INVESTIGATION OF THE GENETIC BACKGROUND OF CHILDHOOD ASTHMA AND ALLERGY

PhD Thesis

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List of Abbreviations

ACT	Actin-like protein
ADAM33	A disintegrin and metalloproteinase domain-containing protein 33
ADAMTS12	ADAM metallopeptidase with thrombospondin type 1 motif 12
ADRB2	β2 adrenergic receptor
Ang1/2	Angiopoietin 1/2
ANKRD5	Ankyrin repeat domain 5
BCA	Bicinchoninic acid assay
BDP	Beclomethasone dipropionate
BIRC5	Baculoviral IAP repeat containing 5
BN-BMLA	Bayesian network based Bayesian multilevel analysis of relevance
CD14	Cluster of differentiation 14
CHML	Choroideremia-like protein
CI	Confidence interval
CNV	Copy number variation
CO_2	Carbon dioxide
COPD	Chronic obstructive pulmonary disease
CTLA4	Cytotoxic T lymphocyte associated protein 4
CYFIP2	Cytoplasmic FMR1 interacting protein 2
CYSLTR2	Cysteinyl leukotriene receptor 2
DAG	Directed acyclic graph
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPP10	Dipeptidyl peptidase 10
DZ	Dizygotic twins
ECL	Electrochemiluminescence
ECM	Extra cellular matrix
ELISA	Enzyme-linked immunosorbent assay
eQTL	Expression quantitative trait locus
EQTN	Equatorin
ESR1	Estrogen receptor 1
FAT4	FAT atypical cadherin 4

FBS	Foetal bovine serum
FCERIA	Gene for high affinity IgE receptor I α
FcεRII	Low-affinity IgE receptor II
FDA	Food and Drug Administration
FDR	False discovery rate
FENO	Fractional exhaled nitric oxide
FEV1	Forced expiratory volume in 1 second
FRMD6	FERM-domain containing 6
FVC	Forced vital capacity
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GBD	Global Burden of Disease Study
GINA	Global Initiative for Asthma Guidelines
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GP	General practitioner
GPR154	G-protein coupled receptor PGR14
GPRA	G-protein coupled receptor for asthma susceptibility
GSTP1	Glutathione S-transferase pi 1
GWAS	Genome-wide association study
H1	Histamine receptor 1
H2	Histamine receptor 2
HDAC	Histone deacetylase
HLA-DQB1	Human leukocyte antigen DQ β 1
HLA-G	Human leukocyte antigen G
HRP	Horseradish peroxidase
HWE	Hardy-Weinberg Equilibrium
IAP	Inhibitor of apoptosis
ICS	Inhaled corticosteroid
IFNγ	Interferon-γ
IFNA	Gene for interferon alpha
IFNG	Gene for interferon gamma
Ig	Immunoglobulin
IL	Interleukin
IL18R1	Interleukin 18 receptor 1

IL7R	Interleukin 7 receptor
IRAK3	Interleukin 1 receptor associated kinase 3
ISAAC	International Study of Asthma and Allergies in Childhood
ITLN1	Intelectin-1
JAG1	Jagged 1
JNK	Janus kinase
KLF15	Kruppel Like Factor 15
KASP	KBioscience Competitive Allele-Specific PCR
LABA	Long-acting beta agonist
LATS1/2	Large tumour suppressor kinase 1/2
LD	Linkage Equilibrium
LIFR	Leukaemia inhibitory factor receptor α
LTA	Lymphotoxin α
MAF	Minor Allele Frequency
MOB1	MOB kinase activator 1
mRNA	Messenger RNA
MS4A2	Membrane spanning 4-domains A2
MST1/2	Mammalian STE20-like protein kinase 1/2
MZ	Monozygotic twins
NAT2	N-acetyltransferase 2
NGS	Next-generation sequencing
NRP1/2	Neurophilin-1/2
NSAID	Non-steroid anti-inflammatory drug
OPN3	Opsin 3
OR	Odds ratio
ORMDL3	Orosomucoid-like 3
OVA	Ovalbumin
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDZ	Post synaptic density protein, Drosophila disc large tumour suppressor, Zonula occludens-1 protein
PGF	Placental growth factor
PGF2α	Prostaglandin F2α

PHF11	PHD Finger Protein 11
PPARGC1B	Peroxisome proliferator-activated receptor gamma coactivator 1-beta
PTGER4	Prostaglandin E receptor 4
PVDF	Polyvinylidene fluoride
Ras	Ras superfamily
Rho	Rho family of GTPases
RIPA buffer	Radioimmunoprecipitation assay buffer
RLT buffer	Guanidine thiocyanate buffer
RNA	Ribonucleic acid
ROS	Reactive oxygen species
rs#	SNP reference number
RT-PCR	Reverse transcriptase PCR
SABA	Short-acting beta agonist
SAV1	Salvador family WW domain containing protein 1
SCIN	Scinderin
SD	Standard deviation
SFRS8	Splicing factor, Arginine/Serine-rich 8
SH3	SRC homology 3
SMAD	SMAD family members
SNP	Single-nucleotide polymorphism
TAD	Transcriptional activation domain
TAZ	Tafazzin
TB	Transcriptional enhancer factor-binding
TCR	T cell receptor
TEAD	TEA domain containing protein
TEK	Gene for tyrosine-protein kinase receptor 2
TFF1	Trefoil Factor 1
Th1	T helper type 1 lymphocyte
Th2	T helper type 2 lymphocyte
Tie2	Tyrosine-protein kinase receptor 2
TLE4	Transducin like enhancer of split 4
TLR9	Toll-like receptor 9
TNFα	Tumour necrosis factor α

TP73	Tumour protein p73
UTR	Untranslated region
VDR	Vitamin D receptor
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
WGS	Whole genome sequencing
YAP1	Yes-associated protein 1
YM155	Sepantronium Bromide
ZFR3	Zinc finger RNA binding protein 3

1. Introduction

1.1. Asthma and Allergy

1.1.1. General Characteristics

Asthma is a chronic inflammatory respiratory disease influenced by a wide range of environmental and genetic factors (Chen, Wong, and Li 2016). It is characterized by airflow obstruction due to smooth muscle constrictions and airway inflammation with symptoms such as coughing, wheezing, tightness in the chest, bronchoconstriction and airway hyperresponsiveness that may remit spontaneously or upon treatment. Further, long term inflammation leads to mostly irreversible structural and functional changes in the airway smooth muscles called airway remodelling that is characterized by bronchial wall thickening and increased vascularity, sub-mucosal gland hyperplasia and hypertrophy as well as extracellular matrix (ECM) deposition and angiogenesis ('New NHLBI guidelines for the diagnosis and management of asthma. National Heart, Lung and Blood Institute', 1997) (Figure 1).

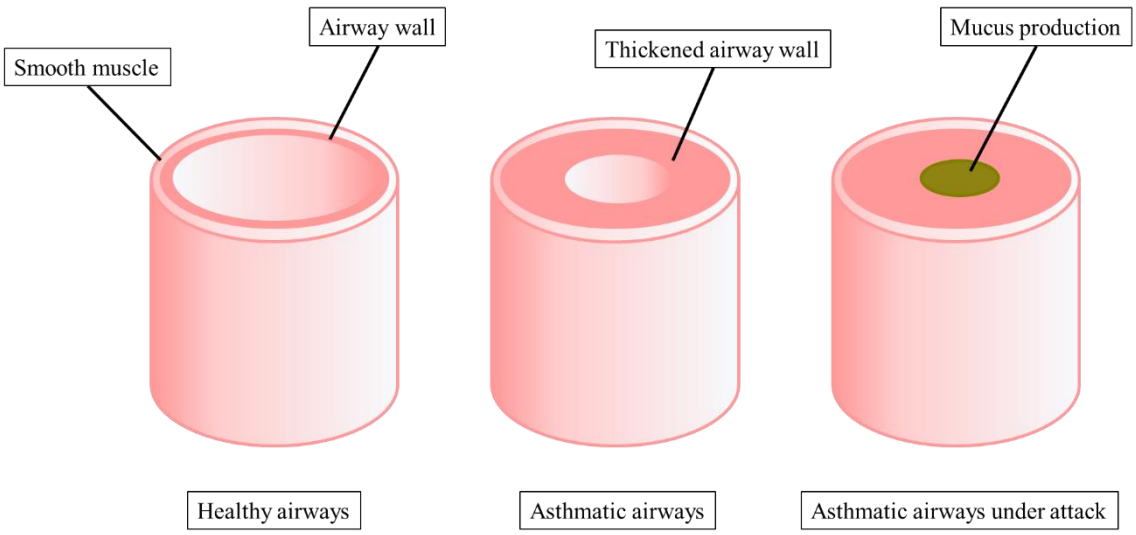

***Figure 1**. Schematic diagram of healthy and asthmatic airways.*

Asthma exacerbations may be caused by different environmental triggers. These factors, among others, may be grouped into indoor and outdoor stimuli, where indoor factors include allergens of dust mites, cockroaches, mice and pets, indoor burning of tobacco, wood and biomass, indoor endotoxins or products from Gram-positive bacteria. Outdoor factors include viral and microbial pathogens, airborne particles, ozone, diesel exhaust particles, pollens, outdoor moulds, tobacco smoke, cold air or humidity.

Environmental stimuli also include exercise, occupation or even diet (Ho 2010; Diette et al. 2008).

1.1.2. Classification of Asthma and Allergy

Based on which cause initiated asthma or the exacerbation, asthma phenotypes can be distinguished. These include allergic-, non-allergic asthma, viral-induced asthma, exercise-induced asthma. Within the limits of this thesis, we have included these four groups in our analyses and have created subgroups for these asthma phenotypes with the help of respiratory specialists (Figure 2). Other asthma types can also be characterized for example aspirin-exacerbated respiratory disease that is a combination of asthma, chronic rhinosinusitis with nasal polyps and a sensitivity to aspirin or other types of non-steroid anti-inflammatory drugs (NSAIDs), pre-asthma wheezing in infants where recurrent episodes of the abnormality is likely due to asthma (Martinez et al. 1995), but other reasons may exist such as allergies, infection or obstructive sleep apnea. Furthermore, there is exacerbation-prone asthma with more frequent visits to the hospital due to recurrent asthma attacks, and asthma associated with apparent irreversible airflow limitation, where irreversibility may only be defined based on longitudinal studies, a progressive development of airway obstruction and treatment irresponsiveness (Pascual and Peters 2009), as well as eosinophilic and neutrophilic asthma (Bruijnzeel, Uddin, and Koenderman 2015; Patterson, Borish, and Kennedy 2015; Pelaia et al. 2015).

We have added subgroups to the pre-existing ones included in our analyses, for example asthma comorbidities of allergic rhinitis and allergic conjunctivitis, as these are the most frequent asthma associated allergic diseases which often occur together (Shaker and Salcone 2016; Rosario and Bielory 2011; Lee et al. 2013). Conjunctivitis is an inflammatory disease of the eye characterized by flushing, swelling, itching, and watering of the eyes whereas rhinitis is an inflammation of the nasal mucosal surface indicated by sneezing, a runny and/or stuffy nose, and post-nasal dripping. In different studies, between 50-65% of patients with rhinitis also had conjunctivitis, but conjunctivitis could also exist without rhinitis (Rosario and Bielory 2011). Our subgroups also include clinical parameters of asthma such as total IgE level and absolute eosinophil concentrations, which have been found to correlate with asthma severity. In children, the most frequent phenotype is the IgE mediated allergic asthma which can also have heterogeneous symptoms.

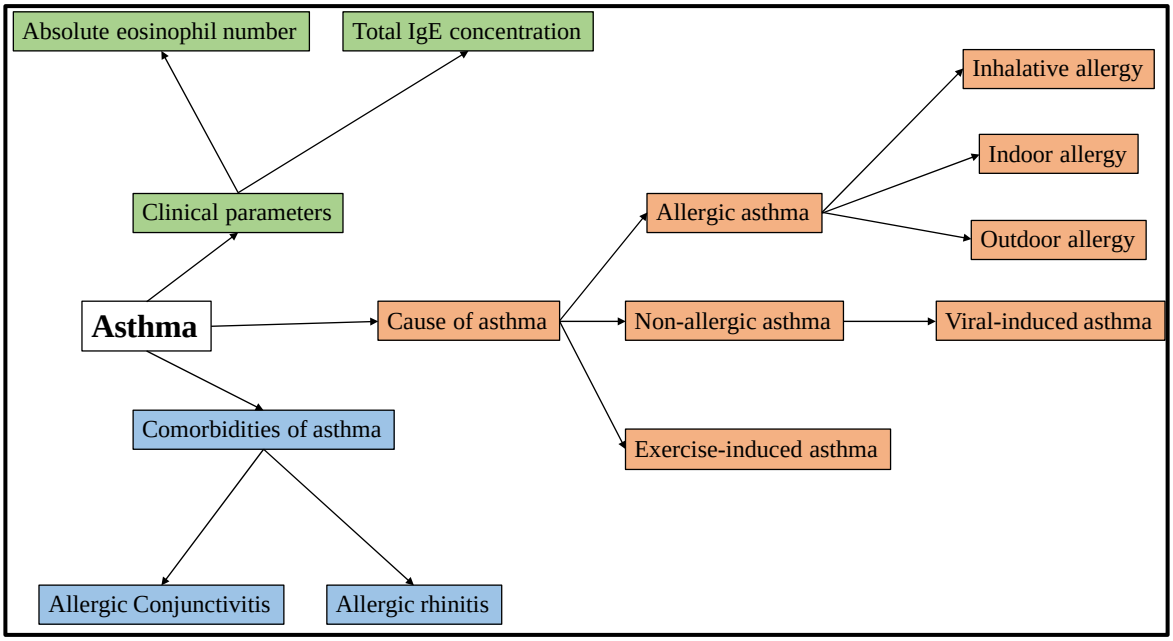

***Figure 2**. Subgroups of asthma phenotypes included in our analyses. Asthma phenotypes may overlap.*

1.1.3. Diagnostic Criteria

It is well-known that asthma is not a single disease but rather a series of overlapping individual diseases or phenotypes, each defined by its unique interaction between genetic and environmental factors (Lötvall et al. 2011; Borish and Culp 2008). Moreover, non-asthmatic disease symptoms may also overlap with asthma. Diagnosis of the disorder may therefore be difficult, but crucial in terms of the therapy applied, morbidity and mortality. There are guidelines, such as The Global Strategy for Asthma Management and Prevention 2015 report update or the National Institutes of Health Guidelines for the Diagnosis and Management of Asthma Expert Panel Report-3, for an easier diagnosis (Global Initiative for Asthma; National Asthma Education and Prevention Program 2007). The detailed history of symptoms and a physical exam aids diagnosis of asthma subtypes. Measurements of forced expiratory volume in 1s (FEV1) and forced vital capacity (FVC) and especially their ratio, FEV1/FVC are good indicators of airflow obstruction. Specialists also examine diffusing capacity or lung volumes and may apply Broncho provocation (Global Initiative for Asthma; National Asthma Education and Prevention Program 2007).

1.1.4. Pathogenesis

It is important to understand the pathophysiology of asthma which has still not been fully elucidated. The description of the course of the disease goes beyond the scope of this paper, therefore, here I only summarize the main aspects of asthma pathogenesis.

Asthma is a chronic inflammatory disorder, where many cells and elements of the immune response play a role in its pathogenesis. Once the body encounters an allergen, virus or a noxious agent the immune system will be activated and in genetically susceptible individuals will over-react. In allergic asthma, dendritic cells, that are antigen-presenting cells, encounter the allergen and migrate to lymph nodes to present the peptide to naïve T lymphocytes that will be activated to mature into T helper 2 (Th2) cells with the aid of other regulatory cells (Kuipers and Lambrecht 2004). T lymphocyte subpopulations among others, include Th1 and Th2 cells with distinctive cytokine profiles that include interleukin-12 (IL-12), interferon-γ (IFNγ) and IL-4, -5, -9 and -13, respectively. There are several factors that determine the Th1/Th2 balance. According to the 'hygiene hypothesis' the Th1/Th2 balance may be skewed towards the cytokine profile of Th2 cells in newborns. This imbalance is usually lifted by infections, the presence of older siblings, rural environment or daycare attendance at an early age, that all entail a Th1 response. On the other hand, urban environment, the use of antibiotics or sensitization to diverse allergens do not involve Th1 cytokines, hence the early imbalance remains making the individual more susceptible to allergies, asthma or other chronic inflammatory diseases (Sears et al. 2003; Horwood, Fergusson, and Shannon 1985; Gern, Lemanske, and Busse 1999; Gern and Busse 2002; Eder, Ege, and von Mutius 2006).

The release of Th2 cytokines activates a cascade of events that lead to airway inflammation and in the long run, airway remodelling. IL-4 aids the differentiation of Th2 cells and along with IL-13 they play a role in the formation of IgE immunoglobulins through the induction of class-switching of B-lymphocytes, hence IgE receptors will be produced once they have become plasma cells. IgE receptors are important actors in hypersensitivity type I and diseases such as allergic asthma, atopic diseases or allergic conjunctivitis. IL-5 and granulocyte-macrophage colony-stimulating factor (GM-CSF) help the maturation of eosinophil granulocytes in the bone marrow and after infiltration to the inflamed airways their prolonged survival, respectively. Furthermore, tumour necrosis factor-α (TNF-α) further enhances the inflammatory processes in the lungs (Chung and Barnes 1999).

Beside eosinophil infiltration, other immune cells, such as neutrophils, macrophages or mast cells also transmigrate into the airways. Eosinophils have increased numbers in asthmatic airways. By releasing pro-inflammatory mediators and cytokines, they contribute to the inflammatory response. It has been shown that higher numbers of eosinophils correlate with asthma severity. Mast cells play a critical role in the pathogenesis of allergic diseases, as having many IgE receptors on their surface allows these immunoglobulins to be physically cross-linked by allergens, hence degranulation of the mast cells begin, which then empty bronchoconstrictors, such as histamine, leukotrienes or prostaglandins into the surrounding tissues (Boyce 2003; Robinson 2004). Histamine mediates oedema and mucus secretion as well via its histamine receptors 1 (H1) and 2 (H2), respectively (White 1990). Leukotrienes not only influence airway smooth muscle, but also recruit neutrophils (Gelfand and Dakhama 2006). Among several types of prostaglandins, PGF2α causes direct constriction of airway smooth muscles. It has been shown that upon PGF2α treatment asthmatics had an 8000-fold increase in sensitivity to it compared to healthy subjects (Mathé et al. 1973). It has been suggested that airway hyperresponsiveness also has a relation to the increased numbers of mast cells found in the airway smooth muscle. Further, mast cells not only release cytokines upon allergen contact, but in exercise-induced asthma they may also be activated by osmotic changes (Brightling et al. 2002). Macrophages may be activated by IgE receptors as well, releasing more inflammatory mediators and other cytokines enhancing the inflammatory response (Peters-Golden 2004). The role of neutrophils remains unclear in the pathogenesis of allergic diseases, but elevated numbers have been found in the airways of more severe asthmatics (Fahy et al. 1995; Wenzel 2006; Wenzel et al. 1997).

Epithelial cells of the airway also play a role in asthma. These cells lining the airways have a barrier function and they also maintain tissue homeostasis (Moheimani et al. 2016). By releasing more pro-inflammatory mediators during the inflammatory processes in asthma, epithelial cells may also suffer injury. Repair mechanisms in asthmatic patients are impaired, further worsening the controlled state of asthma.

Oxidative stress also has an effect on the bronchial epithelium in asthma. Oxidative stress is the imbalance between the production of increased oxidative sources and the impaired mechanisms of detoxifying the reactive intermediates and repairing the caused damage (Holguin 2013). Reactive oxygen species (ROS) are produced either upon environmental exposure to air pollution of gases and particulate matter or the local inflammation will secondarily induce the production of ROS (Bowler 2004; Ghio,

Carraway, and Madden 2012). Oxidative stress is associated with inflammatory cell activation and hence the production of pro-inflammatory mediators (Paredi, Kharitonov, and Barnes 2002; Wood, Gibson, and Garg 2003). The increased amount of ROS results in oxidative lipid peroxidation and DNA damage, further aggravating inflammation and the severity of asthma.

1.1.5. Medication

To date there is no cure for asthma. It is a very complex disease with many factors, pathways, mechanisms that play a role in the pathophysiology of asthma. Which medication an asthmatic individual will take depends on age, what triggered their asthma, symptoms and whether the drug is effective. It is important for patients to have controlled asthma regardless of the severity of their disease. There are several types of long-term and short-term asthmatic medications. Long-term medications help to maintain a controlled asthmatic state on an everyday basis so that the incidence of an asthma attack is lower, while short-term drugs are a quick relief in case of such an attack.

The most essential drug among long-term medications is inhaled corticosteroids (ICS), which are anti-inflammatory medicines (e.g. Budesonide (Pulmicort), Fluticasone (Flovent)). ICSs reduce airway inflammation by down-regulating pro-inflammatory proteins (Adcock, Ito, and Barnes 2004; De Bosscher, Vanden Berghe, and Haegeman 2003), reversing components of airway remodelling, such as increased vascularity of the bronchial wall (Chanez et al. 2004), suppressing the production of chemotactic mediators and adhesion molecules that attract immune cells to the site of inflammation (eosinophils, dendritic cells, mast cells, lymphocytes) and also by inhibiting their survival (Schwiebert, Stellato, and Schleimer 1996). ICSs are better than orally taken corticosteroids, because they locally treat inflammation, rather than causing side-effects. Leukotriene modifiers are also effective oral anti-inflammatory drugs (e.g. Zafirlukast (Accolate), Montelukast (Singulair)), but in some cases, may cause side-effects of depression, aggression or agitation. Long-acting beta agonists (LABAs) (e.g. Salmeterol (Serevent), Formoterol (Foradil)) are inhaled drugs taken with ICSs enhancing their effects by suppressing inflammatory genes (Korn et al. 1998) and increasing the localization of glucocorticoid receptors in the nucleus for a better uptake of the medication (Eickelberg et al. 1999). On the other hand, taken alone, LABAs may increase the risk of severe asthma attacks. Theophylline, also a bronchodilator and anti-inflammatory drug is used in low doses next

to ICSs. Theophylline increases the activity of histone deacetylase (HDAC), which in turn reduces the number of eosinophils. Because ICSs activate HDAC through a different mechanism, it has been suggested that the low dose of theophylline enhances the anti-inflammatory effect of ICSs both in asthma and chronic obstructive pulmonary disease (COPD) (Cosio et al. 2004; Hossny et al. 2016).

Short-term asthma medications include short-acting beta agonists (SABAs) which offer ease of symptoms within minutes once inhaled through a nebulizer directly to the airways (e.g. Metaproterenol, Levalbuterol (Xopenex)). Orally or intravenously taken corticosteroids upon an asthma attack are also very effective in treating episodes of asthma attacks. In such case, the most used corticosteroids are prednisone, prednisolone or methylprednisolone, which should not be taken for long periods of time as may cause side-effects of weakness, weight gain, mood or behaviour changes, etc.

There are of course many approaches to target different factors in asthma that lead to a decrease in the inflammation in the airways. For instance, omalizumab is a humanized antibody (IgG1k) against IgE antibodies, one of the key players in asthma pathogenesis. It has been approved in the 2000s in the United States by the Food and Drug Administration (FDA), as well as in the European Union to treat patients 12 years-of-age or older (Allergic Asthma and CIU Treatment | XOLAIR® (Omalizumab)). It is used in cases of corticosteroid resistance, but due to its higher price and only a few long-term trial studies, it is not yet frequently used nor it is administered for longer time-periods (Chang et al. 2007; Humbert et al. 2014; Normansell et al. 2014; Schulman 2001). Furthermore, several drugs have been developed to target cytokines IL-5, IL-4 or IL-13 with antibody therapy. All of these medications are only effective in eosinophilic asthma phenotypes, but unfortunately minute non-eosinophilic asthma biomarkers are available to use in the search for potent therapies (Guilleminault et al. 2017).

1.2. Epidemiology of Asthma

It is well known that there is an increasing number of persons with asthma and/or allergies. The higher numbers of patients burden the countries both financially and socially. Many factors must be included in order to gain a complete knowledge of the burden of the disease. These include the number of adult and child patients in a country, the number of hospital visits, the cost of their treatment, morbidity (e.g. number of days missed from school or place of work/year) or mortality.

According to the Global Asthma Report 2014 that is based on the Global Burden of Disease Study (GBD) between 2008 and 2010 it has been estimated that around 334 million people suffer from asthma world-wide. Furthermore, there was an increase in the number of asthmatics according to the 2011 report based on 2000-2002 population data, where 235 million people had asthma globally (The Global Asthma Report 2014). This increase may perhaps be due to better and more precise diagnosis of asthma phenotypes (Carr and Bleecker 2016). Moreover, it is estimated that due to incomplete data, these numbers may be higher. The International Study of Asthma and Allergies in Childhood (ISAAC) estimated that around 14% of all the children in the world have asthma each year (Lai et al. 2009). The GBD study found that asthma is the fourteenth in number of years lost to asthma-associated morbidity and mortality world-wide (Walter et al. 2015).

In the USA in 2013 it was predicted that 16.5 million adults (8.3% of population) and 6.1 million children (7% of population) have asthma. The disease is more prevalent in women compared to men, also more common in children, than in adults, furthermore, it is more frequent in boys compared to girls. Asthma was the leading cause of absenteeism in children, where 50% of children missed school for at least 1 day every year, in 2013 this meant 13.8 million missed school days. Asthma morbidity is similar in adults, as 14 million days of work is missed each year. Although in 2014 3651 people died from asthma in the USA, death rates have decreased by 26% since 1999 (https://www.cdc.gov/asthma/pdfs/asthma_facts_program_grantees.pdf, https://www.cdc.gov/asthma/most_recent_data.htm).

The cost of asthma in the USA is $56 billion/year, which means a $3259 per person. The yearly cost of the disease in Europe is €19.3 billion (https://www.cdc.gov/asthma/impacts_nation/), whereas in Asia-Pacific the cost varies between $184 and $1189 per person (www.globalasthmareport.org/burden/burden.php).

The Bulletin of the Hungarian Korányi Pulmonology Institute summarized the epidemiology of several pulmonology related diseases including asthma. According to the study, in which information of registered asthma patients were collected from all Hungarian dispensaries, in 2013, 282 754 people were affected by asthma, which means a 2.8% prevalence in Hungary (Csoma et al. 2016). The yearly increase in number of asthmatic adults is 16 000 - 19 000 (Figure 3).

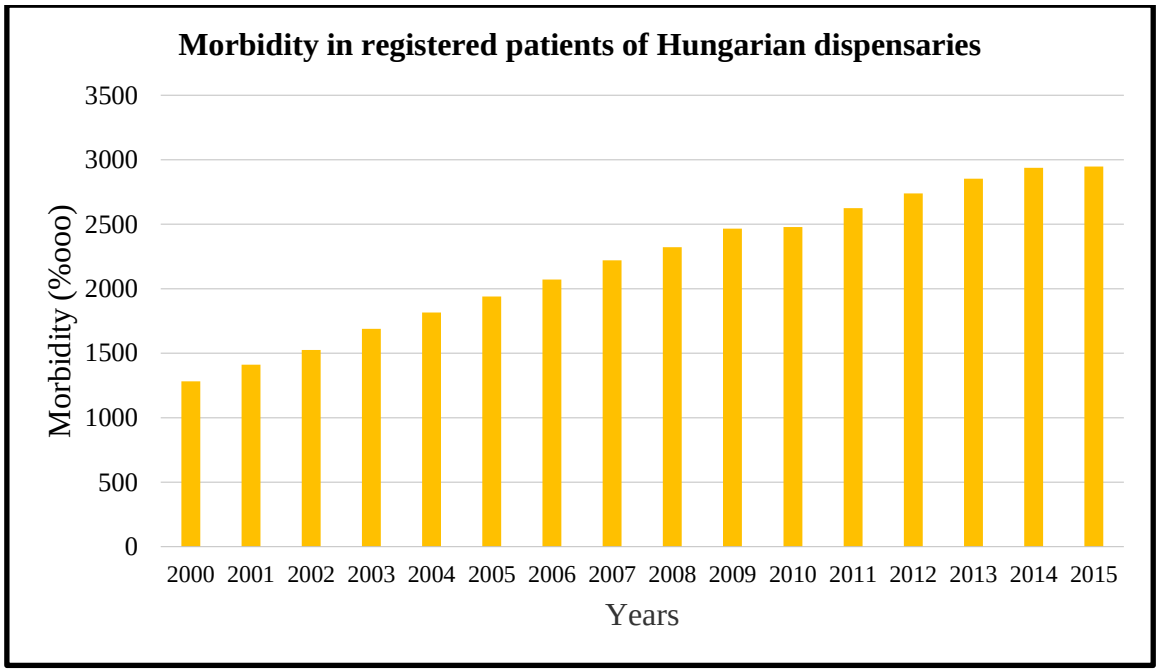

***Figure 3**. Diagram showing morbidity rates of asthma patients collected from all Hungarian dispensaries. (Csoma et al. 2016)*

1.3. Genetic Background

1.3.1. Genetic Methods and Results in the Research of Asthma and Allergy

Asthma is a multifactorial disorder, considering that apart from several small effect genes and variations that may also be inherited in an additive fashion, environmental factors also play a key role in the development of such diseases. With the rapid advances in genetics and genetic technologies, the amount of research of complex disorders have become substantial, however, our understanding of the mechanisms of their inheritance is very limited.

The heritability of asthma is estimated by the comparisons of correlations and concordance rates of monozygotic (MZ) and dizygotic (DZ) twins. According to twin studies, 36-77% of asthma is heritable (Duffy et al. 1990; Harris et al. 1997; Koppelman, Los, and Postma 1999; Nieminen, Kaprio, and Koskenvuo 1991), where the concordance of MZ twins is 0.74 and 0.35 of DZ twins, presenting a significant difference (Liu, Spahn, and Leung 2011). Furthermore, when neither of the parents have asthma, the likelihood of the children having the disease is 11-13%, but when both parents have asthma, this rate is 50-70% (Barnes and Marsh 1998), also showing the importance of the genetic component of asthma.

Essentially, there are three different types of genetic studies to elucidate the genetic background of complex diseases. These include candidate gene association studies, genome-wide association studies (GWASs) and whole genome sequencing (WGS).

Candidate gene association studies analyse the relationship of a prespecified gene or genetic region and a given disease or phenotype by comparing variation frequencies between cases and controls. The previously determined genes are chosen based on the protein's function or role in the disease. There are more than 1000 candidate gene association studies of asthma, from which 120 genes have been identified to have a relationship with the disorder (March, Sleiman, and Hakonarson 2011). There are 54 genes found to be associated with asthma that were reproduced in 2-5 independent studies. Fifteen genes were found to be associated with the disease in 6-10 independent studies, whereas only 10 were found in more than 10 independent investigations. These ten, most likely to indeed have a functional role in asthma, include *IL-4*, *IL-13*, β2 adrenergic receptor (*ADRB2*), major histocompatibility complex, class II, DQ β 1 (*HLA-DQB1*), *TNFα*, lymphotoxin α (*LTA*), high affinity IgE receptor (*FCERIA*), IL-4 receptor (*IL4R*), *CD14*, and a disintegrin and metalloproteinase domain-containing protein 33 (*ADAM33*) (Basehore et al. 2004; Haller et al. 2009; Howard et al. 2002; Kabesch et al. 2006; Liggett 1995; Munthe-Kaas et al. 2008; Potaczek, Okumura, and Nishiyama 2009; Potter et al. 1993; Pykäläinen et al. 2005; Randolph et al. 2004; Suttner et al. 2009; Van Eerdewegh et al. 2002; Vladich et al. 2005; Wu et al. 2010; Zhou et al. 2009).

Unfortunately, candidate gene association studies cannot give a full picture of the complex genetic background of diseases such as asthma, because multifactorial diseases develop through many inherited genetic variations that also influence each other, while environmental factors also contribute to the formation of the disorder. This problem was partially solved by WGS, which is a hypothesis-free research tool examining hundreds of microsatellite markers in affected siblings. Thus, many 10-20 million base pair regions have been identified to contain candidate genes for asthma and atopy. These regions need to be restricted to find the disease susceptibility genes or variations, which is done by positional cloning. This technique begins with taking a linked marker in a region and depicting the area in its proximity by several levels of recombination. This task was daunting until the results of the Human Genome Project were available to search on a computer, for a much easier identification of variations in a given region on the DNA

(Jorde, Carey, and Bamshad 2010). WGS and positional cloning have identified the following among others. *ADAM33*, dipeptidyl peptidase 10 (*DPP10*), G-protein coupled receptor for asthma susceptibility (*GPRA*), human leukocyte antigen G (*HLA-G*), cytoplasmic FMR1 interacting protein 2 (*CYFIP2*) or orosomucoid-like 3 (*ORMDL3*) (Allen et al. 2003; Laitinen et al. 2004; Moffatt et al. 2007; Nicolae et al. 2005; Noguchi et al. 2005; Van Eerdewegh et al. 2002).

Technical advances in genetics, such as microarrays or next-generation sequencing (NGS) have led to the evolution of GWASs. These case-control studies allow millions of markers (single nucleotide polymorphisms (SNPs), or copy number variations, (CNVs)) to be investigated in large populations with a given phenotype (Jorde, Carey, and Bamshad 2010). Many genes and regions have been identified with high-throughput GWASs (Table 1). On the other hand, GWASs also faced a problem of 'missing heritability'. Researchers expected previously identified susceptibility genes with a strong cumulative effect to be replicated during the association study, however the results could not be reproduced. In this regard, the analysis of GWAS results is essential, as well as the development of new techniques for the identification of genetics regions, candidate genes and variations.

Table 1. *Asthma or asthma-associated phenotype susceptibility genes and regions. Adapted from Gu and Zhao, 2011 (Gu and Zhao. 2011).*

Chromo-some	Locus	Candidate genes
1	1p36,1qter,1q23	*FCER1A, OPN3, CHML, IL10*
2	2q14,2q32,2q33,2p	*DPP10, IL18R1, CTLA4, CD28*
3	3q21-q22,3q21.3,3p	*TLR9*
5	5q31-q33,5q31,5p13,5p15,5q23.3	*IL4, IL9, ZFR3, LIFR, PTGER4, ADAMTS12, IL7R*
6	6p21,6q24-q25,6q25.3	*HLAG, ESR1, TNF*
7	7p14-p15,7q	*GPR154*
8	8p23.3-23.2	*NAT2*
9	9p1,9p21,9p22	*TLE4, IFNA*
11	11q13,11q21,11q,11p14	*MS4A2, GSTP1*
12	12q13.12-q23.3,12q13-12q24,12q21,12q24.31,12q24.33	*SFRS8, CD45, IFNG, IRAK3, VDR*
13	13q14,13q	*PHF11, CYSLTR2*
14	14q11.2,14q13-q23,14q24,14q23	*TCR, ACT*
17	17q21	*ORMDL3*
19	19q13,19q13.3	*FCER2*
20	20q13,20p12	*ADAM33, JAG1, ANKRD5*
21	21p21	-
x	Xp, Xq	-

1.3.2. Apoptosis in Asthma

It is well-known, that apoptosis is a key feature in the pathomechanism of asthma (Vignola et al. 1999). The most reviewed process is eosinophil-clearance, which is impaired in asthmatic patients, hence the high numbers of eosinophils accumulated in the bronchial tissues will neither go through apoptosis, nor be cleared by phagocytosis of macrophages (Kankaanranta et al. 2000; Walsh 2000; Woolley et al. 1996). Additionally, it has been shown that the lack of eosinophil apoptosis in asthmatics correlates with disease severity (Duncan et al. 2003).

The balance between cell apoptosis and survival depends on the control and maintenance of different regulatory elements and pathways. For instance, the members of the inhibitor of apoptosis protein (IAP) family not only inhibit apoptotic pathways in a caspase-dependent manner, they also play a role in the regulation of cell cycle and cell division, proving that such regulatory elements have a much more complex function than

initially thought. Furthermore, the aberrant expression of the members of this protein family results in pathologic cell functioning and uncontrolled cell division (Altieri 2010).

Baculoviral IAP repeat containing 5 (BIRC5), also called survivin is an important anti-apoptotic member of the IAP family. BIRC5 has been previously thought to be only expressed in foetal tissues during growth. Moreover, it is abnormally expressed in cancerous tissues, hence being a featured target of therapeutic research (Altieri 2010). Recently, it has been shown that BIRC5 has additional roles in inflammatory mechanisms and disorders, such as asthma (Altznauer et al. 2004; Valentin et al. 2009; Vassina et al. 2006). Furthermore, our research group has found several important aspects of BIRC5 in asthma. Namely, that the mRNA level of *Birc5* in ovalbumin (OVA) induced asthmatic mouse model was significantly increased compared to normal mice (fold-change of 5.94, p=0.001) (Tölgyesi et al. 2009). This result was replicated by Tumes et al, who also found that in mice, the mRNA and protein expression of Birc5 found in the bronchoalveolar fluid correlated with the number of eosinophils (Tumes, Connolly, and Dent 2009). Our research group has further shown, that the gene expression level of *BIRC5* was significantly higher in asthmatic patients compared to healthy controls, and both the gene expression level and one of the studied variations, rs9904341, were significantly correlated with the eosinophil ratio found in the induced sputum of asthmatics (Ungvári et al. 2012a).

Furthermore, our group's previous results have shown that the gene expression of FERM-domain containing 6 (*FRMD6*) is significantly decreased in both the OVA-induced mouse model, as well as asthmatic patients compared to controls. Additionally, a gene polymorphism has been shown to be associated with asthma verified by both frequentist and Bayesian statistical approaches (Ungvári et al. 2012b). FRMD6 is the upstream activator of the Hippo signalling pathway, which also regulates the expression of several proteins, such as BIRC5 (Ungvári et al. 2012a; Ungvári et al. 2012b).

The Hippo pathway is highly conserved from *Drosophila melanogaster* to mammals and regulates organ size via promoting apoptosis and inhibiting cell proliferation in the embryonic stages of development (Huang et al. 2016; Yu, Zhao, and Guan 2015). Its name originates from the *Drosophila* Hippo protein kinase (Hpo), which produces tissue overgrowth or „hippopotamus-like" phenotype upon mutations in its coding gene. It is still not exactly known whether the pathway is regulated by determinants of cell polarity and cell-cell junctions, mechanical cues of the cell, soluble factors regulating Hippo members or metabolic status, like cellular energy and oxygen

stress (Yu, Zhao, and Guan 2015) (Figure 4). However, it has been proposed that FRMD6 (also known as Willin) influences the activity of the Hippo pathway by turning on its central kinase cascade (Angus et al. 2012). The members of this signalling cascade, mammalian STE20-like protein kinase 1 and 2 (MST1/2) and large tumour suppressor kinase 1 and 2 (LATS1/2) with scaffold proteins salvador family WW domain containing protein 1 (SAV1) and MOB kinase activator 1 (MOB1), respectively, phosphorylate one another to inhibit yes-associated protein 1 and tafazzin (YAP1/TAZ), the main effectors of the pathway (Harvey and Tapon 2007; Harvey, Pfleger, and Hariharan 2003; Huang et al. 2005; Jia et al. 2003; Justice et al. 1995; Lai et al. 2005; Lange et al. 2015; Pan 2007; Wu et al. 2003; Xu et al. 1995). YAP1/TAZ, upon phosphorylation on several serine sites by its upstream regulators, are sequestered in the cytoplasm by 14-3-3 proteins, unable to enter the nucleus, then, they may also be degraded by proteasomes (Piccolo, Dupont, and Cordenonsi 2014). YAP1 and TAZ are transcriptional coactivators that bind to transcription factors when active, such as TEA domain containing proteins (TEAD), SMAD family members (SMAD) or tumour protein P73 (TP73), to regulate the expression of anti-apoptotic, (e.g. *BIRC5*) or apoptotic genes that play a role in cell differentiation, survival and migration (Alarcón et al. 2009; Strano et al. 2001; Vassilev et al. 2001).

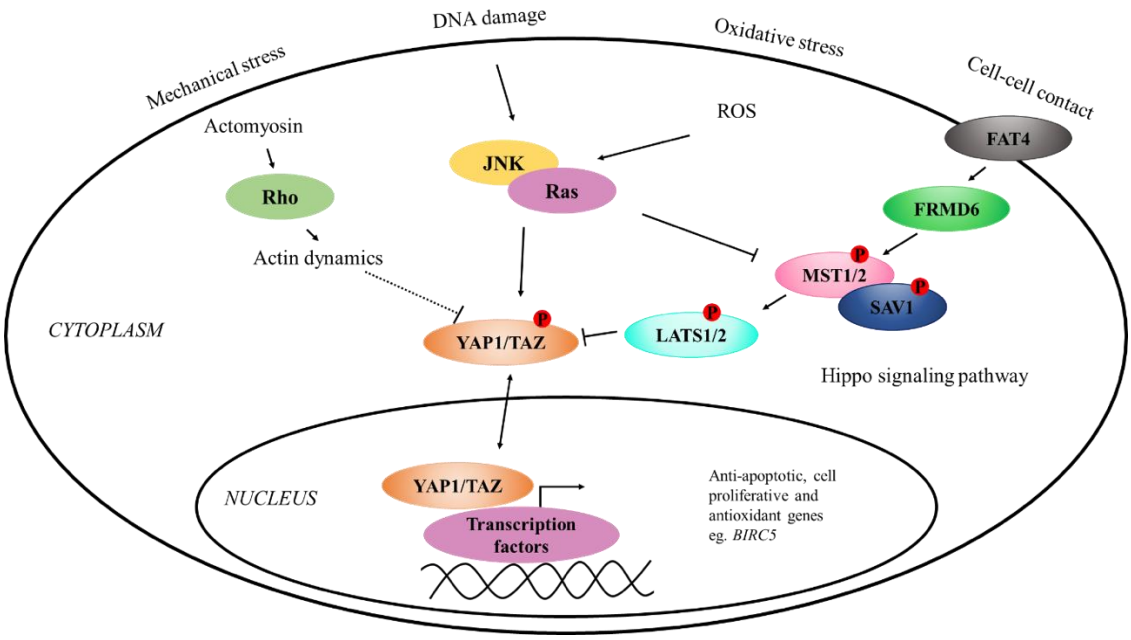

Figure 4. *Examples of signals and pathways regulating YAP1 activity, including the Hippo signalling pathway.*

The gene, *YAP1*, that codes for the main effector of the Hippo pathway, is located on the long arm of chromosome 11. It is a 123 kb gene comprising 10 exons and 9 introns that will be a 54 kDa protein after translation. YAP1 contains a transcriptional enhancer factor-binding domain (TB), a 14-3-3 binding site, two WW domains that aid the binding and interaction with LATS kinases, as well as playing a role in the regulation of transcription, cell transformation and tissue growth (Sudol and Harvey 2010; Zhang et al. 2010). Furthermore, YAP1 has an SRC homology 3 domain (SH3) binding motif, a transcriptional activation domain (TAD), a PDZ binding domain and several serine phosphorylation sites throughout its sequence (Iglesias-Bexiga et al. 2015) (Figure 5).

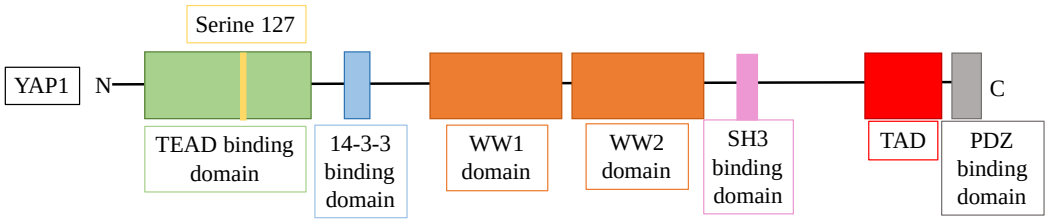

Figure 5. *Simplified schematic diagram of YAP1 protein structure. N: N-terminus, C: C terminus*

YAP1 behaves as an oncogene, that has been investigated and applied as a therapeutic target in different types of cancers, such as liver, prostate, thyroid, gastric, or lung cancer. Besides embryonic tissues, where YAP1 plays an important role in, for example, lung maturation and postnatal airway homeostasis, it is widely expressed in respiratory epithelial cells of the embryonic and mature lung, where the Hippo/YAP1 signalling regulates epithelial cell proliferation and differentiation (Mahoney et al. 2014). Furthermore, in mice it has been demonstrated that YAP is dynamically regulated during regeneration of the airway epithelium following lung injury suggesting a possible role of Hippo/YAP1 signalling in the pathogenesis of acute and chronic lung diseases (Lange et al. 2015).

1.3.3. Angiogenesis in Asthma

Processes such as cytokine production, inflammatory cell infiltration to the lungs, injury to epithelial cells or apoptosis all play an important role in the development and severity of asthma. Structural changes in the airway walls due to both neovascularization

and angiogenesis are also key aspects of asthma (Bischof et al. 2009). Increased vascularity and angiogenesis in asthmatic patients may cause an increase in airway wall thickness, and hence, airway occlusion (Makinde and Agrawal 2011). Moreover, Salvato has found that the number of vessels as well as vascularity have a positive correlation with asthma severity (Salvato 2001).

Angiogenesis is the process where new blood vessels form from pre-existing ones (Madeddu 2005). It takes place in embryonic development, as well as adults, where angiogenesis is an important feature of both many physiological and pathological processes. Physiological processes include hair growth, the female reproductive cycle, wound healing, or bone repair (Carmeliet 2005). On the other hand, uncontrolled angiogenesis is present in cancer, rheumatoid arthritis, diabetes or psoriasis, but poor angiogenesis results in myocardial or brain ischemia or non-healing ulcers (Bellou et al. 2013; Costa and Soares 2013; Zachary and Morgan 2011). Angiogenesis is regulated by several molecules, such as vascular endothelial growth factor (VEGF), which is the most distinct growth factor for the vascular endothelium (Breier et al. 1992; Ferrara 2002; Shweiki et al. 1993). There are several growth factors in this family that either primarily regulate the growth of blood vessels or lymphangiogenesis, furthermore, placental growth factor (PGF) is expressed in the placenta or certain types of tumours (De Falco, Gigante, and Persico 2002; Ferrara 2002; Maglione et al. 1991). The VEFG family function through VEGF receptors (VEGFR), VEGFR-1, -2 and -3 (Ferrara 2002), that also have coreceptors, Neurophilin-1 and -2 (NRP1 and -2) that increase VEGF affinity for VEGFR (Becker et al. 2005; Gluzman-Poltorak et al. 2000; Soker et al. 1998) (Figure 6). Other regulators of angiogenesis are angiopoietins, like Ang1 and Ang2 in humans. These are glycoproteins that have both been characterized by acting as ligands for tyrosine-protein kinase receptor, Tie2 (Saharinen et al. 2008; Yuan et al. 2009). Ang1 and Ang2 have a similar affinity for binding to Tie2. Ang1 stimulates kinase activity by binding to Tie2; on the other hand, Ang2 may act as an agonist or an antagonist for Tie2 in a tissue- and cell-dependent manner (Augustin et al. 2009). Ang1 provides an anti-inflammatory effect on blood vessels during angiogenesis, whereas Ang2 and vascular endothelial growth factor (Vegf) contribute to a pro-inflammatory effect. (Gamble et al. 2000; Makinde and Agrawal 2011)

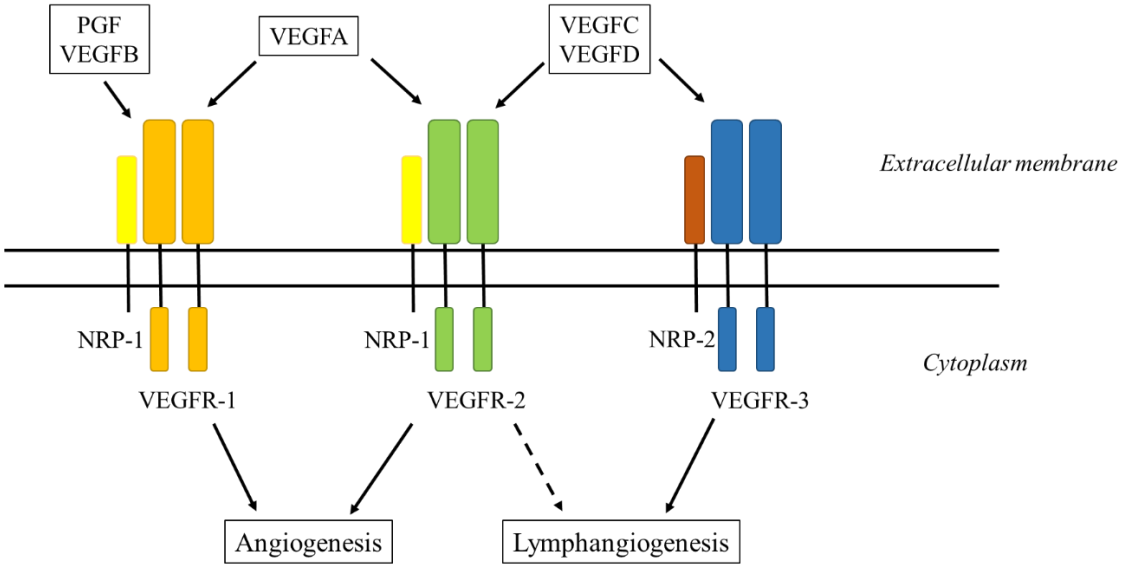

Figure 6. *Schematic diagram of VEGFs, VEGF receptors and their primary functions. Adapted from Detoraki et al, 2010. (Detoraki et al. 2010)*

Tie2 is encoded by the *TEK* gene in humans and is found on the short arm of chromosome 9. It plays a role in angiogenesis, endothelial cell survival, proliferation, migration, adhesion and cell spreading, as well as the reorganization of the actin cytoskeleton or the maintenance of vascular quiescence (Audero et al. 2004; Cascone et al. 2003; Fukuhara et al. 2008; Martin et al. 2008; Saharinen et al. 2008; Yuan et al. 2009; Lee et al. 2004). Tie2 also has an anti-inflammatory effect by averting the leakage of pro-inflammatory elements from the blood vessels into the surrounding tissues. It has been found that Tie2 receptor mRNA and protein are abundantly expressed in the lungs (Kanazawa 2007). In a recent genome wide association study in European American populations the strongest signals were identified at the 9p21.2 locus consisting of four SNPs in weak LD with each other and spanning three genes (*EQTN* (Equatorin), *MOB3B* (MOB kinase activator 3B), *TEK*) (Almoguera et al. 2016). It was hypothesized that, based on its function, variations in the *TEK* gene were responsible for the association. In another study, three non-linked expression quantitative trait locus (eQTL) SNPs were identified in the introns of the *TEK* gene, which all associated with lower *TEK* gene expression in a HapMap3 cohort and increased risk for developing acute respiratory distress syndrome (Ghosh et al. 2016).

The Tie2 pathway also has an important role in the development and function of the eye and the *TEK* gene is highly expressed in the Schlemm's canal endothelium in the eye (Souma et al. 2016). Mutation in the *TEK* gene is associated with primary congenital

glaucoma and Tie2 is a highly-investigated target in different eye diseases like subretinal and choroidal neovascularization, macular oedema or diabetic retinopathy (Campochiaro and Peters 2016; Campochiaro 2015; Tan et al. 2016).

1.4. Previous Results of Ovalbumin-Induced Mouse Model of Asthma

Our research group has previously developed an ovalbumin-induced mouse model of asthma and have carried out a whole genome gene expression microarray analysis on different healthy and asthmatic mouse groups. Using a high-throughput microarray technology may lead to greater insights into new genes and pathways regulating the development of asthma. This study has been carried out by Tölgyesi et al and the details can be found in the GEO database with the record number GSE11911. Therefore, here, I only summarize the experimental setup and findings that are relevant for our following analyses.

Six- to eight-week-old, female, pathogen-free BALB/c mice were used in the experiment that were kept on an OVA-free diet in order to senzitize them to the compound. All together, 4 groups of mice have been created, where one of them received placebo (PBS) during the allergen challenge which comprised the control group. The other asthmatic groups received OVA during the allergen challenge, where groups 1 and 2 comprised the mice from which BALF and lung tissue was collected 4 hours after the first and third allergen challenge on days 28 and 30, respectively. From group 3 mice, we have collected BALF and lung tissue 24 hours after the third allergen challenge on day 31. From lung tissues, RNA was isolated for the whole genome gene expression analysis and mRNA levels have been compared between control and asthmatic mice. As a result, 533, 1554 and 1134 genes showed a larger than 2-fold expression change that were statistically significant in groups 1, 2 and 3 compared to the control group, respectively. Furthermore, 861 transcripts showed a statistically significant, larger than 2-fold difference in gene expression between the asthmatic groups (Tölgyesi et al. 2009). These data have been used for further analysis in order to find orthologous genes in humans that may influence the development of asthma by being differentially regulated on an mRNA or DNA level.

We have chosen 60 genes based on the results of this study. Our main reasons for considering a gene cover several aspects. Firstly, a gene having a larger than 2-fold

expression change between asthmatic and control groups. Secondly, a gene (protein) having a potential role in the pathogenesis of asthma. Thirdly, finding previous scientific results for a given gene. And last, having scientific novelty (Temesi et al. 2014). These criteria led to the selection of a variety of genes that may be found in Table S2.

1.5. Current Shortcomings in the Research of Asthma and Allergy

Based on the above, it is clear that asthma is a prevalent, often severe, complex disorder that is yet impossible to cure. Understanding the mechanisms and pathways of the disease, its pathogenesis, its genetic background will bring solutions to asthma one step closer.

Our research is the very first to identify and hypothesize the Hippo pathway to play a role in a phenotype of asthma, as well as a variation of the *TEK* gene to act in allergic conjunctivitis. Although research of apoptosis and angiogenesis in asthma is a small piece in a bigger picture, nonetheless, such scientific contributions may advance further research in this field.

2. Objectives

Our goal was to study the genetic and genomic background and the pathogenesis of childhood asthma and its associated phenotypes. The main objectives can be summarised as follows.

1. Investigation of the role of the Hippo signalling pathway in asthma
    - Evaluating the differences in gene expressions of seven Hippo pathway genes between asthmatics and healthy subjects based on previous results of Ungvári et al. (Ungvári et al. 2012a; Ungvári et al. 2012b)
    - Assess relationship of polymorphisms (tagSNPs) spanning the whole of *YAP1* gene (based on above results) and asthma and its phenotypes by estimation of allele frequencies between asthma patients and healthy controls. Moreover, gaining further associations through the haplotype analysis of our data and a more extensive Bayesian statistical analysis.
    - Comparing FRMD6, BIRC5 and YAP1 protein levels in induced sputum samples from asthmatics and controls in order to evaluate role of Hippo signalling pathway in asthma through protein expression.
    - Investigating HeLa cells *in vitro* upon BIRC5 antagonist, YM155 treatment in order to find functional roles for Hippo signalling pathway components, BIRC5, YAP1 and FRMD6.

2. Investigation of the role of angiopoietin receptor Tie-2 in asthma and its phenotypes
    - Assessing the incidence of different comorbidities of asthma within our study population.
    - Evaluating tagSNPs of Tie2, encoded by the *TEK* gene, in asthmatic and control subjects to find biomarkers for asthma susceptibility.

3. Investigation of associations between 60 previously identified genes and asthma
    - Sixty genes have been previously identified by gene expression microarray on an ovalbumin-induced mouse model of asthma by our research group (Tölgyesi et al. 2009). Following variation selection, identification of associations by estimation of allele frequencies between asthma patients and healthy controls.

3. Methods

3.1. Subjects

3.1.1. Characteristics of Participants of Sputum Induction and Gene Expression Measurements

The gene expression analysis was done using the induced sputum of 18 asthmatic patients and 10 healthy controls. All subjects completed a detailed, pre-edited questionnaire based on the ISAAC Phase Three Questionnaire. The recent Global Initiative for Asthma guidelines (www.ginasthma.org) were used to diagnose asthma by a respiratory medicine specialist. The evaluation of asthma severity was done at the time of acquisition of induced sputum samples from the patients based on patient history, including number of exacerbations per year, lung function test results, medical treatment applied and response to medication. Asthmatics were divided into four severity groups, but due to low number of patients GINA 1,2 (mild) and GINA 3,4 (moderate-severe) were aggregated. GINA groups of severity from 1-4 are summarised as follows. GINA 1 is 'intermittent' asthma, with symptoms less than once a week and brief exacerbations. Nocturnal symptoms do not occur more than twice a month. FEV1 is more than 80%, where their variability is less than 20%. GINA 2 is 'mild persistent' with symptoms more than once a week but less than once a day and exacerbations may affect the patient's activity and sleep. Nocturnal symptoms occur more than twice a month. FEV1 is more than 80% with variability of less than 20-30%. GINA 3 is 'moderate persistent' with daily symptoms and exacerbations affecting activity and sleep. Nocturnal symptoms occur more than once a week and there is a need for daily use of inhaled short-acting beta-agonist. FEV1 is between 60-80% with a variability of more than 30%. GINA 4 is 'severe persistent' with daily symptoms and frequent exacerbations. There are also frequent nocturnal symptoms of asthma. The patients suffer from the limitation of physical activites. FEV1 is less than 60% with a variability of more than 30%. Out of the 18 asthmatic patients, 14 regularly used inhaled corticosteroids (ICS): <500 μg/day beclomethasone dipropionate (BDP) or equivalent (n = 5), 500–1000 μg/day BDP or equivalent (n = 7) and >1000 μg/day BDP or equivalent (n = 2); while four were considered steroid naive. Healthy volunteers with no previous history of asthma or any airway conditions comprised the control group. Everyone participated in a lung function test (PDD-301/S, Piston Inc., Budapest, Hungary) and were assessed for fractional exhaled nitric oxide (FENO) levels (NIOX MINO, Aerocrine, Solna, Sweden). All

healthy subjects had a normal lung function and had no respiratory tract infection four weeks prior to the analysis. A skin prick test was also performed for common allergens to test the presence of atopy, which is a genetic predisposal for developing allergic rhinitis, atopic dermatitis and/or asthma. The participants' sex, age, smoking habits and allergic statuses were compared between cases and controls and between severity groups, but no statistical significance was found (Ungvári et al. 2012a). Table 1 shows a summary of this study population.

Table 1. *Detailed characteristics of subjects participating in sputum and gene expression analysis.*

Clinical and biological characteristics	Asthmatic patients, n=18	Control subjects, n=10	Statistical difference (p-value)
Age ± SD	43.7 ± 16.7	29.3 ± 4.6	0.01
Sex (Male/Female)	10/8	5/4	1
Asthma severity:			
Mild (GINA 1,2)	11	-	-
Moderate-to-severe (GINA 3,4)	7	-	-
Sputum eosinophil %	13.1 ± 12.4	0 ± 0	0.001
Sputum neutrophil %	20.3 ± 17.9	18.1 ± 9.3	0.8
Sputum macrophage %	59.8 ± 21.0	74.8 ± 8.2	0.2
Sputum bronchial epithelial cell %	1.2 ± 1.6	7.1 ± 5.7	0.0007
ICS dose (μg) ± SD	594.4 ± 527.4	-	-
FENO level (ppb)± SD	22.6 ± 12.5	NA	-
FEV1 level (L) ± SD	2.3 ± 0.7	-	-

GINA: Global Initiative for Asthma; ICS: Inhaled corticosteroid in μg; FENO: Fractional exhaled nitric oxide in parts per billion; FEV1: Forced expiratory volume in 1 second in litres.

3.1.2. Characteristics of Participants of Genotyping Analysis

The genotyping analysis of *YAP1* SNPs included 1233 unrelated individuals, out of which 522 were asthmatic children (mean age±SD: 10.20±5.31 years; 328 males and 194 females) and 711 healthy controls (mean age±SD: 14.0±11.2; 429 males and 282

females). Further, the genotyping analysis of *TEK* SNPs included 1189 unrelated individuals, out of which 435 were asthmatic children (mean age±SD: 10.3 ± 4.4 years; 269 males and 166 females) and 754 healthy controls (mean age±SD: 13.8±11.8 years; 453 males and 297 females). Moreover, the genotyping analysis of 60 previously identified genes (list of genes found in Temesi et al. 2014) included 671 individuals, out of which 311 were asthmatic children (mean age±SD: 10.6 ± 4.8 years; 203 males and 18 females) and 360 healthy controls (mean age±SD: 21.7 ± 13.9 years;181 males and 179 females). The patients were all Hungarian (Caucasian), from which about 5% were of Gypsy origin based on state population data. Asthma was diagnosed based on GINA guidelines, as before (Ungvári et al. 2012a). Atopy was identified by a positive skin prick test and/or positive total or specified serum IgE levels. The total and specified serum IgE levels were evaluated with the 3gAllergy blood tests in the Immulite 2000 Immunoassay system (Tarrytown, NY, USA). The resulting serum IgE levels were either normal or high based on age-specific ranges (kU/litre).

Different types of asthma were defined in patients. A number of participants were excluded from the analysis, where insufficient information was available on asthma phenotypes, hence altogether, 391 and 320 asthmatic children were involved in the phenotype analysis of *YAP1* and *TEK* genes, respectively. Asthma was divided into allergic and non-allergic asthma subtypes. If asthma was not provoked by allergens, but allergy was also present, allergy types were, nonetheless, marked. In allergic patients depending on the types and quantities of allergens, subgroups of indoor, outdoor, or inhalative allergic phenotypes have been designated. Asthma was categorized as exercise-induced asthma when the asthma exacerbation was provoked by exercise in the medical history of the patients. If the onset of asthma or the asthma exacerbations have been associated with an infection related acute respiratory illness the asthma was classified as viral-induced asthma. Non-atopic patients with viral-induced asthma phenotype composed the non-allergic asthma subgroup (Wenzel 2006) (Figure 2). Indoor allergens included dust mites, mould, pet dander and cockroaches, whereas outdoor allergens consisted of different types of pollen. Eosinophil cell counts from blood were measured with the Coulter AXM analyser, where the normal relative range was between 1-6%, and the normal range of absolute eosinophil count was between 50-200/μl. None of the patients had a change of therapy before the blood samples were taken. Neither had they exacerbations or respiratory infections for at least four weeks prior to the blood test. Table

2 shows the detailed characteristics of the asthmatic patients participating in the gene association study.

The samples from the control children were collected at the Orthopaedic Department in the Budai Children's Hospital or at the Urological Department in the Heim Pál Hospital, both in Budapest. None of the controls had any symptoms of asthma or airway conditions, nor any need for medication.

A written informed consent was signed by all patients or by their parent/guardian. The study was carried out according to the Declaration of Helsinki and was approved by the Ethics Committee of the Hungarian Medical Research Council.

Table 2. *Detailed characteristics of asthmatic patients participating in SNP analysis of YAP1 and TEK genes.*

Clinical and biological characteristics of asthmatic patients	**Analysis of YAP1 SNPs**	**Analysis of TEK SNPs**
Number of patients	n=522	n=435
Age ± SD	10.2 ± 5.3	10.3 ± 4.4
Sex (Male/Female)	328/194	269/166
Asthma phenotypes/sensitization status of subjects:	n=486	n=320
Exercise-induced, yes/no[a]	155/233	111/209
Viral-induced, yes/no[a]	181/208	134/186
Allergic asthma, yes/no[a]	56/82	47/60
Inhalative allergy, yes/no[a]	298/99	244/75
Outdoor allergy, yes/no[a]	240/149	202/117
Indoor allergy, yes/no[a]	225/164	190/129
Comorbidity Rhinitis, yes/no[a]	217/173	178/142
Comorbidity Conjunctivitis, yes/no[a]	120/270	100/220
GINA status[a]:		
Number of patients in GINA 1	97	84
Number of patients in GINA 2	241	206
Number of patients in GINA 3	48	28
Number of patients in GINA 4	96	0
Absolute eosinophil count ± SD (number/µl)	300 ± 300	300 ± 300
Number of patients with normal or high absolute eosinophil count (normal/high)	107/154	72/127
IgE ± SD (kU/l)	468 ± 1828	487 ± 2066
Number of patients with normal or high IgE level (normal/high)	88/182	66/143

[a]*Data are available on a limited data set only. Normal absolute eosinophil count is < 200 /µl and high absolute eosinophil count is ≥ 200 /µl. Normal IgE level is < 200 kU/l, high IgE level is ≥ 200 kU/l.*

3.2. Sputum Induction

Induced sputum was used for gene expression assays and Western blot analysis. Participants were first treated with 400µg of inhaled salbutamol, then, they inhaled 4.5%

saline solution generated by a De Vilbiss Nebulizer (Ultra-NebTm 2000 model 200 HI, Somerset, PA, USA) for 5 minutes. This procedure was repeated two more times, where after each sputum induction, the subjects' pulmonary function was assessed. All samples were examined for salivary contamination and only those were used in the study, which were macroscopically free of such contamination. These samples were, then, diluted with phosphate buffered saline (PBS) that contained 0.1% dithiothreitol from Sigma (St Louis, MO, USA). The samples were thoroughly mixed with a vortex and placed on a bench rocker for 30 minutes at room temperature. Then, the samples were filtered with a 40μm Falcon cell strainer and centrifuged at 1500rpm for 10 minutes at room temperature. The cell pellet was resuspended in 1ml PBS. The Trypan Blue Exclusion test was used to determine cell viability in a Burker chamber. After differential cell count by a specialist, cells were stored on lysis buffer at -80°C until use (Ungvári et al. 2012a).

3.3. DNA Isolation

Genomic DNA was isolated from whole blood samples of 1233 individuals using the QIAamp blood DNA midi kit (Qiagen, Maryland, USA) or the iPrep PureLink gDNA Blood Kit on iPrep Purification Instrument (Invitrogen, Carslbad, CA, USA) starting out from 1 ml whole blood. The average DNA concentration of samples was between 30-60ng/μl measured by Nanodrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE).

3.4. RNA Isolation and cDNA transcription

RNA was isolated successfully from the induced sputum samples of 18 patients and 10 control subjects with the Qiagen Mini RNeasy Kit according to the manufacturer's instruction (Qiagen, Maryland, USA). The total RNA quantity was measured by Nanodrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). The cDNA used in the gene expression analysis, was produced with a High Capacity cDNA Reverse Transcription Kit from Thermo Fisher Scientific (Thermo Fisher Scientific, Waltham, MA USA).

3.5. Gene Expression with TaqMan

Real-time quantitative PCR was performed on *LATS1*, *LATS2*, *MST1*, *MST2*, *SAV1*, *YAP1*, *TAZ* and *β-actin* genes using 7900HT Fast Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA USA) according to the manufacturer's instructions with 1.5 µl cDNA/well and final volume of 25µl. *β-Actin* was used as an endogenous control and all results were normalized to it using the delta delta Ct method.

3.6. SNP Selection and Genotyping with Competitive Allele-Specific PCR

SNPs were selected from *YAP1* gene using UCSC Genome browser. Preferably, promoter, missense or UTR SNPs were chosen where MAF in the Caucasian population was higher than 0.1, which was confirmed by HapMap. We also checked for linkage between SNPs and have chosen tagSNPs with Haploview 4.2 program (Broad Institute of MIT, Cambridge, MA, USA). *TEK* gene SNPs were chosen based on the paper of Ghosh et al. (Ghosh et al. 2016).

KBioscience Competitive Allele-Specific PCR (KASP) version 4.0 genotyping assays were used (LGC Genomics, Berlin, Germany) to genotype fourteen SNPs on the *YAP1* gene and three SNPs on the *TEK* gene (Table 3) according to the manufacturer's instructions. PCR reactions were carried out using a 7900HT Fast Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA USA).

Table 3. *Description of selected SNPs on YAP1 and TEK and results of the genotyping.*

Gene	SNP	Position according to the NCBI Genome Build 38	Function	Alleles on the forward strand	MAF in cases	MAF in controls	HWE in controls (p-value)
YAP1	rs1820453	chr11:102109604	promoter	A/C	0.45	0.47	1.00
	rs7106388	chr11: 102110546	5'UTR	C/T	0.45	0.48	0.82
	rs10895257	chr11:102115913	intron	A/G	0.22	0.24	0.75
	rs1426398	chr11:102117330	intron	C/T	0.45	0.46	1.00
	rs11225138	chr11:102123167	intron	C/G	0.10	0.09	0.15
	rs1426394	chr11:102149503	intron	A/G	0.29	0.31	0.05
	rs948737	chr11:102158098	intron	C/T	0.33	0.36	0.08
	rs1942683	chr11:102173916	intron	A/G	0.40	0.42	0.69
	rs11225161	chr11: 102199763	intron	C/T	0.12	0.11	0.35
	rs1894116	chr11:102199908	intron	C/T	0.12	0.12	1.00
	rs11225166	chr11:102219736	intron	C/G	0.11	0.12	0.71
	rs8504	chr11:102232869	3'UTR	A/G	0.42	0.44	0.49
	rs2846836	chr11:102234942	downstream	C/T	0.44	0.46	1.00
	rs7115540	chr11:102267059	downstream	A/G	0.36	0.35	0.32

Gene	SNP	Position according to the NCBI Genome Build 38	Function	Alleles on the forward strand	MAF in cases	MAF in controls	HWE in controls (p-value)
TEK	rs581724	chr9:27187424	intron	T/G	0.39	0.39	0.99
	rs3780315	chr9:27196292	intron	G/A	0.47	0.44	0.000062
	rs7876024	chr9:27202665	intron	A/G	0.24	0.24	0.02

MAF: minor allele frequency; HWE: Hardy-Weinberg Equilibrium

3.7. SNP Selection and Genotyping with Sequenom iPLEX Gold MassARRAY of Previous Mouse Model Based Asthma Investigation, and Statistical Analyses

SNP selection was done based on a previous study of our group on OVA induced mice (Tölgyesi et al. 2009). Briefly, Tölgyesi et al. have used 6-8 weeks old BALB/c female pathogen-free mice, which have been made ovalbumin sensitive and were either treated with OVA or a placebo 1-3 times. On the 28-31st days, 4-24 hours after the first or third treatments mouse lungs were extracted and RNA was isolated from them. Gene expression analysis was done by Agilent Whole Mouse Genome Oligo Microarray 4 x 44 K chip (GSE11911). Based on the microarray results our group has chosen 60 genes with a statistically significant change and a possible role in human asthma and allergy (Tölgyesi et al. 2009). From these genes 90 SNPs have been chosen by using UCSC Genome Browser, where the preferential SNPs were either promoter, missense or UTR region variations. Full list of genes and polymorphisms may be found in Temesi et al. 2014, Supplementary Material 1. To genotype the selected SNPs the Sequenom iPLEX MassARRAY technology was used at the McGill University and Genome Quebec Innovation Centre, in Montreal, Canada (Temesi et al. 2014).

After calculating allele frequencies and deviation from Hardy-Weinberg Equilibrium (HWE) with DeFinetti software (Helmholtz Zentrum München, Institut für Humangenetik, https://ihg.gsf.de/cgi-bin/hw/hwa1.pl), statistical analysis of results was done by Armitage's trend test by DeFinetti software (Temesi et al. 2014).

3.8. Cell Culturing

HeLa cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% foetal bovine serum (FBS), 1% glutamine, 1% antibiotics, 1% nonessential amino acids and 1% pyruvate (all from Thermo Fisher Scientific, Waltham, MA, USA) on 37°C and 5% CO_2 concentration. Culture medium was replaced every 48h incubation time. Cells were harvested and lysed in RIPA lysis and extraction buffer (Thermo Fisher Scientific, Waltham, MA USA) for use as positive control during Western blot analysis.

HeLa cells were cultured on six-well plates to be treated with BIRC5 inhibitor, Sepantronium Bromide (YM155) (Selleck Chemicals, Munich, Germany). Powdered YM155 was dissolved in DMSO according to the manufacturer's instructions. 10^5 cells

were seeded/well. Cells were treated with 1000nM, 100nM, 10nM, 1nM, 0nM YM155 or 1% dimethyl sulfoxide (DMSO) (Sigma Aldrich, St. Louis, MO, USA) as DMSO control. After 48h incubation cells were harvested. 50% of cells/well were treated with RIPA lysis and extraction buffer (Thermo Fisher Scientific, Waltham, MA USA) for further use as protein and 50% of cells were treated with RLT RNeasy Lysis Buffer (Qiagen, Maryland, USA) for gene expression analysis. All lysed samples were stored at -80°C until further extraction of protein or RNA.

3.9. Western Blot Analysis

Western blot analysis was carried out on human induced sputum samples. After sample preparation, cells were lysed in RIPA lysis and extraction buffer (Thermo Fisher Scientific, Waltham, MA USA)) supplied with Halt protease inhibitor (Thermo Fisher Scientific, Waltham, MA USA)) at 1X final concentration and centrifuged at 14,000 x *g* at 4°C for 15 min. Total protein content was determined by the Pierce BCA protein assay kit (Thermo Fisher Scientific, Waltham, MA USA) according to the manufacturer's instructions. Samples were loaded on 4-20% Tris-glycine precast gels (Thermo Fisher Scientific, Waltham, MA USA)) then blotted onto Immun-Blot PVDF membrane (Bio-Rad Laboratories, Hercules, CA, USA). After blocking the following antibodies were used. The primary antibodies were anti-FRMD6, anti-YAP1 and anti-GAPDH (Abcam, Cambridge, UK), respectively. The secondary antibodies were polyclonal donkey anti-rabbit IgG HRP (Abcam, Cambridge, UK) and polyclonal goat anti-mouse immunoglobulins HRP (Dako, Glostrup, Denmark). The membrane was treated with Pierce ECL plus substrate (Thermo Fisher Scientific, Waltham, MA USA) according to the manufacturer's instructions and bands were visualized on standard x-ray film (Kodak, Rochester, NY, USA).

3.10. Bioinformatics

3.10.1. Frequentist Statistical Analysis

For sputum analysis, normalized gene-expression levels of *LATS1*, *LATS2*, *MST1*, *MST2*, *SAV1*, *YAP1*, *TAZ* and *β-actin* genes were compared by Mann–Whitney *U* test or Kruskal–Wallis test, when appropriate. Contingency tables were analysed by Fisher's

exact test. Correlation studies were performed by Spearman non-parametric test. Differences were considered to be significant when $p < 0.05$.

YAP1 SNP allele frequencies were estimated by allele counting and tested for deviation from HWE by the software program DeFinetti between cases and control subjects. For the significant deviation threshold, we used $p < 0.05$ value.

SNP data were analysed using SPSS version 22 (SPSS Inc., Chicago, IL, USA) software. Logistic regression analyses adjusted for age and gender were used to evaluate the association between *YAP1* genotypes and asthma, its intermediate phenotypes, the discretized (normal/high) serum IgE and discretized (normal/high) eosinophil levels (see at Subjects) and the different phenotypes. Additionally, multinomial logistic regression adjusted for age and gender was used for the analysis of *YAP1* SNPs and GINA statuses. Confidence intervals (CIs) were calculated at the 95% level. Additive, dominant and recessive statistical models were used, which are summarized as follows. The additive model compares all three genotype groups, both homozygous ones and the heterozygous with eachother. In the recessive or dominant models, either the recessive homozygous or the wild type homozygous groups are compared to the heterozygous and the remaining homozygous groups taken together, respectively. Multiple comparisons were corrected for using the Benjamini-Hochberg correction, and a new significance level of $p=0.004$ with the FDR < 6.5% was estimated. Haplotype analysis was carried out with the Haploview 4.2 program (Broad Institute of MIT, Cambridge, MA, USA). Odds ratios (ORs) for haplotypes were counted by VassarStats software (http://vassarstats.net/index.html).

HWE was tested for *TEK* SNPs using the chi-square goodness-of-fit test implemented in the online DeFinetti HWE application, as before. The significance level was set to 0.01. SNP data were analysed using SPSS version 19 (SPSS Inc., Chicago, IL, USA) software. Logistic regression analyses adjusted for age and gender were used to evaluate the association between *TEK* genotypes and asthma, its intermediate phenotypes, the discretized (normal/high) serum IgE and eosinophil levels (see at Subjects) and the different asthma phenotypes. Multiple comparisons were corrected for using the Benjamini-Hochberg correction, and a new significance level of $P=0.008$ with the FDR < 4.5% was estimated. Figures were made by using Microsoft Excel 2013 (Microsoft Corporation, Redmond, WA, USA).

3.10.2. Bayesian Statistical Analysis

Bayesian statistical analysis was carried out by the research group of dr. Péter Antal at the Budapest University of Technology and Economics in the Department of Measurement and Information Systems. Earlier, our research groups have together developed an alternative, systems biological statistical method, named Bayesian network based Bayesian multilevel analysis of relevance (BN-BMLA). Bayesian networks offer a rich language for genetic association studies, because they exhaustively and exactly represent the strongly relevant variables and their interactions through the Markov Blanket Set and Markov Blanket Graph features and they are able to evaluate multiple targets. Furthermore, this Bayesian global relevance analysis method provides posteriors, which are direct statements about hypotheses, thus it can also be used to construct probabilistic data analytic knowledge bases in genetic association studies to support complex querying, off-line meta-analysis, and fusion with background knowledge (Antal et al. 2009; Antal et al. 2014; Antal et al. 2008; Hullám et al. 2010).

Previously the BN-BMLA method was described in detail (Gézsi et al. 2015; Lautner-Csorba et al. 2013; Lautner-Csorba et al. 2012; Ungvári et al. 2012b, Ungvári, 2013; Lautner-Csorba, 2013; Gézsi, 2016), thus the following only briefly summarizes this approach.

A Bayesian Network is a directed acyclic graph (DAG) that aids the discovery of various dependency relations between random variables by representing their joint probability distribution. A node in the network represents a variable and edges connecting two nodes represent direct dependency between those variables. To find the dependence relations of the variables, a DAG that best describes the dataset must be found. In most cases, there are many DAGs with non-negligible posteriors, but certain structural features may be extracted accurately. Such feature is based on the concept of strong relevance of a single variable or a set of variables. Bayesian learning allows the evaluation of the strength of the data indicating the presence of a certain feature by evaluating its a posteriori probability.

The a posteriori probability can be calculated for strongly relevant variable sets with regard to a target variable. The strongly relevant variables have direct impact on the target. The a posteriori probability of the strong relevance is between 0 and 1, where 1 means that the target (e.g. phenotypes of asthma) most certainly has a dependency relationship with a predictor (e.g. SNP), on the other hand 0 means there is no such

relationship. Posterior probabilities of strong relevance greater than or equal to 0.5 are regarded as relevant, above 0.75 as convincing.

In this study 29 SNPs all in the *YAP1*, *FRMD6* and *BIRC5* genes (previously genotyped by others with the same methods and on the same populations (Ungvári et al. 2012a; Ungvári et al. 2012b) were involved in the BN-BMLA analysis. Table 4 shows all SNPs included and the characteristics of the patients are detailed in Table 2.

Table 4. *Summary of all SNPs included in the BN-BMLA statistical analysis.*

Gene	SNP	Position according to the NCBI Genome Build 38	Function	Alleles on the forward strand	MAF in cases	MAF in controls	HWE in controls (p-value)	Significant associations			
								Influenced trait	p-value	OR	95% CI
YAP1	rs1820453	chr11:102109604	promoter	A/C	0.455	0.466	0.975	none	>0.05	-	-
YAP1	rs7106388	chr11: 102110546	5'UTR	C/T	0.455	0.476	0.804	none	>0.05	-	-
YAP1	rs10895257	chr11:102115913	intron	A/G	0.222	0.237	0.708	none	>0.05	-	-
YAP1	rs1426398	chr11:102117330	intron	C/T	0.448	0.463	0.944	none	>0.05	-	-
YAP1	rs11225138	chr11:102123167	intron	C/G	0.103	0.087	0.128	none	>0.05	-	-
YAP1	rs1426394	chr11:102149503	intron	A/G	0.294	0.313	0.055	none	>0.05	-	-
YAP1	rs948737	chr11:102158098	intron	C/T	0.326	0.356	0.081	none	>0.05	-	-
YAP1	rs1942683	chr11:102173916	intron	A/G	0.395	0.416	0.685	none	>0.05	-	-
YAP1	rs11225161	chr11: 102199763	intron	C/T	0.117	0.114	0.275	none	>0.05	-	-
YAP1	rs1894116	chr11:102199908	intron	C/T	0.120	0.120	0.788	none	>0.05	-	-
YAP1	rs11225166	chr11:102219736	intron	C/G	0.110	0.117	0.581	none	>0.05	-	-
YAP1	rs8504	chr11:102232869	3'UTR	A/G	0.319	0.338	0.477	none	>0.05	-	-
YAP1	rs2846836	chr11:102234942	downstream	C/T	0.439	0.459	0.979	none	>0.05	-	-
YAP1	rs7115540	chr11:102267059	downstream	A/G	0.360	0.354	0.306	none	>0.05	-	-
FRMD6	rs3751464	chr14:51651174	promoter	C/T	0.259	0.226	0.270	**asthma**	**0.0003**	**1.43**	**1.18–1.75**
FRMD6	rs17666653	chr14:51655759	intron	C/T	0.215	0.162	0.397	none	>0.05	-	-
FRMD6	rs17666689	chr14:51655901	intron	C/T	0.159	0.141	0.491	none	>0.05	-	-

Gene	SNP	Position according to the NCBI Genome Build 38	Function	Alleles on the forward strand	MAF in cases	MAF in controls	HWE in controls (p-value)	Significant associations			
								Influenced trait	p-value	OR	95% CI
FRMD6	rs9671722	chr14:51660341	intron	G/A	0.185	0.168	0.207	none	>0.05	-	-
FRMD6	rs10141001	chr14:51665483	intron	G/A	0.145	0.120	0.800	none	>0.05	-	-
FRMD6	rs2277495	chr14:51720248	synonymous codon	C/T	0.305	0.299	0.547	none	>0.05	-	-
FRMD6	rs2277494	chr14:51720254	synonymous codon	T/C	0.251	0.258	0.201	none	>0.05	-	-
FRMD6	rs7150275	chr14:51728572	3'UTR	A/G	0.251	0.251	0.088	none	>0.05	-	-
FRMD6	rs7149810	chr14:51728602	3'UTR	A/G	0.254	0.251	0.095	none	>0.05	-	-
BIRC5	rs3764384	chr17:78211647	5'UTR	C/T	0.386	0.464	0.019	none	>0.05	-	-
BIRC5	rs3764383	chr17:78212770	3'UTR	A/G	0.280	0.300	0.031	none	>0.05	-	-
BIRC5	rs8073903	chr17:78213673	5'UTR	T/C	0.377	0.354	0.078	**asthma**	**0.004**	**1.46**	**1.13-1.89**
								asthma in females	**0.003**	**1.87**	**1.23-2.84**
								non-allergic asthma in females	**0.005**	**2.81**	**1.37-5.75**
BIRC5	rs17878467	chr17:78214076	5'UTR	C/T	0.114	0.090	0.894	none	>0.05	-	-
BIRC5	rs9904341	chr17:78214286	5'UTR	G/C	0.343	0.333	0.549	**absolute eosinophil count**	**0.004**	**0.92**	**−0.145 to −0.026**
								relative eosinophil level	**0.002**	**0.27**	**−2.132 to −0.468**
BIRC5	rs1508147	chr17:78226507	3'UTR	G/A	0.369	0.482	0.011	**non-allergic asthma in females**	**0.003**	**3.06**	**1.45–6.47**

MAF: Minor allele frequency; HWE: Hardy-Weinberg Equilibrium. Previous results of our research group are also shown for BIRC5 *and* FRMD6 *genes (Ungvári et al. 2012a; Ungvári et al. 2012b).*

4. Results

4.1. Results of the Investigation of the Role of the Hippo Signalling Pathway in Asthma

4.1.1. Results of Gene Expression Analysis of the Hippo Signalling Pathway

In the induced sputum samples of 18 asthmatics and 10 control subjects we measured mRNA expression of 7 members of the Hippo/YAP1 pathway. The expression of all genes could be detected in both cases and controls. The mean gene expression level of *YAP1* was slightly lower in asthmatic than in control patients (p=0.032; Figure 7A). There were no other deviations in this respect. We investigated whether within the asthma group there were differences in gene expression between subgroups of patients defined by their GINA status, but no significant differences were found.

During the correlation studies, we found a significant and positive correlation between *YAP1* mRNA level and the sputum bronchial epithelial cells (r=0.575, p=0.003, Figure 7B). There was a significant and negative correlation between *TAZ* mRNA and sputum neutrophils (r = -0.509, p=0.009) and *MST1* showed a significant and positive correlation with sputum eosinophils (r=0.425, p=0.034). There was no significant correlation between *YAP1, TAZ, LATS1*, *LATS2, SAV1, MST2* or *MST1* gene expression and other cellular components, asthma severity, age, gender, airway inflammation or inhaled corticosteroid dose.

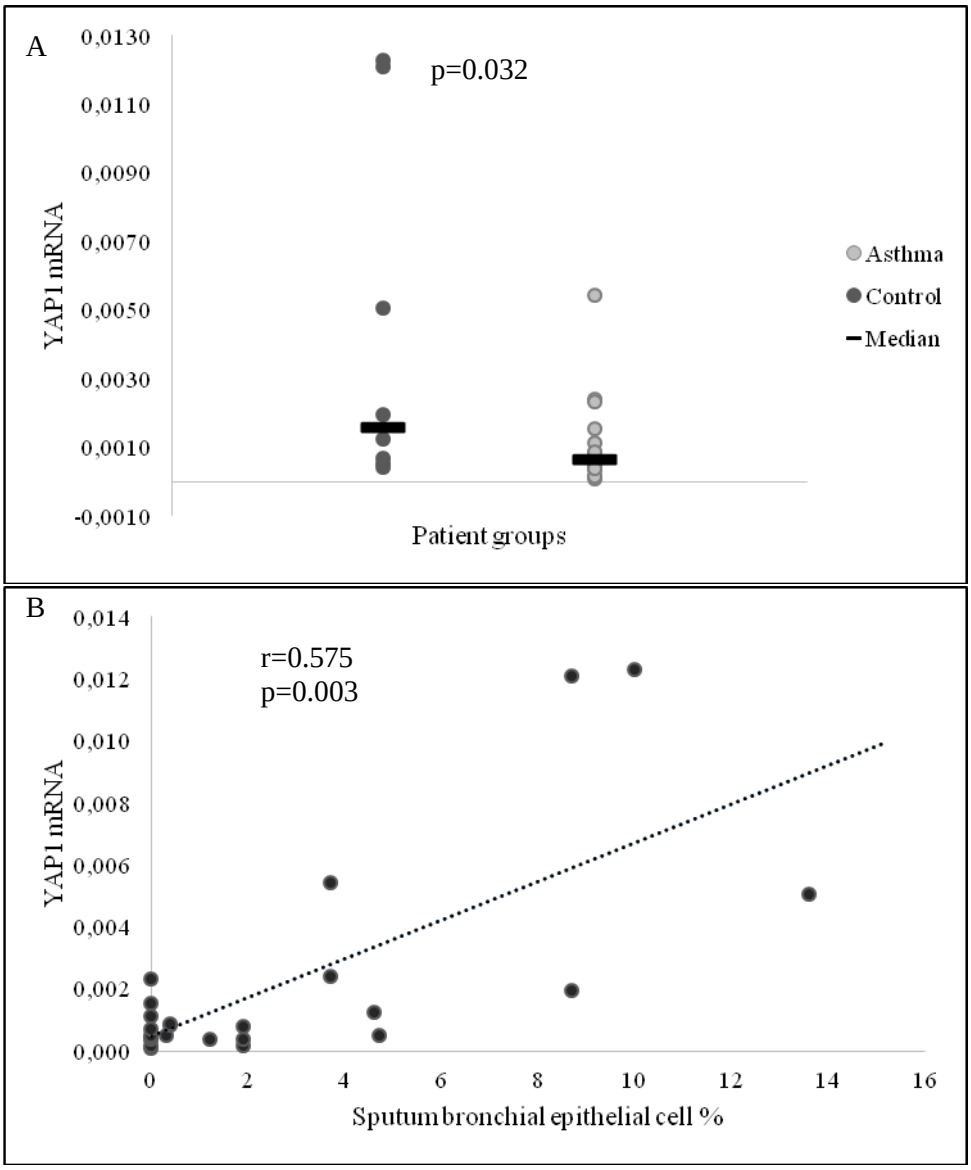

Figure 7. A. YAP1 *mRNA level in the induced sputum of asthmatic patients and controls.* ***B.*** *Relationship between* YAP1 *mRNA and bronchial epithelial cell levels. mRNA levels were normalized and expressed according to the delta delta Ct method. Mann-Whitney U test (A) and Spearmann's non-parametric correlation (B) were used.*

4.1.2. Results of Genotyping Analysis of *YAP1* Gene

We examined whether any of the SNPs in the *YAP1* gene influence the susceptibility of asthma or its associated phenotypes. The statistically significant genotyping results are summarized in Table 5. There was no significant association with any of the SNPs and asthma susceptibility, allergic status, inhalative, outdoor, indoor allergies, allergic and non-allergic asthma, comorbidities of rhinitis and conjunctivitis or

serum IgE and eosinophil levels. However, SNP rs2846836 was significantly associated with exercise-induced asthma (OR=2.1 [1.3-3.4], p=0.004, power=0.83; Table 5; Figure 8A). Additionally, distribution of genotypes of SNP rs11225138 showed a significant difference between GINA 1-2 and GINA 3-4 statuses in a dominant model (OR=2.8 [1.4-5.6], p=0.003, power=0.83, Table 5; Figure 8B).

Table 5. *Significant results of the association analysis of YAP1 SNPs with asthma phenotypes.*

Phenotype	**SNP**	**Model**	**Alleles (1/2)**	**Phenotype**	**Genotypes**			**n**	**p-value**	**OR (95% CI)**
					11 (%)	**12 (%)**	**22 (%)**			
Exercise-induced asthma	rs2846836	ADD	C/T	present	37 (27)	77 (57)	22 (16)	136	0.004	2.1 (1.3-3.4)
				absent	80 (38)	80 (38)	51 (24)	211		
GINA 1,2 vs. 3,4	rs11225138	DOM	G/C	GINA 1 and 2	261 (83)	53 (17)		314	0.003	2.8 (1.4-5.6)
				GINA 3 and 4	87 (70)	37 (30)		124		

In case of rs2846836 genotypes 11, 12 and 22 indicate TT, CT and CC, respectively. In case of rs11225138 genotypes 11, 12 and 22 indicate GG, GC and CC, respectively. ADD: additive model; DOM: dominant model

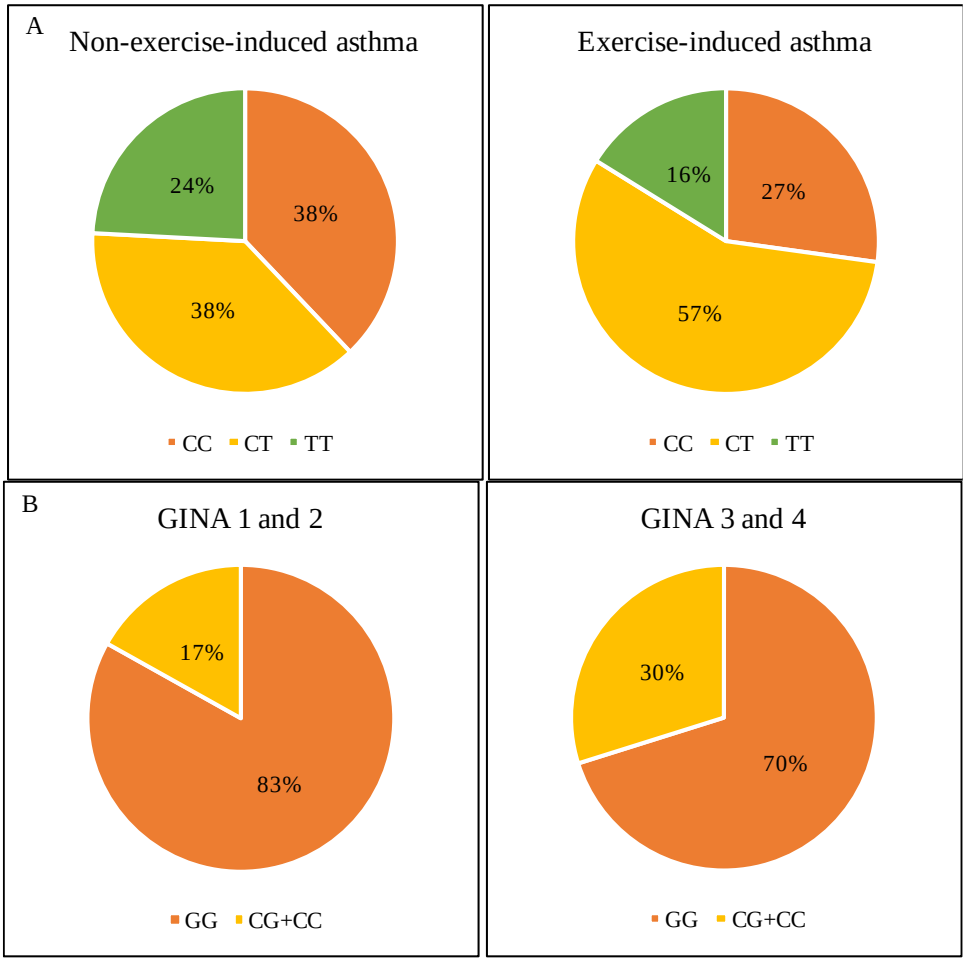

Figure 8. *A. Pie chart of SNP rs2846836 in the non-exercise- and exercise-induced asthma groups, p-value=0.004, OR=2.01 (1.26-3.43). B. Pie chart of SNP rs11225138 in the mild and moderate-to-severe asthma groups. p-value=0.003, OR=2.80 (1.40-5.57). Percentages were calculated from case numbers.*

4.1.3. Bayesian Results of Genotyping Analysis of *YAP1* Gene

Based on the results after genotyping 29 SNPs in *YAP1*, *FRMD6* and *BIRC5* genes, the laboratory data and the characteristics of the asthmatic participants detailed in Table 2, the a posteriori probabilities of relevance between the variables with respect to target variables were calculated by BN-BMLA.

Table 6 shows the most relevant variables with high posteriori probabilities according to the BN-BMLA analysis. As expected, e.g. eosinophil levels and allergic conjunctivitis are highly relevant to allergic rhinitis. In the case of genetic variations, no direct SNP-SNP or gene-gene interactions were found. The most relevant association was

between rs9671722 in the *FRMD6* gene and exercise-induced asthma with a posterior probability of strong relevance of 0.99. Figure 9 shows the most likely subgraph of the dependence structure of the variables. This structure suggests a direct relevance of rs9671722 to exercise-induced asthma, while another SNP (rs3751464) of the *FRMD6* gene was found to be directly relevant to allergic rhinitis and transitively associated through allergic rhinitis with exercise-induced asthma.

Table 6. *The most relevant results of the BN-BMLA statistical method. Numbers show the a posteriori probability of the strong relevance of a given variable with respect to the target variable*

		a posteriori probability
Target variable		**Exercise-induced asthma**
Variable	Non-allergic asthma	0.90
	Allergic conjunctivitis	0.78
	Allergic rhinitis	0.99
	rs9671722 (*FRMD6*)	0.99

Target variable		**Non-allergic asthma**
Variable	IgE level	0.75
	Inhalative allergy	1.00

Target variable		**Allergic rhinitis**
Variable	Inhalative allergy	0.88
	Allergic conjunctivitis	1.00
	Eosinophil number	1.00

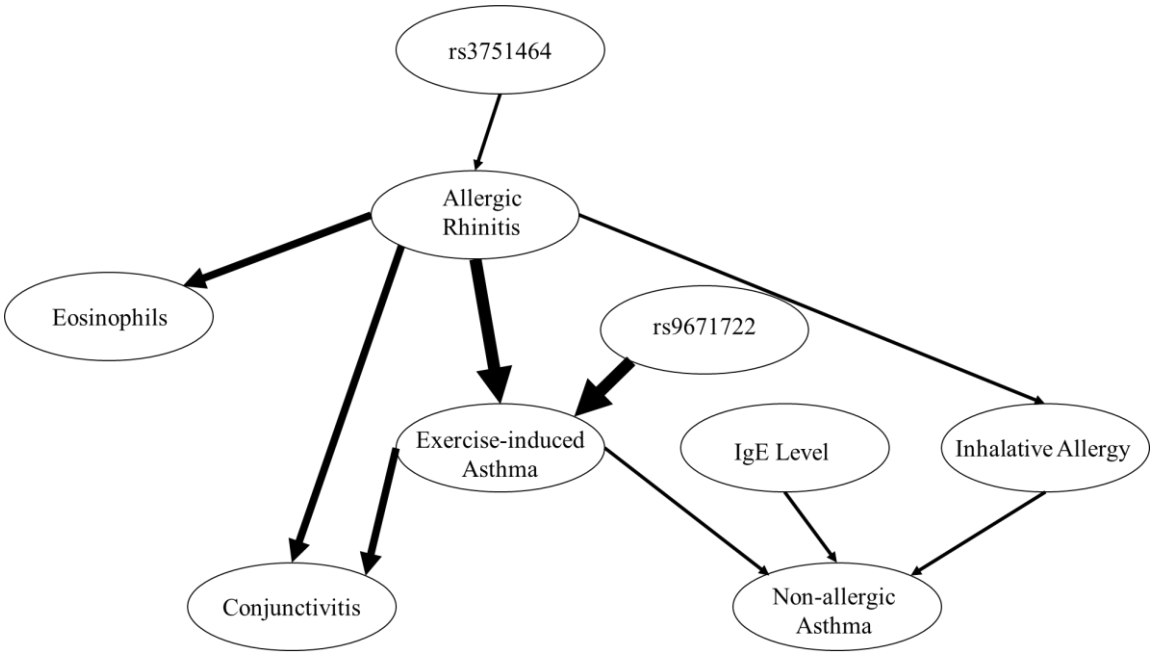

***Figure 9**. Directed acyclic graph of the most likely relations of variants and targets. The directed edges represent only probabilistic relationships between the variables which are not necessary causal. SNPs rs3751464 and rs9671722 are* FRMD6 *polymorphisms.*

The relationship was confirmed by logistic regression which showed that patients with GG genotypes of the rs9671722 SNP had the highest likelihood of having both allergic rhinitis and exercise-induced asthma (OR=18.0 (5.9-54.9); p=3.7E-7); Table 7). The interaction term in the logistic regression model was significant (p = 0.01).
In Figure 10, another type of graph is presented which shows a dendrogram of the subsets of strongly relevant variables with respect to allergic rhinitis.

***Table 7.** Verification of Bayesian results by logistic regression regarding exercise-induced asthma and rs9671722 (*FRMD6*) as variables and allergic rhinitis as a target variable*

Exercise-induced asthma	**rs9671722**	**OR (95%CI)**	**p-value**
Not present	GG	1	
Present	GG	18.0 (5.9-54.9)	3.7E-07
Not present	GA or AA	1.6 (0.8-3.1)	0.17
Present	GA or AA	3.0 (0.8-11.4)	0.11

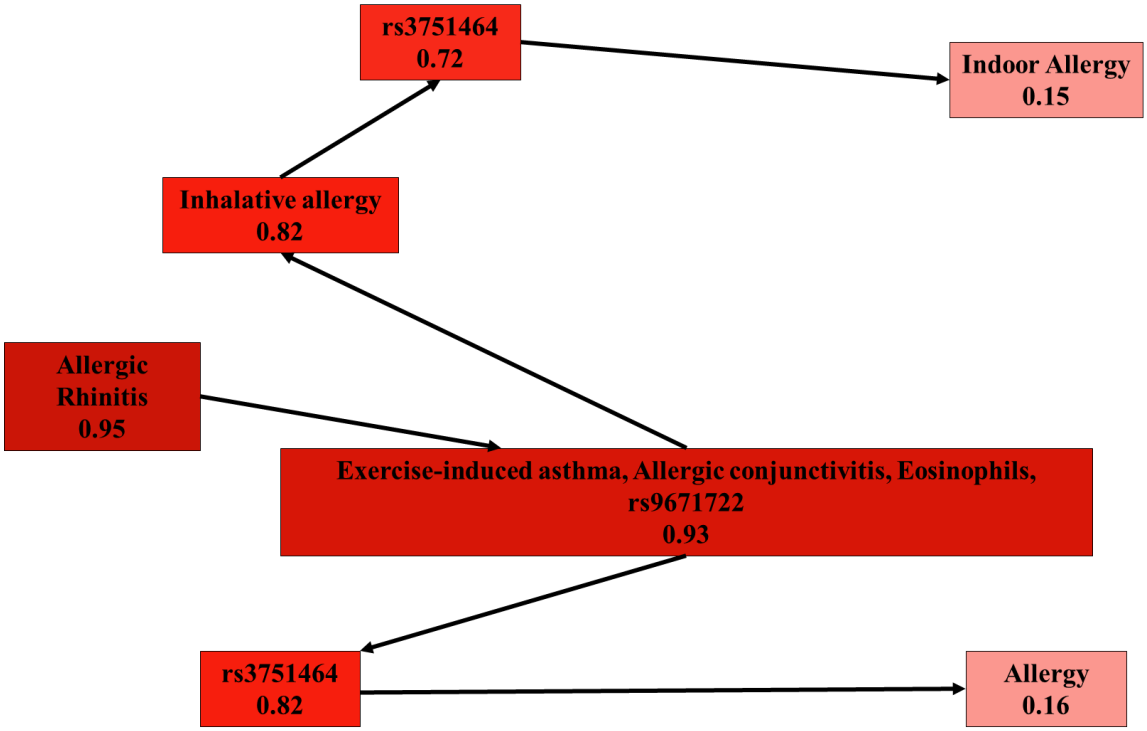

Figure 10. *Dendrogram of the subsets of strongly relevant variables with respect to allergic rhinitis. The nodes in the dendrogram represent strongly relevant subsets of all variables, and the arrows denote the broadening of the subsets. The numbers in the nodes show the a posteriori probability that all variables in a given subset are strongly relevant. SNPs rs3751464 and rs9671722 are* FRMD6 *polymorphisms.*

4.1.4 Haplotype Analysis of *YAP1* SNPs

In order to find more evidence for the associations, we also conducted haplotype analyses. There is a significant difference between patients of GINA 2 and GINA 3 when we compared the frequencies of a haplotype formed by the rare alleles of SNPs rs1426398 and rs11225138, where the frequency of TC haplotype was more prevalent in GINA 3 than in GINA 2 (28% vs. 8%; $p=10^{-7}$). Furthermore, the CA haplotype from SNPs rs11225138 and rs1426394, also showed a significant difference when patients in the two GINA statuses were compared (26% vs. 7%, $p=10^{-7}$). When more than two SNPs were included in the analysis, additional associations were found. Corresponding results can be found in the Table S1 in the supporting information's section and Figure 11.

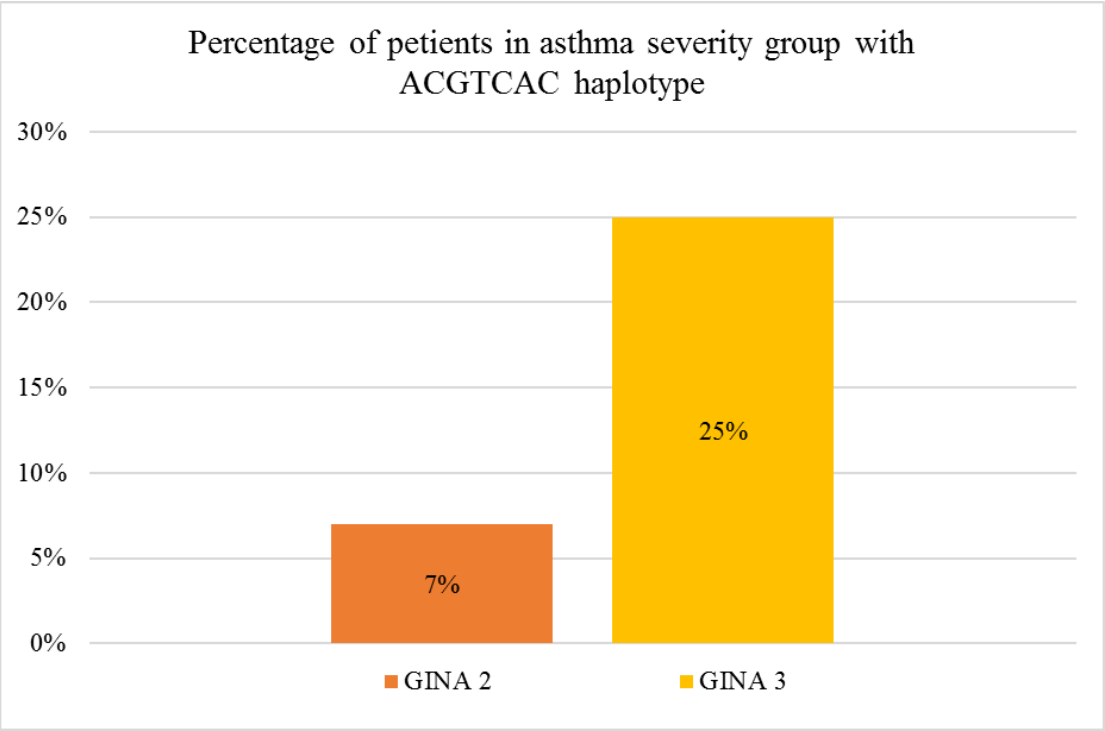

Figure 11. *Diagram showing significant difference in the largest haplotype found among the examined SNPs. The haplotype of ACGTCAC of* YAP1 *SNPs rs1820453, rs7106388, rs10895257, rs1426398, rs11225138, rs1426394, rs948737 between mild (GINA 2) and moderate (GINA 3) asthma severity groups (p=0.000002; OR=4.257, 95% CI=2.393-7.573).*

4.1.5. Results of Western Blot Analysis

Western blots were carried out on proteins of the Hippo/YAP1 pathway that showed significant associations with asthma or phenotypes when their genetic variations were examined. Earlier our research group found a strong significant association between a genetic variation in the *FRMD6* gene and asthma ($p < 0.001$; Table 4 (Ungvári et al. 2012b), as well as between several genetic variations in the *BIRC5* gene and asthma (for all, $p < 0.005$; Table 4 (Ungvári et al. 2012a). Furthermore, we found that genetic variations and haplotypes in the *YAP1* gene are associated with different phenotypes of asthma.

The signal for the FRMD6 protein could be detected in all sputum samples from both asthmatic and control patients. Unfortunately, the BIRC5 protein could not be detected in any of the healthy or asthmatic samples. Interestingly, however, the YAP1 protein could not be detected in the sputum samples of the healthy controls, it was well-

seen in the sputum samples of the mild asthmatics (GINA 1,2) and was also absent from the sputum of severe (GINA 3,4) asthmatics (Figure 12).

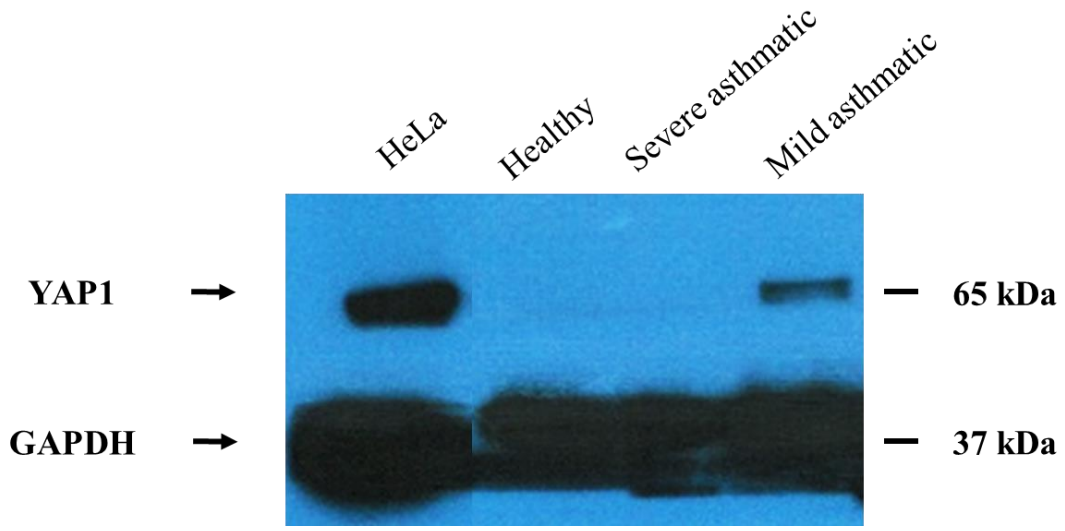

Figure 12. *Western blot analysis of YAP1 and GAPDH proteins. HeLa: HeLa cell extract*

4.1.6. Results of HeLa Cell Treatment with YM155

After 48h incubation time, YM155 treatment seemed successful. As a result, in a Nikon light microscope we found that the higher the concentration of YM155 was, the less cells were found in a given well. DMSO and negative controls were similar in number of cells, therefore DMSO had no visible effect on the cells. After protein expression analysis with Western blot, none of the investigated proteins, BIRC5, FRMD6 or YAP1 could be detected.

4.2. Results of Investigation of the Role of Angiopoietin Receptor Tie-2 in Asthma and its Phenotypes

4.2.1. Results of Genotyping Analysis of *TEK* Gene

4.2.1.1. Prevalence of Co-morbidities Among the Asthmatic Patients

The clinical characteristics of the asthmatic patients are presented in Table 2. Altogether, 435 asthmatic children and 754 healthy controls participated in the genotyping study. Among the fully phenotyped 320 asthmatic patients 178 (55.6%) had allergic rhinitis and 100 (31.3%) had conjunctivitis. Among the rhinitis patients 98 (55.1%) also had conjunctivitis. And two patients had conjunctivitis without rhinitis.

4.2.1.2. Comparison of Gene Expressions in a Mouse Model of Asthma

Our research group has previously carried out measurements of gene expression levels in the lungs of mice with allergic airway inflammation as well as control mice by Agilent Whole Mouse Genome Oligo Microarray 4x44K chips (GSE11911 record number in GEO database) (Tölgyesi et al. 2009). We compared the expression level of the genes in the lungs of mice with OVA-induced allergic airway inflammation and control mice. We looked at Tek, Angpt2 and Vegf genes from the Tie2 pathway, but none of them showed a significant difference, however the expression of Ang1 (Angpt1) was significantly lower in the lungs of mice with allergic airway inflammation (on average with 2.3-fold; corrected $p = 0.001$).

4.2.1.3. SNP Association Study

To investigate whether SNPs in the *TEK* gene influence the susceptibility to asthma or any associated phenotypes, three SNPs have been genotyped. The distribution of the genotypes has been checked for deviation from the Hardy-Weinberg Equilibrium (HWE). Unfortunately, one of the three SNPs, rs3780315, showed a significant deviation from the HWE in the control population (p=1.3E-5), therefore it was excluded from our analysis.

We found no association between either of the two SNPs, rs581724 or rs7876024, and asthma (Table 8). However, SNP rs581724 was significantly associated with allergic conjunctivitis in a recessive way (p=0.007; OR=2.4 (1.3-4.4) (Table 8). More specifically

the homozygote carriers of the rare allele (AA genotype) had a significantly increased risk of developing allergic conjunctivitis (Figure 13) within the asthmatic population. The risk remained significant when the whole population without conjunctivitis was involved in the calculation ($p = 0.003$; OR = 2.1 (1.3-3.6)).

No other phenotypes (GINA status, viral- or exercise-induced asthma, allergic asthma, indoor, outdoor, inhalative allergies, IgE and absolute eosinophil levels, allergic rhinitis) were associated with either of the two SNPs in the statistical analysis.

Table 8. *Results of association of* TEK *SNPs rs581724 and rs7870624 with asthma and allergic conjunctivitis.*

Phenotype	SNP	Phenotype	MAF	Genotype[a] 11 n (%)	Genotype[a] 12 n (%)	Genotype[a] 22 n (%)	Difference between allelic frequencies		Recessive model (11+12 vs. 22)	
							P	OR(95%CI)	P	OR(95%CI)
Asthma	rs581724	Present	0.4	138 (35)	188 (48)	66 (17)	0.3	1.1 (0.9-1.3)	0.4	1 (0.8-1.3)
		Absent	0.39	246 (38)	311 (47)	98 (15)				
	rs7876024	Present	0.24	208 (58)	126 (35)	26 (7)	0.7	1 (0.8-1.3)	0.9	1 (0.6-1.7)
		Absent	0.25	386 (59)	216 (33)	48 (8)				
Allergic conjunctivitis	rs581724	Present	0.4	31 (35)	34 (38)	24 (27)	0.2	1.3 (0.9-1.8)	**0.007**	**2.4 (1.3-4.4)**
		Absent	0.46	68 (33)	107 (53)	28 (14)				
	rs7876024	Present	0.25	51 (62)	21 (26)	10 (12)	1	1 (0.7-1.5)	0.045	2.6 (1-6.5)
		Absent	0.25	104 (55)	74 (39)	10 (6)				

[a]*In case of rs581724 genotypes 11, 12 and 22 correspond to CC, AC and AA genotypes, respectively. In case of rs7876024 genotypes 11, 12 and 22 correspond to AA, AG and GG genotypes, respectively. MAF: Minor Allele Frequency*

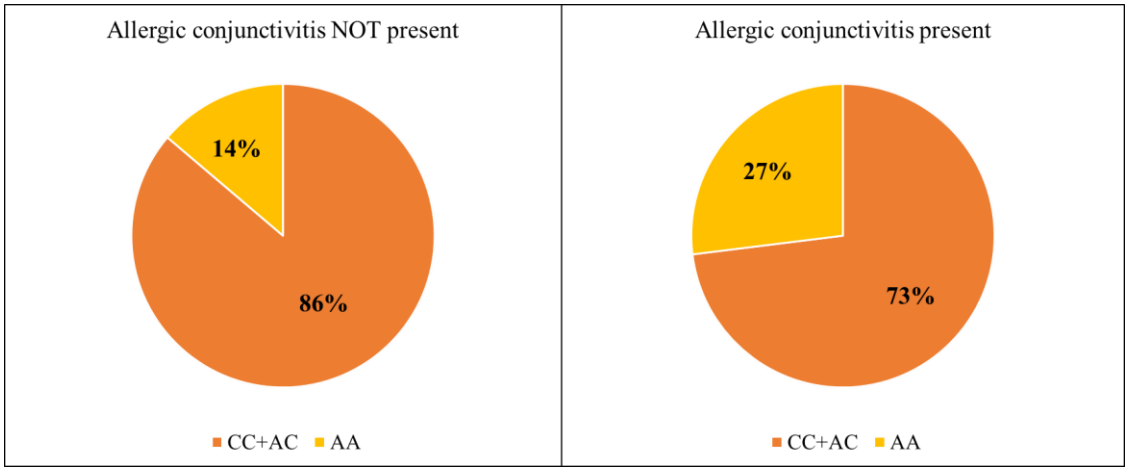

Figure 13. *Pie chart of SNP rs581724 in the subject groups with and without allergic conjunctivitis, p-value=0.007, OR=2.4 (1.3-4.4).*

4.3. Results of SNP Association Study Based on Previous Results from Mouse Model of Asthma

4.3.1. Genotyping Results of 90 SNPs

Due to missing ratios or significant deviation from HWE, 6 SNPs were excluded from the analysis. Besides identifying 4 SNPs on two genes to differ significantly between cases and controls after frequentist statistical analysis which also included correction for multiple testing, which are rs2240572, rs2240571 and rs3735222 on *SCIN* and rs32588 on *PPARGC1B* (Temesi et al. 2014), we have identified two other polymorphisms that may be of interest in the elucidation of asthmatic mechanisms. Table 9 shows the characteristics of the two SNPs.

Table 9. *Characteristics of SNPs rs9862203 and rs1508147*

Gene	SNP	Position	Function	Alleles
KLF15	rs9862203	Chr3:126058362	intronic	A/G
BIRC5	rs1508147	Chr17:76222588	near-gene-3	A/G

The table has been adapted from Temesi et al. 2014. Supplementary material 1.

Table 10 shows the HWE results of SNP rs9862203 of *KLF15* (Kruppel Like Factor 15) and rs1508147 of *BIRC5*. Both SNPs show a significant deviation between cases and controls on the Armitage's trend test, as well as in the difference of allele

frequencies (rs9862203: p=0.04984, OR=1.293 for allele G; p=0.04359, respectively; rs1508147: p=0.04928, OR=1.26 for allele A; p=0.04148, respectively). These SNPs showed significance previous to the multiple testing correction, but have lost significance after it. Nonetheless, these polymorphisms may be an important finding due to their roles in the pathogenesis of asthma (Temesi et al. 2014).

Table 10. *Detail of results of statistical analysis of 90 SNPs*

Phenotype	SNP	Phenotype	MAF	Genotype[a] 11 n (%)	Genotype[a] 12 n (%)	Genotype[a] 22 n (%)	Difference between allelic frequencies		Armitage's trend test	
							P	OR(95%CI)	P	OR(95%CI)
Asthma	rs9862203	Present	0.4	149	110	27	0.04	1.3 (1.0-1.7)	0.049	1.3
		Absent	0.39	176	101	19				
	rs1508147	Present	0.24	116	135	45	0.04	0.8 (0.6-0.9)	0.049	0.8
		Absent	0.25	114	146	72				

The table has been adapted from Temesi et al. 2014. Supplementary material 2

5. Discussion

During our studies, we aimed to study the role of the Hippo/YAP1 signalling pathway in asthma or its associated phenotypes. We aimed to examine the ever-growing population of our asthmatic biobank in terms of genetic variations, gene expression and protein expression levels of the Hippo/YAP1 pathway. We also investigated polymorphisms of the angiopoietin receptor gene, *TEK*, in the pathogenesis of asthma.

Recently, it has been shown that inflammation caused by tissue damage or microbial invasion has an important role in host defence mechanisms, as well as inducing regeneration and repairs. Furthermore, Chan et al showed that house dust mite-induced asthma leads to a significant increase in reactive oxygen species (ROS) production and DNA damage in lung tissues, especially in the bronchial epithelium (Chan et al. 2016). However, the mechanisms by which inflammation, ROS and DNA damage trigger regenerative responses, remain unclear.

Initially, it was thought that the Hippo/YAP1 pathway played an important role in the regulation of organ size, on the other hand, recently it has been indicated that YAP1 protein could also be detected in peripheral respiratory epithelial cells of the adult mouse lung (Lange et al. 2015). Further, it was shown, that the distribution and intensity of YAP1 staining were increased after the depletion of club cells in the lungs. After 10 days, when the regeneration of the bronchiolar epithelium was complete, the YAP1 level and distribution was similar to that in the uninjured airway (Lange et al. 2015). Additionally, several indications link the Hippo pathway with oxidative stress or ROS-initiated signalling pathways and various pathological processes. ROS triggered signalling is also mediated by YAP1, the major Hippo downstream target (Mao et al. 2015). These findings in mice suggest that the Hippo/YAP1 pathway can also be a player in the regeneration processes in human asthma.

Earlier our research group identified the *FRMD6* gene through a partial genome screening in paediatric asthma as well as showing that it was most consistently associated with asthma susceptibility and its function in the asthmatic processes was also confirmed in an OVA-induced mouse model (Ungvári et al. 2012b). In an independent study our group found the genetic variations of *BIRC5* to influence asthma susceptibility, additionally the gene expression of *BIRC5* changed significantly during asthma both in animal and human studies (Ungvári et al. 2012a). Since it has been speculated that *FRMD6* is a possible upstream mediator of the Hippo pathway and *BIRC5* is a

downstream target gene of YAP1, these findings also indicated that the Hippo/YAP1 pathway might have a role in asthma.

In the present study as well as in earlier studies we found all important members of the FRMD6/Hippo/YAP1 pathway to be expressed in the human induced sputum in both asthmatics and healthy persons. The gene expression levels of the various components of the Hippo pathway showed correlations with diverse cell types. This may be suggestive of the main sources of these mRNA in the sputum samples. It may also be proposed that the regulation of these mRNA expressions may be different in the implied cells and/or the genes have additional, diverse functions in these cells.

The gene expression level of *YAP1* was found to be correlated with sputum bronchial epithelial cell number suggesting its possible origin. There was no correlation found between *YAP1* mRNA level and the severity of asthma or other asthma phenotype. Interestingly, YAP1 protein could only be detected in mild asthmatics and could not be seen in controls or in severe asthmatics on Western blot.

Initially, it has been suggested that YAP1 is regulated by the Hippo pathway, a kinase cascade that eventually phosphorylates and hence inhibits the protein. Recently, several studies have suggested that Hippo-mediated YAP1 phosphorylation is an essential input for YAP1 regulation but it is not the only one. YAP1 phosphorylation and activity can be regulated by inflammation, DNA damage, ROS or mechanical signals that represent separate signals with partly independent pathways (Figure 4) (Chan et al. 2016; Mao et al. 2015; Moleirinho et al. 2013; Piccolo, Dupont, and Cordenonsi 2014; Yin and Zhang 2015).

The mechanisms of YAP1 inhibition by phosphorylation are nuclear exclusion, sequestration in the cytoplasm or proteasomal degradation. We detected *YAP1* mRNA in all samples, but YAP1 protein could be detected only in mild asthmatics. Although the detection level of the RT-PCR is lower than that of the Western blot, this finding is in agreement with the previous notion that YAP1 activity is also and perhaps mainly regulated on the protein level. Of course, due to the low number of patients and the type of detection method used this can only be regarded as a preliminary finding. On the other hand, based on the above described observations, the appearance of YAP1 in the mild asthmatics can be explained by the asthma-associated tissue damage induced regeneration where the Hippo/YAP1 pathway can have an important role (Beasley et al. 1989; Jeffery et al. 1989; Laitinen et al. 1985). Inflammation, DNA damage and elevated ROS in the airway epithelium were all found to be associated with increased level of YAP1 protein

(Mao et al. 2015). Presently, it cannot be explained why the YAP1 protein could not be detected in the sputum samples of the severe asthmatics. However, it can be hypothesized that by an unknown mechanism YAP1 is not (or less) activated in the lung of severe asthmatics which can result in an impaired regeneration process in the airways which can contribute to the irreversible organ damage and the severity of airway remodelling in these patients.

Here, we have to mention some limitations to our study. Firstly, although Western blot can determine the molecular weight of the protein and in this way, has a higher specificity comparing to e.g. ELISA, it is less sensitive and less capable of quantitative measurement. Secondly, in this study we did not differentiate between dephosphorylated and phosphorylated YAP1. Furthermore, because we have no available data on the exact time points inhaled corticosteroid (ICS) were administered, we cannot exclude the possibility that the different time intervals between ICS administration and sputum induction may influence our results.

Finding a genetic variation in the *YAP1* gene to be associated with exercise-induced asthma as well as finding a significant difference in the distributions of certain haplotypes and different asthma GINA statuses further supports the possible role of the Hippo/YAP1 pathway in asthma. The latter observation, in line with the lack of YAP1 protein in the induced sputum of severe asthmatics, also supports the finding that haplotypes in the *YAP1* gene associate with the severity of the disease. It must be mentioned, however, that there may be differences in childhood and adult asthma and thus the genetic associations must be confirmed in a well characterized adult population.

Based on our genotyping results and the characteristics of the asthmatic patients, we searched for the most probable interaction networks with respect to different target variables. We also wanted to know whether there were interactions between the three genes (*FRMD6*, *YAP1* and *BIRC5*) whose genetic variations associated with asthma or asthma phenotypes in this population. Using the BN-BMLA method there was no interaction between these genes implying that the genetic variations in the three genes influenced the disease susceptibility independently from each other or the studied population was too small to detect such interactions.

Two genetic variations in the *FRMD6* gene proved to be the most relevant to exercise-induced asthma and allergic rhinitis within the asthma group. The two SNPs are in epistatic interaction with each other through allergic rhinitis and exercise-induced asthma. The term exercise-induced asthma describes the transient narrowing of the

airways after exercise. Presently, the exact mechanism of exercise-induced asthma is not known but as breathing through the mouth is common during exercise, there is an increased penetration of pollutants, cold air and allergens into the airways which can lead to epithelial damage, inflammation, and remodelling (Boulet and O'Byrne 2015; Weiler et al. 2010). Based on the literature and of our findings it is not possible to explain the connection between the variations in the *FRMD6* gene, rhinitis and exercise-induced asthma, but a possible hypothesis may be that the variations in the gene can weaken the regeneration capacity of the Hippo pathway which can lead to persistent epithelial damage and asthmatic symptoms in genetically susceptible individuals.

In our next study, we investigated whether eQTL SNPs in the *TEK* gene influenced the risk for asthma or associated phenotypes. We did not find any associations between these SNPs and asthma in our population, however, one of the variations showed a rather strong association with allergic conjunctivitis. To the best of our knowledge this is the first study to show that a genetic variation associates only with allergic conjunctivitis and not with other atopic diseases like allergic rhinitis or asthma. Figure 14 shows the possible role of Tie2 signalling in allergic conjunctivitis.

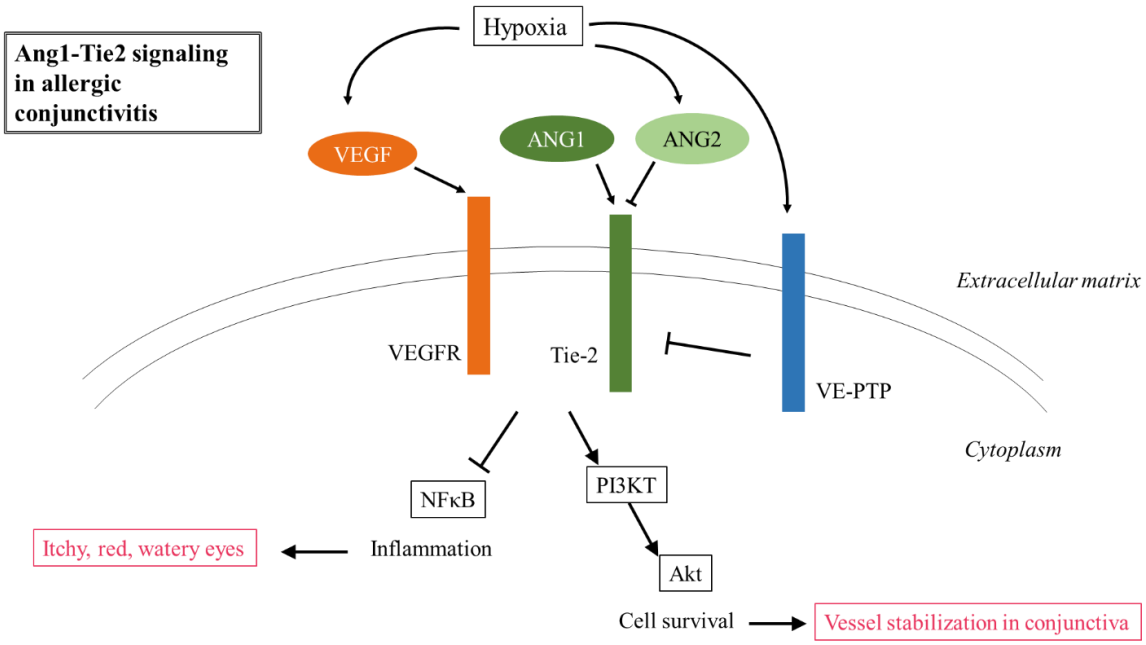

Figure 14. *Possible role of Ang1-Tie2 signalling pathway in allergic conjunctivitis.*

However, a number of evidence indicates, that the Tie2 pathway may have a role in asthma. The gene expression of the main ligand of the Tie2, angiopoietin 1 was significantly reduced in our OVA-induced mouse model of asthma and our results were

also supported by Simoes et al (Simoes et al. 2008). As Ang1 has an anti-inflammatory role in the lung by inhibiting leukocyte transendothelial migration, cytokine production and vascular permeability, its reduced expression may contribute to the development of the asthmatic airway inflammation (Simoes et al. 2008).

Furthermore, in a recent large genome wide association study four low frequency SNPs on chromosome 9p21.2 were found to be significantly associated with asthma. Although the detected SNPs were closest to the *EQTN* gene, they were in LD with a missense variant in the *TEK* gene however being physically quite far from it. Because of this linkage and the known function of the Tie2, the *TEK* gene was suggested as a candidate gene. However, fine mapping the region showed no eQTL effects in any of the tissues relevant to asthma (Almoguera et al. 2016). In our study, we tested three eQTL SNPs within the *TEK* gene with known respiratory disease association, whether they influenced the risk for asthma or any associated phenotypes. We found no association with asthma, but a quite strong association with allergic conjunctivitis.

Possibly, allergic conjunctivitis is the least well studied atopic disease. Because it occurs together with rhinitis in most cases, they are often studied together as rhinoconjunctivitis and most studies report only on either the proportion of rhinitis patients suffering from ocular symptoms or the associated burden (Klossek et al. 2012). However, not all rhinitis patients develop conjunctivitis and conjunctivitis can exist without rhinitis indicating a partially different genetic background. In contrast, there is hardly any published genetic study in allergic conjunctivitis and there are no genetic variants which associate only or mainly with allergic conjunctivitis. Although several studies illustrate that ocular symptoms could have greater negative impact on the quality of life of the patients than nasal problems, they are underappreciated and often under-treated (Pitt et al. 2004; Smith et al. 2005). The presence of ocular symptoms has been shown to be correlated with sleep impairment, limitations in daily activities and emotional distress (Stull et al. 2009).

In our study population, the prevalence of allergic conjunctivitis among the patients with rhinitis (55.1%) was within the range of the results of other studies (50-65%) (Rosario and Bielory 2011). The gene for the Tie2 receptor is a plausible candidate gene in allergic conjunctivitis. It is highly expressed in the eye; its mutations can cause congenital glaucoma and it is a potential drug target in different eye diseases (Souma et al. 2016). The associated SNP is located in an intron near the transcription start site and annotated as an endothelial cell specific enhancer region, associated with heavily

acetylated histones and/or endothelial cell specific euchromatin. The rare allele of the rs581724 SNP which is associated with the increased risk to conjunctivitis is also associated with reduced Tie2 expression in HapMap3 population.

Presently it cannot be explained how exactly this SNP increases the risk to conjunctivitis, but it has been shown that Tie2 participates in the regulation of the barrier function of the endothelial cells (David et al. 2013; Rübig et al. 2016). It can be hypothesized that the rs581724 SNP, which is associated with a lower Tie2 expression, can weaken this barrier function of the endothelial cells. Moreover, it is well known that the inflammatory response to allergens causes nearby blood vessels to dilate and become more permeable. It can be speculated that this second hit to the microvascular barriers already weakened by reduced Tie2 expression may increase the risk of the activated inflammatory cells leaking out of the blood vessels into the surrounding tissues in the eye causing the characteristic symptoms of the allergic conjunctivitis.

Although there are different types of ophthalmic anti-allergic medications available for the treatment of the patients with allergic conjunctivitis, it is generally accepted that the patients are under-treated. E.g. in a large French study it was shown that despite having received treatment for ocular symptoms, in more than 20% of the patients the symptoms persisted indicating that the treatments were not effective (Klossek et al. 2012). Based on our results, the Tie2 pathway can play a role in the pathomechanism of the disease and it is a potential novel therapeutic target in allergic conjunctivitis. In the last decade, several different agents that can activate the Tie2 pathway have been investigated in diseases where leaky blood vessels and/or downregulated Tie2 receptor contributed to their development. These diseases include sepsis, acute kidney injury, influenza, stroke and eye diseases, and it was shown that these drugs could provide additional benefit to the prevailing therapy (Bourdeau et al. 2016; Cui et al. 2013; David et al. 2013; Rübig et al. 2016; Sugiyama et al. 2015). It was also shown that compensatory changes in Ang1 expression might help preserve basal Tie2 signalling when *Tie2* gene expression is low. Our results suggest that these or similar drugs might also be potential candidates in allergic conjunctivitis.

In our following study, we have used a previous investigation of asthma based on an OVA-induced mouse model by Tölgyesi et al. This study is discussed in another Doctoral Thesis by Temesi, therefore I only focus and discuss my own scientific findings here. After gene choice, we genotyped 90 SNPs on our human biobank of asthmatics and controls (Temesi et al. 2014). We have identified two SNPs, one of them, rs9862203 is

an intronic SNP on the *KLF15* gene and the other, rs1508147 is a near-gene SNP on the 3' end of the *BIRC5* gene. Both of them showed a significant deviation between asthmatics and controls, but the significance was lost after multiple testing correction. Nonetheless, both SNPs and genes may have a role in the pathogenesis of asthma, hence I find it important to elaborate these findings.

Identifying a difference between asthmatics and healthy controls in a regulatory region of *BIRC5* provides further evidence for its role in asthma. Tölgyesi et al have previously shown that *Birc5* gene expression increases in OVA-induced mice in the Th2-type inflammation model (Tölgyesi et al. 2009). The group's results were confirmed in a mouse model of asthma, where the mRNA level of *Birc5* significantly correlated with the eosinophil level found in the mice's bronchoalveolar lavage (Tumes, Connolly, and Dent 2009). Furthermore, Ungvári et al has shown that *BIRC5* gene expression increase in asthma also remains in humans, as found in the induced sputum samples of asthmatics and controls, as well as identifying a polymorphism that may play a role in the asthmatic mechanisms (Ungvári et al. 2012a). Ungvári et al has also identified the same SNP, rs1508147 to be slightly associated with asthma in females (OR=1.683, CI=1.096-2.585, p=0.017) (Ungvári et al. 2012a). This polymorphism has been found to have the highest impact on *BIRC5* expression (Dixon et al. 2007). Ungvári et al hypothesize, that due to its position near the 3'end of the gene, rs1508147 may also disrupt or create miRNA binding sites, although they could not confirm this assumption because of the available miRNA predicting tool (Ungvári et al. 2012a).

KLF15, Kruppel Like Factor 15, a transcription factor has been implicated to play a role in the regulation of vascular smooth and cardiac muscle functions. Recently, it has been shown by expression profiling that during the identification of target genes of glucocorticoids - that play a major role in the treatment of asthma symptoms - in human airway smooth muscle cells, *KLF15* has a differential gene expression in the presence of the drug (Masuno et al. 2011) They have also confirmed the result on other airway smooth muscle cell lines by qPCR. Masuno et al have shown, that the difference *in vitro* has an *in vivo* function, as they have treated wild type and *Klf15*-/- mice after OVA induction with a synthetic glucocorticoid, and have found that the *Klf15* deficient mice had a reduced AHR associated with the OVA challenge. They hypothesize that Klf15 in mice play a role in the contractility of the airways by regulating apoptosis and proliferation (Masuno et al. 2011). Their findings have been confirmed by Himes et al, who have used RNA-seq to identify airway smooth muscle transcriptome in response to glucocorticoids.

KLF15 was one of the differentially expressed genes they found, providing further evidence for its role in asthma pathogenesis (Himes et al. 2014). In addition to these findings, Tölgyesi et al have created an allergic airway inflammatory mouse model of asthma, with 3 groups of mice with a differential OVA-challenge protocol (Tölgyesi et al. 2009). In group 1, they have seen a quick increase in neutrophil cell count (neutrophil infiltration) in the inflammatory cell composition from isolated BALF, however in groups 2 and 3, eosinophilic infiltration was seen, as the Th2-type eosinophilic airway inflammation and eventually airway hyperresponsiveness has developed towards the end of their protocol in group 3 (Tölgyesi et al. 2009). After the microarray gene expression experiment a significant decrease in *Klf15* mRNA was seen in group 2 and a slight increase but nonetheless significant reduction in group 3 compared to control mice without OVA-challenge, but this was not seen in group 1 (Group 2 vs Control group: corrected p-value=0,0043, normalized log2 ratio=-1,39; Group 3 vs Control group: corrected p-value=0,0073, normalized log2 ratio=-1,51) (Tölgyesi et al. 2009). Therefore, in mice, *Klf15* gene expression was reduced significantly in a systemic allergic status, but in comparison in the allergic airway inflammatory disease, *Klf15* expression has slightly increased. These results further support the role of KLF15 in asthma.

Both genes may play a role in asthma, but their functional studies are needed to better understand asthma processes and to find new potential therapeutic targets.

6. Conclusions

Our study provides additional evidences that the FRMD6/Hippo/YAP1 pathways might have a role in asthma and its different subtypes.

We showed that all investigated Hippo signalling pathway members were detectable in the induced sputum of both asthmatics and controls.

We showed that *YAP1* mRNA expression is significantly lower in asthmatics compared to controls and found also that the main source of *YAP1* in asthmatics may be the bronchial epithelial cells. Additionally, more correlations have been revealed between Hippo member gene expressions and various cell types, suggesting their source of origin.

We found genetic variations on the *YAP1* gene to be associated with exercise-induced asthma, and asthma severity. The latter result was also confirmed by the haplotype analysis. Of course, the genetic associations must be confirmed in independent populations.

The BN-BMLA revealed a direct relevance of SNP rs9671722 on the *FRMD6* gene to exercise-induced asthma, while another SNP, rs3751464 from the same gene was found to be directly relevant to allergic rhinitis and transitively associated through allergic rhinitis with exercise-induced asthma, suggesting an increased importance of the FRMD6/Hippo/YAP1 pathway in the pathogenesis of asthma and its associated phenotypes.

We found that YAP1 protein was only expressed in mild asthmatics, but neither in controls nor in severe asthmatics. It would be also interesting to reveal how exactly the activity of YAP1 protein is regulated in the airways of the asthmatic patients. If additional studies can confirm that the YAP1 associated pathways have a role in the regeneration processes in airway inflammations, these pathways can be potential novel therapeutic targets in asthma and other inflammatory airway diseases.

Although several lines of evidences indicate that the Tie2 pathway might have a role in asthma, the investigated variations in the *TEK* gene, which are associated with lower Tie2 expression, did not influence the susceptibility to the disease.

We found however, that the homozygote carriers of the rs581724 SNP had significantly increased risk to allergic conjunctivitis. If additional studies can confirm the role of the Tie2 pathway in allergic conjunctivitis, this can be a potential novel therapeutic target in the disease.

We investigated 90 SNPs on 60 genes chosen based on a previous study on OVA-induced mice. We have identified two SNPs, one of them, rs9862203 is an intronic SNP on the *KLF15* gene and the other, rs1508147 is a near-gene SNP on the 3' end of the *BIRC5* gene. Both of them showed a significant deviation between asthmatics and controls, but the significance was lost after multiple testing correction. Nonetheless, both SNPs and genes may have a role in the pathogenesis of asthma due to their functions. Both genes may play a role in asthma but their functional studies are needed to better understand asthma processes and to find new potential therapeutic targets.

7. Summary

Asthma is a chronic inflammatory respiratory disease influenced by a wide range of environmental and genetic factors. We investigated the possible roles of the Hippo/YAP1 associated pathway, variations in the *TEK* gene and 90 SNPs based on the results of an OVA-induced mouse model of asthma in paediatric asthma and/or associated phenotypes. Several lines of evidences indicate that the Hippo/YAP1 pathways might play a role in asthma. We compared the level of gene and protein expression of several members of the Hippo/YAP1 pathway. The mRNA of all the members of the Hippo/YAP1 pathway could be detected in the induced sputum of both controls and cases. The YAP1 protein could not be detected in the sputum samples of the healthy controls and severe asthmatics but it was detectable in mild asthmatics. Fourteen SNPs in the *YAP1* gene were genotyped on our study population. The rs2846836 of the *YAP1* gene was significantly associated with exercise-induced asthma (OR=2.1 [1.3-3.4], p=0.004). The association was confirmed by haplotype analysis. With Bayesian network based Bayesian multilevel analysis of relevance (BN-BMLA) two genetic variations in the *FRMD6* gene proved to be the most relevant to exercise-induced asthma and allergic rhinitis.

The Tie2 receptor is an important player in angiogenesis. The *Tie2* mRNA and protein are abundantly expressed in the lungs and the associated pathway also has an important role in the development and function of the eye. Recently, variations in the *TEK* gene, encoding Tie2, have been found associated with asthma. Three SNPs in the *TEK* gene were genotyped on our study population. The genotyped SNPs showed no association with asthma. However, SNP rs581724 was significantly associated with allergic conjunctivitis in a recessive way (p=0.007; OR=2.3 (1.3-4.4)) within the asthmatic population. The risk remained significant when the whole population without conjunctivitis was involved in the calculation (p = 0.003; OR = 2.1 (1.3-3.6)). The rare allele of the rs581724 SNP which is associated with the increased risk to conjunctivitis is also associated with reduced Tie2 expression. If additional studies can confirm the role of the Tie2 pathway in allergic conjunctivitis, it can be a potential novel therapeutic target in the disease.

The association analysis of asthma and 90 SNPs based on a previous study of OVA-induced mouse model of asthma resulted in two nominally significant associations within the *BIRC5* gene providing further evidence for the role of the FRMD6/Hippo/YAP1 pathway in asthma, and *KLF15* gene which may play a role in the contractility of airway smooth muscle by regulating apoptosis and cell proliferation.

8. Összefoglaló

Az asztma krónikus gyulladásos légúti megbetegedés, amit különféle környezeti és genetikai tényezők is befolyásolnak. Azért, hogy jobban megértsük a betegség kialakulását, megvizsgáltuk a Hippo/YAP1 szignál útvonal, a *TEK* gén genetikai variációinak és egy korábbi ovalbumin indukált asztmás egér modell vizsgálat eredményei alapján 90 SNP lehetséges szerepét az asztmában és a hozzá kapcsolódó fenotípusokban. Számos bizonyíték jelzi, hogy a Hippo/YAP1 útvonal szerepet játszhat az asztmában. Összehasonlítottuk a Hippo útvonal több tagjának gén illetve fehérje expresszióját. A Hippo/YAP1 útvonal tagjainak indukált köpet mRNS szintjét egészségesekben is és asztmásokban is detektáltuk. A YAP1 fehérjét nem tudtuk kimutatni sem egészségesek sem pedig súlyos asztmások indukált köpet mintájában, viszont az enyhe asztmásokban találtunk a fehérjéből. Tizennégy SNP-t genotipizáltunk a beteg populáción. A *YAP1* gén rs2846836 SNP-je szignifikánsan asszociált terheléses asztmával (OR=2,1 [1,3-3,4], p=0,004). Az asszociációt haplotípus elemzés is megerősítette. A Bayesi (BN-BMLA) elemzés során az *FRMD6* gén két genetikai variációja bizonyult a legrelevánsabbnak terheléses asztmában és allergiás rhinitisben.

A Tie2 receptor az angiogenezis egy fontos szereplője. A Tie2 mRNS-e és fehérjéje széles körben fejeződik ki a tüdőben, és a hozzá kapcsolódó útvonal fontos szerepet tölt be a szem fejlődésében és működésében. Nemrég a *TEK* (Tie2 kódoló gén) variációit hozták kapcsolatba az asztmával. Három *TEK* SNP-t genotipizáltunk beteg populáción. A genotipizált SNP-k nem asszociáltak az asztmával. Azonban, az rs581724 szignifikáns asszociációt mutatott az allergiás conjunctivitis-szel (p=0,007; OR=2,3 (1,3-4,4)) az asztmás populációban. A kockázat akkor is megmaradt, amikor az egész populációt belevettük a számításba (p = 0,003; OR = 2,1 (1,3-3,6)). Az rs581724 ritka allélja, ami kapcsolatban van a conjunctivitis megemelkedett kockázatával, szintén csökkent Tie2 expresszióval asszociál. Amennyiben további tanulmányok igazolják a Tie2 szerepét az allergiás conjunctivitis-ben, potenciális új terápiás célponttá válhat.

Az asszociációs vizsgálat során az asztmát és 90 SNP-t vizsgáltunk egy korábbi OVA indukált asztmás egér tanulmány eredményei alapján. Két, nominálisan szignifikáns asszociációt találtunk, az egyik SNP-t a *BIRC5* génen, tovább biztonyítva az FRMD6/Hippo/YAP1 útvonal szerepét az asztmában, és a másikat a *KLF15* génen, amely a légúti sima izom kontraktilitásban játszik szerepet a sejtek apoptózisának és proliferációjának szabályozásával.

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10. Publications

10.1. Publications related to doctoral dissertation

1. **Fodor LE**, Gézsi A, Ungvári L, Semsei ÁF, Gál Z, Nagy A, Gálffy G, Tamási L, Kiss A, Antal P, Szalai C. Investigation of the Possible Role of the Hippo/YAP1 Pathway in Asthma and Allergy. Allergy Asthma Immunol Res. 2017 May;9(3):247-256. doi:10.4168/aair.2017.9.3.247 IF: 2.957

2. **Fodor LE**, Gézsi A, Gál Z, Nagy A, Kiss A, Bikov A, Szalai C. Variation in the TEK Gene is Not Associated with Asthma but with Allergic Conjunctivitis. Int J Immunogenet. 2018 Apr 18. doi: 10.1111/iji.12365. [Epub ahead of print] IF: 1.093

3. Temesi G, Virág V, Hadadi É, Ungvári I, **Fodor L E**, Bikov A, Nagy A, Gálffy G, Tamási L, Horváth I, Kiss A, Hullám G, Gézsi A, Sárközy P, Antal P, Buzás E, Szalai C. Novel genes in Human Asthma Based on a Mouse Model of Allergic Airway Inflammation and Human Investigations. Allergy Asthma Immunol Res. 2014;6(6):496-503. doi:10.4168/aair.2014.6.6.496. IF: 2.160

10.2. Publications not related to doctoral dissertation

1. Balla Bernadett, **Fodor Lili**, Lakatos Péter: Az obesitas genetikája c. fejezet. Klinikai obezitológia. Budapest: Semmelweis Kiadó, 2017. pp. 31-48. ISBN:9789633313435

2. Sági, J. C., Kutszegi, N., Kelemen, A., **Fodor, L. E**., Gézsi, A., Kovács, G. T. Erdélyi, D. J., Szalai, C., Semsei, Á. F. Pharmacogenetics of anthracyclines. Pharmacogenomics 2016:17(9): 1075-1087. doi:10.2217/pgs-2016-0036 IF: 2.350

3. Sándor N, Schilling-Tóth B, Kis E, **Fodor L**, Mucsányi F, Sáfrány G, Hegyesi H. TP53inp1 Gene Is Implicated in Early Radiation Response in Human Fibroblast Cells. Piva T, ed. Int J Mol Sci. 2015;16(10):25450-25465. doi:10.3390/ijms161025450. IF: 3.226

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Table S1. *Summary of all YAP1 SNPs included in haplotype analysis and corresponding results.*

SNPs involved in the haplotype analysis														Haplotype	GINA2 Status in asthma	GINA3 Status in asthma	p-value	OR	95%CI
rs1820453	rs7106388	rs10895257	rs1426398	rs11225138	rs1426394	rs948737								ACGTCAC	7%	25%	0.000002	4.257	2,393-7,573
rs1820453	rs7106388	rs10895257	rs1426398	rs11225138	rs1426394	rs948737								ACATGGT	21%	14%	0.121	0.591	0,316-1,103
rs1820453	rs7106388	rs10895257	rs1426398	rs11225138	rs1426394	rs948737								CTGCGAC	56%	50%	0.313	0.792	0,511-1,228
rs1820453	rs7106388	rs10895257	rs1426398	rs11225138	rs1426394	rs948737								ACGTGGT	5%	7%	0.454	1.501	0,628-3,590
	rs7106388	rs10895257	rs1426398	rs11225138	rs1426394	rs948737								CGTCAC	7%	25%	0.000002	4.257	2,393-7,573
	rs7106388	rs10895257	rs1426398	rs11225138	rs1426394	rs948737								CATGGT	21%	14%	0.122	0.606	0,324-1,132
	rs7106388	rs10895257	rs1426398	rs11225138	rs1426394	rs948737								TGCGAC	56%	50%	0.313	0.792	0,511-1,228
	rs7106388	rs10895257	rs1426398	rs11225138	rs1426394	rs948737								CGTGGT	6%	7%	0.634	1.325	0,560-3,138
		rs10895257	rs1426398	rs11225138	rs1426394	rs948737								GTCAC	8%	23%	0.000039	3.576	1,998-6,400
		rs10895257	rs1426398	rs11225138	rs1426394	rs948737								ATGGT	20%	14%	0.155	0.614	0,328-1,147
		rs10895257	rs1426398	rs11225138	rs1426394	rs948737								GCGAC	56%	52%	0.500	0.846	0,546-1,313
		rs10895257	rs1426398	rs11225138	rs1426394	rs948737								GTGGT	6%	6%	0.810	1.124	0,451-2,800
		rs10895257	rs1426398	rs11225138	rs1426394									GTCA	8%	25%	0.000005	4.009	2,265-7,095
		rs10895257	rs1426398	rs11225138	rs1426394									ATGG	20%	14%	0.155	0.622	0,333-1,162
		rs10895257	rs1426398	rs11225138	rs1426394									GCGA	57%	55%	0.652	0.890	0,573-1,381
		rs10895257	rs1426398	rs11225138	rs1426394									GTGG	5%	5%	1.000	0.964	0,361-2,576
			rs1426398	rs11225138	rs1426394	rs948737								TCAC	8%	23%	0.000039	3.576	1,998-6,400
			rs1426398	rs11225138	rs1426394	rs948737								TGGT	26%	20%	0.245	0.712	0,414-1,225
			rs1426398	rs11225138	rs1426394	rs948737								CGAC	56%	52%	0.500	0.846	0,546-1,313
			rs1426398	rs11225138	rs1426394									TCA	8%	25%	0.000007	3.895	2,206-6,877
			rs1426398	rs11225138	rs1426394									TGG	26%	18%	0.118	0.621	0,354-1,090
			rs1426398	rs11225138	rs1426394									CGA	57%	55%	0.653	0.897	0,578-1,393
rs1820453	rs7106388													AC	42%	50%	0.142	1.410	0,909-2,188
rs1820453	rs7106388													CT	57%	50%	0.216	0.746	0,481-1,158
	rs7106388	rs10895257												CG	21%	36%	0.001024	2.220	1,386-3,554
	rs7106388	rs10895257												CA	21%	14%	0.094	0.584	0,313-1,089
	rs7106388	rs10895257												TG	58%	50%	0.176	0.721	0,465-1,119
		rs10895257	rs1426398											GT	21%	32%	0.017	1.799	1,112-2,910
		rs10895257	rs1426398											AT	21%	14%	0.094	0.584	0,313-1,089
		rs10895257	rs1426398											GC	58%	55%	0.572	0.860	0,554-1,336

SNPs involved in the haplotype analysis														Haplotype	GINA2 status	GINA3 status	p-value	OR	95%CI
			rs1426398	rs11225138										TC	8%	28%	0.000001	4.324	2,494-7,496
			rs1426398	rs11225138										TG	34%	18%	0.002349	0.425	0,244-0,742
			rs1426398	rs11225138										CG	58%	55%	0.572	0.860	0,554-1,336
				rs11225138	rs1426394									CA	7%	26%	0.000001	4.497	2,540-7,960
				rs11225138	rs1426394									GA	66%	55%	0.063	0.648	0,415-1,009
				rs11225138	rs1426394									GG	26%	19%	0.155	0.659	0,380-1,144
					rs1426394	rs948737								AC	68%	74%	0.332	1.308	0,798-2,145
					rs1426394	rs948737								GT	27%	23%	0.448	0.797	0,475-1,335
					rs1426394	rs948737								AT	4%	3%	0.782	0.745	0,217-2,559
						rs948737	rs1942683							CG	14%	23%	0.030	1.842	1,072-3,164
						rs948737	rs1942683							TA	7%	3%	0.176	0.412	0,124-1,368
						rs948737	rs1942683							CA	55%	51%	0.575	0.861	0,555-1,335
						rs948737	rs1942683							TG	24%	23%	0.896	0.938	0,558-1,577
							rs1942683	rs11225161						AC	62%	51%	0.068	0.649	0,418-1,009
							rs1942683	rs11225161						GT	9%	12%	0.349	1.387	0,704-2,734
							rs1942683	rs11225161						GC	29%	33%	0.391	1.246	0,781-1,990
								rs11225161	rs1894116					CT	90%	78%	0.001122	0.377	0,213-0,667
								rs11225161	rs1894116					TC	10%	22%	0.001122	2.654	1,499-4,699
									rs1894116	rs11225166				CC	10%	22%	0.001122	2.654	1,499-4,699
									rs1894116	rs11225166				TG	90%	78%	0.002252	0.404	0,229-0,713
										rs11225166	rs8504			CG	9%	23%	0.000130	3.198	1,802-5,675
										rs11225166	rs8504			GG	58%	48%	0.071	0.658	0,424-1,021
										rs11225166	rs8504			GA	32%	30%	0.632	0.877	0,543-1,417
											rs8504	rs2846836		GT	10%	28%	0.000009	3.538	2,071-6,045
											rs8504	rs2846836		GC	58%	40%	0.001576	0.481	0,307-0,752
											rs8504	rs2846836		AT	32%	32%	1.000	0.997	0,624-1,592
												rs2846836	rs7115540	CA	52%	34%	0.001695	0.482	0,305-0,762
												rs2846836	rs7115540	TA	14%	23%	0.029	1.874	1,090-3,223
												rs2846836	rs7115540	TG	29%	39%	0.068	1.563	0,991-2,466
												rs2846836	rs7115540	CG	6%	4%	0.632	0.705	0,242-2,058

Table S2. *Summary of 90 SNPs included in the genetic association study. Adapted from Temesi et al. 2014.*

Gene	**SNP**	**Position**	**Functional Category**	**Alleles**	**Contorl MAF**	**Case MAF**
ACSBG1	rs3813577	15:78527253	near-gene-5(GVS)	C/T	37,94%	33,39%
AGR2	rs706072	7:16844663	utr-variant-5-prime(dbSNP)	T/C	18,75%	19,84%
AGR2	rs1459564	7:16846146	near-gene-5(GVS)	A/G	33,28%	30,16%
AGR2	rs706075	7:16846354	near-gene-5(GVS)	G/T	25,22%	24,09%
AGR2	rs10261011	7:16856487	intergenic(GVS)	G/A	51,02%	49,02%
AIF1	rs2857600	6:31582287	near-gene-5(GVS)	T/C	10,66%	3,26%
ATP6V0A4	rs10258719	7:138455988	missense(dbSNP)	A/G	28,34%	29,84%
BIRC5	rs1508147	17:76222588	near-gene-3(GVS)	A/G	43,67%	38,01%
C1QC	rs6690827	1:22967496	near-gene-3(GVS)	A/G	29,76%	35,32%
C1QC	rs294179	1:22974928	downstream-variant-500B(dbSNP)	T/C	42,65%	48,67%
CCL2	rs2530797	17:32586094	near-gene-3(GVS)	C/T	33,53%	37,03%
CCL8	rs1821142	17:32649988	near-gene-3(GVS)	T/C	3,60%	4,58%
CCNE1	rs7257330	19:30301823	near-gene-5(GVS)	A/G	39,49%	38,62%
CD6	rs1050922	11:60785352	synonymous-codon(dbSNP)	G/A	31,54%	33,39%
CD84	rs1055880	1:160517692	utr-variant-3-prime(dbSNP)	T/C	34,24%	33,50%
CHIA	rs17027410	1:111861822	synonymous-codon(dbSNP)	A/G	11,64%	11,26%
CLEC4E	rs7299659	12:8696661	intergenic(GVS)	A/G	19,05%	18,61%
COL6A2	rs2839110	21:47538960	missense(dbSNP)	G/A	22,92%	24,17%

Gene	SNP	Position	Functional Category	Alleles	Contorl MAF	Case MAF
CREB3L4	rs11264743	1:153941514	missense(dbSNP)	T/C	31,12%	30,03%
CXCL1	rs3117604	4:74734668	near-gene-5(GVS)	T/C	29,30%	31,25%
CXCL5	rs352045	4:74864687	near-gene-5(GVS)	T/G	11,77%	13,11%
CSF2	rs27438	5:131413255	near-gene-3(GVS)	A/G	22,73%	22,50%
E2F7	rs310830	12:77419593	synonymous-codon(dbSNP)	G/A	10,06%	13,87%
FABP3	rs16834408	1:31837942	near-gene-3(GVS)	A/G	15,92%	22,02%
FABP3	rs10914367	1:31846206	near-gene-5(GVS)	A/G	24,09%	22,20%
FXYD4	rs4245604	10:43866528	near-gene-5(GVS)	A/C	32,41%	31,64%
GPR160	rs4955711	3:169753570	intergenic(GVS)	G/A	27,96%	28,14%
ICOS	rs3923093	2:204798020	intergenic(GVS)	T/C	24,15%	25,17%
IL17RB	rs2289205	3:53878616	intron-variant(dbSNP)	T/C	29,94%	31,38%
IL1A	rs1878320	2:113544467	upstream-variant-2kb(dbSNP)	C/T	30,15%	28,31%
IL1A	rs3783520	2:113544339	upstream-variant-2kb(dbSNP)	T/C	29,41%	27,93%
IL1B	rs16944	2:113594867	upstream-variant-2kb(dbSNP)	G/A	39,02%	33,50%
IL1RL1	rs12905	2:102960007	utr-variant-3-prime(dbSNP)	A/G	25,00%	26,99%
IL6	rs2069827	7:22765456	utr-variant-3-prime(dbSNP)	T/G	11,05%	10,98%
IL6	rs2069832	7:22767433	intron-variant(dbSNP)	A/G	38,52%	41,45%

Gene	SNP	Position	Functional Category	Alleles	Contorl MAF	Case MAF
ITGAX	rs11150614	16:31366016	upstream-variant-2kb(dbSNP)	A/G	29,31%	28,95%
ITLN1	rs4656958	1:160856964	intergenic(GVS)	A/G	32,56%	25,90%
ITLN1	rs2274910	1:160852046	intron-variant(dbSNP)	T/C	33,43%	27,30%
KLF15	rs1358087	3:126078890	intergenic(GVS)	C/T	36,36%	35,88%
KLF15	rs9862203	3:126058362	intergenic(GVS)	A/G	23,48%	28,67%
LAPTM5	rs3762296	1:31231386	near-gene-5(GVS)	G/A	48,02%	45,07%
LGALS3	rs7160110	14:55594635	upstream-variant-2kb(dbSNP)	G/A	40,54%	37,16%
LGMN	rs9791	14:93170993	synonymous-codon(dbSNP)	T/C	42,21%	35,22%
LY86	rs760894	6:6656198	near-gene-3(GVS)	G/A	32,48%	30,62%
LY9	rs509749	1:160793560	missense(dbSNP)	A/G	50,94%	47,05%
LY9	rs474131	1:160793442	synonymous-codon(dbSNP)	A/G	46,44%	39,29%
MAFB	rs6102095	20:39320751	intergenic(GVS)	A/G	17,25%	15,24%
MAP3K6	rs11247639	1:27679692	intron-variant(dbSNP)	G/A	32,65%	30,54%
MARCO	rs6748401	2:119698057	near-gene-5(GVS)	G/A	50,45%	44,43%
MAT1A	rs3827869	10:82031278	downstream-variant-500B(dbSNP)	T/C	21,00%	16,44%
MAT1A	rs10887711	10:82034842	synonymous-codon(dbSNP)	A/G	31,53%	39,53%
MAT1A	rs10887708	10:82027988	intergenic(GVS)	A/G	28,40%	30,51%

Gene	SNP	Position	Functional Category	Alleles	Contorl MAF	Case MAF
MAT1A	rs10749550	10:82031197	downstream-variant-500B(dbSNP)	A/G	35,99%	37,54%
MKI67	rs11016071	10:129901393	missense(dbSNP)	C/T	14,88%	16,83%
MKI67	rs10082432	10:129901722	synonymous-codon(dbSNP)	A/G	17,70%	18,12%
MKI67	rs8473	10:129899578	missense(dbSNP)	C/T	45,22%	45,73%
MKI67	rs2152143	10:129906980	missense(dbSNP)	A/G	30,78%	28,85%
MS4A7	rs10750936	11:60144180	near-gene-5(GVS)	G/A	36,76%	34,75%
OSGIN1	rs2432561	16:83982670	intergenic(GVS)	A/G	16,74%	10,82%
PPARGC1B	rs32588	5:149200043	synonymous-codon(dbSNP)	C/T	23,46%	14,73%
PTPN7	rs4359077	1:202129112	utr-variant-5-prime(dbSNP)	A/G	10,59%	10,70%
RETNLB	rs3811687	3:108476519	near-gene-5(GVS)	T/C	29,97%	29,61%
RETNLB	rs10933959	3:108476205	near-gene-5(GVS)	G/A	23,11%	19,18%
RETNLB	rs9870145	3:108477874	near-gene-5(GVS)	T/A	14,93%	16,45%
RETNLB	rs11708527	3:108475974	missense(dbSNP)	A/G	29,94%	29,67%
SAA1	rs4638289	11:18285774	intergenic(GVS)	A/T	38,37%	41,48%
SAA1	rs11603089	11:18282051	intergenic(GVS)	G/A	16,13%	15,08%
SAA2	rs7130337	11:18270605	near-gene-5(GVS)	A/G	24,42%	26,66%
SCIN	rs3173628	7:12627245	intron-variant(dbSNP)	A/G	41,28%	49,83%
SCIN	rs2240572	7:12610594	missense(dbSNP)	G/A	48,69%	37,66%
SCIN	rs2240571	7:12609988	near-gene-5(GVS)	C/G	39,80%	50,00%
SCIN	rs3735222	7:12609679	near-gene-5(GVS)	A/G	48,69%	37,87%

Gene	SNP	Position	Functional Category	Alleles	Contorl MAF	Case MAF
SIGLEC1	rs625372	20:3684729	missense(dbSNP)	T/C	32,04%	31,94%
SLAMF9	rs16831153	1:159920719	near-gene-3(GVS)	A/G	17,85%	18,90%
SLC26A4	rs2248465	7:107303628	intron-variant(dbSNP)	C/T	26,33%	29,17%
SLC26A4	rs2701684	7:107299527	near-gene-5(GVS)	A/G	32,36%	34,65%
SLC26A4	rs2701685	7:107299584	near-gene-5(GVS)	A/G	23,51%	25,99%
SLC26A4	rs2712228	7:107300340	near-gene-5(GVS)	C/A	30,15%	30,53%
TBXAS1	rs12532701	7:139521534	intron-variant(dbSNP)	G/A	43,07%	46,73%
TFF1	rs184432	21:43787562	near-gene-5(GVS)	A/G	31,83%	28,29%
TFF1	rs225359	21:43787436	near-gene-5(GVS)	A/G	33,28%	30,96%
TFF2	rs225340	21:43772947	near-gene-5(GVS)	T/C	44,77%	41,86%
TFF2	rs3814896	21:43771711	near-gene-5(GVS)	G/A	33,92%	30,84%
TFF2	rs225333	21:43764496	near-gene-3(GVS)	A/G	28,14%	27,21%
TIMP3	rs137487	22:33259104	intron-variant(dbSNP)	A/G	47,28%	45,86%
TK1	rs1065769	17:76170735	utr-variant-3-prime(dbSNP)	T/C	32,44%	31,17%
TSLP	rs3806932	5:110405675	near-gene-5(GVS)	G/A	38,89%	40,58%
UBE2T	rs14451	1:202304868	synonymous-codon(dbSNP)	C/T	44,31%	47,56%
ULBP1	rs1853665	6:150298842	intergenic(GVS)	T/C	19,58%	17,07%
ULBP1	rs4425606	6:150284435	near-gene-5(GVS)	G/A	18,39%	17,83%