatics, our next step was to search for this pathway and new molecular mechanisms in a broader level in the same patients. Therefore, plasma EVs was a good candidate for this purpose since they play an essential role in intracellular communication processes supported by a huge variety of surface marker proteins and a precious cargo of RNAs, lipid, and proteins (van Niel et al. 2018). MiRNAs are one of the principal components of the EVs, which shuttle them to exert their post-transcriptional effects in the recipient cells (Alashkar Alhamwe et al. 2021).

EVs were first isolated from plasma of the same blood samples from the same subjects of the previous study (i.e., 10 low type-2 obese asthmatics, 10 low type-2 non-obese asthmatics and 10 normal weight healthy controls). EVs were further characterized by means of NTA analysis in order to measure the size and concentration of the isolated EVs. Diameter size of the plasma EVs did not show a huge difference between obese asthmatics, non-obese asthmatics and healthy controls (~160-175 nm). On the other side, the miRNA content varied remarkably between the three groups, with the highest miRNA proportion of 63.9% of total mapped RNAs was observed in obese asthmatic individuals. Based on the PCA analysis, a unique clustering and obvious separation were observed between the three study groups. This indicates the difference of the circulating EVs and their shuttled miRNAs pointing to subtle pathogenesis differences and molecular mechanisms of specific asthma phenotypes.

Further analyses of the differentially expressed miRNAs revealed potential unique functions of these miRNAs. In obese asthmatics versus healthy controls and obese asthmatics versus non-obese asthmatics comparisons, inflammation, adipocyte differentiating and EMT were among the enriched functions. The latter function is one of the distinct features of the airway remodeling in asthma, in which the epithelial cells lose their basic functions and change into a mesenchymal state, which could in turn contribute to epithelial barrier damage and induce severe asthma exacerbations (Sun et al. 2020; Ijaz et al. 2014). At the same time, signals of neutrophil differentiation were enriched in obese asthmatics versus healthy controls comparison, which might indicate a potential role of neutrophils in the low type-2 obese asthmatics.

miRNA clusters are essential in regulating many cellular processes (Lai and Vera 2013). Clusters of miR-2392 and miR-106b in obese asthmatics versus healthy controls comparison were assigned to *IFN signaling pathways* and *viral infection*, respectively. The three members of the cluster miR-106b (i.e., miR-106b-3p, miR-93-5p and miR-25-3p) are located on the intron 13 of the 7q22 locus (Sárközy et al. 2018) and have been associated with several viral infections such as respiratory syncytial and hepatitis B viruses' infections (Wang et al. 2017; Tan et al. 2014). In addition, miR-25-3p have been shown to play a role in asthma pathogenesis (Szklarczyk et al. 2019). These results were concordant with those in the CD4+ T cells signature pathways. In vitro experiments have found that miR-16b could drive CD4+ T cells to differentiate into Th1 subsets, while upon OVA stimulation and treatment with miR-106b inhibitors, CD4+ T cells have been skewed into a Th2 polarization manner (Tang et al. 2015).

miR-424 and miR-6749 clusters were found also to be associated with *IFN signaling* and *viral infection pathways*, respectively, in obese asthmatics versus non-obese asthmatics comparison.

In summary and conclusion, conducting preliminary studies to investigate the pre-analytical effects on blood processing is required to obtain optimal results from bigger studies. Timing of specific cell populations' isolation after *ex vivo* storage is very controversial. Delayed processing of blood CD4+ T cells was shown to remarkably affect the transcriptomic and epigenetic profiles of the cells of atopic patients specifically. RNA degradation genes such as *CNOT6L* and *CNOT1* might have a potential role in returning the CD4+ T cells phenotype from a diseased back to the healthy state.

Low type-2 obesity-associated asthma is a unique phenotype, in which patients were distinguished from their non-obese counterparts not only based only on BMI on the clinical level, but also even more on the molecular level. *IFN signaling* and *viral infection pathways* were exclusively enriched in obese asthmatics, while *gap junction* and *GPCR ligand binding pathways* were enriched in low type-2 asthmatic regardless of their BMI values.

Obese asthmatics showed the highest proportion of EV miRNAs and lowest EVs size diameter. Differentially expressed miRNAs of obese asthmatics contributed to *EMT* function. *IFN signaling* and *viral infection pathways* were affected by miRNA clusters such as miR-2392, miR-106b, miR-424 and miR-6749. In contrast, a plenty of down- and up- regulated individual miRNAs were identified to target most of the candidate ISGs in CD4+ T cells. With the focus on down-regulated miRNAs, miR-148a and miR-665, which target *OAS2* and *EIF2AK2*, respectively, have been associated with asthma pathogenesis (Specjalski and Jassem 2019; Weidner et al. 2019; Pattarayan et al. 2018).

Taken together, ISGs of the *IFN signaling pathways* and their associated miRNAs play a crucial role in the underlying mechanism of low type-2 obesity-associated asthma and might be a target for biological and stratified therapies in the future.

Next steps, making it possible to learn also the regulatory mechanisms behind the observed transcriptional changes of blood CD4+ T cells and EVs miRNAs on the epigenetic landscape by examining specific histone modifications or DNA methylation

(Alashkar Alhamwe et al. 2020). This could be further expanded by studying miRNA levels directly in CD4+ T cells. Ultimately, proteomic analyses could follow from blood CD4+ T cells or from plasma EVs (Xu et al. 2020). Moreover, it would be worthwhile to validate the role of IFN signaling pathways *in vivo* using well-established HFD (high fat diet) and HDM mouse models. *In vitro* experiments testing inhibitor molecules that target some of the candidate ISGs or several members of the five miRNA clusters could be also a potential goal for future work.

As clearly shown at the level of CD4+ T cells transcriptome that obesity affects not only asthma disease, but rather leads to several individual asthma phenotypes with specific molecular features. Better understanding requires identification of highly specific biomarkers to which our studies made a substantial contribution.

Further, a mechanistic understanding of the underlying pathomechanisms will help to develop stratified therapy concepts that will optimally treat patients suffering from these specific asthma phenotypes.

7. Summary

In the evolution of personalized medicine and stratified therapies, comprehensive understanding of the molecular mechanisms underlying different disease phenotypes, such as asthma phenotypes including obesity-associated asthma, are urgently needed. Biological pathways and functions of differentially expressed genes and miRNAs can play an essential role in the development and severity of obesity-related asthma.

To fulfill this purpose, a preliminary work was initiated to establish optimal experimental conditions by investigating the effects related to different timings of CD4+ T cells processing from peripheral blood. Thus, 2 peripheral blood samples were drawn from each of 3 atopic patients and 3 healthy donors (12 samples), CD4+ T cells isolation was conducted from the first blood sample within two hours (Immediate) and from the second blood sample after 24 hours (Delayed), transcriptomic profiles of CD4+ T cells were examined using RNA-Seq analysis and readouts were verified on the epigenetic level through the H3K27ac ChIP-Seq analysis. After a successful establishment of ideal experimental conditions, peripheral blood was drawn from 10 obese, non-atopic asthmatic adults with a high body mass index (BMI; 36.67 ± 6.90), 10 non-obese, non-atopic asthmatic adults with normal BMI (23.88 ± 2.73), and 10 healthy controls with normal BMI (23.62 ± 3.74). All asthmatic patients were considered to have a low type-2 asthma phenotype according to blood eosinophils counts, FeNO and IgE levels criteria. Peripheral blood CD4+ T cells were isolated, mRNA sequencing was conducted. Moreover, plasma was also taken from the same blood samples of the same previous individuals, EVs were isolated, EVs RNA was extracted and small/microRNA-Seq was performed.

The transcriptomic profiles of delayed processed CD4+ T cells showed only 3 differentially expressed genes at FDR < 0.1 when comparing atopic with healthy individuals. CD4+ T cells of healthy donors were not harshly affected by the delayed ex vivo blood incubation, while a drastic change has been shown in CD4+ T cells of atopic patients following the delayed blood processing accompanying by downregulation atopy-related biological pathways such as IL-2 and IL-17 signaling pathways. Concordant results were observed on the epigenetic level through H3K27ac profiles. IFN signaling pathways dominated the CD4+ T cells responses solely in low type-2 obese asthmatics represented by upregulation of different ISGs such as IFITM3,

IFIT3, OAS2, OAS3, EIF2AK2, MX1, USP18, GBP3 genes, which correlated positively with lung function parameters including FEV1, FVC, VC max, TIFF, IC and PEF and negatively with the airway inflammation marker; FeNO. Viral infection pathways were also enriched for low type-2 obese asthmatics augmented by upregulation of different toll-like receptor genes such as TLR1, TLR-2, TLR-3, TLR-4, TLR-6, and TLR-8. Furthermore, obesity gene markers like IL15 and SOCS3 were also up-regulated in CD4+ T cells from obese asthmatics compared to both non-obese asthmatics and healthy controls. On the other hand, gap junction and GPCR ligand binding pathways were enriched in both low type-2 asthma groups. EVs miRNA clusters such as miR-2329 and miR-106b seemed to be assigned to IFN signaling and viral infection pathways, respectively, in low type-2 obese asthmatics. In addition, single Plasma EV downregulated miRNAs including miR-665, miR-4419b, miR-4769-3p, miR-6893-5p, miR-4743-3p, miR-6721-5p, miR-1207-5p, miR-6132, miR-4700-3p, miR-4731-5p, miR-5089-5p, miR-502-5p, miR-6088, miR-148a-5p and miR-373-3p seemed to target most of the enriched ISGs of IFN signaling pathway in CD4+ T cells.

In conclusion, the dominance of the IFN signaling pathways and their association with viral infection pathways in CD4+ T cells response could underpin the underlying molecular mechanism of low type-2 obesity-associated asthma. The IFN signaling pathway enriched ISGs, their associated miRNAs and other miRNA clusters might be a target for biological and stratified therapies for this unique asthma phenotype.

Zusammenhang

Im Zuge der Entwicklung der personalisierten Medizin und stratifizierter Therapien ist ein umfassendes Verständnis der molekularen Mechanismen, die verschiedenen Krankheitsphänotypen zugrunde liegen, wie z. B. dem Asthma-Phänotyp, einschließlich des mit Fettleibigkeit assoziierten Asthmas, dringend erforderlich. Biologische Pfade und Funktionen von unterschiedlich exprimierten Genen und miRNAs können eine wesentliche Rolle bei der Entwicklung und dem Schweregrad von fettleibigkeitsbedingtem Asthma spielen.

Um diesen Zweck zu erfüllen, wurde eine Vorarbeit durchgeführt, um optimale Versuchsbedingungen zu schaffen, in der die Auswirkungen verschiedener Zeitpunkte für die Verarbeitung von CD4+ T-Zellen aus peripherem Blut untersucht wurden. So

wurden jeweils zwei periphere Blutproben von drei Atopikern und drei gesunden Spendern (12 Proben) entnommen. CD4+ T-Zellen wurden aus der ersten Blutprobe innerhalb von zwei Stunden (sofort) und aus der zweiten Blutprobe nach 24 Stunden (verzögert) isoliert, transkriptomische Profile der CD4+ T-Zellen mittels RNA-Seq-Analyse untersucht und die Ergebnisse auf epigenetischer Ebene durch die H3K27ac ChIP-Seq-Analyse verifiziert. Nach erfolgreicher Etablierung idealer Versuchsbedingungen wurde peripheres Blut von 10 fettleibigen, nicht-atopischen asthmatischen Erwachsenen mit hohem Body-Mass-Index (BMI; 36,67 ± 6,90), 10 nicht fettleibigen, nicht-atopischen asthmatischen Erwachsenen mit normalem BMI (23,88 ± 2,73) und 10 gesunden Erwachsenen mit normalem BMI (23,62 ± 3,74) abgenommen. Alle Asthmapatienten wurden anhand der Kriterien Eosinophilenzahl im Blut, FeNO und IgE-Spiegel als Patienten mit einem niedrigen Typ-2-Asthma-Phänotyp eingestuft. CD4+ T-Zellen aus dem peripheren Blut wurden isoliert, und es wurde eine mRNA-Sequenzierung durchgeführt. Darüber hinaus wurde Plasma aus denselben Blutproben derselben Personen entnommen, extrazelluläre Vesikel (EVs) isoliert, EVs-RNA extrahiert und small/microRNA-Seq durchgeführt.

Die transkriptomischen Profile der verzögert prozessierten CD4+ T-Zellen zeigten beim Vergleich zwischen atopischen und gesunden Personen nur 3 differentiell exprimierte Gene bei FDR < 0.1. CD4+ T-Zellen gesunder Spender wurden durch die verzögerte Ex-vivo-Blutinkubation nicht stark beeinträchtigt, während bei CD4+ T-Zellen von Atopikern nach der verzögerten Blutverarbeitung eine drastische Veränderung festgestellt wurde. Diese zeigte eine Herunterregulierung von biologischen Signalwegen wie IL-2 und IL-17, welche mit Atopie assoziert sind.. Übereinstimmende Ergebnisse wurden auf epigenetischer Ebene durch H3K27ac-Profile beobachtet. IFN-Signalwege dominierten die CD4+ T-Zellen-Reaktionen ausschließlich bei adipösen Asthmatikern mit niedrigem Typ-2-Gehalt, was sich in einer Hochregulierung verschiedener IFN-stimulierte Gene (ISGs) wie IFITM3, IFIT3, OAS2, OAS3, EIF2AK2, MX1, USP18 und GBP3-Genen zeigte, die positiv mit Lungenfunktionsparametern wie FEV1, FVC, VC max, TIFF, IC und PEF und negativ mit dem Atemwegsentzündungsmarker FeNO korrelierten. Virale Infektionswege waren bei adipösen Asthmatikern mit niedrigem Typ-2-Gehalt ebenfalls angereichert, was durch die Hochregulierung verschiedener Toll-like-Rezeptor-Gene wie TLR1, TLR-2, TLR-3, TLR-4, TLR-6 und TLR-8 verstärkt wurde. Darüber hinaus wurden auch Adipositas-Genmarker wie IL-15 und SOCS3 in CD4+ T-Zellen von adipösen

Asthmatikern im Vergleich zu nicht adipösen Asthmatikern und gesunden Kontrollpersonen hochreguliert. Andererseits waren Gap Junction und GPCR-Ligandenbindungswege in beiden Gruppen mit niedrigem Typ-2-Asthma angereichert. EVs miRNA-Cluster wie miR-2329 und miR-106b schienen bei adipösen Asthmatikern mit niedrigem Typ-2-Asthma den IFN-Signalwegen bzw. den Virusinfektionswegen zugeordnet zu sein. Darüber hinaus wurden einzelne Plasma EV herunterregulierte miRNAs wie miR-665, miR-4419b, miR-4769-3p, miR-6893-5p, miR-4743-3p, miR-6721-5p, miR-1207-5p, miR-6132, miR-4700-3p, miR-4731-5p, miR-5089-5p, miR-502-5p, miR-6088, miR-148a-5p und miR-373-3p schienen die meisten der angereicherten ISGs des IFN-Signalwegs in CD4+ T-Zellen anzugreifen.

Zusammenfassend lässt sich sagen, dass die Dominanz der IFN-Signalwege und ihre Assoziation mit viralen Infektionswegen in der Immunantwort von CD4+ T-Zellen, den zugrunde liegenden molekularen Mechanismus des mit Fettleibigkeit assoziierten Asthmas untermauern könnte. Die angereicherten ISGs in IFN-Signalwegen, ihre zugehörigen miRNAs und andere miRNA-Cluster könnten ein Ziel für biologische und stratifizierte Therapien für diesen einzigartigen Asthma-Phänotyp sein.

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10. Verzeichnis der akademischen Lehrer

Philipps Marburg Universität

Meine akademischen Lehrer waren die Damen und Herren:

Holger Garn, Daniel P. Potaczek, Ho-Ryo Chung, Clemens Thölken, Timm Greulich, Harald Renz, Till Adhikary and Hani Harb.

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