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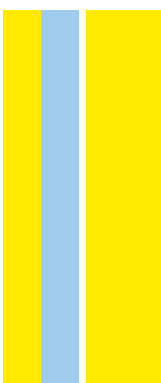
PATOLOGIA E GENÉTICA MOLECULAR

# Emerging risk factors from cardiovascular disease and asthma control.

Margarida Cortez e Castro

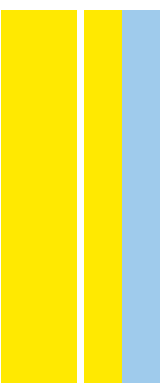
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**2017**



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*Margarida Cortez e Castro*



**U. PORTO**



INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR  
UNIVERSIDADE DO PORTO

Margarida Cortez e Castro

## **EMERGING RISK FACTORS FROM CARDIOVASCULAR DISEASE AND ASTHMA CONTROL**

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## **EMERGING RISK FACTORS FROM CARDIOVASCULAR DISEASE AND ASTHMA CONTROL**

Tese de Candidatura ao Grau de Doutor em  
Patologia e Genética Molecular submetida ao  
Instituto de Ciências Biomédicas Abel Salazar  
Universidade do Porto, ICBAS-UP

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Salazar da Universidade do Porto

## **1.Acknowledgments**

I thank my supervisor Prof Dr.Manuel Bicho for the enthusiasm and the dedication to the scientific investigation within the medical school that created school within the department of genetics that now directs. A word to my co-supervisor Prof Rui Medeiros who always knew to print calm, scientific rigor and focus in the essential to the good scientific research. I thank all Masters students who have contributed to the approach of new endotypes in asthma and who have shown an interest in the study of this disease. A thank to the MsC Andreia Matos, BSc Joana Freitas and MsC Angela Gil for the commitment, scientific rigor, friendship and for the scientific challenges that this work in Asthma research has brought to the Genetic team. I also thank Martyna Wesserling for the contribution in the study of asthma and Tadeusz Pawelczyk for the cooperation in the study of the Polish cohort. To Prof Holloway and Prof Howarth a cheerful appreciation for showing how grateful scientific research could be. I also thank all the Lab technicians namely Conceição Afonso who had rigorously performed the genetics laboratory work. A thank to the Director of the Immunology Service at CHLN-HSM Lisbon (Prof M.A. Barbosa) and to all the patients. A last and grateful thank to my family.

## FINANCIAL SUPPORT

This thesis has been approved and supported by a FCT grant: SFRH/BD/78517/2011

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## **1.Publications, presentations and Premiums**

### **Publications in full text**

**-Lelp-1, Its Role in Atopic Dermatitis and Asthma: Poland and Portugal-** Margarida Cortez<sup>1,2</sup>, Andreia Matos, Martyna Wesserling, Tadeusz Pawelczyk, Magdalena Trzeciak and Manuel Bicho- J Allergy Ther 2016, 7:1

**-Haptoglobin polymorphism and bronchial asthma -** Margarida Cortez, Andreia Matos, Joana Ferreira and Manuel Bicho - International Journal of Current Advanced Research Research ;

Vol 5, Issue 7, pp 1047 -1052, July 2016.

- "DETERMINANTES GENÉTICOS DA RESPOSTA ALÉRGICA E DA ASMA BRÔNQUICA"; M.Cortez- FML-27/4/09- In : **Arquivos Portugueses de Ciências Biológicas**;2009; pag. 24-26.

### **Publications in abstract**

**-Pathophysiology of asthma: From endotype to phenotype-**Margarida Cortez e Castro, Andreia Matos, Joana Ferreira, José Albuquerque, Manuel Bicho; European Respiratory Journal 2014 44: P3826; DOI:

**-Inducible nitric oxide synthase polymorphism in asthmatic patients-** Margarida Cortez e Castro, Andreia Matos, Joana Ferreira, Ridhi Prabhudas, Manuel Bicho; European Respiratory Journal 2014 44: P3837; DOI:

**-eNOS polymorphism in asthmatic patients-** Margarida M.T.C.Cortez e - Castro,JoanaFerreira,,JoséAlbuquerque, ManuelBicho.. Human Immunology Volume 73, Supplement, **October 2012, Page 108.**

**-Asthma and myeloperoxidase gene promoter region polymorphism-** Margarida Cortez e Castro, Andreia Matos, Joana Ferreira, Ridhi Prabhudas, Manuel Bicho; European Respiratory Journal 2014 44: P832; DOI:

**- ACE Polymorphism in asthmatic patients-**. MargaridaCortez,JoanaFerreira, LeonorLopes, ManuelPereira-Barbosa,ManuelBicho. Human Immunology Volume 74, Issue 5, **May 2013, Page 484.**

**- YKL-40 as a Biomarker of Airway Disease in Severe Asthma-**M. Cortez, G.A. Lockett, L. Lau, P.H. Howarth, J.W. Holloway-AJRCCM-ATSJournal-2017

## Poster and oral presentations

**-The role of type 1 angiotensin 2 receptor polymorphism in asthma-**Castro M.M.T.C.C., Matos A., Joana F., Lopes L., Manuel B.- ISAF,Gottenburg Sweden; 2012

**- Ace polymorphism in asthma-** Castro M.M.T.C.C., Matos A., Joana F., Lopes L., Manuel B.- ISAF,Gottenburg Sweden; 2012

**-Can NOS3 and ACE polymorphisms modulate the immune response in a Portuguese Asthmatic population?-** Cortez, Margarida; Matos, Andreia; Ferreira, Joana; Albuquerque, José; Lopes, Leonor; Bicho, Manuel -SPI-Porto,2012

**-Polimorfismo da sintase do óxido nítrico indutível em doentes asmáticos -** Castro M.M.T.C.C., Matos A., Joana F., Prabhudas R., Manuel B. -SPAIC, Porto 2013

**-Papel do polimorfismo na região do gene promotor da MPO em doentes asmáticos-** Margarida Cortez e Castro, Andreia Matos , Joana Ferreira, Ridhi Prabhudas , Manuel Bicho- -SPAIC, Porto 2013

**-ACE polymorphism in asthmatic patients-**

Castro M.M.T.C.C., Matos A., Joana F., Lopes L., Manuel B.- EAACI-WAO-2013; Milan, Italy

**-The role of type 1 angiotensin 2 receptor polymorphism in asthmatic patients-**

Cortez e Castro M.M.T.C.C., Andreia M., Albuquerque J., Ferreira J., Bicho M.- EAACI-WAO-2013: Milan, Italy

**-The role of beta2 adrenergic receptor polymorphism in bronchial asthma**

Cortez e Castro M.M.T.C.C., Andreia M., Albuquerque J., Ferreira J., Bicho M.- EAACI-WAO-2013; Milan, Italy

**-Pathophysiology of asthma : from endotype to phenotype-** Cortez e Castro, Margarida ; Matos, Andreia ; Ferreira, Joana; Prabhudas, Ridhi

; Lopes, Leonor; Albuquerque, José; Bicho, Manuel- EAACI 2014-Copenhagen,Denmark

**-Novel gene polymorphisms in the pathophysiology of asthma : mechanistic approach -**

Cortez e Castro, Margarida ; Matos, Andreia ; Prabhudas, Ridhi; Ferreira, Joana; Gil, Angela ; Bicho, Manuel- EAACI 2014-Copenhagen,Denmark

**-Novel gene polymorphisms in the pathophysiology of asthma”,** Cortez e Castro, Margarida ; Matos, Andreia ; Prabhudas, Ridhi; Ferreira, Joana; Gil, Angela ; Bicho, Manuel-Interasma-World Asthma Congress,Mexico,2014

**-The Role of Myeloperoxidase Gene Promoter Region Polymorphism in Asthmatic Patients,** Castro, Margarida ; Matos, Andreia ; Prabhudas, Ridhi; Ferreira, Joana; Gil, Angela ; Bicho, Manuel- Interasma-World Asthma Congress,Mexico,2014



**-Novel biomarkers in the pathophysiology of asthma-**M.Cortez e Castro , A.Matos , J.Ferreira , R.Prabhudas , L.Lopes , J.Albuquerque , M.Bicho - Interasma-World Asthma Congress,Mexico,2014

**-Inducible Nitric Oxide Synthase Polymorphism in Asthmatic Patients-** Castro, Margarida<sup>1</sup> ; Matos, Andreia ; Prabhudas, Ridhi; Ferreira, Joana; Bicho, Manuel.

**-The role ofGSTM1 and GSTT1 gene polymorphisms in bronchial asthma-** Cortez e Castro, Margarida ; Matos, Andreia ; Lourenço,Marta ; Ferreira, Joana ; Bicho, Manuel-Vienna-Austria-EAACI,2016

**Inducible nitric oxide synthase polymorphism in asthmatic patients-** Cortez e Castro, Margarida<sup>1</sup> ; Matos, Andreia ; Prabhudas, Ridhi; Ferreira, Joana; Gil, Angela ; Bicho, Manuel- Vienna-Austria-EAACI,2016

**-The role of Lelp-1 polymorphism in asthmatic patients -** Cortez e Castro, Margarida ; Weserling, Martyna; Matos, Andreia ; Gil, Angela ; Ferreira, Joana ; Bicho, Manuel- Vienna-Austria-EAACI,2016

**-The role of myeloperoxidase gene promoter region polymorphism in asthma -** Cortez e Castro, Margarida ; Matos, Andreia ; Prabhudas, Ridhi; Ferreira, Joana; Gil, Angela ; Bicho, Manuel- Vienna-Austria-EAACI,2016

**-The role of eNOS, iNOS and ACE polymorphisms in bronchial asthma- Endothelial dysfunction in asthma.** Cortez e Castro M., Ferreira J., Gil A. , Matos A. , Bicho M.; WIRM Switzerland, 2017

**-YKL-40 as a Biomarker of Airway Disease in Severe Asthma-**M. Cortez., G.A. Lockett, L. Lau, P.H. Howarth, J.W. Holloway-ATS,USA,Washington,2017

## **Premiums**

- In June 1990 won a scholarship for the EGID *meeting of the European Group for Immunodeficiencies Oxford*

- December 1990 won the National Magazine Award with the work: "*infection by HTLV-I and common variable immunodeficiency*

- FCT grant: SFRH/BD/78517/2011 with the project:” polymorphisms in CVD and asthma”

- *In 2014 received an award on EAACI- poster presentation*

- *In 2017 received an award on WIRM- poster presentation*

- *In 2017 received an award on ATS- poster presentation*

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### **3.Glossary**

Aa- Amino Acids

AA-Arachidonic acid

ACE-Angiotensin I-converting enzyme

AD-Atopic Dermatitis

ADRB2- beta-2 adrenergic receptor

AGTR1-Angiotensin II type 1 receptor

ACQ7- Asthma control questionnaire 7

APCs- Antigen-Presenting Cells

Apo A1- Apolipoprotein A1

APRE- Acute phase response elements

Asp- Aspergillus

BCR- B cell antigen receptor

BR- Biliverdin reductase

CCL20-C-C ligand motif 20

CCL22-C-C chemokine motif 22

CCR6 Chemokine Receptor Type 6

CD4- Thelper Cells

CD4 + CD25 + Treg- Regulatory Cells

CD5- B cell IgM receptor

CD6- Cluster of differentiation belonging to the family of cysteine-rich capturing receptors

CD8-cytotoxic T cells

CD11 / CD18- family of  $\beta$ -integrins

CD22- Cluster of differentiation 22 transmembrane receptor of the B cells

CD23- Low affinity IgE receptor

CD40 - co-stimulatory protein of APCs

CD54- Co-stimulatory Protein of Antigen-Presenting Cells

CD86- Co-stimulatory Protein of Antigen-Presenting Cells

CD163- Hp-Hb complex receptor

CD206- Mannose receptor

CIC-Immune complexes

CO-Carbon monoxide

COX- Cyclooxygenase

CVD-Cardiovascular Disease

CXCL10-CXC motif chemokine 10

DAMPs- Molecular patterns associated with injury

Dcs- Dendritic cells  
Dc1- Dendritic cells type 1  
Dc2- Type 2 dendritic cells  
CVD-Cardiovascular Disease  
DEP- Diesel exhaust particles  
Derp1-Molecular allergen 1 of the dematofagoides pteronyssinus  
Derp2- Molecular Allergen 2 of the dematofagoides pteronyssinus  
DNA- Deoxyribonucleic acid  
ECP- Eosinophil cationic protein  
ECM-extracellular matrix  
EGF- Epidermal growth factor  
Eos-Eosinophils  
FOXP3 Forkhead box p3: Tregs marker  
Fe<sup>2+</sup> - Ferrous iron  
Fe<sup>3+</sup> + Ferric Iron  
FeNO-Exhaled nitric oxide  
FLG-Filaggrin  
FOXP3- Forkhead box p3: Tregs marker  
FEV1 Forced expiratory volume in 1 second  
GATA3-trans-specific transcription factor T 3 cells  
GINA- Guidelines for the Global Asthma Initiative  
GM-CSF- colony stimulating factor granulocyte-macrophages  
GREs- Glucocorticoid and response elements  
GSTM1- GSTM1 glutathione S-transferase mu 1  
GSTT1- glutathione S-transferase theta 1  
Hb- Hemoglobin  
HbSR- Hb Capturing Receptor  
HDL-High Density Lipoprotein  
12-HETE-12-Hydroxy eicosatetraenoic  
HIF1 $\alpha$ -transcription factors inducible by hypoxia 1 $\alpha$   
HIF-2 $\alpha$ -transcription factors inducible by hypoxia 2 $\alpha$   
HIF1AN- Hypoxia-inducible factor 1-alpha inhibitor  
HIV-1-Human Immunodeficiency Virus type 1  
HO- Heme-oxygenase  
Hp- Haptoglobin  
Hp \* 1-Haptoglobin allele 1  
Hp \* 2- Allele 1 of Haptoglobin

Hp1 -1- Homozygous for Haptoglobin Allele 1  
Hp2-1- Heterozygotes for Haptoglobin allele1 and 2  
Hp2-2 - Homozygous for Haptoglobin Allele 2  
Hp-Hb-complex Hp-Hb  
ICAM-1- Intercellular adhesion molecule 1  
IFN $\gamma$ - Interferon gamma  
IgE- Immunoglobulin E  
IL-1RI- Type 1 IL-1 receptor  
IL-1-RII-IL-1 receptor type 2  
IL-1RA- IL1 Type 1 Receptor Antagonist  
IL-2 Interleukin 2  
IL-4-- Interleukin 4  
IL-5-- Interleukin 5  
IL-6-- Interleukin 6  
IL-10- Interleukin 10  
IL-12-Interleukin 12  
IL-13-- Interleukin 13  
IL-17-- Interleukin 17  
IL-18- Interleukin 18  
IL-21-- Interleukin 21  
IL-23-- Interleukin-23  
IL-27-- Interleukin 27  
iNOS- Inducible nitric oxide synthase  
ISAC- *Immuno Solid-phase Allergen Chip*  
iTreg- Induced / adaptive regulatory T cells  
LBA-Bronchoalveolar lavage  
LELP-1- late cornified envelope-like proline-rich 1  
LO- Lipoxygenase  
LPS- Lipopolysaccharide  
LTA- Lymphotoxin  $\alpha$   
M1- Classically-activated macrophages  
M2- Activated type 2 macrophages  
M2a- Macrophages activated by the alternating route type 2a  
M2b- Activated macrophages type 2b  
M2c- Activated macrophages type 2c  
M4-macrophages inducible by platelet chemokines  
M-DC: macrophages that originate dendritic cells

Mb-Macrophage Inducible by Hp-Hb Complexes  
Mc-Mastocytes  
M-CSF- macrophage colony stimulating factor  
MCP1-Monocyte Chemoattractant Protein  
MDDCs - dendritic cells derived from monocytes  
MD2 - myeloid differentiation factor 2  
M-HA: Macrophages associated with hemorrhage  
MHC-Major Histocompatibility System  
MMP2- Matrix metalloproteinase 2  
MMP9- Matrix metalloproteinase 9  
Mo-Monocytes  
M-ox: Spongy cells  
MPO- Myeloperoxidase  
MRNA-messenger RNA  
MTHFR- Methylene tetrahydrofolate reductase  
NADPH oxidase -Nicotinamide adenine dinucleotide phosphate oxidase  
NF-E2- Factor 2 related to erythroid nuclear factor2  
NF-kB- nuclear factor  $\kappa$ B  
NGF- neuronal growth factor  
NK-Natural killer cells  
NLRs- Nod-Like-Receptors  
NO- Nitric Oxide  
NO2- Nitrogen dioxide  
NOS- Nitric oxide synthase  
NOS2A- Inducible nitric oxide synthase  
NOS3- Endothelial nitric oxide synthase  
NTBI- Plasma labile iron not bound to transferrin  
NTreg- Natural regulatory T cells  
NU-Neutrophils  
O3-Ozone  
24p3- Acute Phase Protein  
PAF- platelet activating factor  
PAGE - polyacrylamide gel electrophoresis  
PAI-1 Plasminogen activator inhibitor 1  
PALMs - lipid mediators associated with pollens  
PAMPs- Molecular patterns associated with pathogens  
PAQLQ- Quality of life questionnaire for asthmatic patients in pediatrics

PAR-2-Protease Activated Receptor  
PCR-C-reactive protein  
PD20- Dose of provocation test that decreases respiratory rates of 20%  
PDc2-Plasmacytoid dendritic cells  
PGE2- Prostaglandin E2  
PGF2 $\alpha$ - Prostaglandin F2 $\alpha$   
PKC-protein kinase C  
PPRs- Pathogen Recognition Receptors  
PST- Proline Serine Threonine  
RAST- Radioallergosorbent test  
ROR $\gamma$ T- Transcription factor related to the ortho-retinoic acid receptor  
RNI- Reactive nitrogen species  
ROI- Reactive Oxygen Intermediates  
ROS - Reactive Oxygen Species  
SCD163-Soluble receptor for the Hp-Hb complex  
SCRC- Cysteine-rich capturing receptors  
SP-A- Surfactant Protein A  
SP-D- Surfactant Protein D  
SPRR- small proline rich proteins  
STAT3- Signal transducer and transcription activator 3  
Syk- Tyrosine Kinase  
TAM-macrophage associated with tumors  
Tbet- T cell transcription factor  
Tconv- Conventional T cells  
TCR-T cell receptor  
TGF $\beta$ - transforming factor of growth  $\beta$   
Th0- naive T cells  
Th1- Thelper type 1 cells  
Th2-Thelper type 2 cells  
Th3- T helper 3 regulatory cells  
Th17- Thelper 17 type cells  
TLRs-Toll-Like-Receptors  
TNF $\alpha$ - Tumor necrosis factor  $\alpha$   
Tr1- Regulatory cells type 1  
Treg- Regulatory T cells  
TSLP-Lymphopietin of thymic stroma  
TWEAK-factor-like receptor for apoptosis-inducing weak tumor necrosis

TxA<sub>2</sub>-Thromboxane A<sub>2</sub>

VEGF- Vascular endothelial growth factor

VOC- Volatile Organic Compounds

#### 4- Abstract

**Background:** Atherosclerosis and CVD might be associated with the traditional CV risk factors such as age and hypertension as well as nontraditional risk factors comprising current inflammation associated with asthma and genetic polymorphisms that predisposes to different status of oxidative stress and inflammation. In this multiple risk factor assessment, the risk charts that are based only on traditional risk factors are insufficient to capture CV risk extent in bronchial asthma and CVDs in general. The purpose of this thesis is to analyze the role of the different polymorphisms as emerging risk factors for CVD and asthma control and severity.

**Material and Methods:** It has been performed a clinical case-control study between asthmatics patients (356) and volunteer controls (153) for the polymorphisms of: Renin-angiotensin system; NO associated system; BETA2- adrenergic receptors; Detoxification; Atopic march; Epigenetics. It has been done a literature revue and meta-analysis accomplished with comprehensive meta-analysis software version 2 for CVD and the polymorphisms of: Renin-angiotensin system; NO associated system; BETA2- adrenergic receptors; Detoxification; Atopic march; Epigenetics. Genomic DNA Isolation and quantification. Genotyping: PCR, PCR-RFLP; PCR – multiplex. PAGE for haptoglobin polymorphism. Serum Hp concentration was determined by nephelometry. Determination of plasma concentration of MPO was performed using the ELISA technique. All statistical analysis was carried out using the SPSS 21.0 software. The results were considered statistically significant for  $p < 0.05$ . The Genetic Risk score of: endothelial dysfunction ((ACE) and nitric oxide (NOS) gene polymorphisms); endothelial dysfunction and epigenetics (MTHFR+(ACE) and nitric oxide (NOS) gene polymorphisms); uncontrolled asthma (NOS2-IVS16+ 88T>G , ACE I/D and age) and allergic asthma (MTHFR and GSTT1 and ACE and gender).

**Results:** For the different polymorphisms of: Renin-angiotensin system; NO associated system; BETA2- adrenergic receptors; Detoxification; Atopic march; Epigenetics; the systematic literature review and meta-analysis; the case control clinical study; the haplotype and epistatic interactions; the endothelial dysfunction in asthma and genetic risk scores, drive to novel disease classification/endotypes and emergent non-traditional risk factors and phenotypes in CVD and asthma severity. The role of ACE (I/D) polymorphism, in asthmatic patients is a controversy risk factor to the severity of asthma, but we concluded that those who has an allele D have protection of having asthma, in this hospital - based population and there are more genotypes II in the asthmatics than controls. In the Systematic literature review for CVD if the DD genotype is present there is susceptibility to Heart disease. In this study group there is not a significant evidence, that AGTR1 gene A1166C polymorphism could be a genetic marker for the pathophysiology of asthmatic disease. In the Systematic literature review for CVD the risk of having heart condition in those that express allele C there is a trend to be decreased although n.s..For haptoglobin asthmatics had lower levels of the circulating Hp when compared to the control-group and that this difference is associated with Hp 2-2 genotype. In asthmatics, Hp levels are different between genotypes (with age  $\geq 15$  years). In the Systematic literature review for CVD if the Hp2.2 genotype is present there is higher susceptibility to Heart condition. For Intron 4 polymorphism of the eNOS gene those who express allele b (ab+bb) have an increased risk of having asthma. In the Systematic

literature review for CVD the risk of having heart condition in this eNOs polymorphism is n.s.. For NOS2 polymorphism (exon 16-14CT) those who are homozygous for the allele C have a protection of having asthma. NOS2 polymorphism (intron 16 - 88GT) there is no increased risk of having asthma. NOS2 polymorphism (intron 20 - IVS20 + 524 GA) those who express allele A (AG+AA) have protection of having asthma when compared with controls. In the Systematic literature review for CVD *usually, there is no inducible nitric oxide synthase (NOS2) in the normal heart, however macrophages associated with inflammation could express it.* For MPO Polymorphism (- 463 GA) those who are homozygous for the allele G (higher enzyme activity) have protection of having asthma. There are differences in MPO levels by genotypes being the AA and GA with lower MPO levels than GG and associated with gender. In the Systematic literature review for CVD there is a higher risk and significant if the AA genotype is present in *MPO (-463G>A (rs2333227) polymorphism* and susceptibility to Heart condition. For the Polymorphism on the Receptor beta2adrenergic Arg16 Gly (rs1042713) Those who have allele A as homozygous or heterozygous and younger than 30 years, have an increased risk of asthma. In the Systematic literature review for CVD the risk of having heart condition if the GG genotype is present in Beta2 adrenoreceptor polymorphisms: Arg16Gly (rs1042713). For detoxification enzymes the GSTT1\*0 is more frequent in asthmatics and GSTT1+ in controls and there is an increased risk of being allergic asthmatics if they are GSTT1\*0 and women. There are no differences in the frequencies of genotypes between asthmatics and controls for GSTM1. For the literature review and CVD and GSTT1 associated with overall effect significant if the null genotype is present and susceptibility to Heart condition For the literature review and CVD and GSTM1 the overall effect is non significant n.s. and susceptibility to Heart condition. There is not a significant evidence, that LELP1 polymorphism (rs7534334) could be a genetic marker for atopic asthma ( $p>0.05$ ) in this hospital-based population. For the literature review and CVD the induction of SPRR expression by IL-6 cytokines could be a central mechanism of an 'innate' defense system in response to stress and induction of SPPR genes may serve a novel cell protective strategy in CVD. For MTHFR polymorphism : C677T (rs1801133) There is a trend to have statistical differences between asthmatics and controls, being the CC more frequent in asthmatic allergic patients. In the literature review for CVD is associated with a significant overall effect if the genotype CC is present and susceptibility to Heart condition. Significant SNPs in asthma vs controls; controlled/uncontrolled asthma; allergic vs non-allergic asthma were used to create different genetic models and construct haplotype, epistatic interaction and genetic risk score for each participant by analyzing the coefficients for each of the resulting variables after statistical analysis.

**Conclusion:** The mapping of genetic susceptibility by candidate genes approach and the mechanistic approach of asthma and CVD is considered a hallmark of asthma and CVD, putting the emerging biomarkers and genetic susceptibility to disease in relation to asthma and CVD as the mainstream in omics profiling and its response to target therapy and precision Medicine.



## 5- Resumo

**Introdução:** A aterosclerose e DCV podem estar associadas aos factores de risco de CV tradicionais, como idade e hipertensão, bem como factores de risco não tradicionais que compreendem a inflamação atual associada à asma e polimorfismos genéticos que predis põem a diferentes condições de stress oxidativo e inflamação. Nesta avaliação de factores múltiplos de risco, os scores de risco que se baseiam apenas em fatores de risco tradicionais são insuficientes para capturar a extensão do risco CV na asma brônquica e CVDs em geral. O objetivo desta tese é analisar o papel dos diferentes polimorfismos como factores de risco emergentes para CVD, controlo e gravidade da asma.

**Material e métodos:** Foi realizado um estudo clínico caso-controlo entre pacientes asmáticos (356) e controlos voluntários (153) para os polimorfismos de: sistema Renina-angiotensina; sistema associado ao NO; Receptores Beta2-adrenérgicos; isoenzimas Glutatião S-transferases - enzimas de fase II ou de reacões de conjugação; Marcha atópica; Epigenética. Foi realizada uma revisão de literatura e meta-análise realizada com o software de meta-análise versão 2 para CVD e os polimorfismos de: sistema Renina-angiotensina; sistema associado ao NO; Receptores Beta2-adrenérgicos; Glutatião S-transferases isoenzimas- enzimas de fase II ou de reacões de conjugação; Marcha atópica; Epigenética. Isolamento e quantificação de DNA genómico. Genotipagem: PCR, PCR-RFLP; PCR - multiplex. PAGE para o polimorfismo de haptoglobina. A concentração de Hp foi determinada por nefelometria. A determinação da concentração plasmática de MPO foi realizada utilizando a técnica ELISA. Todas as análises estatísticas foram realizadas com o software SPSS 21.0. Os resultados foram considerados estatisticamente significativos para  $p < 0,05$ . O índice de risco genético de: disfunção endotelial ((ACE) e polimorfismos de genes de óxido nítrico (NOS)); Disfunção endotelial e epigenética (MTHFR + (ACE) e polimorfismos do gene de óxido nítrico (NOS)), asma não controlada (NOS2-IVS16 + 88T> G, ACE I / D e idade) e asma alérgica (MTHFR ,GSTT1 e ACE e género).

**Resultados:** Para os diferentes polimorfismos de :sistema Renina-angiotensina; sistema associado ao NO; Receptores Beta2-adrenérgicos; Glutatião S-transferases isoenzimas-enzimas de fase II ou de reacões de conjugação; Marcha atópica; Epigenética: revisão sistemática da literatura e metanálise; Estudo clínico de caso-controlo; haplótipo e as interações epistáticas; a disfunção endotelial na asma e os scores de risco genético, conduzem a novas classificações / endótipos de doença e fatores de risco não-tradicionais emergentes associados a fenótipos em DCV e gravidade da asma. O papel do polimorfismo ACE (I / D), em asmáticos é um fator de risco controverso para a gravidade da asma, mas concluiu-se que aqueles que têm um alelo D têm proteção de ter asma, nesta população hospitalar e existem mais genótipos II nos asmáticos do que nos controlos. Na revisão da literatura sistemática para DCV se o genótipo DD estiver presente, existe uma susceptibilidade para doença cardíaca. Neste grupo de estudo não há evidências significativas, que o polimorfismo no gene AGTR1 (A1166C) poderia ser um marcador genético para a fisiopatologia da doença asmática. Na revisão da literatura sistemática para DCV, o risco de ter doença cardíaca naqueles que expressam o alelo C há uma tendência para estar diminuída, embora n.s.. Nos asmáticos o estudo da haptoglobina revelou níveis mais baixos de Hp circulante quando comparados ao grupo controlo e que essa diferença está associada ao genótipo Hp 2-2. Nos asmáticos, os níveis de Hp são diferentes entre os genótipos (com idade  $\geq 15$  anos). Na revisão da literatura sistemática para DCV se o

genótipo Hp2.2 estiver presente, há maior susceptibilidade à doença cardíaca. Para o polimorfismo Intron 4 do gene eNOS, aqueles que expressam o alelo b (ab + bb) têm um risco aumentado de ter asma. Na revisão da literatura sistemática para DCV, o risco de ter doença cardíaca neste polimorfismo eNOS é n.s. Para o polimorfismo NOS2 (exão 16-14CT), aqueles que são homozigóticos para o alelo C têm proteção de asma. Polimorfismo NOS2 (intron 16 - 88GT) não há risco aumentado de ter asma. O polimorfismo NOS2 (intron 20 - IVS20 + 524 GA) aqueles que expressam o alelo A (AG + AA) têm proteção de asma quando comparados com os controles. Na revisão sistemática da literatura para DCV geralmente, não há sintase induzível do óxido nítrico (NOS2) no coração normal, no entanto, os macrófagos associados à inflamação podem expressá-lo. Para o polimorfismo MPO (-463 GA), aqueles que são homozigóticos para o alelo G (maior atividade enzimática) têm proteção de asma. Existem diferenças nos níveis de MPO por genótipos que são AA e GA com níveis mais baixos de MPO do que GG e associados ao gênero. Na revisão sistemática da literatura para DCV, existe um risco maior e significativo se o genótipo AA estiver presente no polimorfismo MPO (-463G> A (rs2333227) e susceptibilidade à doença cardíaca. Para o Polimorfismo no Receptor beta2adrenérgico Arg16 Gly (rs1042713) aqueles que têm alelo A como homozigoto ou heterozigoto e com menos de 30 anos, apresentam um risco aumentado de asma. Na revisão sistemática da literatura para DCV, o risco de ter doença cardíaca se o genótipo GG estiver presente nos polimorfismos dos receptores Beta2 adrenérgico: Arg16Gly (rs1042713). Para as isoenzimas Glutatio S-transferases - enzimas de fase II ou de reações de conjugação, o GSTT1 \* 0 é mais frequente em asmáticos e GSTT1 + nos controles e há um risco aumentado de serem asmáticos alérgicos se forem GSTT1 \* 0 e gênero feminino. Não há diferenças nas frequências de genótipos entre asmáticos e controles para GSTM1. Para a revisão da literatura e DCV e GSTT1 está associado com o efeito global significativo se o genótipo nulo estiver presente e a suscetibilidade à doença cardíaca. Para a revisão da literatura e DCV e GSTM1, o efeito global não é significativo n.s. e susceptibilidade para doença cardíaca. Não há evidências significativas de que o polimorfismo LELP1 (rs7534334) possa ser um marcador genético para a asma atópica nesta população hospitalar. Para a revisão da literatura e DCV, a indução de expressão de SPRR por citocinas como a IL-6 poderia ser um mecanismo central de um sistema de defesa "inato" em resposta ao stress e a indução de genes SPRR pode servir para uma nova estratégia de proteção celular na DCV. Para o polimorfismo MTHFR: C677T (rs1801133). Existe uma tendência para ter diferenças estatísticas entre asmáticos e controles, sendo o CC mais frequente em doentes alérgicos asmáticos. Na revisão da literatura para DCV está associada a um efeito global significativo se o genótipo CC estiver presente e susceptibilidade para doença cardíaca. Para os SNPs significativos na asma versus controles; asma controlada / não controlada; asma alérgica versus não alérgica foram utilizados diferentes modelos genéticos para construir haplótipos, interação epistática e score de risco genético para cada participante, analisando os coeficientes para cada uma das variáveis resultantes da análise estatística.

**Conclusão:** O mapeamento da susceptibilidade genética pela abordagem dos genes candidatos e a abordagem mecanística da asma e DCV são consideradas uma característica da asma e das doenças cardiovasculares, colocando os biomarcadores emergentes e a susceptibilidade genética para a doença na análise de endótipo/fenótipo destas doenças e na possível resposta a terapêuticas alvo no âmbito da medicina de precisão.

**CHAPTER I-Introduction**

## I.1.Epidemiology

Non communicable diseases (NCDs) (1) such as cardiovascular diseases, cancer, diabetes and respiratory diseases, among others, are considered the leading cause of worldwide morbidity and mortality. They share, among them, the fact that they are associated with certain lifestyles, specific modifiers of each individual (genetic susceptibility) and environmental exposure (epigenetic susceptibility).

The risk factors (1,2) of the NCDs with the greatest impact are hypertension (HT), smoking, alcohol, dyslipidemia, obesity, atherogenic diet and sedentary lifestyle.

Asthma is a disease that is closely linked to atopy evolving with paroxysmal episodes that may be associated with a pattern of severity in which airway remodeling and inflammation (mainly Th2 high or Th2 low) predominate (3).

Each of the hallmark characteristics - *canonical pathways* - of asthma (4–13) (inflammation, remodeling, airway hyperreactivity, oxidative stress, innate and adaptive immunity) is the expression of a complex network of molecules, very diverse both within any given patient in time and between any patients.

The susceptibility to asthma and its more severe forms as well as the association and or predisposition to cardiovascular diseases accompanies the development of new biomarkers (6,14,15), associated with the markers of genetic susceptibility that define and characterize the different endotypes and asthma phenotypes.

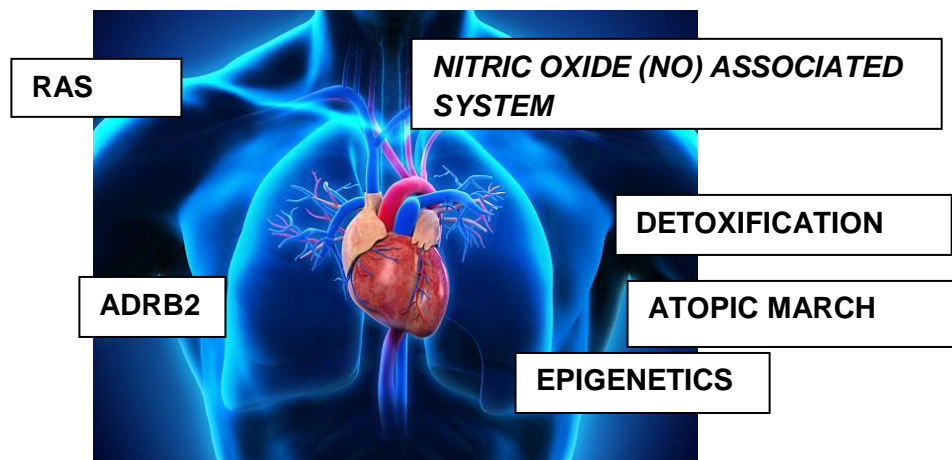
According to some authors (6,12,14) the biomarkers can be characterized as relevant for the pathobiological knowledge of the disease, response to therapy and prognostic evolution – putting the genetic factors and biomarkers as central and required in future studies of susceptibility and surveillance of diseases.

The mapping of genetic susceptibility by candidate genes and the mechanistic approach of asthma is considered as a constituent of the analysis of the emerging biomarkers in relation to asthma and its response to therapy.

The present thesis focuses on the genetic determinants of atopy, asthma and CVD, trying to approach a study of candidate genes and susceptibility to asthma through a case-control study conducted in a cohort of ambulatory asthma patients with various types of asthma: Intermittent and persistent moderate or severe, which after a period of optimization of two months and adequate clinical follow-up according to the severity of the disease were characterized as controlled and uncontrolled (ACQ-7 and PAQLQ). The susceptibility to Cardiovascular disease is evaluated through a systematic review of the literature for candidate genes under study.

**I.2. Physiopathology of Asthma and CVD and polymorphisms studied: a) Renin-Angiotensin-System; b) Nitric Oxide (NO) associated systems; c) Beta-2 adrenergic receptors; d) Detoxification; e) Atopic march; f) Epigenetics**

We sought to understand the functional and regulatory pathways that play central roles in the pathobiology of asthma and CVD and to understand the overlap between the pathways that orchestrate inflammation and endothelial dysfunction in both diseases (Fig.I.2.1).

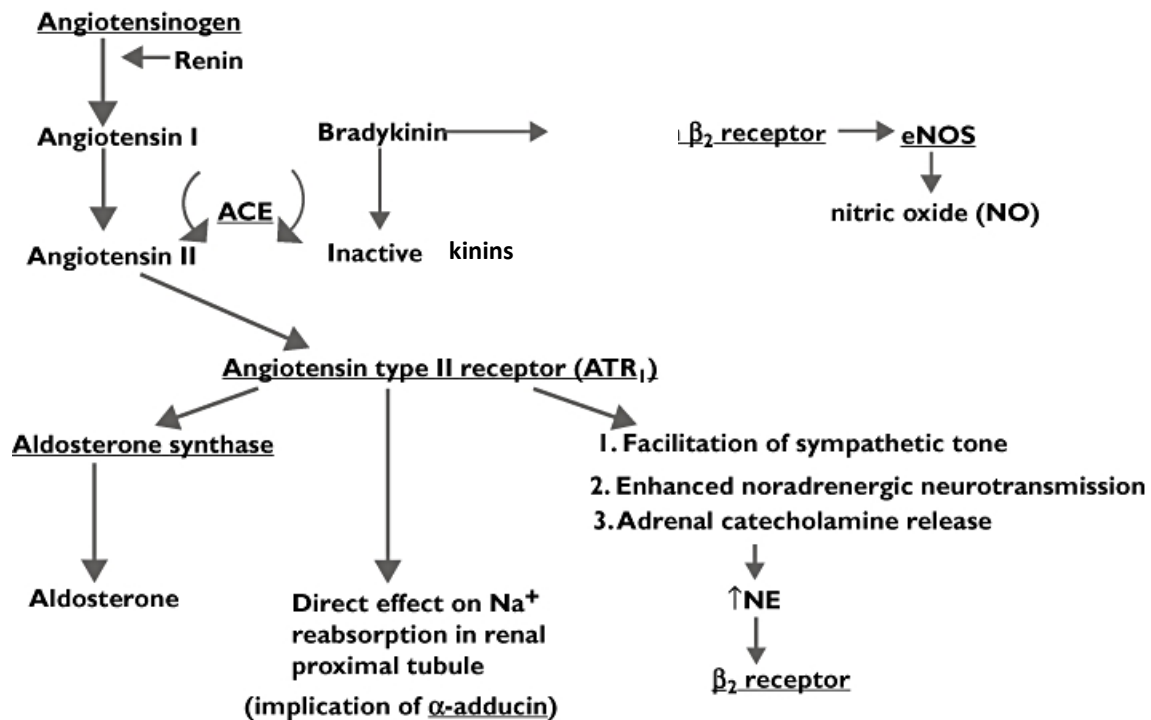


**Fig.I.2.1:** The different candidate genes/SNPs and pathways studied in this thesis.

The genetic constituents of the renin-angiotensin-aldosterone system (RAAS), a neurohormonal pathway that is activated in many Cardiovascular diseases such as: IHD; CAD; MI; cardiomyopathy; MI and HTA among others, has also an important role in asthma and CVD as will be showed in this thesis.

ACE also inactivates bradykinin, substance P and neurokinin A, which plays important roles in the pathogenesis of asthma specially in neurogenic inflammation. There are 3 genotypes: insertion homozygote II; deletion homozygote DD; heterozygotes DI. The serum ACE levels with DD is reported to be double that of II type and intermediate in DI type (16–18) .

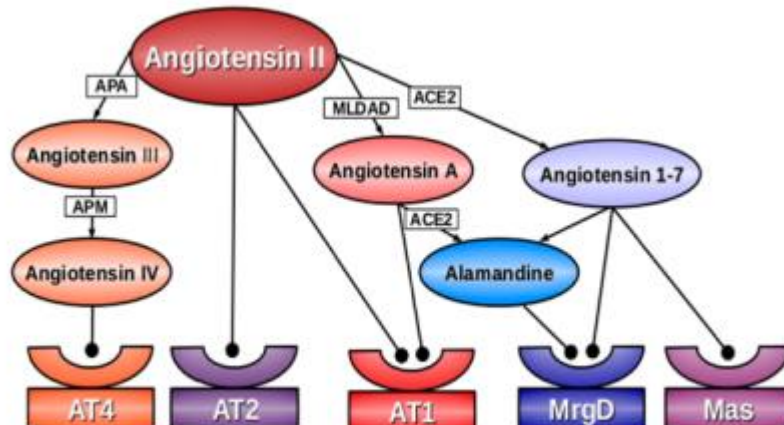
RAS (Fig.I.2.2) has been associated with HTA and with inflammatory response, pulmonary hypertension, fibrosis and asthma. Angiotensin II is a profibrotic and Immunomodulator molecule through TGF beta. ACE is involved in the catabolism of bradykinins and tachykinins. The C-terminal domain is the major catalytic site in cleaving the angiotensin I into angiotensin II in inflammatory response. Bioavailability of nitric oxide (NO) associated with RAS is also an important factor in the pathophysiology of asthma (16-18).



**Fig.I.2.2:** The different genes coding for the components of these pathways are included in this study and related with Heart diseases such as : IHD; CAD; MI; cardiomyopathy; MF and HTA among others ( *adapted from Jakubiak et al 2008* (18)) and the pathophysiology of asthma.

ACE plays a vital role in the renin-angiotensin-system (RAS) which regulates blood pressure by converting angiotensin I into a powerful vasoconstrictor and bronchoconstrictor, angiotensin II, that also has an important role in airway remodeling and in inactivation of bradykinin and tachykinins which are potent bronchoconstrictors and mediators of inflammatory reactions. (II with lower levels of ACE; II < ID < DD).

The renin-angiotensin system (RAS) plays a crucial role in the pathophysiology of cardiovascular conditions, such as hypertension, heart failure, endothelial dysfunction, and endothelial and airway remodeling in diseases such as asthma (19)(20).



**Fig.I.2.3:** RAS with opposing biological functions and its receptors.( *adapted from Hrenak et al 2016 (19)*).

The RAS incorporates molecules with opposing biological actions (Fig.I.2.3). In a way we have the vasoconstrictive, bronchoconstrictive, pro-proliferative, and pro-inflammatory molecules, such as ACE, angiotensin II (Ang II), and AT1 receptors. The opposing effect involves ACE2, angiotensin 1–7 (Ang 1–7), angiotensin type 2 (AT2) receptor, Mas receptor or *N*-acetyl-seryl-aspartyl-lysyl-proline (Ac-SDKP), which counteracts the SDKP and being the opposing arm of the potentially harmful actions of RAS (21–24). Angiotensin-converting enzyme (24) cleaves angiotensin I, bradykinin, neurotensin and other substrates. In part, this is due to its two independent catalytic domains: angiotensin II is produced by the ACE C-domain; the anti-fibrotic peptide AcSDKP, are substrates only of the ACE N-domain.

Endothelial-mesenchymal transition is a mechanism that regulates fibrosis with a potential role in development of diseases such hypertension and asthma because of the acquiring of stem cell properties and generating connective tissue, and its potential as a novel mechanism for tissue regeneration and remodeling (24,25). The Myoendothelial Junction and the Smooth Muscle to Endothelial Cell Communication and Endothelial Cell to Smooth Muscle Communication is a key feature in disease severity namely asthma and CVD as it is the heme-iron bioavailability to the also very important modulation of NO signaling, cell-cell communication, endothelial cell function, and vascular tone (25)(26).

The signaling pathways (27), related with ACE polymorphism regulating the cytokine production by T cells, could induce a different Th profile modulating the immune response in asthma by interfering with TGF beta and Tregs.

ACE could interfere with NO release 28–31 either by direct activation of the B1 receptor or indirectly, through bradykinin effects on the B2 receptor. Potentiation of kinins might happen when ACE had lower activity.

It is known that type 1 angiotensin II (Ang II) receptor (AGTR1) (30,31) could be related with the pathogenesis of bronchial asthma. It is involved in Th polarization, through different signaling pathways modulating allergic airway inflammation, and also may participate in airway remodeling and bronchoconstriction, and regulation of ACE activity that could be related with AGTR1 polymorphism. The purpose of this study is to analyse the association between AGTR1 1166A/C (rs5186) gene polymorphism with asthma severity.

RAS is reportedly activated in severe acute attacks of asthma, as evidenced by elevations of plasma renin and Ang II levels and Ang II causes a bronchoconstriction in patients with mild asthma. AT1(29,30) receptors are involved in the effects of Ang II including a bronchoconstriction

The ROS produced by NADPH (32–36) oxidases seem to have 2 general downstream physiological roles. Superoxide produced by NOX 2 is required for the respiratory burst that occurs in phagocytes. Superoxide and hydrogen peroxide (NOX derived ROS) derived from NADPH oxidase proteins/family could activate downstream signaling pathways that regulates: cell growth; differentiation, apoptosis; vascular tone and remodeling.

p42/44 ERK (MEK-1/2) –is the main contribute to the Ang II-elicited bronchial smooth muscle (BSM) hyperresponsiveness (37,38).

The mechanism of Ang II in bronchoconstriction(38,39) is related with the fact that in the mammalian cells , they express multiple mitogen-activated protein kinases (MAPKs) including the well characterized extracellular signal-regulated kinase (ERK) pathway.

p42/44 ERK (34,38,39), which is critical in the mitogenic response, is phosphorylated by mitogen-activated protein/ ERK kinase (MEK1/2). MAPK activation is typically associated with cell growth, but recent findings indicate that the pathway is also responsible for vascular contraction. The bronchoconstriction induced by Ang II was mediated by AT1 receptor phosphorylation of p42/44 ERK pathway MAPKs - in bronchial smooth muscle.

NO produced in the environment could lead to nitrosative stress(40) and then airway inflammation and remodeling. nitrotyrosine formation is common via the reaction between NO and O<sub>2</sub><sup>-</sup> in addition to the myeloperoxidase-mediated pathway (41).

Because reactive oxygen and related species including nitric oxide (NO) have a potent proinflammatory action, these molecules may be involved in the airway inflammatory process in asthma(42–44) (Fig.I.2.4).

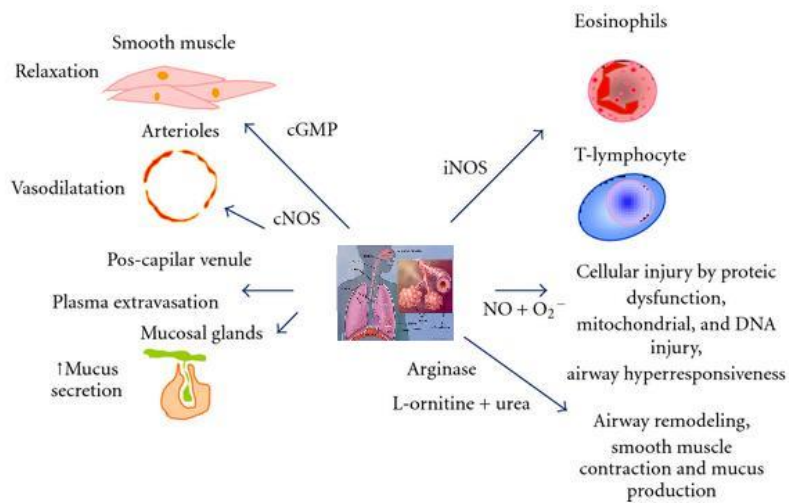
Large amounts of NO and ONOO<sup>-</sup> may target numerous proteins and enzymes critical for cell survival and signaling. These include signaling molecules involved in cytokine signaling like JAK or STAT proteins, as well as MAPK pathways, some G proteins and transcription



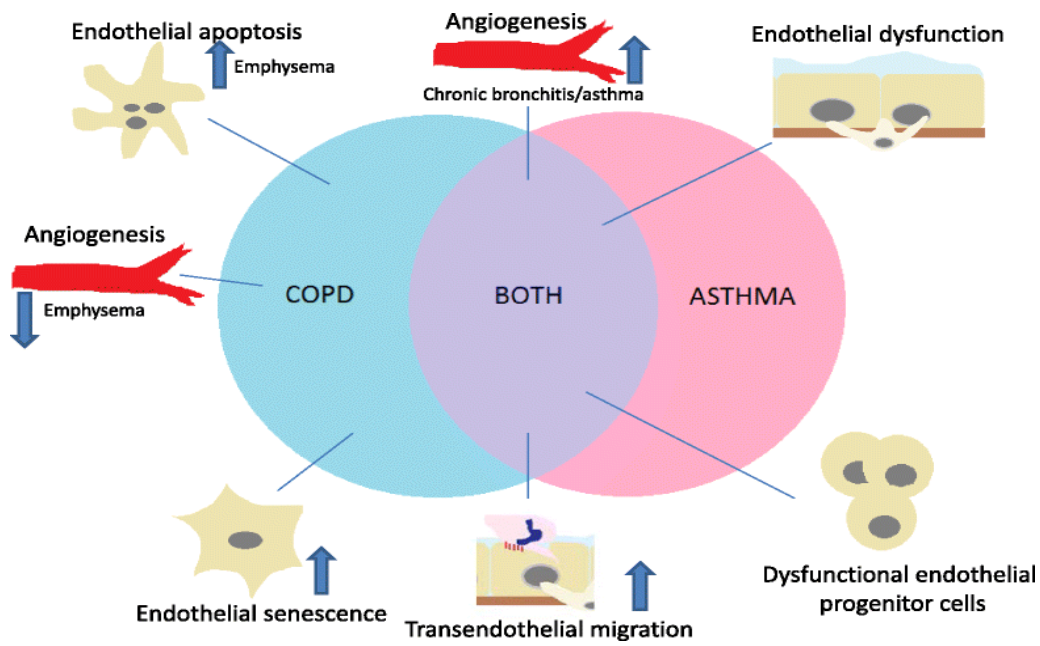
factors. Nitration of cysteines in these proteins may lead to their activation or inactivation (41,43,44) .

After allergen exposure and asthmatic airway inflammation we could have NO, O<sub>2</sub><sup>-</sup> and peroxynitrite-induced nitration(41).

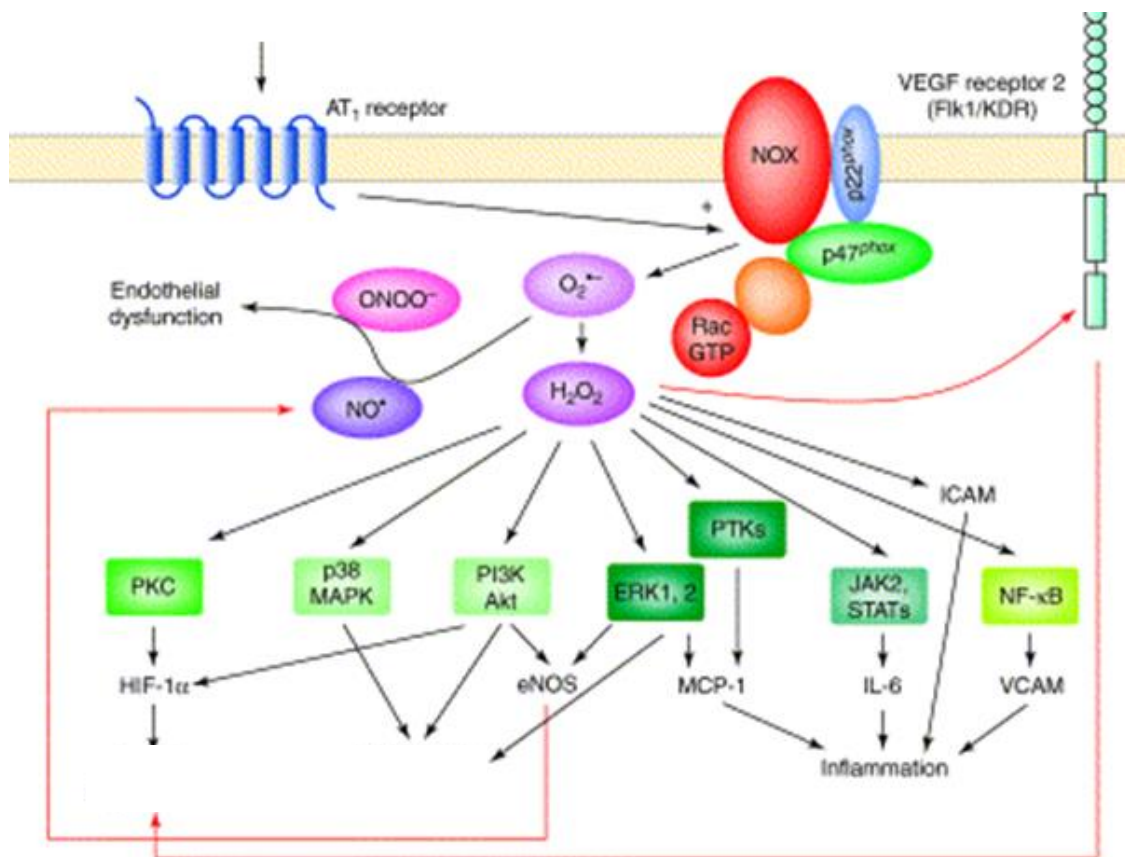
Endothelial dysfunction is related to abnormalities in nitric oxide (NO) and in the activation of the renin-angiotensin system. Angiotensin converting enzyme (ACE) and nitric oxide (NOS) gene polymorphisms, are important in endothelial dysfunction and in the pathophysiology of asthma and CVD(102) (Fig.I.2.5) (45).



**Fig.I.2.4:** Nitric oxide in asthma. (adapted from Clempus et al ; 2006 (46))



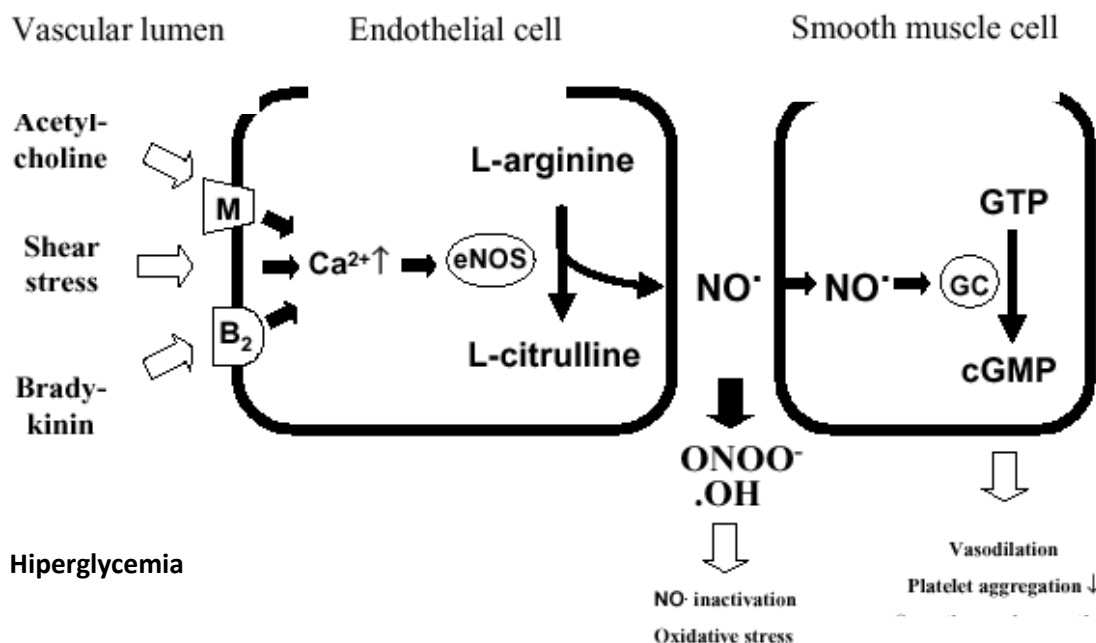
**Fig.I.2.5:** Endothelial dysfunction in respiratory diseases ( *adapted from Green et al 2017(47)*)



**Fig.I.2.6:** Angiotensin II-stimulated activation of vascular NAD(P)H oxidases (NOXs); (adapted from Cai et al 2003(48); Holgate et al 2009 (49)).

Activation of the angiotensin Type 1 receptor of Angiotensin II (AGTR1) leads to phosphorylation of p47phox (Fig.I.2.6). Between AT1 receptor stimulation and phosphorylation of p47phox there is the activation of phospholipase D (PLD), protein kinase C (PKC) and c-Src tyrosine kinase. Phosphorylation of p47phox and its binding to the membrane oxidase components; c-Src also activates in trans the epidermal growth factor receptor (EGFR), which leads to activation of phosphatidylinositol 3-kinase (PI3K) and Rac-1. Translocation of Rac-1 to the membrane activates the NOXs and sustains its function.

The mechanism of Ang II in bronchoconstriction is related with the fact that in the mammalian cells, they express multiple mitogen-activated protein kinases (MAPKs) including the well characterized extracellular signal-regulated kinase (ERK) pathway. p42/44 ERK, which is critical in the mitogenic response, is phosphorylated by mitogen-activated protein/ ERK kinase (MEK1/2). MAPK activation is typically associated with cell growth, but recent findings indicate that the pathway is also responsible for vascular contraction. The bronchoconstriction induced by Ang II was mediated by AT1 receptor phosphorylation of p42/44 ERK pathway MAPKs - in bronchial smooth muscle (37,38,48) .



### Lipid peroxidation

**Fig.I.2.7:** Pathways of endothelial dysfunction in the airways.(*adapted from Brandes et al 2005(34)*)

Many single nucleotide polymorphisms involved in CVD are also hallmarks of asthma and could affect the susceptibility and outcome of asthma severity that will be addressed in this Thesis: A) **RENIN-ANGIOTENSIN SYSTEM:** ACE polymorphism of I / D (287 bp, on chromosome 17q23, intron 16 (rs1799752); Polymorphism for Type 1 receptor of Angiotensin II (AGTR1) 1166A / C (rs5186); B) **Nitric Oxide ASSOCIATED SYSTEM:** Haptoglobin polymorphism (1.1, 2.1, 2.2): Intron 4 polymorphism of the eNOS gene; NOS2 polymorphism (exon 16-14CT): NOS2 polymorphism (intron 16 - 88GT); NOS2 polymorphism (intron 20 - IVS20 + 524 GA); MPO Polymorphism (- 463 GA); C) **BETA2ADRENERGIC RECEPTORS:** Polymorphism Receptor beta2adrenergic Arg16 Gly (rs1042713); D) **DETOXIFICATION:** GSTM1 polymorphism: search for null genotypes (homozygous for the allele GSTM1 \* 0) (M-); GSTT1 polymorphism: null genotype search (Homozygous homing for the GSTT1 \* 0 allele) (T-); E) **ATOPIC DERMATITIS (AD) AND ASTHMA-ATOPIC MARCH:** LELP1; F) **EPIGENETICS:** MTHFR polymorphism MTHFR: C677T (rs1801133).

It is known that NO has a relevant role in inflammation, vascular and muscular tonus in asthma. Inducible nitric oxide synthase (iNOS) modulates the amount of NO that could be related with iNOS polymorphism. The purpose of this study is to analyze the association between inducible nitric oxide synthase (iNOS) gene polymorphism iNOS intron 20 (IVS20 + 524 G>A-rs944722; exon 16-14CT- rs 2297518; intron 16 - 88GT-(rs9282801) ) with asthma severity when compared with a control group of healthy blood donors. The major

pathogenesis of asthma is chronic inflammation. In asthmatic airways, activated mast cells, eosinophils and T helper 2 lymphocytes (Th2) are predominant. Although nitric oxide (NO) hyperproduction due to inducible NO synthase (iNOS) is observed in asthma and diseases like COPD, nitrotyrosine formation is common via the reaction between NO and O<sub>2</sub><sup>-</sup> in addition to the myeloperoxidase-mediated pathway. Because reactive oxygen and related species including nitric oxide (NO) have a potent proinflammatory action, these molecules may be involved in the airway inflammatory process in asthma. Large amounts of NO and ONOO<sup>-</sup> may target numerous proteins and enzymes critical for cell survival and signaling. These include signaling molecules involved in cytokine signaling like JAK or STAT proteins, as well as MAPK pathways, some G proteins and transcription factors. Nitration of cysteines in these proteins may lead to their activation or inactivation. Nitric oxide is produced by a family of NOS isoforms that convert L-arginine into NO and L-citrulline using oxygen and NADPH as cosubstrates. Three NOS isozymes have been identified to date: neuronal NOS (nNOS or NOS I), inducible NOS (iNOS or NOS II) and endothelial NOS (eNOS or NOS III)(44).

One of the functional polymorphisms with relevance to iNOS enzyme activity constitutes a transition from C to T located at exon 16 (position 2087) and which causes a substitution of the serine amino acid for leucine at codon 608 (Ser608Leu). This variation increases the expression of the enzyme in the target cells, resulting in high levels of NO, being responsible for the genetic susceptibility and severity of various respiratory diseases. The gene encoding the NOS2 enzyme is called NOS2A and is found on the long arm of chromosome 17q11.2-12. It consists of 27 exons that span 37 Kb and encodes a 131 kDa protein (1145 amino acids). The iNOS gene has a transcription start site in exon 2 and a stop codon in exon 27, exhibiting two different functional catalytic domains, the oxygen domain encoded by exons 1-13 and reductase domain by exons 14-26 responsible for susceptibility, severity and disease modifying endotype/phenotype of several diseases (44).

Intron 16 + 88 G> T (rs9282801): one of the functional polymorphisms with relevance to iNOS enzyme activity constitutes a transition from T to G in intron 16 (rs9282801). They might affect the splicing or protein expression of the gene increasing NOS2 activity and NO synthesis. The gene encoding the NOS2 enzyme is called NOS2A and is found on the long arm of chromosome 17q11.2-12. It consists of 27 exons that span 37 Kb and encodes a 131 kDa protein (1145 amino acids). The iNOS gene has a transcription start site in exon 2 and a stop codon in exon 27, exhibiting two different functional catalytic domains, the oxygen domain encoded by exons 1-13 and reductase domain by exons 14-26 responsible for susceptibility, severity and disease modifying endotype/phenotype of several diseases (44).

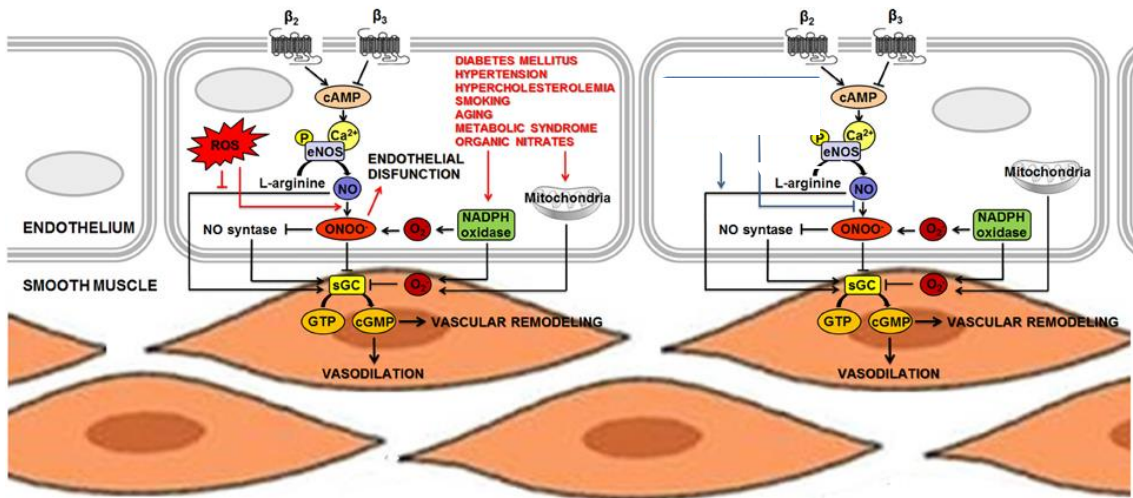
One of the functional polymorphisms with relevance to iNOS enzyme activity constitutes a transition from G to A in intron 20 (rs944722). It might affect the splicing or protein expression of the gene increasing NOS2 activity and NO synthesis.

The gene encoding the NOS2 enzyme is called NOS2A and is found on the long arm of chromosome 17q11.2-12 . It consists of 27 exons that span 37 Kb and encodes a 131 kDa protein (1145 amino acids). The iNOS gene has a transcription start site in exon 2 and a stop codon in exon 27, exhibiting two different functional catalytic domains, the oxygen domain encoded by exons 1-13 and reductase domain by exons 14-26 responsible for susceptibility, severity and disease modifying endotype/phenotype of several diseases (44).

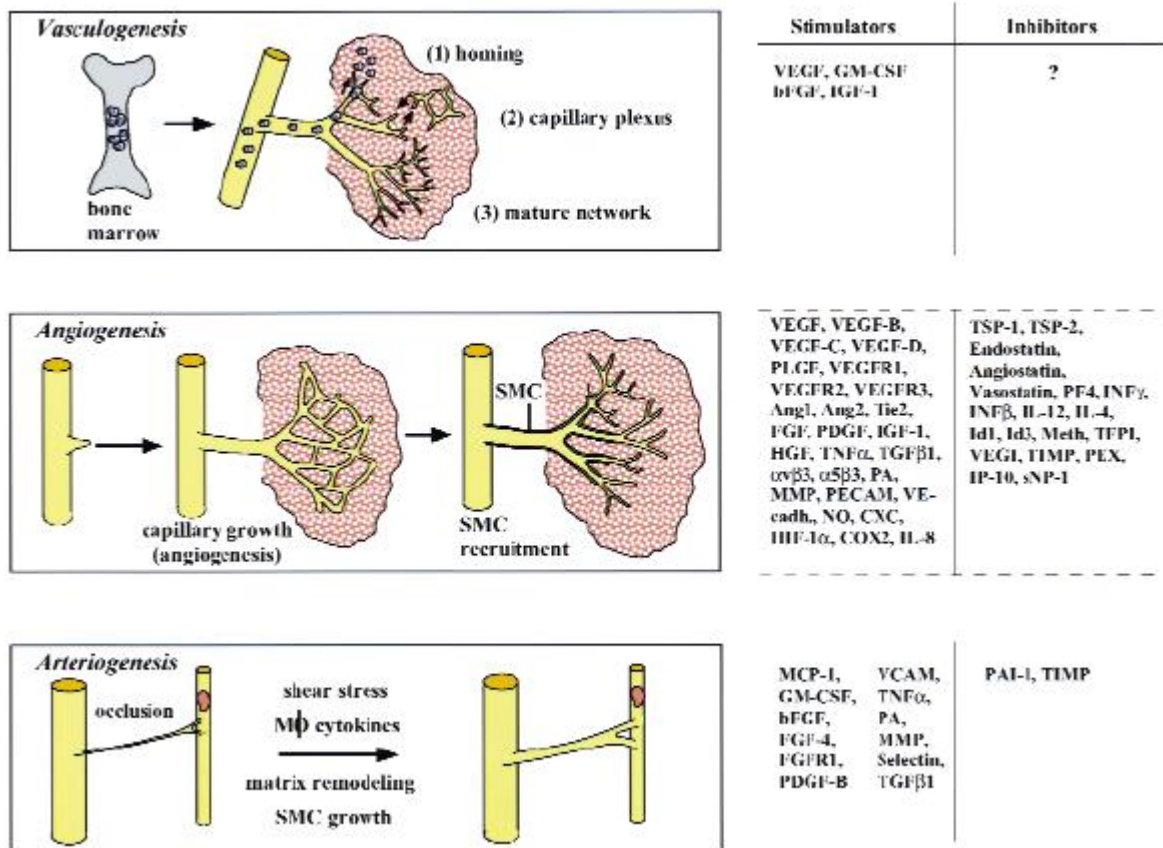
eNOS polymorphism is important in asthma because it plays an important role in bronchial hyperreactivity, interferes with immunomodulation and airway inflammation by down-regulating Th1 cells at sites of chronic inflammation and polarizing the Th2 response, eosinophilic inflammation, and are related with higher levels of IgE, it also interacts with No availability on endothelial and is potential role on smooth muscle cells and on endothelial dysfunction in the airways.

eNOS plays an important role in bronchial hyperreactivity, interferes with immunomodulation and airway inflammation by down-regulating Th1 cells at sites of chronic inflammation and polarizing the Th2 response, eosinophilic inflammation, and are related with higher levels of IgE. Endothelial Nitric Oxide synthase is expressed in vascular endothelium, airway epithelium and other cell types is able to generate NO, which performs important functions in respiratory diseases such asthma. In humans, the gene encoding eNOS is located on chromosome 7q35-36, with 26 exons spanning 21 Kb and produces a protein with about 1203 aminoacids . Most of the variations described in the eNOS gene occur in introns. A 27 base pair (bp) repeat polymorphism was identified in intron 4 of the gene. This polymorphism influences the mRNA expression, and thus the protein concentration and its enzymatic activity. eNOS polymorphism is important in asthma because it plays an important role in bronchial hyperreactivity, interferes with immunomodulation and airway inflammation by down-regulating Th1 cells at sites of chronic inflammation and polarizing the Th2 response, eosinophilic inflammation, and are related with higher levels of IgE (50,51).

Candidate gene-association studies put NOS and ACE enzymes as important regulators of oxidative stress, bronchial hyperresponsiveness , endothelial homeostasis and vascular remodeling through the up-regulation of angiogenic factors and the release of angiogenic mediators (Fig.I.2.7 and Fig.I.2.8). The endothelium (Fig.I.2.9) has emerged as a key regulator of vascular homeostasis, with its barrier and active signal/cytokine transducer for circulating and tissue influences that could modify the endothelial phenotype from quiescent to activated endothelial phenotype and orchestrate remodeling and the physiopathology of asthma (50,51).



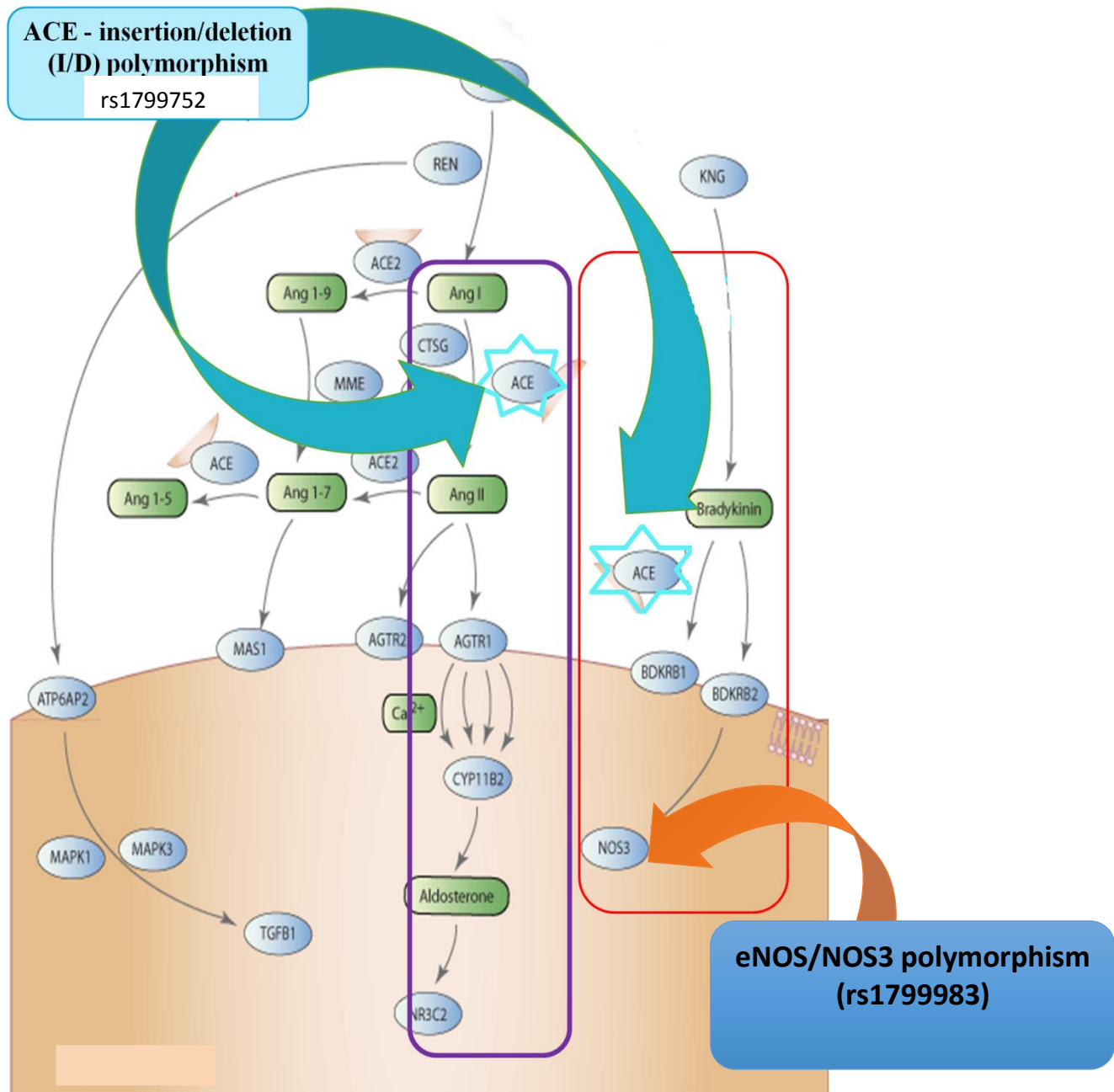
**Fig.I.2.8:** Endothelial homeostasis and dysfunction in disease ( CVD and asthma). (*adapted from Conti et al; 2013(52)*)



**Fig.I.2.9:** Vasculogenesis; angiogenesis and arteriogenesis (*adapted from Carmeliet et al, 2000*) (53).

The Candidate gene-association and GWAS studies put NOS, ACE enzymes (Fig.I.2.10) and ADRB2 as important regulators of oxidative stress, bronchial hyperresponsiveness and endothelial dysfunction. Even over, the activation of ADRB2 caused by beta agonists could lead to uncoupling of eNOS and overproduction of ROS such as peroxynitrite and endothelial dysfunction (50,51).





**Fig.I.2.10:** ACE, AGTR1 and eNOs polymorphisms- involved signaling pathways Nitric oxide in asthma. (adapted from Bergeron et al 2010(50); Manuyakorn et al 2011(51))

Myeloperoxidase (MPO) is a lysosomal heme-containing enzyme catalyzing the conversion of superoxide-generated hydrogen peroxide into hypochlorous acid is important in regulating oxidative stress through production of hypohalogenic oxidants that may further mediate oxidative modification of lipids, proteins and DNA, and also has a key role in innate immune system (54).

MPO may also be involved in the pathophysiology of irreversible airflow obstruction in asthmatics (54).

The MPO -463 G/A polymorphism, which is localized in the promoter region of the myeloperoxidase gene, plays a role in regulating myeloperoxidase activity. While the G allele provides high myeloperoxidase activity, the mutant A allele causes loss of this activity. The purpose of this study is to analyze the association between single nucleotide polymorphism in the MPO promoter region of the gene, -463G>A (rs2333227)

located on chromosome 17q23.1 with asthma severity when compared with a control group of healthy blood donors and its relation with MPO levels (54).

Haptoglobin (Hp), an alpha2-sialoglycoprotein known to bind free hemoglobin (Hb) has been implicated in modulation of Th1/Th2 response, intervening in innate and adaptive immune response. The Hp locus is situated at 16q22 chromosome, being in humans, polymorphic for the alpha chain. The alpha chain of Hp has 2 major co-dominant alleles Hp\*1 and Hp\*2, with 3 genotype variants, Hp1-1, Hp2-1, Hp2-2. Asthma is considered a heterogeneous disease, characterized most of the times by a Th2 inflammatory response. Haptoglobin (Hp), is an alpha2-sialoglycoprotein known to bind free hemoglobin (Hb) and has been implicated in modulation of Th1/Th2 response, Intervening in innate and adaptive immune response. The Hp locus is situated at 16q22 chromosome, being in humans, polymorphic for the alpha chain. The alpha chain of Hp has 2 major co-dominant alleles Hp\*1 and Hp\*2, with 3 genotype variants, Hp1-1, Hp2-1, Hp2-2. The aim of the study is to establish a relation between Hp genotypes and Hp levels (intermediate phenotype), and the pathophysiology of asthma when compared with a control group of healthy blood volunteers. Haptoglobin (Hp), known to bind free hemoglobin (Hb) could also be related with NO bioavailability and inflammation of the airways (55-59).

ADRB2-beta 2 adrenergic receptor is a G-protein-coupled receptor encoded by the ADRB2 gene located at 5q31-32. ADRB2 is polymorphic with more than 49 polymorphisms and 2 variants Ins/Del identified. The frequencies of polymorphisms Arg16Gly could be 59% and Gln27Glu 29%. These polymorphisms could be associated with altered expression, ligand binding, coupling, or regulation phenotypes. Clinical studies to date have revealed that some of these polymorphisms have a significant disease modifying effect or alter the response to treatment. In this study we focus on polymorphisms that alter the protein sequence at nucleotide 46: Glycine to Arginine at codon 16 (Gly16Arg) (A > G); minor allele frequency

approximately(MAF) 0.4–0.5. Patients who are homozygous for Arg16 and/or Glu 27 may be more susceptible to tachyphylaxis with chronic use of beta2- agonists (60,61).

In ADRB2-beta 2 adrenergic receptor polymorphisms some clinical studies to date have revealed that some of the polymorphisms have a significant disease modifying effect or alter the response to treatment.

In this study we focus on one polymorphism that alter the protein sequence at nucleotide 46: Glycine to Arginine at codon 16 (Gly16Arg) (A > G). Minor allele frequency approximately (MAF) 0.4–0.5. Arg/Arg homozygotes for the Arg16Gly polymorphism in the beta2 adrenoreceptor gene (ADRB2) have a reduced response to short acting beta2 agonists. A codon 16 Arg/Arg genotype may identify patients at risk for deleterious or nonbeneficial effects of regularly scheduled therapy with inhaled beta2 –agonists. These patients may be candidates for alternative schedules of therapy, earlier initiation of anti- inflammatory agents, or both. Patients who are homozygous for Arg16 may be more susceptible to tachyphylaxis with chronic use of beta2- agonists. LABA don't seem to affect it, although they had less benefit if are homozygous Arg16 (60,61).

Phase II detoxification enzymes (62-64) particularly the glutathione S-transferases (GSTs), are important in the inflammatory responses associated with xenobiotic or reactive oxygen compounds.

The inability of GST variants of the enzymes to detoxify the reactive oxygen species (ROS) contributes to the activation of the inflammatory process, bronchoconstriction, and asthma symptoms.

An increasing risk for asthmatic disease and an increase in individual susceptibility to pro-allergy effects associated with xenobiotics have been demonstrated to be linked to functional polymorphisms.

GSTT1 is located on chromosome 22q11.23, with eight thousand one hundred and seventy-nine bases, and also has two alleles, one wildtype (GSTT1 \* 1) and one null allele (GSTT1 \* 0). Individuals homozygous for the null allele (Homozygous homing the GSTT1 \* 0 allele:T-) can not express the GSTT1 protein that is why they could be a candidate gene when studying respiratory diseases.

Phase II detoxification enzymes, particularly the glutathione S-transferases (GSTs), are important in the inflammatory responses associated with xenobiotic or reactive oxygen compounds. The inability of GST variants of the enzymes to detoxify the reactive oxygen species (ROS) contributes to the activation of the inflammatory process, bronchoconstriction, and asthma symptoms. An increasing risk for asthmatic disease and an increase in individual susceptibility to pro-allergy effects associated with xenobiotics have been demonstrated to be linked to functional polymorphisms of GST enzymes, in particular, *GSTM1* and *GSTT1*

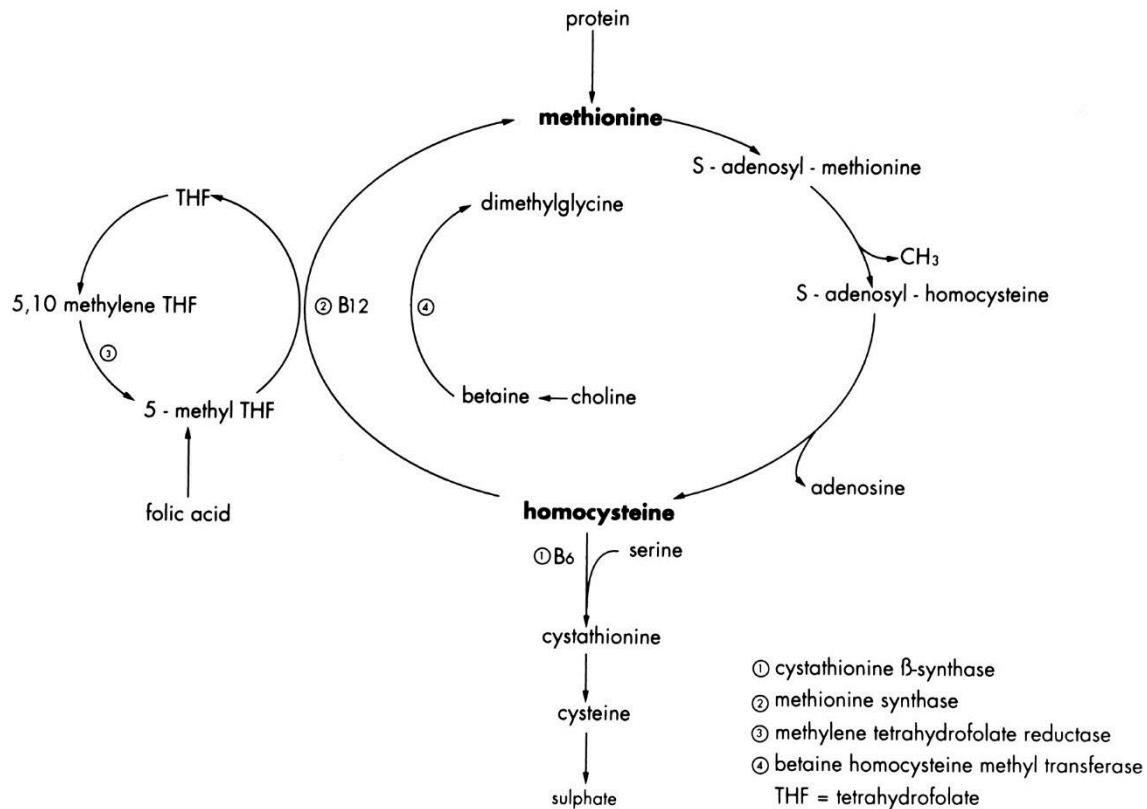
null polymorphisms. One of these genes, *GSTM1*, encodes for a class m GST isoenzyme involved in polycyclic aromatic hydrocarbons (PAHs) detoxification. Another polymorphic gene of the same family is *GSTT1* which encodes for a class q GST that catalyzes the conjugation of halomethanes in human erythrocytes (62-64).

*GSTM1* is a gene located on chromosome 1p13.3, with twenty-one thousand two hundred and forty-four bases and has two alleles, one wildtype (*GSTM1* \* 1) and one non-functional null allele (*GSTM1* \* 0). Homozygous individuals for the null allele of *GSTM1* have a deletion that leads to non-transcription of messenger RNA and non-translation of *GSTM1* protein; *GSTM1* is expressed in the airways that is why it could be a candidate gene when we are studying respiratory diseases. Phase II detoxification enzymes, particularly the glutathione S-transferases (GSTs), are important in the inflammatory responses associated with xenobiotic or reactive oxygen compounds. The inability of GST variants of the enzymes to detoxify the reactive oxygen species (ROS) contributes to the activation of the inflammatory process, bronchoconstriction, and asthma symptoms. An increasing risk for asthmatic disease and an increase in individual susceptibility to pro-allergy effects associated with xenobiotics have been demonstrated to be linked to functional polymorphisms of GST enzymes, in particular, *GSTM1* and *GSTT1* null polymorphisms. One of these genes, *GSTM1*, encodes for a class m GST isoenzyme involved in polycyclic aromatic hydrocarbons (PAHs) detoxification (62-64).

DNA methylation (65), an epigenetic feature of DNA like acetylation could modulate gene expression involves methyltransferases that use the methyl donor S-adenosyl-L-methionine. Methylene tetrahydrofolate reductase (*MTHFR*) catalyzes the synthesis of 5-methyltetrahydrofolate (5-methylTHF), the methyl donor for synthesis of methionine from homocysteine and precursor of S-adenosyl-L-methionine. About 85% of the general population carries a variant such as *C677T* mutation in the *MTHFR* gene associated with higher blood homocysteine also a risk factor for cardiovascular disease. *T/T* genotypes had a diminished level of DNA methylation.

*MTHFR* *C677T* polymorphism (Fig.1.2.11) which is associated with decreased enzyme activity, and thus increases the availability of 5,10-methylene tetrahydrofolate for DNA synthesis, which partially explains the reduced methylation in those with *TT* genotype. Methylene tetrahydrofolate reductase (*MTHFR*) is an enzyme in folate metabolism and plays a key role in DNA biosynthesis and epigenetics through methylation. The role of this enzyme is to catalyse the reaction of 5,10-methyl-tetrahydrofolate (*MTHF*) to 5-*MTHF*, which is part of the folate metabolism. 5,10-*MTHF* is required for DNA synthesis, whereas its product 5-*MTHF* is the methyl donor for regeneration of methionine from homocysteine for methylation. *MTHFR* *C677T* polymorphism in the *MTHFR* gene have recently been the focus of studies on disease risk. The nucleotide polymorphism 677 C>T (rs1801133) is located within the

coding region for the catalytic domain of MTHFR and results in an amino acid substitution from an alanine to a valine at codon position 222 (exon 4). The 677 C>T variant has been associated with a reduced enzyme activity (65).



**Fig.I.2.11:** A common (thermolabile) variant: MTHFR polymorphism MTHFR: C677T (rs1801133) associated with elevated plasma homocysteine, itself could be an independent risk factor for CVD and other diseases related with remethylation cycle (*adapted from* Gallagher et al 1996 (66)).

Studies of association of genes in Atopic Dermatitis (AD) (67) put in evidence the cluster of the EDC and other barrier candidates, but the most important associations were related to FLG (filaggrin) and two null mutations (R510X and 2282del4). In this study we have studied the role of LELP1 (another EDC gene) polymorphism (late cornified envelope-like proline-rich 1) [rs7534334]. The molecular signature of AD is mainly associated with Th2, IgE high (extrinsic) and IgE low (intrinsic) mediated by keratinocyte, thymic stromal lymphopoietin (TSLP) regulating dendritic cells. This Th2 activation contributes to barrier dysfunction by impairing FLG and other skin barrier genes expression. IL-22 and IL-33 play also its role, in

this Th2 driven inflammation by allergens, associated with FLG and other EDC gene polymorphisms that lead to barrier dysfunction and could contribute to AD and atopic march. The chromosomal region 1q21 has been linked to allergy and atopic dermatitis in previous studies, with a peak linkage overlying the epidermal differentiation complex (EDC). The EDC contains various important genes, such as involucrin (IVL), loricin (LOR), the small proline-rich protein (SPRR) gene family, profilaggrin (FLG) and trichohyalin (THH) encoding structural components of the epidermis, and the S100 gene family encoding calcium-binding proteins involved in signaling. The next gene to be identified as a possible factor in the development of AD and that might also be involved in atopic asthma, is LELP-1 (late cornified envelope-like proline-rich 1). This gene encodes a late cornified envelope-like proline-rich protein and the (SNP) rs7534334, an intron variant on gene of LELP1 might be related with atopic disease (67).

A phenotype (14,15,68,69) is defined as the “observable properties of an organism that are produced by the interactions of the genotype and the environment”. The concept of the phenotype has been suggested to be the prelude to that of the ‘endotype’, wherein a specific biological pathway is identified that explains the observable properties of a phenotype.

The definition of a true phenotype (or endotype) requires a unifying and consistent natural history, consistent clinical and physiological characteristics, an underlying pathobiology with identifiable biomarkers and genetics and a predictable response to general and specific therapies.

*Mechanistic and common pathway* approach based on *candidate genes* and on the sufficient cause framework of disease try to find *new biomarkers* and to explain what the current guidelines for asthma diagnosis and management do not recognize.

GWAS (70) studies may show some limitations namely the need of meticulous *phenotypic* classification of disease cases and selection of an appropriate disease-free *control* group are critical initial steps in complex disease mapping, adding to the lack of functional *biological plausibility* using in vivo or in vitro experimental models

The identification of distinct *asthma phenotypes* has fostered the concept of specific *targeted or personalized therapies* – *PRECISION MEDICINE* based in the emerging *endotype-driven therapeutic strategies*.

Each of the hallmark characteristics - *canonical pathways* - of asthma (inflammation, remodeling, airway hyperreactivity, oxidative stress, innate and adaptive immunity) is the expression of a complex network of molecules, very diverse both within any given patient in time and between any two patients.

Although asthma has been considered as a single disease for years, recent studies have increasingly focused on its *heterogeneity*.

The characterization of this heterogeneity has promoted the concept that asthma consists of *multiple phenotypes* or consistent groupings of characteristics.

Asthma phenotypes were initially focused on combinations of clinical characteristics, but they are now evolving to *link biology to phenotype*.

Recently, several genes and genetic loci has been associated with asthma and have been described as common *susceptibility factors* for the disease.

In complex diseases such as asthma and CVD (68,69,14,15,18,19), a large number of *molecular and cellular components* may interact through *complex networks involving gene–gene and gene–environment* interactions.

If we think that asthma is mainly a Th2 high or Th2 low, disease then hypertension and CVD might be associated with a Th1 or Th17 profile (71-74).

For each SNP, in this thesis different genetic models: additive\_1 , additive\_2 and potential dominant and recessive effects were evaluated by combining homozygote and heterozygote variant carriers for comparison with reference in susceptibility to asthma, severity and allergic status.

**CHAPTER II- Objectives**



## **II.1. Objectives**

### **General Objectives**

**This thesis intends to identify non-traditional and emergent risk factors of CVD and susceptibility to asthma control and severity of the disease.**

### **Specific Objectives**

**In this thesis it has been evaluated the impact of inflammation and endothelial dysfunction on the development of asthma susceptibility and control and concurrent CVD.**

For CVD we had done a literature review and meta-analysis; for asthma we had done an epidemiological case-control study and estimation of genetic risk score

According to that we studied the different polymorphisms related with different biochemical and signaling pathways in CVD and bronchial asthma :a)renin-angiotensin system ( ACE polymorphism of I / D (287 bp, on chromosome 17q23, intron 16 (rs1799752); Polymorphism for Type 1 receptor of Angiotensin II (AGTR1) 1166A / C (rs5186); b)NO associated system ( Haptoglobin polymorphism (1.1, 2.1, 2.2); Intron 4 polymorphism of the eNOS gene; NOS2 polymorphism (exon 16-14CT); NOS2 polymorphism (intron 16 - 88GT); NOS2 polymorphism (intron 20 - IVS20 + 524 GA); MPO Polymorphism (- 463 GA; c)beta2 adrenergic receptors (Receptor beta2adrenergic polymorphism Arg16 Gly (rs1042713); d)detoxification (GSTM1 polymorphism and GSTT1 polymorphism; e) atopic dermatitis and asthma LELP1 (rs7534334); f)Epigenetics(Methylations of DNA and Histones) and homocysteine levels( MTHFR polymorphism C677T (rs1801133).

**CHAPTER III. Material and Methods**

### III.1. Material and Methods for The Literature review and meta-analysis in Cardiovascular disease

For the CVD study on the impact of inflammation and endothelial dysfunction associated with CVD we performed a literature review and meta-analysis that was accomplished with comprehensive meta-analysis software version 2. The Polymorphisms studied were:

#### A) RENIN-ANGIOTENSIN SYSTEM

ACE polymorphism of I / D (287 bp, on chromosome 17q23, intron 16 (rs1799752)

Polymorphism for Type 1 receptor of Angiotensin II (AGTR1) 1166A / C (rs5186)

#### B) NO ASSOCIATED SYSTEM

Haptoglobin polymorphism (1.1, 2.1, 2.2)

Intron 4 polymorphism of the eNOS gene

NOS2 polymorphism (exon 16-14CT)

NOS2 polymorphism (intron 16 - 88GT)

NOS2 polymorphism (intron 20 - IVS20 + 524 GA)

MPO Polymorphism (- 463 GA)

#### C) BETA2ADRENERGIC RECEPTORS

Polymorphism Receptor beta2adrenergic Arg16 Gly (rs1042713)

#### D) DETOXIFICATION

GSTM1 polymorphism: search for null genotypes (homozygous for the allele GSTM1 \* 0) (M-)

GSTT1 polymorphism: null genotype search (Homozygous homing for the GSTT1 \* 0 allele) (T-)

#### E) ECZEMA AND ASTHMA

LELP1

#### F) EPIGENETICS

MTHFR polymorphism MTHFR: C677T (rs1801133)

For the review of the literature, a research was done in EBSCOhost using Mesh descriptors: "Polymorphisms entitled for the thesis" - terms of the subject and "cardiovascular disease"; Dates: no temporal limit; Academic journals; English language. Those that are removed or were repeated or did not have in the title the reference to the SNPs or CVD. Additional research was conducted whose research intercepted these topics with the subtopics of "genetic determinants of cardiovascular disease". The references selected for this article from the literature review are representative and do not include all the bibliographic research carried out and had not temporal limit. Statistical analysis was performed by using the software comprehensive meta-analysis version 2.

### III.2. Material and Methods for Clinical study in asthmatic patients vs controls:

Type of study: Epidemiologic study, Case / Control study for bronchial asthma. Study group: Asthma patients at the CHLN-HSM Allergy Clinic; N = 153;

Control group: healthy volunteers; N = 356

**Sample Characterization:** The sample will consist of both controlled and uncontrolled asthmatic patients and the control group of healthy volunteers. For a power of 90% and with

$p < 0.05$ , with an allele frequency in the control population of 40% and with  $OR > 1.5$  with 95% CI, it is estimated a sample that approaches the 140 individuals (75).

**Informed consent:** signed by all patients.

**The participants were:** the control group with 356 healthy volunteers and 153 asthmatic patients from the Immunology Service of the Santa Maria Hospital-CHLN. The diagnosis of asthma will be classified according to the guidelines of the Global Initiative for Asthma (GINA) (76), , taking into account the episodic nature of dyspnea, wheezing, cough, chest tightness, frequency of symptoms, Presence of nocturnal symptoms, frequency of exacerbations, use of relief medication, FEV1 and PEF values, and daily variability. The definition of patients with uncontrolled asthma and patients with controlled asthma was performed by assessing the level of asthma control by a validated instrument: Asthma Control Questionnaire - ACQ7 The cut-point considered is clinical practice 0.75 (for Who are aged > 17 years), and PAQLQ Pediatric Asthma Quality of Life Questionnaire-Portuguese version by Juniper (7-17 years) (<4 implies uncontrolled asthma)(<http://www.qoltech.co.uk/questionnaires.htm>).

This evaluation will be performed after patient selection, and submission to an 8-week (3-appointments) of therapeutic optimization period according to GINA guidelines(125). Patients who maintain at least one uncontrolled asthma indicator within the last month (frequent use of relief medication, nocturnal asthma wakes, frequent wheezing / dyspnea with limitation of daily activities, exacerbations leading to the use of the Emergency department, FEV1 <80% and no improvement or worsening of the score (Asthma Control Questionnaire) - ACQ-7 or PAQLQ, will be classified as having uncontrolled asthma. The remaining patients will be considered as controlled.

The exclusion criteria are: individuals who do not agree to participate in the study; noncompliance with anti-asthmatic therapy (assessed through completion of the patient diary card-Asthma Clinical Research Network); The existence of other comorbidities that may interfere with the severity of the respiratory disease; The existence of a diagnosis of Chronic Obstructive Pulmonary Disease or other lung disease; Smoking; Patients with HIV infection, parasitized or otherwise infected ,with anemia, renal failure or chronic liver disease.

All asthmatic participants will be characterized in relationship to socio-demographic variables (ethnicity, gender, age, country of origin, place of residence, profession, years of schooling), clinical history, longevity of asthmatic disease, absence of other pulmonary pathology and others exclusion criteria; Non-specific inhaled challenge test with positive methacholine and/or positive bronchodilation test; Skin prick tests, total IgE, RAST and ISAC, FeNO.

The condition of controlled and uncontrolled asthma will be related to: a) the type of allergens to which they are sensitized Skin tests in Prick performed according to the recommendations

of the European Academy of Allergy and Clinical Immunology (EAACI) using the ALK allergen battery -Abello. Saline serum as a negative control and 10 mg / ml histamine as a positive control. Skin tests are considered positive if at least one allergen has a papule greater than 3 mm in diameter after subtraction of the negative control; b) Asthma severity (Intermittent and Persistent: mild / moderate / severe) according to GINA guidelines ; for asthma severity levels after therapeutic optimization period (Controlled / Partially Controlled / Uncontrolled); C) classification of asthma exacerbations according to GINA guidelines (mild, moderate, severe and imminent respiratory arrest); D) FEV1 and PD20 in the non-specific inhalation provocation test with methacholine according to ATS standards. Inhaled provocation test is performed with methacholine, administered through a dosimeter M.E.F.A.R. MB3, with an aerosol flow rate of 39 $\mu$ L / 5 nebulizations, the patient inhales each concentration of methacholine 5 times from the residual volume to the total lung capacity. Respiratory function is measured 3 minutes after the inhalation of each methacholine dose through forced expiration maneuvers with a spirometer. The test is considered complete when a reduction of FEV1  $\geq$ 20% or after inhalation of the last dose of methacholine is measured for the first time. The dose of methacholine that causes a reduction of FEV1 = 20% (PD20 FEV1) is calculated by linear interpolation of the last two points of the dose-response curve; E) total IgE levels measured by fluoroenzyme immunoassay (Pharmacia, Uppsala, Sweden and specific RAST-In vitro-RadioAllergoAbsorbent (RAST) test for the detection of specific IgE antibodies circulating in serum-EIA (Pharmacia, Uppsala, Sweden) and molecular allergen diagnostic with the Immuno-Solid phase Allergen Chip (ISAC) - ISAC microarray (ThermoFisher Phadia) that allows simultaneous detection of allergen specific IgE to a wide range of molecular allergen components ; F) Nitric oxide in the exhaled air-Nitric oxide assay of the air exhaled for 6 seconds with Nioxmino.ADULTS: FeNO (ppb) <5; 5-25 (Improbable) ; 25-50 (Present but moderate); > 50 (significant); CHILDREN: FeNO (ppb) <5; 5-20 (Unlikely) ; 20-35 (Present but moderate); > 35 (significant) - g) consumption of beta2 mimetics short acting (Salbutamol; Terbutaline) and long acting (Salmeterol; Formoterol); H) inhaled corticosteroids (Beclomethasone dipropionate, Budesonide / Budesonide suspension for nebulization, Fluticasone / Fluticasone nebulizers and dose, i) administration of anticholinergics / Parasympatholytics (ipratropium bromide, tiotropium); J) Methylxanthines (aminophylline; theophylline); L) leukotriene receptor antagonists (montelukast; zafirlukast); M) Oral corticosteroids (Prednisone, Prednisolone, Methylprednisolone, Deflazacort, Betamethasone); N) Anti-IgE monoclonal antibody (IgG1k) (Omalizumab) or anti IL5-mepolizumab; O) existence (active / in the past) or not of specific immunotherapy .p) levels of peripheral blood ECP and eosinophil count.

**Asthmatic patients are compared with a group of healthy volunteers according to the polymorphisms studied and a correlation is made by systematic and comprehensive**

**review of the literature with the same polymorphisms for cardiovascular disease.** The study population consisted of healthy volunteers individuals in the control group and asthmatics from a Portuguese cohort.

Written informed consent was obtained from all participating individuals. Patients were diagnosed by physicians for asthma according to the guidelines of GINA (76). and as having atopy or not according to WAO/ EAACI guidelines (77) they were examined for a self-reported history of breathlessness, wheezing, and other atopic comorbidities such as rhinitis, atopic dermatitis, drug allergy, food allergy, urticarial among others, and family history. Atopic individuals have a positive skin prick test (SPT) for at least one of the common environmental allergens or the presence of specific IgE, associated with high serum IgE levels estimated using enzyme-linked immunosorbent assay and suffered from asthma. The level of control of asthmatic disease was evaluated with instrument validated for the Portuguese version by Juniper ACQ7 and PAQLQ.( <http://www.goltech.co.uk/questionnaires.htm> ) The exclusion criteria are: individuals who do not agree to participate in the study; Noncompliance with anti-asthmatic therapy (assessed through completion of the patient diary card-Asthma Clinical Research Network); The existence of other comorbidities that may interfere with the severity of the respiratory disease; The existence of a diagnosis of Chronic Obstructive Pulmonary Disease or other lung disease; Smoking; Patients with HIV infection, parasitized or otherwise infected; with anemia, renal failure or chronic liver disease. The diagnosis of asthma is based on clinical symptoms, physical examination and airway reversibility with FEV1 and/or FVC response of 200 mL or greater and 12% improvement from baseline after bronchodilator as albuterol(ATS).

## **Genomic DNA Isolation and quantification**

### **I) Extraction of genomic DNA**

Whole blood samples were collected in 10 ml EDTA tubes and stored at -20 ° C. DNA was obtained from 2 ml of blood using a non-enzymatic method by the salting-out technique adapted from the method of DK Lahiri and JI Nurnberger Jr.(78).

### **II) DNA quantification**

Quantification in (ng /  $\mu$ l) and determination of purity of genomic DNA (ratio between absorbances at 260 and 280 nm) was performed on a full-spectrum spectrophotometer (NanoDrop® ND-2000). The DNA was solubilized in TE buffer (Tris EDTA pH = 8) and stored at 4 ° C.

## **Genotyping ( Annexes Figs:1a-12a)**

### **ACE polymorphism of I / D (287 bp, on chromosome 17q23, intron 16 (rs1799752)- Fig1a- Annexes**

The polymorphism I/D of ACE gene was detected using the forward primer 5'-CTGGAGACCACTCCCATCCTTTCT-3' and the reverse primer 5'-GATGTGGCCATCACATTCGTCAGAT-3'. The PCR reaction was performed in a final volume of 25 µL using 200 ng of genomic DNA, 10 pmol of both primers, 12.5 µl of DreamTaq Green PCR Master Mix (2X) (Thermo Scientific®). The PCR reaction started with an initial denaturation at 94°C for 2 minutes, followed by 35 cycles for 45 seconds at 94°C, 45 seconds at 58°C and 45 seconds at 72°C, with a final extension of 5 minutes at 72°C. Fragments were separated by electrophoresis on a 2% agarose gel for 60 minutes at 110V and visualized by ethidium bromide staining.

There was one fragment of 477 bp for homozygous I/I, two fragments of 477 bp and 190 bp for heterozygous I/D and one fragment of 190 bp for homozygous D/D.

### **Polymorphism for Type 1 receptor of Angiotensin II (AGTR1) 1166A / C (rs5186) -Fig2a- Annexes**

The A1166C polymorphism at the AT1R locus was detected using the forward primer 5'-ATAATGTAAGCTCATCCACC – 3' and the reverse 5'- GAGATTGCATTTCTGTCTAGT– 3'. The underlined base in the sense strand primer is a base mismatch, introduced to produce a (*DdeI*) restriction site, whenever the cytosine (C) of the A1166C polymorphism is present in the template. If adenine (A) is present, there is no restriction site. PCR was performed in a total reaction volume of 50 µl containing 10 pmol of each primer, 0.2 mM of each dNTP, 3 µl of 1.5 mM MgCl<sub>2</sub>, 5 µl of 10 x incubation buffer and 0.5 U AmpliTaq DNA polymerase, and 400 ng genomic DNA. The PCR reaction started with an initial denaturation step at 94°C for 5 min, 35 cycles of PCR consisting of 94°C for 30 sec, 51°C for 30 sec and 72°C for 45 sec. A final extension step at 72°C for 7 min ended the PCR. Twenty microliters of the reaction product were then exposed to 10 U of the enzyme (*DdeI*). Fragments were separated by electrophoresis on a 2% agarose gel for 60 minutes at 110V and visualized by ethidium bromide staining.

### **Haptoglobin polymorphism (1.1, 2.1, 2.2) -Fig3a- Annexes**

The determination of the genetic polymorphism of haptoglobin was performed from Plasma Hp by polyacrylamide gel electrophoresis (PAGE)(79).

Determination of the genetic polymorphism of haptoglobin is performed from plasma Hp by 4.7% polyacrylamide gel (PAGE) in 0.504M TRIS-HCl buffer solution, pH 8.9. Samples for gel application (10µL) are prepared using 40% (w / v) sucrose, 28.2mg / mL Hb and 3: 2: 4 plasma at a final volume of 45µL. For the coloring of the resulting bands of electrophoresis the contact staining method is used, using 16mM o-dianisidine in 50% (v / v) acetic acid and

thereafter 0.6% (v / v) hydrogen peroxide. Hp phenotypes are determined by polyacrylamide gel electrophoresis, and the corresponding genotype is assigned. **Determination of the Haptoglobin (Hp) plasmatic concentration**

Serum Hp concentration was determined by nephelometry- (BN ProSpec from Siemens Healthcare Diagnostics) in mg / dL.

#### **Intron 4 polymorphism of the eNOS gene)-Fig4a- Annexes**

The polymorphism 4 b/a of NOS3 gene was detected using the forward primer 5'-AGGCCCTATGGTAGTGCCCTTT-3' and the reverse primer 5'-TCTCTTAGTGCTGTGGTCAC-3'. The PCR reaction was performed in a final volume of 25  $\mu$ L using 200 ng of genomic DNA, 10 pmol of both primers, 12.5 ml of DreamTaq Green PCR Master Mix (2X) (Thermo Scientific®). The PCR reaction started with an initial denaturation at 94°C for 2 minutes, followed by 35 cycles for 30 seconds at 94°C, 30 seconds at 53°C and 45 seconds at 72°C, with a final extension of 5 minutes at 72°C. Fragments were separated by electrophoresis on a 3% agarose gel for 120 minutes at 110V and visualized by ethidium bromide staining.

There was one fragment of 420 bp for homozygous b/b, two fragments of 393 bp and 420 bp for heterozygous a/b and one fragment of 393 bp for homozygous a/a.

#### **NOS2 polymorphism (exon 16-14CT), NOS2 polymorphism (intron 16 - 88GT)**

#### **NOS2 polymorphism (intron 20 - IVS20 + 524 GA)-Fig5a; -Fig6a; -Fig7a- Annexes**

**The polymorphisms -14 C/T in exon 16 and -88 G/T in intron 16 of NOS2 gene** were detected using the forward primer 5'-TAAACCAACTTCCGTGGTGGG-3' and the reverse primer 5'-AGCTGGAGAATGGAGCTGGAC-3'. The PCR reaction was performed in a final volume of 50  $\mu$ L using 200 ng of genomic DNA, 10 pmol of both primers, 25  $\mu$ L of DreamTaq Green PCR Master Mix (2X) (Thermo Scientific®). The PCR reaction started with an initial denaturation at 94°C for 2 minutes, followed by 35 cycles for 45 seconds at 94°C, 45 seconds at 61°C and 45 seconds at 72°C, with a final extension of 5 minutes at 72°C. The PCR products were digested with 10U of TaSI (Thermo Scientific®) for 16 hours at 65°C for exon 16 and with 10U of Adel (Thermo Scientific®) for 16 hours at 37°C for intron 16.

Fragments were separated by electrophoresis on a 2% agarose gel for 90 minutes at 85V and visualized by ethidium bromide staining.

**For -14 C/T in exon 16**, there were two fragments of 285 bp and 170 bp for homozygous without mutation (CC), four fragments of 285 bp, 170 bp, 137 bp and 33 bp for heterozygous CT and two fragments of 285 bp and 137 bp for homozygous with mutation (TT).

**For -88 G/T in intron 16**, there was an undigested fragment of 455 bp for homozygous without mutation (GG), three fragments of 455 bp, 263 bp and 192 bp for heterozygous GT and two fragments of 263 bp and 192 bp for homozygous with mutation (TT).



**The polymorphism 524 G/A in intron 20 of NOS2** gene was detected using the forward primer 5'-TTATCCCAATCCCAGCCACTCG-3' and the reverse primer 5'-GCCAGGCTCTGTTTCTCTGATCC-3'. The PCR reaction was performed in a final volume of 50 µL using 200 ng of genomic DNA, 10 pmol of both primers, 25 µl of DreamTaq Green PCR Master Mix (2X) (Thermo Scientific®). The PCR reaction started with an initial denaturation at 94°C for 2 minutes, followed by 35 cycles for 45 seconds at 94°C, 45 seconds at 59°C and 45 seconds at 72°C, with a final extension of 5 minutes at 72°C. The PCR product was digested with 10U of HinfI (Thermo Scientific®) for 16 hours at 37°C. Fragments were separated by electrophoresis on a 4% agarose gel for 90 minutes at 85V and visualized by ethidium bromide staining.

There were three fragments of 75 bp, 54 bp and 39 bp for homozygous without mutation (GG), four fragments of 129 bp, 75 bp, 54 bp and 39 bp for heterozygous GA and two fragments of 129 bp and 39 bp for homozygous with mutation (AA).

#### **MPO Polymorphism (- 463 GA)- Fig8a- Annexes**

MPO polymorphism was analyzed by PCR-RFLP in genomic DNA. PCR was performed with the following reaction mixture: 20 pmol of forward and reverse primers of sequences 5'-GTATAGGCACACAATGGTGAG-3' and 5'-GCAATGGTTCAAGCGATTCTTC-3', respectively; 200µM PCR Nucleotide Mix, containing four dNTPs; 25mM MgCl<sub>2</sub>; 1 U of Taq polymerase; And 200ng of genomic DNA, to a final volume of 50µl. The PCR conditions used were hot start at 94 ° C for 2 minutes, followed by 35 cycles of 1 minute at 94 ° C (denaturation), 1 minute at 59 ° C (annealing), 1 minute at 72 ° C (extension), adding a 7 minute extension to 72 ° C.

The reaction product is 350 bp and was digested with Ssi I (Acyl) 5U / µl endonuclease (Thermo Scientific) at 37 ° C for 16 hours. The hydrolyzed fragments were subjected to 3% (m / v) agarose gel (SeaMem® LE Agarose) electrophoresis in TAE (20 mM Tris-Acetate, 1 mM EDTA, pH 8.0) with 10 µg / ml ethidium bromide, For 90 min at 85V.

#### **MPO levels**

Determination of plasma concentration of MPO was performed using the "Human Myeloperoxidase Immunoassay" Kit from "R & D Systems". This method is based on the ELISA technique, an enzyme-linked immunosorbent assay.

#### **Polymorphism of the Receptor beta2adrenergic Arg16 Gly (rs1042713)- Fig9a- Annexes**

The polymorphism 16ArgGly of ADRB2 gene was detected using the forward primer sequence 5'-CCTTCTTGCTGGCACCCCAT-3' and the reverse primer 5'-GGAAGTCCAAAACCTCGCACCA-3'. The PCR reaction was performed in a final volume of 25 µL using 200 ng of genomic DNA, 10 pmol of both primers, 12,5 µl of DreamTaq Green PCR Master Mix (2X) (Thermo Scientific®). The PCR reaction started with an initial denaturation

at 94°C for 2 minutes, followed by 35 cycles for 30 seconds at 94°C, 30 seconds at 60°C and 45 seconds at 72°C, with a final extension of 5 minutes at 72°C. The PCR product was digested with 10U of NcoI (Thermo Scientific®) for 16 hours at 37°C.

Fragments were separated by electrophoresis on a 3% agarose gel for 90 minutes at 85V and visualized by ethidium bromide staining.

There was one fragment of 308 bp for homozygous without mutation (ArgArg), three fragments of 308 bp, 291 bp and 17 bp for heterozygous ArgGly and two fragments of 291 bp and 17 bp for homozygous with mutation (GlyGly).

### **GSTT1 and GSTM1 polymorphisms- Fig.10 a- Annexes**

The two polymorphisms in the (GSTM1 and GSTT1) genes were performed by PCR-Multiplex technique using 5 primers. Primers 1 and 2 are specific for GSTM1 and GSTM4 with the following sequences respectively 5'-GCCATCTTGTGCTACATTGCCCG-3' and 5'-ATCTTCTCCTCTTCTGTCTCCCC-3'. (GSTM4 will serve as a control for DNA amplification because it has no polymorphisms). Primer 3 is specific for GSTM1 and has the sequences 5'-TTCTGGATTGTAGCAGATCATGCC-3'. Primers 4 and 5 are specific for GSTT1 and have the following sequences respectively 5'-TTCCTTACTGGTCCTCACATCTC-3' and 5'-TCACCGGATCATGGCCAGCA-3'. The PCR reaction was performed in a final volume of 50 µL using 200 ng of genomic DNA, 10 pmol of each primer, 25 µl of DreamTaq Green PCR Master Mix (2X) (Thermo Scientific®) and 2.5 µl Of DMSO (dimethylsulfoxide). The PCR reaction started with an initial denaturation at 94°C for 2 minutes, followed by 40 cycles for 45 seconds at 94°C, 45 seconds at 58°C and 45 seconds at 72°C, with a final extension of 5 minutes at 72°C. Fragments were separated by electrophoresis on a 3% agarose gel for 120 minutes at 100V and visualized by ethidium bromide staining.

In the analysis of the PCR product, we observed 3 bands: one with 230 base pairs, resulting from the action of primer 1 and 3 (which will identify the GSTM1 sequence), a band with 157 pairs of bases Resulting from the action of primers 1 and 2 (which identifies the GSTM4 sequence) and a band with 480 base pairs resulting from the action of primers 4 and 5 (which identifies GSTT1).

### **Atopic march- LELP1 -Fig.11a- Annexes**

The LELP-1 [rs7534334]genotypes were determined by the polymerase chain reaction and restriction fragment length polymorphism (PCR-RFLP) technique, the polymorphic region was amplified in a 50 µl reaction mixture: 10 mM of each primer (forward: 5'-CCTCCACCATGTACAACGCT-3'; and reverse: 5'- TTGCATTAAACCCATGCAGCC-3'), 200 ng of genomic DNA and 0.2 mM of PCR nucleotide Mix Thermo Scientific® DreamTaq Green containing 10 mM dNTPs, 1.5 mM MgCl<sub>2</sub>, 1 U Taq polymerase. PCR conditions involved an initial denaturation of DNA at 94°C for 3 min, followed by 35 cycles of amplification at 94°C for 30 s, 53°C for 45 s, 72°C for 1 min and 30 s and one cycle at 72°C for 5 min. The

amplified fragments of 506 bp were then digested by the restriction endonuclease MwoI at 60°C for 3 hr according to the manufacturer's recommendations. The digestion products were analyzed by electrophoresis in 3% agarose gel stained with ethidium bromide (10 µg/mL) for 60 minutes, with 80 volts. With this process we are able to differentiate genotypes: the TT genotype gives rise to one single band of 506 bp; the CC genotype appears as two bands, one with 339 bp, and other with 167 bp; the CT genotype has all the three bands.

#### **MTHFR polymorphism: C677T (rs1801133)- Fig12a- Annexes**

The C/T polymorphism of MTHFR (C677T) gene was detected using the forward primer : 5'-TGAAGGAGAAGGTGTCTGCGGGA – 3' and the reverse primer 5'-AGGACGGTGCGGTGAGAGTG – 3'. The PCR reaction was performed in a final volume of 50 µL using 200 ng of genomic DNA, 10 pmol of both primers, 25 µl of DreamTaq Green PCR Master Mix (2X) (Thermo Scientific®). The PCR reaction started with an initial denaturation at 94°C for 2 minutes, followed by 30 cycles for 30 seconds at 94°C, 30 seconds at 61°C and 60 seconds at 72°C, with a final extension of 7 minutes at 72°C. The PCR product was digested with 10U of HinfI (Thermo Scientific®) for 16 hours at 37°C.

Fragments were separated by electrophoresis on a 4% agarose gel for 90 minutes at 85V and visualized by ethidium bromide staining.

There was one fragment of 198 bp for homozygous without mutation (CC), three fragments of 198 bp, 175 bp and 23 bp for heterozygous CT and two fragments of 175 bp and 23 bp for homozygous with mutation (TT).

#### **Statistical Analysis**

Descriptive data of continuous and categorical variables is presented as descriptive statistics with frequencies, percentage and central tendency measures. Normal distribution is assessed with normality tests. After the assumption or not of the normality: parametric and non-parametric tests were used, and logistic regression models were built and adjusted for age and gender.

The Hardy-Weinberg equilibrium is based on the distribution of the contrasting alleles p (wild allele) and q (mutant allele), according to the development of Newton's binomial ( $p^2 + 2pq + q^2 = 1$ ). Observed genotype frequencies were tested for deviation from Hardy-Weinberg equilibrium (HWE) with the Chi-square goodness-of-fit test. This test was also used to evaluate the significant differences between groups, in and within the two populations, in order to know if the odds ratio (OR) test was justifiable.

The frequencies of the various genotypes of the polymorphisms studied in the different categories of samples were determined and tabulated according to contingency tables. The analyzes of these were made through the chi-square  $\chi^2$  analysis, to study the genotypic and allelic differences, between the cases and the controls. Homozygous and heterozygous individuals for each of the alleles, variants for each polymorphism, were compared to homozygotes for the wild-type allele.

In the two cohorts OR for patients risk and the corresponding 95% confidence intervals (95% CI) were calculated using logistic regression analysis. This test was applied to the polymorphisms, to analyze its risk factor individually. All statistical analyses were carried out using the SPSS 21.0 software. The results were considered statistically significant for  $p < 0.05$ .

Genetic Risk score of: endothelial dysfunction ((ACE) and nitric oxide (NOS) gene polymorphisms)); endothelial dysfunction and epigenetics (MTHFR+(ACE) and nitric oxide (NOS) gene polymorphisms)); uncontrolled asthma (IVS16+ 88T>G, ACE I/D and age) and allergic asthma (MTHFR and GSTT1 and ACE and gender).

Significant SNPs in asthma vs controls; controlled/uncontrolled asthma; allergic vs non-allergic asthma were used to create different genetic models and construct a genetic risk score for each participant by summing the coefficients for each of the resulting variables after statistical analysis with Stepwise multivariate logistic regression with backward elimination.

**CHAPTER IV. Results**

#### **IV.1.Literature review and meta-analysis with the SNPs entitled for this Thesis and its role in CVD**

The systematic literature review and meta-analysis for the polymorphisms for CVD included in the study are : a) Renin-Angiotensin-System; b) Nitric Oxide (NO) associated systems; c) Beta-2 drenergic receptors ; d) Detoxification; e) Atopic march; f) Epigenetics

The pathophysiology of Cardiovascular diseases and the chronic stimulation of the renin-angiotensin-aldosterone system (RAAS), affects the cardiovascular system through, increased vasoconstriction, sodium and water retention, heart remodeling and myocardial fibrosis. The use of angiotensin-converting-enzyme inhibitors (ACEIs), angiotensin II type 1 receptor blockers (ARBs) and aldosterone antagonists among others as therapeutic targets . The variance in the genetic constitution of this pathway might represent a predisposing factor to Cardiovascular diseases and be implicated in the risk of disease manifestation and response to therapy in certain patients

The genetic constituents of the renin-angiotensin-aldosterone system (RAAS) and NO, a neurohormonal pathway that is activated in many Cardiovascular diseases such as: IHD; CAD; MI; cardiomyopathy; MI and HTA among others, has also an important role in asthma as will be showed in this thesis.

Haptoglobin namely Hp2.2 genotype is a susceptibility risk to Heart diseases such as: IHD; CAD; MI; cardiomyopathy; MF and HTA among others.

The pathophysiology of Cardiovascular diseases and the chronic stimulation of the renin-angiotensin-aldosterone system (RAAS), affects the cardiovascular system through, increased vasoconstriction, sodium and water retention, heart remodeling and myocardial fibrosis. The use of angiotensin-converting-enzyme inhibitors (ACEIs), angiotensin II type 1 receptor blockers (ARBs) and aldosterone antagonists among others as therapeutic targets. The variance in the genetic constitution of this pathway might represent a predisposing factor to Cardiovascular diseases and be implicated in the risk of disease manifestation and response to therapy in certain patients.

In the EDC the SPRR proteins are downstream target of gp130 cytokine receptor signaling, confers cardiomyocyte protection in response to environmental stress. SPRR could be downstream effectors of the stress response mediated by IL-6 family cytokines. Therefore, the induction of SPRR expression by IL-6 cytokines could be a central mechanism of an 'innate' defense system in response to stress and induction of SPPR genes may serve a novel cell protective strategy.

DNA methylation, an epigenetic feature of DNA like acetylation could modulate gene expression involves methyltransferases that use the methyl donor S-adenosyl-L-methionine. Methylenetetrahydrofolate reductase (MTHFR) catalyzes the synthesis of 5-methyltetrahydrofolate (5-methylTHF), the methyl donor for synthesis of methionine from

homocysteine and precursor of S-adenosyl-L-methionine. About 85% of the general population carries a variant such as C677T mutation in the MTHFR gene associated with higher blood homocysteine also a risk factor for cardiovascular disease. T/T genotypes had a diminished level of DNA methylation compared with those with the C/C but according to folate status, only the T/T subjects with low levels of folate accounted for the diminished DNA methylation- folate dependent. An elevated plasma level of the amino acid homocysteine is a significant and independent risk factor for the development of coronary heart disease.

Individuals with the MTHFR 677 TT genotype had a higher risk of CHD compared with individuals with the CC genotype. There was significant heterogeneity between the results obtained in European populations compared with American populations (which might largely be explained by interaction between the MTHFR 677C->T polymorphism and folate status if we are considering the hypomethylation status as a susceptibility to CVD.

In this thesis there will be a comprehensive literature review and meta-analysis for the different polymorphisms studied in asthma and its importance in CVD. Polymorphisms studied:

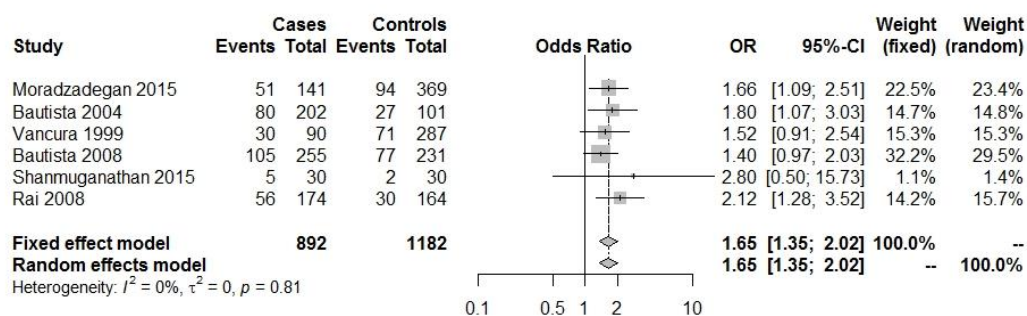
Many single nucleotide polymorphisms could affect the outcome of CVD pathophysiology and severity that will be addressed in this thesis: A) RENIN-ANGIOTENSIN SYSTEM: ACE polymorphism of I / D (287 bp, on chromosome 17q23, intron 16 (rs1799752); Polymorphism for Type 1 receptor of Angiotensin II (AGTR1) 1166A / C (rs5186); B) NO ASSOCIATED SYSTEM: Haptoglobin polymorphism (1.1, 2.1, 2.2): Intron 4 polymorphism of the eNOS gene; NOS2 polymorphism (exon 16-14CT): NOS2 polymorphism (intron 16 - 88GT); NOS2 polymorphism (intron 20 - IVS20 + 524 GA); MPO Polymorphism (- 463 GA); C) BETA2ADRENERGIC RECEPTORS: Polymorphism Receptor beta2adrenergic Arg16 Gly (rs1042713); D) DETOXIFICATION: GSTM1 polymorphism: search for null genotypes (homozygous for the allele GSTM1 \* 0) (M-); GSTT1 polymorphism: null genotype search (Homozygous homing for the GSTT1 \* 0 allele) (T-); E) ECZEMA AND ASTHMA-ATOPIIC MARCH: LELP1; F) EPIGENETICS: MTHFR polymorphism MTHFR: C677T (rs1801133).

## IV.1.Literature review and meta-analysis with the SNPs entitled for this Thesis and its role in CVD

### A) RENIN-ANGIOTENSIN SYSTEM

ACE polymorphism of I / D (287 bp, on chromosome 17q23, intron 16 (rs1799752)

The pathophysiology of Cardiovascular diseases and the chronic stimulation of the renin-angiotensin-aldosterone system (RAAS)(1,2), affects the cardiovascular system and different phenotypes and severity of the disease.



Number of studies combined:  $k = 6$

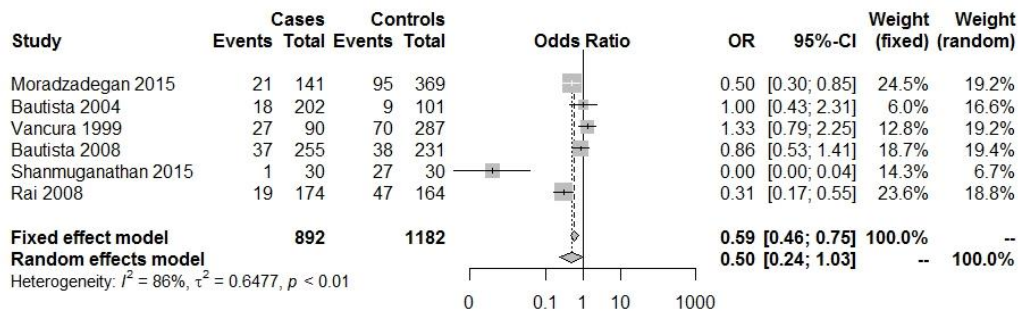
	OR	95%-CI	z	p-value
Fixed effect model	1.6529	[1.3526; 2.0198]	4.91	$< 0.0001$
Random effects model	1.6488	[1.3485; 2.0159]	4.87	$< 0.0001$

**Fig. IV.1.1:** Forest plot with the Genetic model : DD/total( heart diseases(HDs) (CAD; MI; cardiomyopathy; HT)(80-85).

Genetic model ): DD/total( heart diseases(HDs) (CAD; MI; cardiomyopathy, HT)

These studies had low heterogeneity (0%) the values of fixed and random effect are very similar and is associated with significant overall effect: OR: 1.6529 [1.3526; 2.0198]  $z=4.91$   $p < 0.0001$ , if the DD genotype is present and susceptibility to Heart condition(CAD; EAM; cardiomyopathy; HT) The risk of having heart condition in those that are DD is almost 2.





Number of studies combined: k = 6

	OR	95%-CI	z	p-value
Fixed effect model	0.5880	[0.4638; 0.7457]	-4.38	< 0.0001
Random effects model	0.4951	[0.2389; 1.0258]	-1.89	0.0586

**Fig. IV.1.2:** Forest plot with the Genetic model: II/total( heart diseases(HDs) (CAD; MI; cardiomyopathy; HT) 80-85.

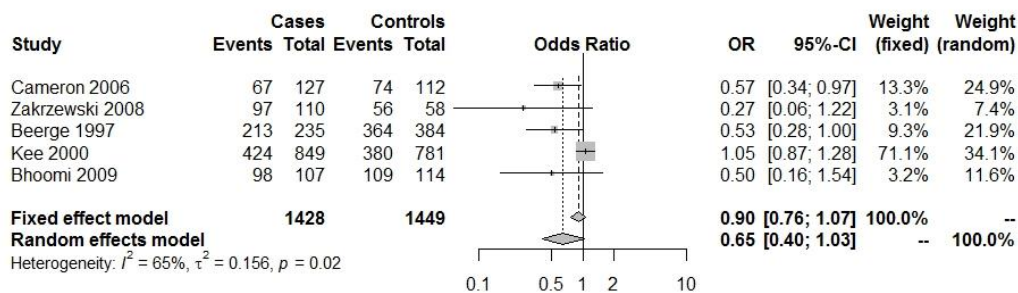
Genetic model ): II/total( heart diseases(HDs) (CAD; MI; cardiomyopathy; HT)

These studies had high heterogeneity (86%) the values of fixed and random effect are very similar and is associated with non significant overall effect if we consider the random effect, although a trend might be present: OR: 0.4951 [0.2389; 1.0258] z=-1.89 p=0.0586 ,if the II genotype is present and susceptibility to Heart condition(CAD; MI; cardiomyopathy; HT) The risk of having heart condition in those that are II is 53,5% decreased.

## A) RENIN-ANGIOTENSIN SYSTEM

### Polymorphism for Type 1 receptor of Angiotensin II (AGTR1) 1166A / C (rs5186)

The pathophysiology of Cardiovascular diseases and the chronic stimulation of the renin-angiotensin-aldosterone system (RAAS) (18,19), affects the cardiovascular system and different phenotypes and severity of the disease.



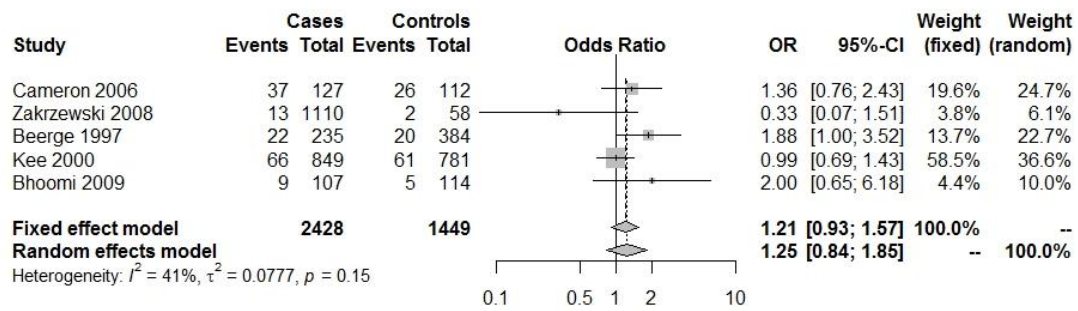
Number of studies combined: k = 5

	OR	95%-CI	z	p-value
Fixed effect model	0.8985	[0.7576; 1.0655]	-1.23	0.2186
Random effects model	0.6452	[0.4047; 1.0287]	-1.84	0.0656

**Fig. IV.1.3:** Forest plot with the Genetic model: AC+CC/total (heart diseases(HDs) (HT, CAD, MI,HF.)(18,86-89).

Genetic model ): AC+CC/total ( heart diseases(HDs) (HT, CAD, MI,HF.)

These studies had high heterogeneity (65%) the values of fixed (OR:0.90)and random effect (OR:0.65) are very similar and are associated with decreased risk of Heart Disease (HT, CAD, MI, etc..) if the Allele C (AC+CC) is present in AGTR1 polymorphism and susceptibility to HDs and susceptibility to Heart condition (HT, CAD, MI,HF) .The risk of having heart condition in those that express allele C there is a trend to be decreased although n.s..



Number of studies combined:  $k = 5$

	OR	95%-CI	z	p-value
Fixed effect model	1.2076	[0.9268; 1.5735]	1.40	0.1624
Random effects model	1.2455	[0.8378; 1.8517]	1.09	0.2779

**Fig. IV.1.4:** Forest plot with the Genetic model: CC /total ( heart diseases(HDs) (HT, CAD, MI, HF) (18,86-89).

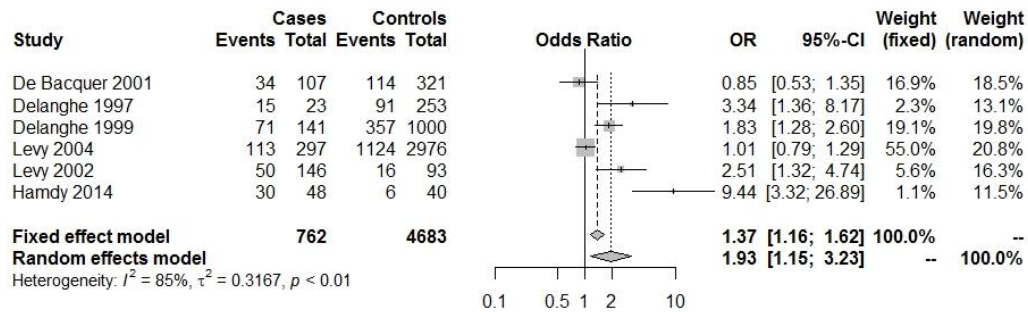
Genetic model): CC /total (heart diseases(HDs) (HT, CAD, MI, HF)

These studies had moderately high heterogeneity (41%) the values of fixed (OR:1.21) and random (OR:1.25) effect are very similar and favors increased risk if the CC genotype is present in AGTR1 polymorphism and susceptibility to HDs (HT, CAD, MI, HF) .The risk of having heart condition in those that express CC genotype is non significant.

## B) NO ASSOCIATED SYSTEMS

### Haptoglobin polymorphism (1.1, 2.1, 2.2)

The Hp phenotypes have different biochemical and biophysical characteristics and functional efficiencies that account for their distinct antioxidant and immunomodulatory capacities in asthma and CVD (90).



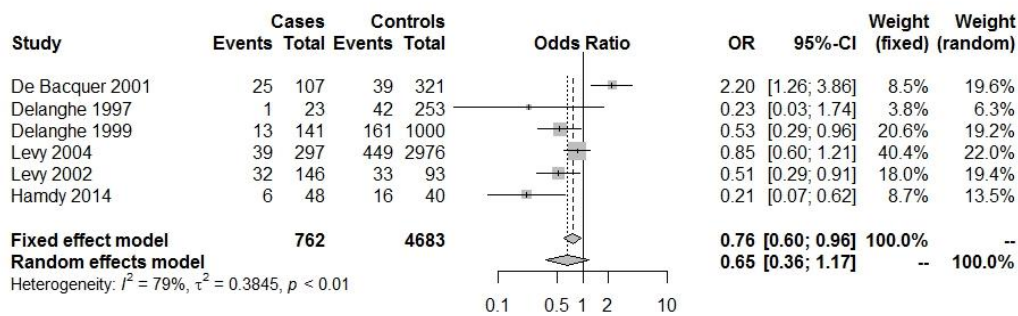
Number of studies combined:  $k = 6$

	OR	95%-CI	z	p-value
Fixed effect model	1.3664	[1.1557; 1.6155]	3.65	0.0003
Random effects model	1.9288	[1.1524; 3.2283]	2.50	0.0124

**Fig. IV.1.5:** Forest plot with the Genetic modelHp2.2/total( heart diseases(HDs) (CHD; CHD and DM;CAD mortality; CABG<45 years)(91-96).

Genetic model): Hp2.2/total (heart diseases(HDs) (CHD; CHD and DM;CAD mortality; CABG<45 years)

These studies had high heterogeneity (79%) the values of fixed and random effect are slightly different and is associated with significant overall effect : OR: 1.9288 [1.1524; 3.2283]  $z=2.50$   $p=0.0124$ ,if the Hp2.2 genotype is present and susceptibility to Heart condition (CHD; CHD and DM;CAD mortality; CABG<45 years). The risk of having heart condition in those that are Hp 2.2 is almost 2.



Number of studies combined: k = 6

	OR	95%-CI	z	p-value
Fixed effect model	0.7596	[0.6030; 0.9568]	-2.34	0.0195
Random effects model	0.6484	[0.3583; 1.1731]	-1.43	0.1521

**Fig. IV.1.6:** Forest plot with the Genetic model: Hp1.1/total (heart diseases(HDs) (CHD; CHD and DM;CAD mortality; CABG<45 years) (91-96).

Genetic model): Hp1.1/total (heart diseases(HDs) (CHD; CHD and DM; CAD mortality; CABG<45 years)

These studies had high heterogeneity (79%) the values of fixed and random effect are and are associated with non significant overall effect :OR: 0.6484 [0.3583; 1.1731]; z=-1.43 ; p= 0.1521,if the Hp1.1 genotype is present and susceptibility to Heart condition (CHD; CHD and DM;CAD mortality; CABG<45 years). The risk of having heart condition in those that are Hp 1.1 has although an effect tendentially to be decreased .

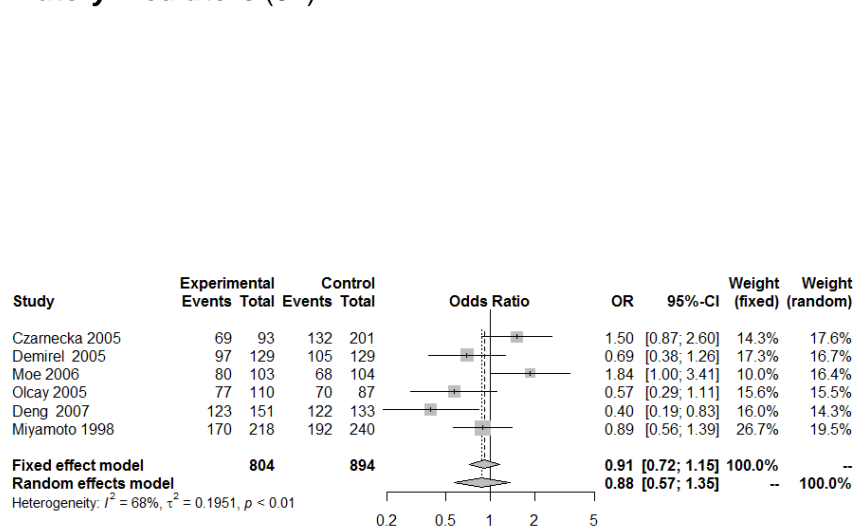
## B) NO ASSOCIATED SYSTEMS

### Intron 4 polymorphism of the eNOS gene ( rs1799983)

NOS catalyzes the conversion of L: -arginine to L: -citrulline and NO and under particular circumstances reactive oxygen species (ROS) can be generated due to NO uncoupling.

Nitric oxide synthase is involved in nitric oxide's metabolism most nitric oxide synthase activity in the normal heart is present in endothelium and related with eNos- endothelial

isoform of nitric oxide synthase. Neuronal nitric oxide synthase is uncertain associated with nerves and ganglion cells. **Usually, there is no inducible nitric oxide synthase (NOS2) in the normal heart, however macrophages associated with repair following various forms of cardiac damage contain this isoform-NOS2 and its expression is induced by pro-inflammatory mediators (97).**



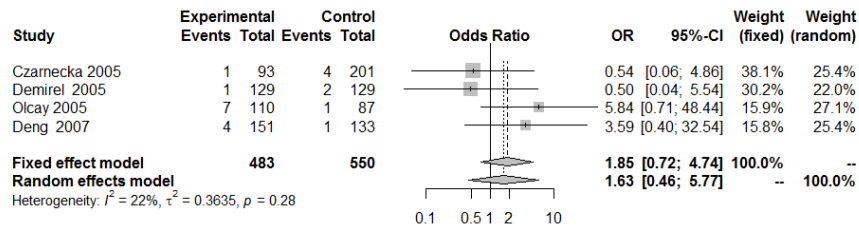
Number of studies combined: k = 6

	OR	95%-CI	z	p-value
Fixed effect model	0.9086	[0.7209; 1.1452]	-0.81	0.4170
Random effects model	0.8755	[0.5693; 1.3463]	-0.61	0.5447

**Fig. IV.1.7:** Forest plot with the Genetic model:bb/total ( heart diseases(HDs) (HT) (98-103).

Genetic model ): bb/total ( heart diseases(HDs) (HT)

These studies had high heterogeneity (68%) the values of fixed (OR:0.91)and random effect (OR:0.88) are very similar and are associated with decreased risk of Heart Disease (HT) if the genotype bb is present in eNOS polymorphism and susceptibility Heart condition (HT) although n.s..



Number of studies combined:  $k = 4$

	OR	95%-CI	z	p-value
Fixed effect model	1.8504	[0.7219; 4.7428]	1.28	0.2001
Random effects model	1.6333	[0.4627; 5.7654]	0.76	0.4459

**Fig. IV.1.8:** Forest plot with the Genetic model: aa/total (heart diseases(HDs) (HT) (98,99,101,103).

Genetic model): aa/total (heart diseases(HDs) (HT)

These studies had low heterogeneity (22%) the values of fixed (OR:1.85)and random effect (OR:1.63) are very similar and are associated with increased risk of Heart Disease (HT) if the genotype aa is present in eNOS polymorphism and susceptibility Heart condition(HT) although n.s..

## B) NO ASSOCIATED SYSTEMS

NOS2 polymorphism (exon 16-14CT)

NOS2 polymorphism (intron 16 - 88GT)

NOS2 polymorphism (intron 20 - IVS20 + 524 GA)

**Usually, there is no inducible nitric oxide synthase (NOS2) in the normal heart, however macrophages associated with repair following various forms of cardiac damage contain this isoform-NOS2 and its expression is induced by pro-inflammatory mediators (44,104).**

However, some authors refer that in some inflammatory diseases such as rheumatoid arthritis there is a the potential contribution of inducible and endothelial nitric oxide synthase (iNOS/ NOS2) gene polymorphisms to cardiovascular (CV) events.

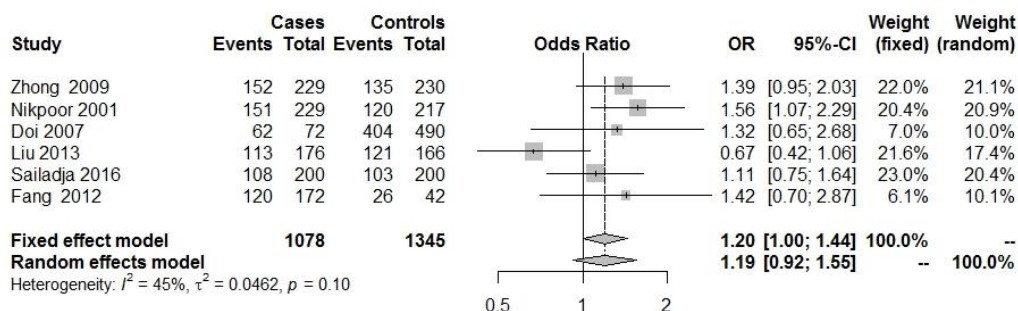
Several candidate genes (EGF, LTA, HIF1A, HIF1AN, MMP2, MMP9, iNOS, NOS3 and VEGF) play a role in angiogenesis and endothelial dysfunction. Polymorphisms in angiogenesis-related

genes have been associated with CVD and respiratory diseases such as asthma. Inducible nitric oxide synthase (iNOS) catalysis the synthesis of nitric oxide (NO), which can be proangiogenic and iNOS is overexpressed in some diseases (e.g.: asthma) and some inflammatory cells such as macrophages.

Accelerated atherosclerosis and CVD might be associated with the traditional CV risk factors such as age and hypertension as well as non-traditional risk factors comprising current inflammation associated with asthma and genetic polymorphisms that predisposes to different status of oxidative stress and inflammation. In this multiple risk factor assessment, the risk charts that are based only on traditional risk factors are insufficient to capture CV risk extent in bronchial asthma.

## B) NO ASSOCIATED SYSTEM

### MPO Polymorphism (- 463 GA)



	OR	95%-CI	z	p-value
Fixed effect model	1.2007	[0.9983; 1.4442]	1.94	0.0521
Random effects model	1.1928	[0.9193; 1.5477]	1.33	0.1845

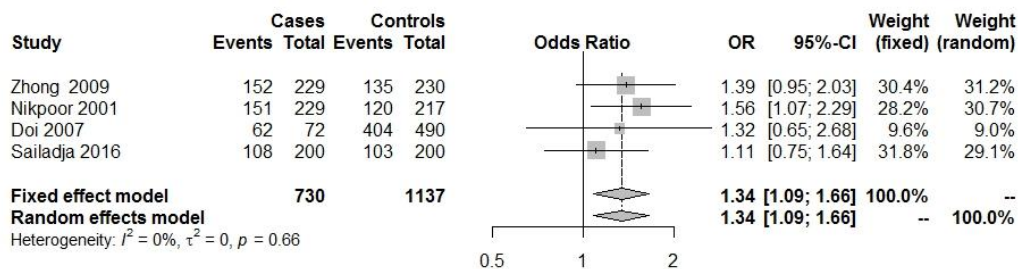


**Fig. IV.1.9:** Forest plot with the Genetic model: (AA+GA vs GG): GG/total (heart diseases(HDs) (CAD; Nephrosclerosis hypertensive; HT; Carotid atherosclerosis)-Dominant(105-110).

Genetic model (AA+GA vs GG): GG/total(heart diseases(HDs) (CAD; Nephrosclerosis hypertensive; HT; Carotid atherosclerosis)-Dominant

These studies had medium heterogeneity (45%) the values of fixed and random effect are very similar and is associated with higher overall effect and a trend to be significant ( $p=0,0521$ ) if the GG genotype is present in MPO (-463G>A (rs2333227) polymorphism and susceptibility to Heart condition CAD; Nephrosclerosis hypertensive; HT; Carotid atherosclerosis) in the Fixed effect model. The random overall effect is non significant (n.s.)( $p=0.1845$ ).

Genetic model (GG+AG vs AA): AA/total ( heart disease (CAD; Nephrosclerosis hypertensive; HT; Carotid atherosclerosis) Recessive



Number of studies combined:  $k = 4$

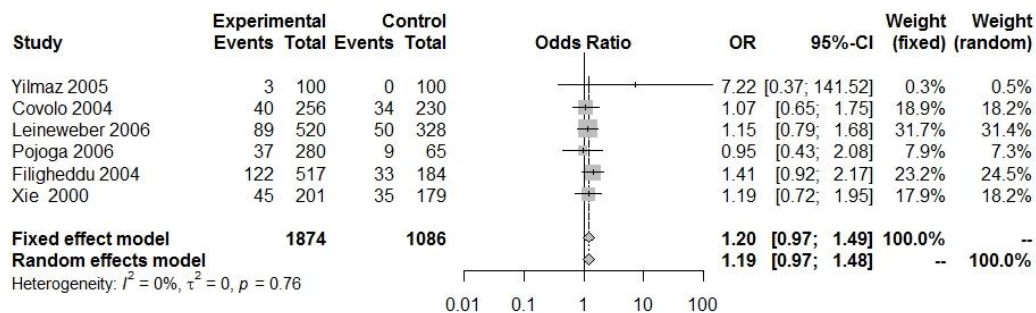
	OR	95%-CI	z	p-value
Fixed effect model	1.3418	[1.0857; 1.6583]	2.72	0.0065
Random effects model	1.3420	[1.0857; 1.6588]	2.72	0.0065

**Fig. IV.1.10:** Forest plot with the Genetic model :(GG+AG vs AA): AA/total (heart disease (CAD; Nephrosclerosis hypertensive; HT; Carotid atherosclerosis) Recessive (105-107,109).

Interpretation: These studies had low heterogeneity (0%) the values of fixed and random effect are very similar and is associated with higher risk and significant (if we consider the fixed effect model) if the AA genotype is present in MPO (-463G>A (rs2333227) polymorphism and susceptibility to Heart condition CAD; Nephrosclerosis hypertensive; HT; Carotid atherosclerosis) in the Fixed effect model ( $p = 0.0065$ ). This CI95% shows that the overall effect is significant with a global risk effect (fixed effect) is 1,34 of having a heart condition (CAD; Nephrosclerosis hypertensive; HT; Carotid atherosclerosis) if the AA genotype is present. The study of Nikpoor et al with great weight (28,2%) the CI95% shows that the effect is significant with OR 1,56 of having a heart condition.

### C) BETA2ADRENERGIC RECEPTORS

#### Polymorphism of beta2adrenergic receptor Arg16 Gly (rs1042713)



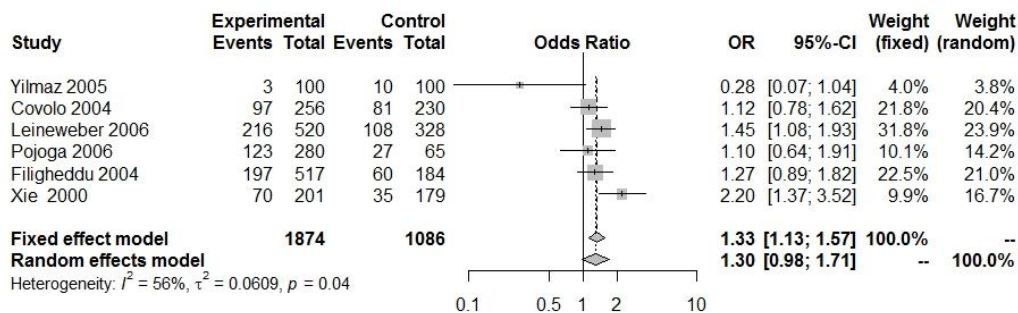
Number of studies combined:  $k = 6$

	OR	95%-CI	z	p-value
Fixed effect model	1.2038	[0.9750; 1.4864]	1.72	0.0847
Random effects model	1.1937	[0.9660; 1.4751]	1.64	0.1011

**Fig. IV.1.11:** Forest plot with the Genetic model :(GG+AG vs AA): AA/total( heart disease (MI; CHF and HF; HT)-Dominant (111-116).

Genetic model (GG+AG vs AA): AA/total (heart disease (MI; CHF and HF; HT)-Dominant  
 These studies had low heterogeneity (0%) the values of fixed and random effect are very similar and is associated with higher risk although not significant if the AA genotype is present in Beta2 adrenoreceptor polymorphisms: Arg16Gly (rs1042713) and susceptibility to Heart condition (MI; CHF and HF; HT). This CI95% shows that the overall effect is non significant(ns). Even in the study of Leineweber et al with major weight (31,7%) the CI95% shows that the effect is non significant (n.s.).

Genetic model (AA+AG vs GG): GG/total (heart disease (MI; CHF and HF; HT)-Recessive



Number of studies combined: k = 6

	OR	95%-CI	z	p-value
Fixed effect model	1.3303	[1.1267; 1.5708]	3.37	0.0008
Random effects model	1.2952	[0.9835; 1.7055]	1.84	0.0655

**Fig. IV.1.12:** Forest plot with the Genetic model :( AA+AG vs GG): GG/total( heart disease (MI; CHF and HF; HT)-Recessive (111-116).

These studies had moderately high heterogeneity (56%) the values of fixed and random effect are very similar and is associated with higher risk and significant (if we consider the fixed effect model) if the GG genotype is present in Beta2 adrenoreceptor polymorphisms: Arg16Gly (rs1042713) and susceptibility to Heart condition (MI; CHF and HF; HT). This CI95% shows that the overall effect is significant with a global risk effect (fixed effect) is 1,33 of having a heart condition (MI; CHF and HF; HT) if the GG genotype is present, and significant. Two studies of Leineweber et al with major weight (31,7%) and Xie et al (weight 9,9%) the CI95% shows that the effect is significant with OR 1,45 and 2.20 respectively of having a heart condition.

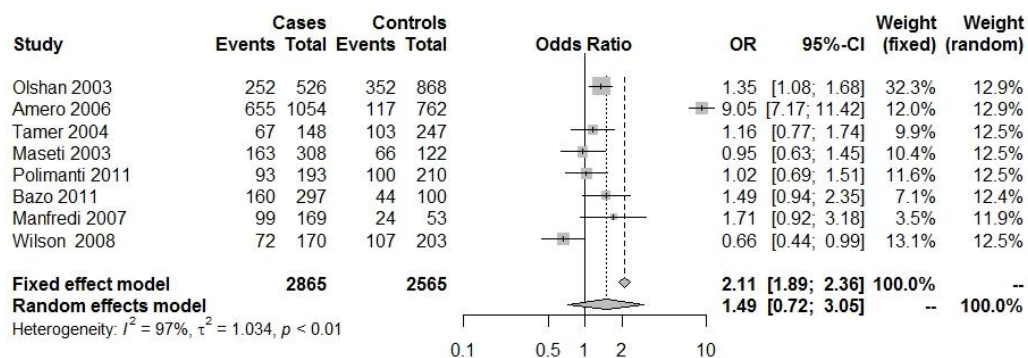
#### D) DETOXIFICATION

GSTM1 polymorphism: search for null genotypes (homozygous for the allele GSTM1 \* 0) (M-)

GSTT1 polymorphism: null genotype search (Homozygous homing for the GSTT1 \* 0 allele)

(T-)

#### GSTM1



Number of studies combined: k = 8

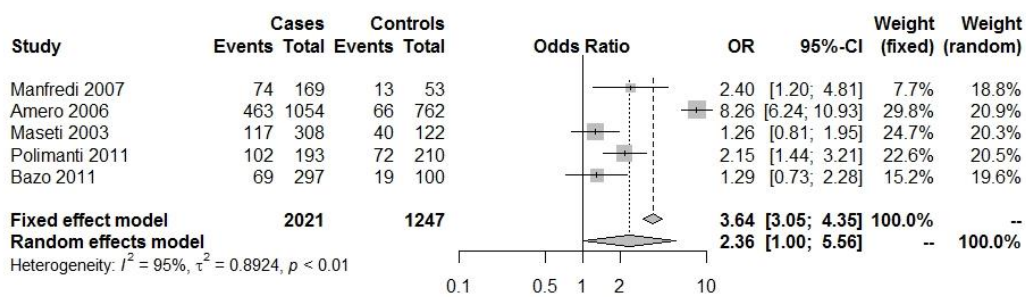
	OR	95%-CI	z	p-value
Fixed effect model	2.1079	[1.8856; 2.3564]	13.12	< 0.0001
Random effects model	1.4878	[0.7248; 3.0543]	1.08	0.2789

**Fig. IV.1.13:** Forest plot with the Genetic model : ( GSTM1 \* 0/total( heart diseases(HDs) (CAD associated or independent of smoking status; HT (117-124).

Genetic model): GSTM1 \* 0/total (heart diseases(HDs) (CAD associated or independent of smoking status; HT)

These studies had high heterogeneity (96%) the values of fixed and random effect are different and is associated with overall effect non significant n.s. (p=0,2789) if the null genotype is present in and susceptibility to Heart condition (CAD associated or independent of smoking status; HT). Random effects model 1.4878 [0.7248; 3.0543] z=1.08; p= 0.2789.

### GSTT1



Number of studies combined: k = 5

	OR	95%-CI	z	p-value
Fixed effect model	3.6413	[3.0498; 4.3476]	14.29	< 0.0001
Random effects model	2.3599	[1.0018; 5.5595]	1.96	0.0495

**Fig. IV.1.14:** Forest plot with the Genetic model : (GSTT1 \* 0/total( heart diseases(HDs) (CAD associated or independent of smoking status; HT) (118,120-123).

Genetic model): GSTT1 \* 0/total (heart diseases(HDs) (CAD associated or independent of smoking status; HT)

These studies had high heterogeneity (95%) the values of fixed and random effect are different and is associated with overall effect significant (p=0,0495) if the null genotype is present in and susceptibility to Heart condition (CAD associated or independent of smoking status; HTA). Random effects model 2.3599 [1.0018; 5.5595] z=1.96; p= 0.0495; with OR of having heart condition of 2 if they are GSTT1 \* 0.

## **E) ECZEMA AND ASTHMA**

### **LELP1**

Epidermal keratinocytes(125) undergo a terminal differentiation and programmed cell death (physiological apoptosis) known as cornification . Cornification leads to the cornified layer, and different genes proceed in an organized sequence to provide this outermost skin barrier in the spinous and granular layers that express proteins like keratins (namely: K1, K2 and K10) and non- keratin proteins like filaggrin (FLG), loricrin (LOR), involucrin (IVL) and small proline rich proteins (SPRRs). These proteins are cross-linked in the cornified cell envelope by transglutaminase enzymes, and this insoluble envelope associated with the keratin-containing macrofibrils fills corneocytes and with the lipids ,forms the skin barrier that protect from dehydration and environment allergens(67) .

Atopic dermatitis (AD)(126) is a skin disease often associated with a progression for other atopic comorbidities such as asthma in what we know as atopic march. It affects 20% of children and 60% of them will have asthma. The mechanistic approach to AD involves genes of a defective skin barrier such as EDC and those genes associated with immunedisregulation and allergic disease. Besides FLG mutations (FLG-null mutations R501X and 2282del4) that predisposes to increased risk of AD other proteins like SPRR where LELP-1 polymorphism is included might also have an important role in modulation of AD and asthma.

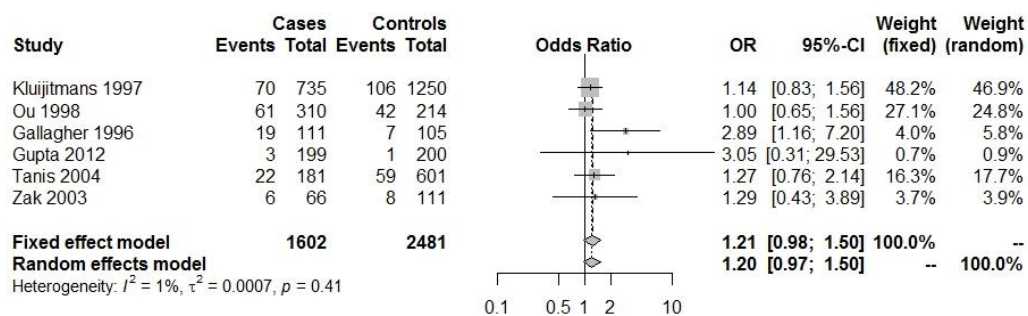
The association of SPRR proteins and CVD is because they are a downstream target signaling, and confer cardiomyocyte protection in response to environmental stress. SPRR could be downstream effectors of the stress response mediated by IL-6 family cytokines. Therefore, the induction of SPRR expression by IL-6 cytokines could be a central mechanism of an 'innate' defense system in response to stress and induction of SPRR genes may serve a novel cell protective strategy in CVD.

## F) EPIGENETICS

### MTHFR polymorphism MTHFR: C677T (rs1801133)

DNA methylation (127), an epigenetic feature of DNA like acetylation could modulate gene expression involves methyltransferases that use the methyl donor S-adenosyl-L-methionine. Methylenetetrahydrofolate reductase (MTHFR) catalyzes the synthesis of 5-methyltetrahydrofolate (5-methylTHF), the methyl donor for synthesis of methionine from homocysteine and precursor of S-adenosyl-L-methionine. About 85% of the general population carries a variant such as C677T mutation in the MTHFR gene associated with higher blood homocysteine also a risk factor for cardiovascular disease. T/T genotypes had a diminished level of DNA methylation compared with those with the C/C but according to folate status, only the T/T subjects with low levels of folate accounted for the diminished DNA methylation- folate dependent. An elevated plasma level of the amino acid homocysteine is a significant and independent risk factor for the development of coronary heart disease.

Individuals with the MTHFR 677 TT genotype had a higher risk of CHD compared with individuals with the CC genotype. There was significant heterogeneity between the results obtained in European populations compared with American populations (which might largely be explained by interaction between the MTHFR 677C-->T polymorphism and folate status if we are considering the hypomethylation status as a susceptibility to CVD).



Number of studies combined:  $k = 6$

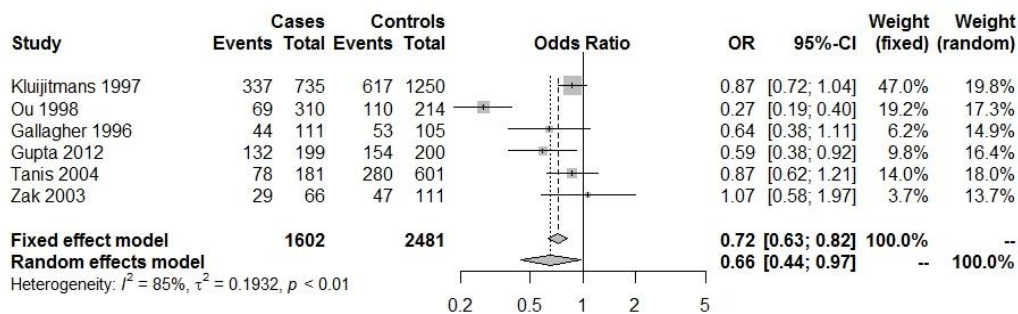
	OR	95%-CI	z	p-value
Fixed effect model	1.2114	[0.9756; 1.5042]	1.74	0.0824
Random effects model	1.2030	[0.9656; 1.4988]	1.65	0.0994

**Fig. IV.1.15:** Forest plot with the Genetic model : TT/total ( heart diseases(HDs) (CHD; CAD;MI; CAD <45 years)(128-133).

MTHFR polymorphism MTHFR: C677T (rs1801133)

Genetic model): TT/total (heart diseases(HDs) (CHD; CAD;MI; CAD <45 years)

These studies had low heterogeneity (1%) the values of fixed (OR:1,21) and random effect (OR:1,20) are very similar and is associated with a non significant overall effect: 1.2114 [0.9756; 1.5042] z=1.74 ; p=0.0824, if the genotype TT is present and susceptibility to Heart condition (CHD; CAD;MI; CAD <45 years).



Number of studies combined: k = 6

	OR	95%-CI	z	p-value
Fixed effect model	0.7196	[0.6311; 0.8206]	-4.91	< 0.0001
Random effects model	0.6552	[0.4428; 0.9694]	-2.12	0.0344

**Fig. IV.1.16:** Forest plot with the Genetic model : CC/total( heart diseases (HDs) (CHD; CAD;MI; CAD <45 years) (128-133).

MTHFR polymorphism MTHFR: C677T (rs1801133)

Genetic model): CC/total (heart diseases(HDs) (CHD; CAD;MI; CAD <45 years)

These studies had high heterogeneity (85%) the values of fixed (OR:0,72) and random effect (OR:0,66) are very similar and is associated with a significant overall effect: Random effects model: OR:0.6552 [0.4428; 0.9694] z=-2.12; p= 0.0344, if the genotype CC is present and susceptibility to Heart condition (CHD; CAD;MI; CAD <45 years), there is a decreased risk of HDs.



## IV.2. Clinical study: Case-Control study with the SNPs entitled for this Thesis

### DEMOGRAPHIC CHARACTERISTICS OF THE STUDY POPULATION

In this sample we had studied 356 (69,9%) individuals in the control group and 153 (30,1%) in the asthma group.

For the age if we display the results by 3 groups (<15 years; 15-30 years and >30 years) we will obtain these results

**Table IV.2.1:** Participant's demographic and clinical characteristics.

	<b>Controls</b>	<b>Asthma</b>	<b>p value</b>
N (%)	356 (69.9)	153 (30.1)	n.a.
Female	198 (55.6)	101(66.0)	<b>0.037*</b>
Male	158 (44.4)	52 (34.0)	
Age (years; mean±; min-max)	(43,46±13,01;18;85) (n=356)	(38,30±18,52;7;86)(n=151)	0.001†
< 15	0 (0.0)	18 (11.9)	
15-30	64 (18.0)	42 (27.8)	<b>&lt;0.001*</b>
> 30	292 (82.0)	91 (60.3)	
Atopy/No Atopy	n.a.	128/23 (84.8/15.2)	n.a.
Asthma controlled / Asthma not controlled	n.a.	107/44(70.9/29.1)	n.a.

Bolded results are less than 0.05 of statistical significance; p\*, p value of  $\chi^2$  test values, the values represent absolute frequencies (relative frequencies, %); p†, Independent sample T-test, the values represent means  $\pm$  standard deviation (SD); n.a., non applicable.

There are differences in the age between patients and controls (p<0.001) being the asthmatics younger than controls: (Control group: <15: 0(0,0%); 15-30:64(18%),>30:292(82%); Asthmatics: <15: 18(11,9%); 15-30:42(27,8%),>30:91(60,3%) );

The mean age  $\pm$  SD; min, max, in asthmatics (38,30±18,52;7;86). The mean age  $\pm$  SD; min, max, in control group (43,46±13,01;18;85)

There are differences in the gender distribution between patients and controls ( $p=0.037$ ); **(Table V.1.1)** 198 females (55,6%) and 158 males in the control group (44,4%) vs 101 females (66%) and 52 (34%) males in the control group; there are more females in the asthmatics.

We had 107 (70.9%) asthmatic patients with controlled asthmatic symptoms and 44 (29.1%) with uncontrolled asthmatic symptoms: Control of asthma assessed by (ACQ7 and PAQLQ).

We had 23 (15.2%) asthmatic non-allergic patients and 128 (84.8%) asthmatic allergic patients.

#### **A) RENIN-ANGIOTENSIN SYSTEM**

ACE polymorphism of I / D (287 bp, on chromosome 17q23, intron 16 (rs1799752))

Asthmatics:  $n=98$ ; were compared with a control group of  $n=187$  healthy volunteers. The (I/D) polymorphism was determined by PCR- Polymerase chain reaction. The **Control** group is in HWE ( $\chi^2=1.517$ ;  $p=0.468$ ).

**Table IV.2.2:** Demographic and clinical characteristics of the study population.

(ACE) insertion/deletion (I/D) polymorphism (287 base pairs, on chromosome 17q23, intron 16(rs4340))	<b>Controls</b>	<b>Asthma</b>	<b>p value</b>
N (%)	187 (65.6)	98 (34.4)	n.a.
Female	63 (33.7)	64 (65.3)	<b>&lt;0.001</b>
Male	124 (63.3)	34 (34.7)	
Age (years)	(41,34±11,75)	(38,96±17,6)	0.229 <sup>†</sup>
< 15	0 (0.0)	9 (9.2)	
15-30	42 (22.5)	27 (27.6)	<b>&lt;0.001</b>
> 30	145 (77.5)	62 (63.3)	
Atopy/No Atopy	n.a.	82/16 (83.7/16.3)	n.a.
Asthma controlled / Asthma not controlled	n.a.	67/31 (68.4/31.6)	n.a.

Bolded results are less than 0.05 of statistical significance.

$p^*$   $\chi^2$  test values; the values represent absolute frequencies (relative frequencies, %).

$p^\dagger$  Independent sample T-test; the values represent means  $\pm$  standard deviation (SD).

n.a.: non applicable.

We had studied for ACE polymorphism (287 bp, in chromosome 17q23, intron 16 (rs1799752) 187 individuals in the control group and 98 in the asthma group (Table V.1.2).

We had studied 63 (33,7%) females and 124 (66,3%) males in the control group; 64 (65,3%) females and 34 (34,7%) males in the asthma group. We had more females in the asthmatics ( $p < 0.001$ ).

We had studied 16 (16.3%) asthmatic non-allergic patients and 82 (83.7%) asthmatic allergic patients.

We had studied 67 (68.4%) asthmatic patients with controlled asthmatic symptoms and 31 (31.6%) with uncontrolled asthmatic symptoms: Control of asthma assessed by (ACQ7 and PAQLQ).

**Table IV.2.3:** Distribution of alleles and genotypes by groups in ACE polymorphism (287 bp, in chromosome 17q23, intron 16 (rs1799752)

ACE I/D	Controls	Asthma	p value	OR(95%CI) <sup>a</sup>
Allele I	(0.33)	(0.38)	0.290	1.235[0.862-1.769]
Allele D	(0.67)	(0.62)		
DD n (%)	88(47.1)	49(50.0)	0.006	
ID n (%)	73(39.0)	23(23.5)		
II n (%)	26(13.9)	26(26.5)		

In asthmatics the frequencies of genotypes are: DD (50%); ID (25,3%); II (26, 5%). In the controls the frequencies of genotypes are: DD (41,7%); ID (39%); II (13,9%). There are statistical differences between these groups ( $p = 0.006$ ). There are more genotypes II in the asthmatics than controls.

In asthmatics the frequencies of allele I were 0,38 and allele D: 0,62. In control group, the frequencies of allele I were 0,33 and for allele D: 0,67. There is no statistical differences between these groups ( $\chi^2$  test: 1,120; OR: 1,235; CI95%:[0,862;1,769]; ( $p = 0.290$ ).

There is statistical difference between the distribution of genotypes by gender in asthmatics vs control group ( $p < 0,001$ ). There are more men in the control group (66,3%) and more women in the asthmatics (65,3%).

There is no statistical difference between the distribution of genotypes by controlled and uncontrolled asthma groups ( $p=0,781$ ).

There is no statistical difference between the distribution of genotypes by allergic and non-allergic asthma groups ( $p=0,180$ ).

**Genetic models (Table IV.2.4):** in the Dominant model( ID+IIvs DD) those who are homozygous for the alleleD have no increased risk of having asthma( crude: OR: 1,148; 95%CI :[0,703;1,876];  $p$ value=0,580;adjusted values: OR: 11,130; 95%CI :[0,674;1,895];  $p^b$  value=0,644).

In the recessive model those who express allele D (*ID+DD*) have protection of having asthma ( decreased risk of 53,5% (crude) of having asthma) (OR crude:0,465; 95%CI :[0,251;0,861];  $p^a$  value=0,015);(OR adjusted:0,496; 95%CI :[0,259;0,949];  $p^b$  value=0,034); (decreased risk of 50,4% (adjusted) of having asthma).

In the additive model 1(DD vs ID) those who are heterozygous with genotype ID have no increased risk of asthma when compared with control although that might be a *trend* in the crude OR for protection of having asthma ( crude:OR :0,566; 95%CI :[0,315;1,015];  $p$ value=0,056; adjusted: OR: 0,590; 95%CI :[0,318;1,095];  $p$ value=0,095).

In the additive model 2(DD vs II) those who are homozygous for the allele I (genotypes II) have no increase of the risk of asthma when compared with controls (crude OR:1,796; 95%CI :[0,941;3,427];  $p^a$ value=0,076);(OR adjusted: 1,757; 95%CI :[0,890;3,470];  $p^b$ value=0,104).

**Table IV.2.4:** Genetic models in ACE polymorphism (287 bp, in chromosome 17q23, intron 16 (rs1799752) and risk of asthma-susceptibility of disease.

Dominant model	N cases/controls	OR crude <sup>a</sup> [95%CI]; p <sup>a</sup> value	OR adjusted <sup>b</sup> [95%CI]; p <sup>b</sup> value
II+ID	48/99	referent	
DD	49/88	1,148[0,703;1,876]; p <sup>a</sup> value=0,580	1,130[0,674;1,895]; p <sup>b</sup> value=0,644

Recessive Model	N cases/controls	OR crude[CI]; p <sup>a</sup> value	OR adjusted[CI]; p <sup>b</sup> value
II	72/161	referent	
ID+DD	25/26	0,465[0,251;0,861]; pvalue=0,015	0,496[0,259;0,949]; pvalue=0,034

Additive1	N cases/controls	OR crude[CI]; p <sup>a</sup> value	OR adjusted[CI]; p <sup>b</sup> value
DD	49/88	referent	
ID	23/73	0,566[0,315;1,015]; pvalue=0,056	0,590[0,318;1,095]; pvalue=0,095

Additive2	N cases/controls	OR crude[CI]; p <sup>a</sup> value	OR adjusted[CI]; p <sup>b</sup> value
DD	49/88	referent	
II	26/26	1,796[0,941;3,427]; p <sup>a</sup> value=0,076	1,757[0,890;3,470]; p <sup>b</sup> value=0,104

**Conclusion:** The role of ACE (I/D) polymorphism, in asthmatic patients is a controversy risk factor to the severity of asthma, but we concluded that those who has an allele D have protection of having asthma (OR crude: 0,465[0,251;0,861]; pvalue=0,015); 53,5% decreased risk;(OR adjusted: 0,496[0,259;0,949]; pvalue=0,034; 50,4% decreased risk) in this hospital - based population. Genotypes II are more prevalent in the asthmatics than controls.

## A)RENIN-ANGIOTENSIN SYSTEM

### Polymorphism of the type 1 Angiotensin II receptor (AGTR1) 1166A/C(rs5186)

Asthmatic patients: n=97 were compared with a control group of n = 33 healthy blood donors. The AGTR1 1166A/C polymorphism was determined by PCR-RFLP . The control group is in HWE ( $\chi^2$  test=0,247; p value=0,884).

**Table IV.2.5:** Demographic and clinical characteristics of the study population

(Polymorphism of the type 1 Angiotensin IIreceptorI(AGTR1) 1166A/C(rs5186)	Controls	Asthma	p value
N (%)	33 (25.4)	97 (74.6)	n.a.
Female	25 (75.8)	64(66.0)	0.408
Male	8 (24.2)	33 (34.0)	
Age (years)	(38,55±17,54 )	(39,27±17,54 )	0.802 <sup>†</sup>
< 15	0 (0.0)	9(9.3)	
15-30	11 (33.3)	26 (26.8)	0,177
> 30	22 (66.7)	62 (63.9)	
Atopy/No Atopy	n.a.	82/15 (84.5/13.5)	n.a.
Asthma controlled /			
Asthma not controlled	n.a.	67/30(69.1/30.9)	n.a.

Bolded results are less than 0.05 of statistical significance.

p\*  $\chi^2$  test values; the values represent absolute frequencies (relative frequencies, %).

p<sup>†</sup> Independent sample T-test; the values represent means ± standard deviation (SD).

n.a.: non applicable.

We had studied (**Table IV.2.5**) 33 individuals in the control group and 97 asthmatics. There is no statistical difference in the mean age between groups(p=0,802), and by subgroups of age(p=0,177).

There is no statistical difference for gender between asthmatics and controls frequencies for Polymorphism of the type 1 Angiotensin II receptor I(AGTR1) 1166A/C(rs5186) (p=0 ,408).

There is no statistical difference between the distribution of genotypes by controlled and uncontrolled asthma groups (p=0 ,585)

There is no statistical difference between the distribution of genotypes by allergic and non-allergic asthma groups ( $p=0,418$ ).

**Table IV.2.6:** Distribution of alleles and genotypes by groups in Polymorphism of the type 1 Angiotensin II receptor I (AGTR1) 1166A/C(rs5186)

AGTR1 1166A/C(rs5186)	Controls	Asthma	p value	OR(95%CI) <sup>a</sup>
Allele A	0.55	0.57	0.872	0.916[0.523- 1.607]
Allele C	0.45	0.43		
AA n (%)	9(27.3)	27(27.0)	0.873	
AC n (%)	18(54.5)	56(57.7)		
CC n (%)	6(18.2)	14(14.4)		

In asthmatics (**Table IV.2.6**) the frequencies of genotypes are: AA(27,8%); AC(57,7%); CC(14,4%). In the controls the frequencies of genotypes are : AA(27,3%); AC(54,5%); CC(18,2%). There are no statistical differences between these groups ( $p=0.873$ ). The allelic frequencies are, for the control group: Allele A:0,55; Allele C: 0,45; for the asthmatics: Allele A:0,57; Allele C: 0,43. There is no increased risk of being asthmatic: OR: 0,916; 95%CI :[0,523;1,607];  $p$ value=0,872.

**Genetic models:** There is no increased risk of being asthmatics for those that are homozygous for AA: Dominant model(CC+ACvsAA): n.s. (OR: 1,029; 95%CI: [0,424;2,493];  $p^a$ value=0,950) crude; n.s. (OR:0,951; 95%CI :[0,386;2,345];  $p^b$ value=0,913) adjusted (age and female gender).

There is no increased risk of being asthmatics for those that have allele A in the genotypes (AC+AA): Recessive model ( CCvsAA+AC): n.s.( OR: 1,317, 95%CI :[0,461;3,766];  $p^a$ value=0,607)crude; n.s.(OR: 1,312; 95%CI :[0,450;3,821];  $p^b$ value=0,619) adjusted (age and female gender).

There is no increased risk of being asthmatics for those that are homozygous for allele A vs heterozygous AC genotypes (Additive 1 model): n.s. (OR: 1,037; 95%CI :[0,412;2,609];  $p^a$ value=0,938)crude; n.s. (OR: 1,155; 95%CI :[0,446;2,990];  $p^b$ value=0,766) adjusted (age and female gender).

There is no increased risk of being asthmatics for those that are homozygous for allele A vs homozygous for allele C genotypes (Additive 2 model): n.s. (OR: 1,286; 95%CI: [0,380;4,347]; p<sup>a</sup>value=0,686)crude; n.s.(OR: 1,324; 95%CI: [0,380;4,616]; p<sup>b</sup>value=0,660) adjusted (age and gender).

**Conclusion:** In this study group there is not a significant evidence, that AGTR1 gene A1166C polymorphism could be a genetic marker for the pathophysiology of asthmatic disease

## B) NO ASSOCIATED SYSTEMS

### Haptoglobin polymorphism (1.1, 2.1, 2.2)

In a group of 113 asthmatic patients and 50 controls we studied the Hp levels that were determined by nephelometry and genotypes by polyacrylamide gel electrophoresis (PAGE). Statistical analysis was performed with statistical software PASW version 18, having established a level of significance of p< 0.05. The control group is in HWE( $\chi^2=1.282$ ; p=0.527).

### Haptoglobin polymorphism (Hp1-1, Hp2-1, Hp2-2)

**Table IV.2.7:** Participant's demographic and clinical characteristics

<i>Haptoglobin</i> polymorphism- <b>Hp1-1, Hp2-1, Hp2-2</b>	<b>Controls</b>	<b>Asthma</b>	<b>p value</b>
N (%)	50 (30.7)	113 (69.3)	n.a.
Female	45 (90.0)	68(60.2)	<b>&lt;0.001</b>
Male	5(10.0)	45 (39.8)	
Age (years)	(50.4±13.44 )	(40.6±18,43 )	<b>0.001<sup>†</sup></b>
< 15	0 (0.0)	11(9.8)	
15-30	5 (10.0)	27 (24.1)	<b>0.004</b>
> 30	45 (90.0)	74 (66.1)	
Atopy/No Atopy	n.a.	82/14 (85.4/14.6)	n.a.
Asthma controlled /			
Asthma not controlled	n.a.	61/35(63.5.3/36.5)	n.a.

Bolded results are less than 0.05 of statistical significance.

p<sup>\*</sup>  $\chi^2$  test values; the values represent absolute frequencies (relative frequencies, %).

p<sup>†</sup> non-parametric Mann-Whitney; the values represent means ± standard deviation (SD).

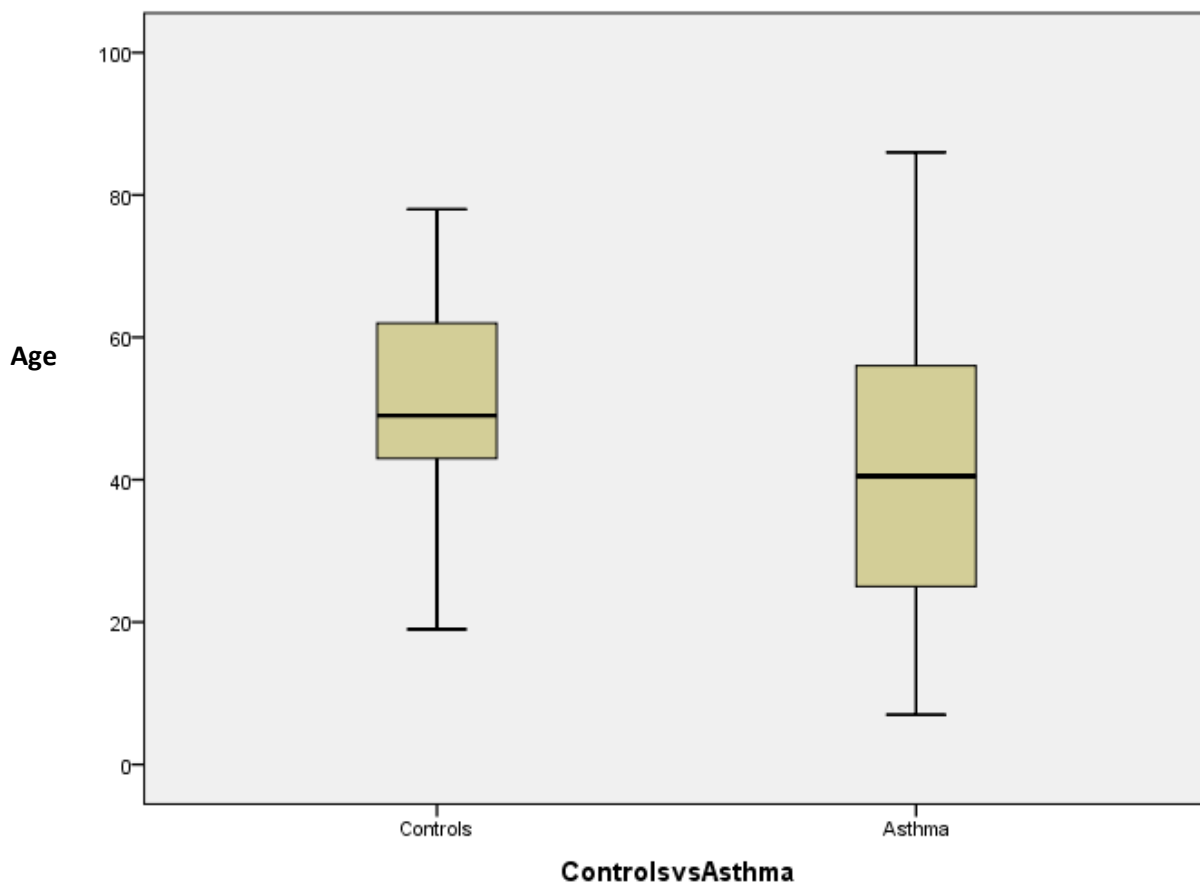
n.a.: non applicable.



We had studied 50 individuals in the control group and 113 in the asthmatics (**Table IV.2.7**).

There are differences in the distribution of gender between asthmatics (F/M:68(60.2%); 45(39.8)) and controls (F/M:45(90.0%); 5 (10.0%)) (**<0.001**). being more females in the controls and more males in the asthma group.

The mean age is different across controls and asthma ( $p=0.001$ ). There are also differences when we stratify by age being youngest the asthmatics and older the control group ( **$p=0.004$** ).



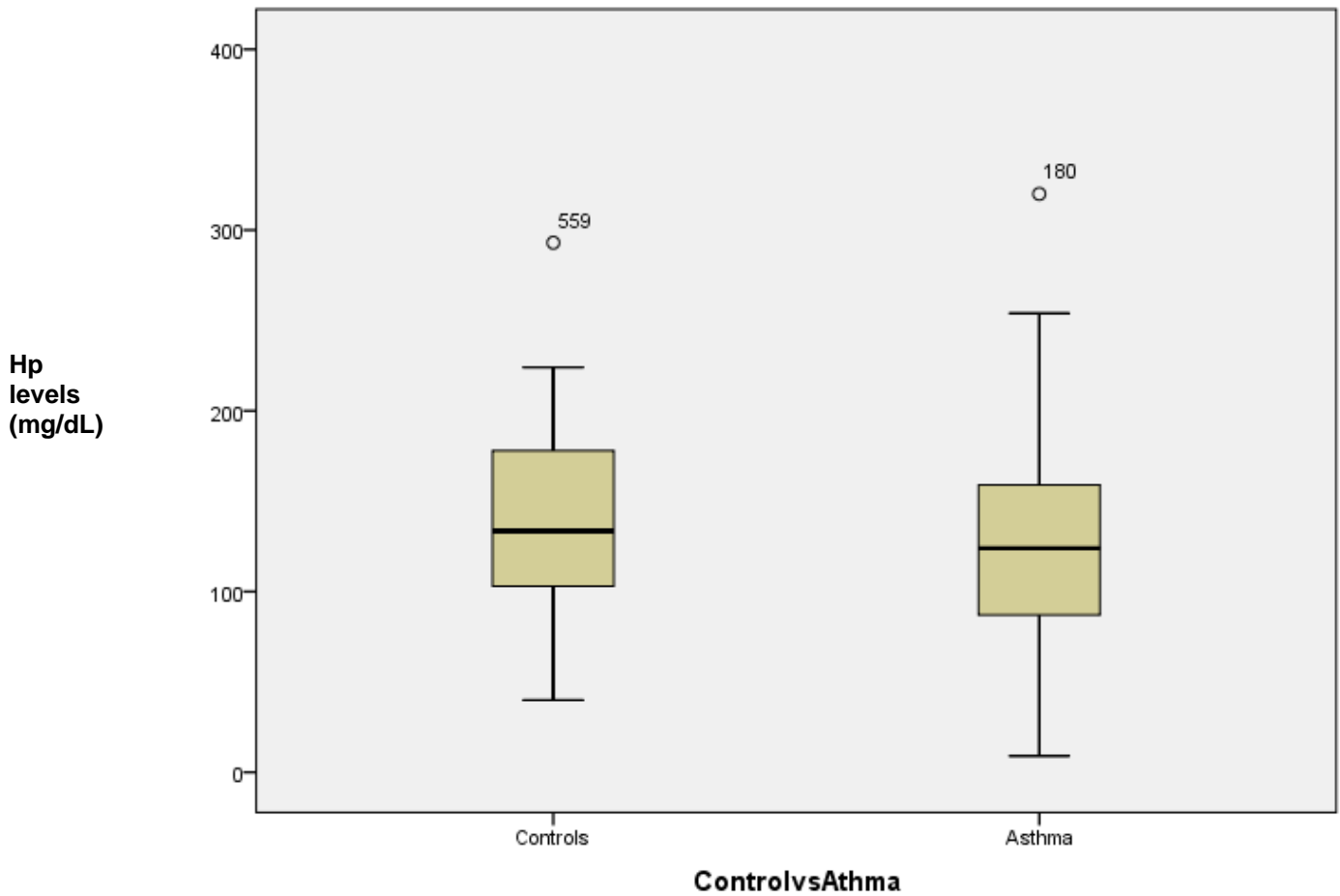
**Fig.IV.2.1:** - Distribution of age between asthma and controls ( $p=0,001$ ; Mann-Whitney, non-parametric test).

**Table IV.2.8:** Distribution of alleles and genotypes by groups in *Haptoglobin* polymorphism- Hp1-1, Hp2-1, Hp2-2

<i>Haptoglobin</i> polymorphism- Hp1-1, Hp2-1, Hp2-2	Controls	Asthma	p value	OR(95%CI) <sup>a</sup>
Hp*1	0.39	0.58	0,641	0.866 [0.535-1.400]
Hp*2	0.61	0.42		
Hp1-1n (%)	5(10.0)	21(18.6)	0,311	
Hp2-1 n (%)	29(58.0)	54(47.8)		
Hp2-2 n (%)	16(32.0)	38(33.6)		

Hp genotype (Hp 1-1(21(18.6%)) , Hp 2-1(54(47.8%)) , Hp 2-2(38(33.6%)) ) distribution in asthmatics, are not statistical different from control group (Hp 1-1(5(10%)) , Hp 2-1 (29(58%)), Hp 2-2(16(32%)) )(p= 0.311).

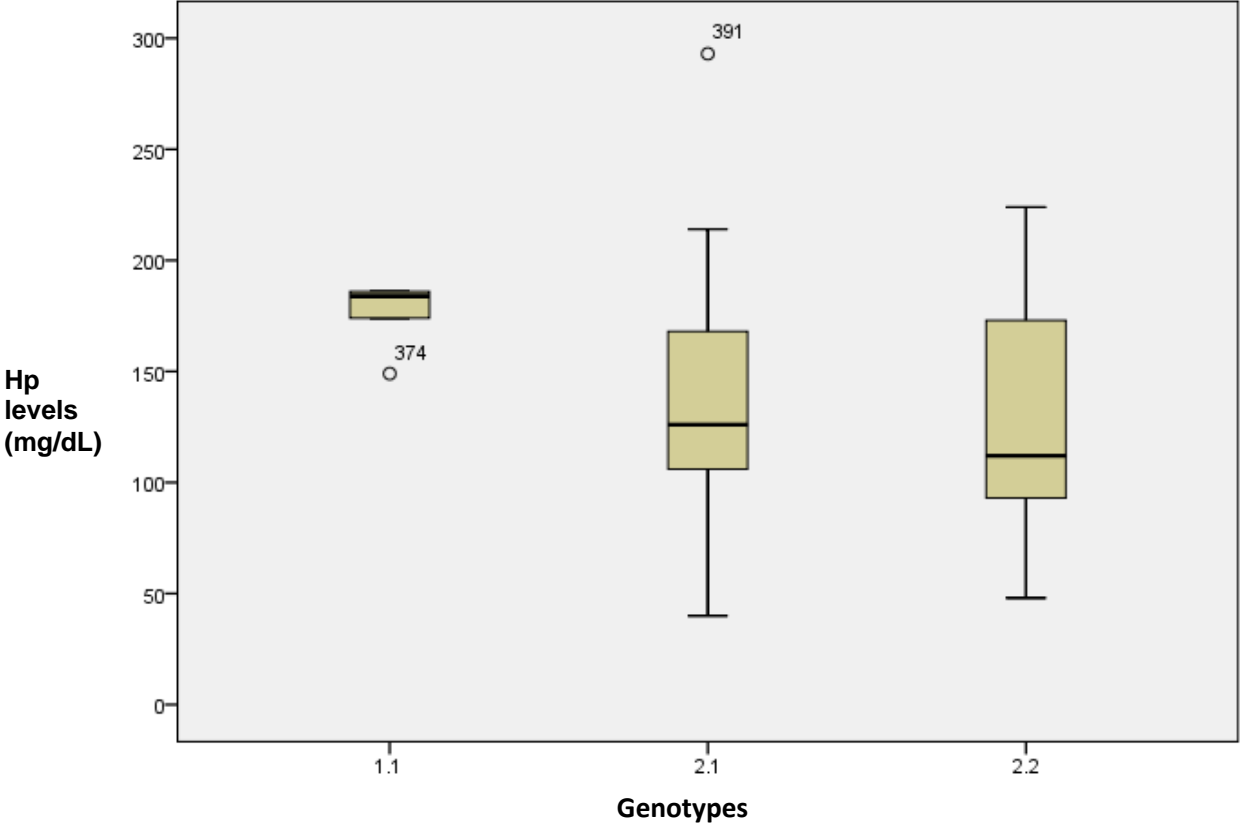
Allelic (Hp\*1:0.39 and Hp\*2:0.61 ) in controls and Allelic (Hp\*1:0.58 and Hp\*2:0.42) in asthma. Allelic (Hp\*1 and Hp\*2 ) in asthma vs control group: there is no increased risk of asthma in those with Hp\*2 vs Hp\*1 : (OR: 0.866; 95%CI: [0.535-1.400]; p=0.641).



**Fig.IV.2.2:**Distribution of Hp levels between asthma and controls ( $p=0.132$ ; Distribution of Hp levels between asthma and controls ( $p=0.132$ ; parametric Student's T test).

When we compared asthmatics with control group we verified that in asthma, the levels of Hp are always lower than in the control group although there is no statistical difference between groups ( $124,73 \pm 51,00$  vs  $137,88 \pm 51,39$  mg/dL) ( $p=0.132$ ).

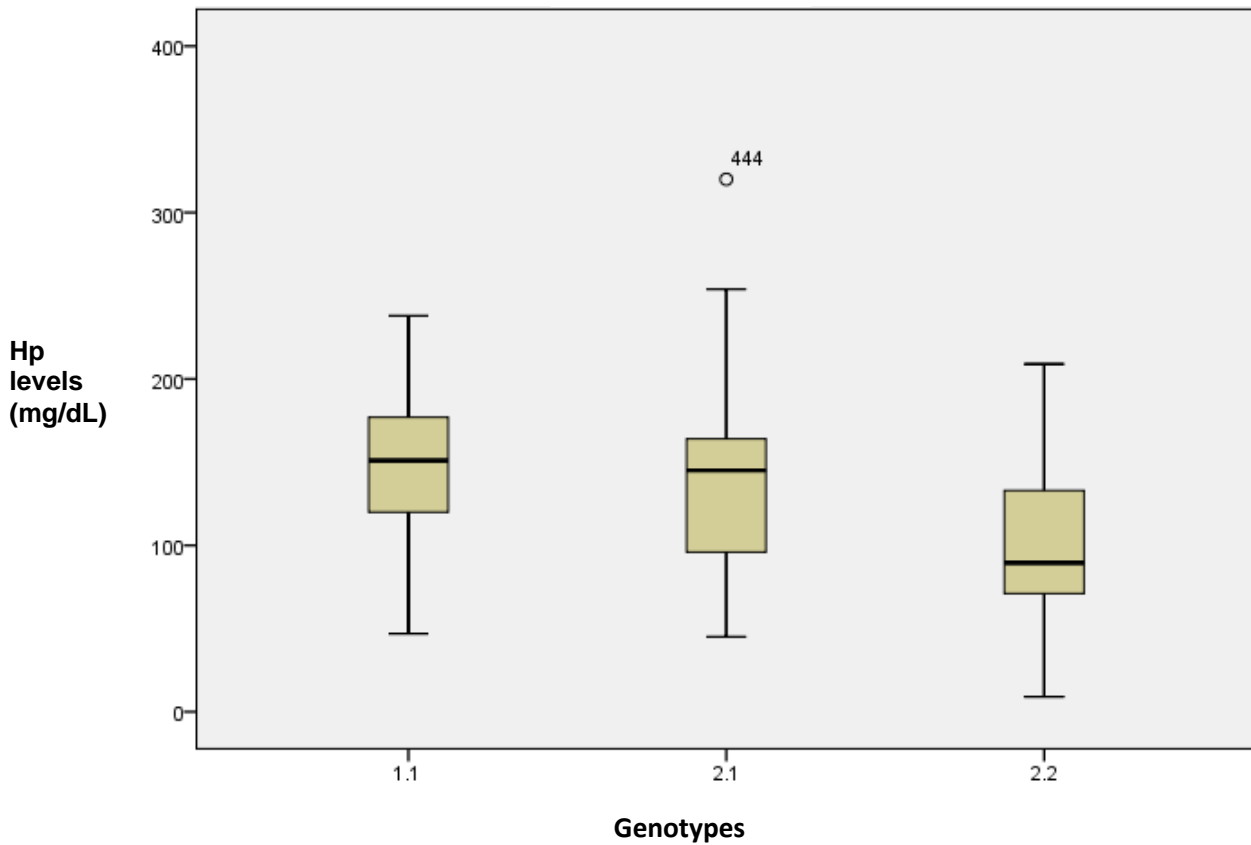
**Hp levels by Genotypes in controls**



**Fig.IV.2.3:** Distribution of Hp levels by genotypes in control group (p=0,075; Mann-Whitney non-parametric test).

There are no differences in Hp levels by genotype in control group: Hp 1-1(175.80±15.79mg/dL) , Hp 2-1(136.55±53.47mg/dL), Hp 2-2(128.44±51.44mg/dL)

### Hp levels by Genotypes in asthma



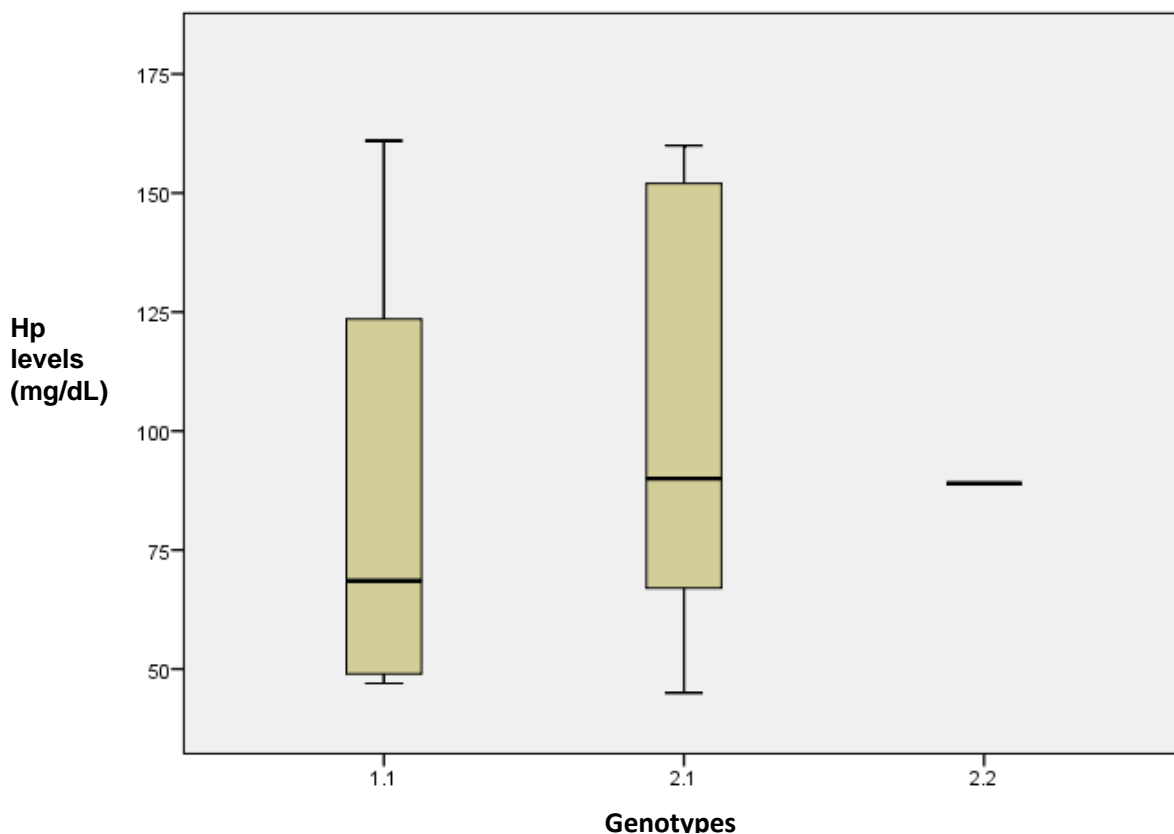
**Fig.IV.2.4:**Hp levels by genotype in asthma group ( $p < 0.001$ ; ANOVA parametric test).

In asthma group: those who express Hp 2-2 had the lower levels of the circulating protein when compared with Hp 2-1 and Hp 1-1 (Hp 1-1 :  $144,95 \pm 48,23$  mg/dL vs Hp 2-1 :  $137,37 \pm 49,58$  mg/dL vs Hp 2-2 :  $95,61 \pm 41,94$  mg/dL) ( $p < 0.001$ ) (pos-hoc analysis).

In the control group: There are no differences of Hp levels by genotypes (Hp 1-1 :  $175,8 \pm 15,79$  mg/dL vs Hp 2-1 :  $136,55 \pm 53,47$  mg/dL vs Hp 2-2 :  $128,44 \pm 51,43$  mg/dL) ( $p = 0,075$ ).

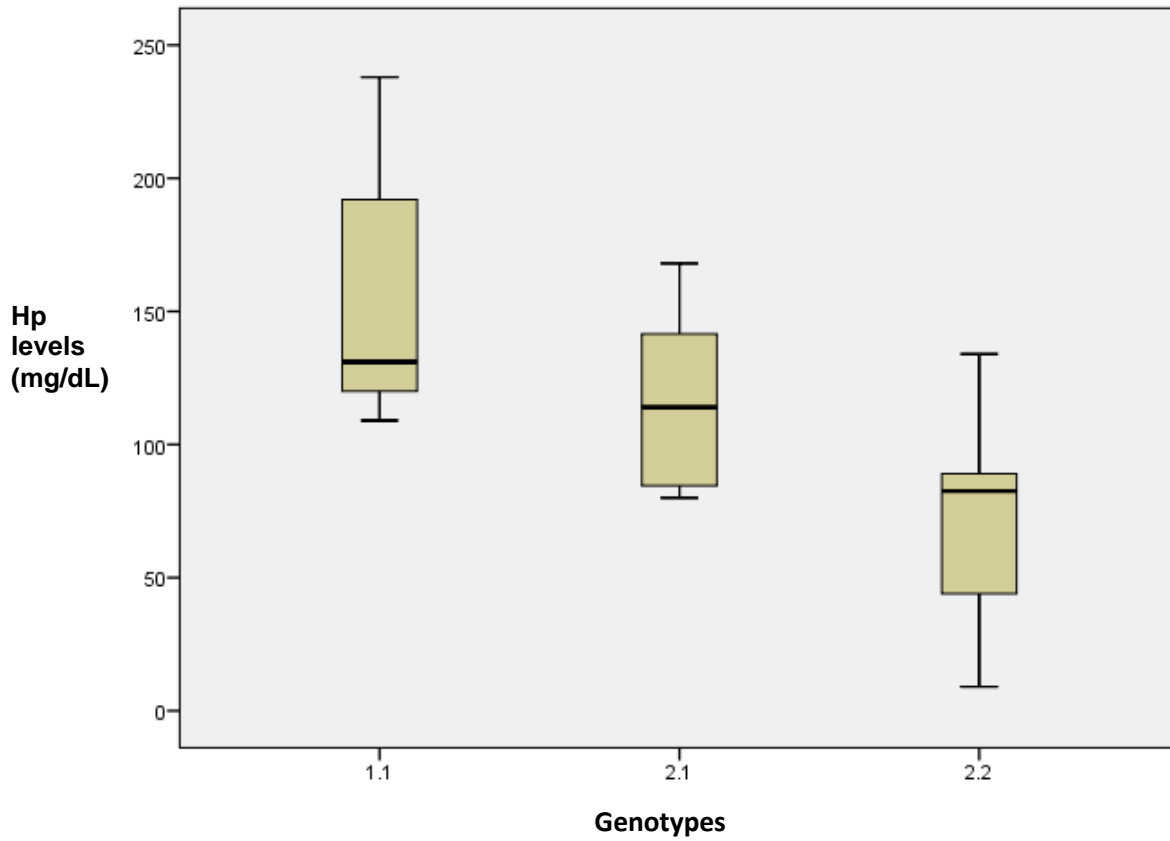
## Hp levels by genotype according to age

There are no differences in the frequencies of genotypes across asthma ( $p=0,347$ ) or control group ( $p=0,134$ ) according to age cutoff (<15 years; 15-30 years; >30 years).



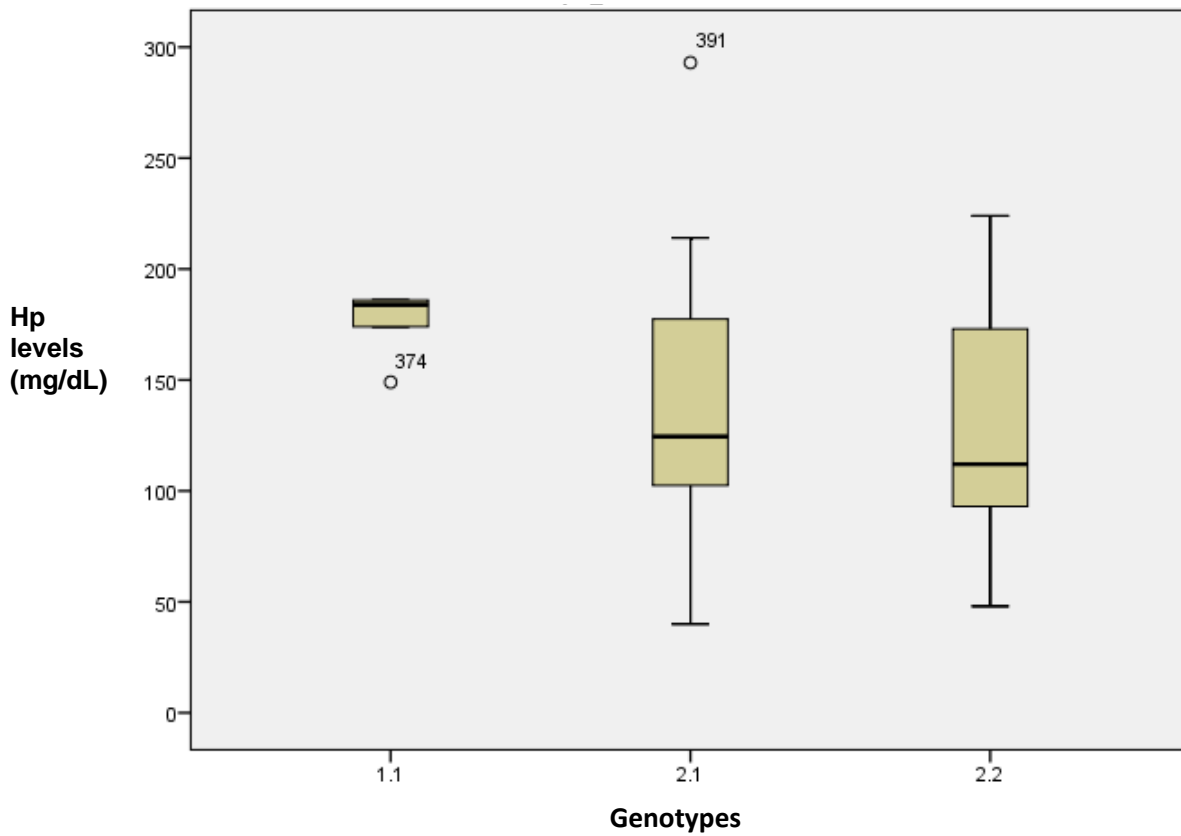
**Fig.IV.2.5:** Hp levels across genotypes in asthma (ages <15 years).

There are no differences across genotypes by asthma (<15 years) ( $p=0,897$ -ANOVA parametric test). (Hp 1-1 :  $86,25 \pm 52,82$  mg/dL vs Hp 2-1 :  $100,67 \pm 46,80$  mg/dL vs Hp 2-2 :  $89,00$  mg/dL).



**Fig.IV.2.6:**Hp levels across genotypes in asthma (ages 15-30 years).

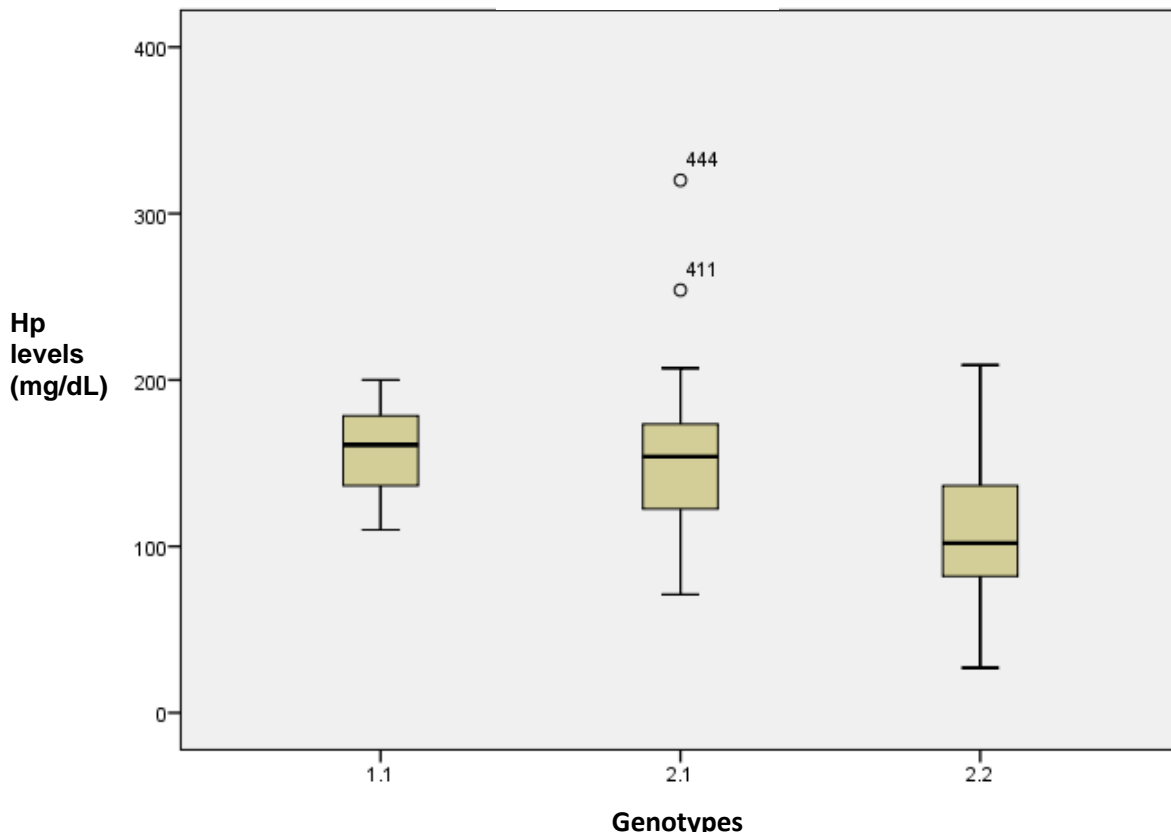
There are differences across genotypes by asthma (15-30 years)( $p=0,002$ -ANOVA parametric test). ( Hp 1-1 : $158,00 \pm 55,07$  mg/dL vs Hp 2-1 : $115,33 \pm 29,57$  mg/dL vs Hp 2-2 : $74,7 \pm 38,19$ )mg/dL.



**Fig.IV.2.7:** Hp levels across genotypes in controls (>30 years).

There are no differences across genotypes by controls (>30 years)( $p=0,104$ -Kruskal Wallis non- parametric test). ( Hp 1-1 : $175,80 \pm 15,79$  mg/dL vs Hp 2-1 : $138,38 \pm 58,04$  mg/dL vs Hp 2-2 : $128,44 \pm 51,44$ )mg/Dl.





**Fig.IV.2.8:** Hp levels across genotypes in asthma (>30 years).

There are differences across genotypes by asthma (>30 years) ( $p < 0.001$ -Kruskal Wallis non-parametric test). (Hp 1-1 :  $159,08 \pm 28,58$  mg/dL vs Hp 2-1 :  $152,69 \pm 49,94$  mg/dL vs Hp 2-2 :  $103,59 \pm 41,98$  mg/dL).

In those asthmatics with age  $\geq 15$  years Hp levels are different by genotype ( $p < 0.05$ ): 1-1 and 2-1 differ from 2-2. [ In those patients 15-30 years ( $p = 0,002$ ); in those patients  $> 30$  years ( $p < 0,001$ )]. Those patients with age  $< 15$  years, Hp levels were no different between genotypes ( $p > 0,05$ ) ( $p = 0,897$ ).

### **Conclusions:**

In asthma group: those who express Hp 2-2 had the lower levels of the circulating protein when compared with Hp 2-1 and Hp 1-1 (Hp 1-1 :  $144,95 \pm 48,23$  mg/dL vs Hp 2-1 :  $137,37 \pm 49,58$  mg/dL vs Hp 2-2 :  $95,61 \pm 41,94$  mg/dL) ( $p < 0.001$ ) (pos-hoc analysis).

In the control group: There are no differences of Hp levels by genotypes (Hp 1-1 :  $175,8 \pm 15,79$  mg/dL vs Hp 2-1 :  $136,55 \pm 53,47$  mg/dL vs Hp 2-2 :  $128,44 \pm 51,43$  mg/dL) ( $p = 0,075$ ).

There are differences across genotypes by asthma (15-30 years) ( $p = 0,002$ -ANOVA parametric test). ( Hp 1-1 :  $158,00 \pm 55,07$  mg/dL vs Hp 2-1 :  $115,33 \pm 29,57$  mg/dL vs Hp 2-2 :  $74,7 \pm 38,19$  mg/dL. There are differences across genotypes by asthma ( $> 30$  years) ( $p < 0.001$ -Kruskal Wallis non- parametric test). ( Hp 1-1 :  $159,08 \pm 28,58$  mg/dL vs Hp 2-1 :  $152,69 \pm 49,94$  mg/dL vs Hp 2-2 :  $103,59 \pm 41,98$  mg/dL. In those asthmatics with age  $\geq 15$  years Hp levels are different by genotype ( $p < 0.05$ ): 1-1 and 2-1 differ from 2-2. [ In those patients 15-30 years ( $p = 0,002$ ); in those patients  $> 30$  years ( $p < 0,001$ )]. Those patients with age  $< 15$  years, Hp levels were no different between genotypes ( $p > 0,05$ ) ( $p = 0,897$ ).

### **B) NO ASSOCIATED SYSTEM**

Intron 4 polymorphism of the eNOS gene (rs1799983)

Asthmatic patients ( $n = 159$ ) were compared with a control group ( $n = 108$ ); the polymorphisms were analyzed by PCR (Polymerase chain reaction). Control of asthma assessed by validated instrument (ACQ7 and PAQLQ). Statistical analysis was performed with PASW version 18 establishing a significance level of  $p < 0.05$ . The control group is not in HWE ( $\chi^2 = 7.699$ ;  $p = 0.021$ ).

**Table IV.2.9:** Participant's clinical and demographic characteristics.

eNOS polymorphism (rs1799983)	Controls	Asthma	p value
N (%)	159 (59.6)	108 (40.4)	n.a.
Female	77 (48.4)	69(63.9)	<b>0.018</b>
Male	82(51.6)	39 (36.1)	
Age (years)	(39,81±10,9 )	(37,06±18,46 )	0.085 <sup>†</sup>
< 15	0 (0.0)	14 (13.0)	
15-30	35 (22.0)	31 (28.7)	<b>&lt;0.001</b>
> 30	124 (78.0)	63 (58.3)	
Atopy/No Atopy	n.a.	89/19 (82.4/17.6)	n.a.
Asthma controlled /			
Asthma not	n.a.	80/28(74.1/25.9)	n.a.
controlled			

Bolded results are less than 0.05 of statistical significance.

p\*  $\chi^2$  test values; the values represent absolute frequencies (relative frequencies, %).

p<sup>†</sup> non-parametric Mann-Whitney; the values represent means  $\pm$  standard deviation (SD).

n.a.: non applicable.

We had studied 159 individuals in the control group and 108 in the asthmatics (**Table IV.2.9**).

There are differences in the distribution of gender between asthmatics (F/M:69(63.9%); 39(36.1)) and controls (F/M:77(48.4%); 82(51.6%)) (p=0,018) being more females in the asthmatics and more males in the control group.

The mean age is not different across controls and asthma(p=0.085). Although there are differences when we stratify by age being youngest the asthmatics and older the control group (**<0.001**).

**Table IV.2.10:** Distribution of alleles and genotypes by groups in eNOS polymorphism (rs1799983)

eNOS polymorphism (rs1799983)	Controls	Asthma	p value	OR(95%CI) <sup>a</sup>
Allele b	0.85	0.79	0.098	0.663 [0.420-1.048]
Allele a	0.15	0.21		
bb n (%)	107(67.3)	76(70.4)	<b>0,009</b>	
ab n (%)	36(22.6)	24(28.7)		
aa n (%)	16(10.1)	8(0.9)		

In asthmatics the frequencies of allele b were 0.85 and allele a: 0.15. In control group, the frequencies of allele b were 0.79 and for allele a: 0.21; (OR: 0.663; 95%CI: [0.420-1.048]; p=0.098). There is no statistical difference between these groups (p=0.098) and no increased risk of being asthmatic for those that express the Allele a in asthmatics vs controls.

Genotypes in asthmatics were: bb: 70.4%; ab: 28.7%; aa: 0.9% and for control group: bb: 67.3%; ab: 22.6%; aa: 10.1%. There is statistical difference between these groups (p=0.009). Being the the genotypes aa less frequent in asthmatics.

**Genetic models (Table IV.2.11)** In the genetic models we had considered as Major allele: b and minor a. In the Dominant model (aa+ab vs bb) those who are homozygous for the allele b have no increased risk of having asthma (crude: OR: 1.154[.680;1.960]; p<sup>a</sup>value =0.596; adjusted values: OR: 1.228[.715;2.108]; p<sup>b</sup>value =0.456). In the recessive model those who express allele b (ab+bb) have an increased risk almost 12 times (crude) and

almost 14 times (adjusted) of having asthma when compared with control (OR: 11,972[1,563;91,683]; p<sup>a</sup>value =0,017 ;OR: 13,582[1,757;104,967]; p<sup>b</sup>value =0,012).

In the additive model 1(bb vs ab) those who are heterozygous with genotype ab have no increased risk of asthma when compared with control (crude:OR 1,212[0,690;2,129]; p<sup>a</sup>value =0,503 ; adjusted: OR: 1,161[0,653;2,064]; p<sup>b</sup>value =0,610).

In the additive model 2(bb vs aa) those who are homozygous for the allele a (genotypes aa) have protection of having asthma when compared with control( crude:OR 0,088[0,011;0,678]; p<sup>a</sup>value =0.020); adjusted:OR: 0,076[0,010;0,596]; p<sup>b</sup>value =0.014).

**Table IV.2.11:**Genetic models for eNOS polymorphism (rs1799983)

Dominant model	N cases/controls	OR crude[CI]; p <sup>a</sup> value	OR adjusted[CI]; p <sup>b</sup> value
aa+ab	32/52	referent	
bb	76/107	1.154[.680;1.960]; p <sup>a</sup> value =0.596	1.228[.715;2.108]; p <sup>b</sup> value =0.456

Recessive Model	N cases/controls	OR crude[CI]; p <sup>a</sup> value	OR adjusted[CI]; p <sup>b</sup> value
aa	1/16	referent	
ab+bb	107/143	11,972[1,563;91,683]; p <sup>a</sup> value =0,017	13,582[1,757;104,967]; p <sup>b</sup> value =0,012

Additive1	N cases/controls	OR crude[CI]; p <sup>a</sup> value	OR adjusted[CI]; p <sup>b</sup> value
bb	76/107	referent	
ab	31/36	1,212[0,690;2,129]; p <sup>a</sup> value =0,503	1,161[0,653;2,064]; p <sup>b</sup> value =0,610

Additive2	N cases/controls	OR crude[CI]; p <sup>a</sup> value	OR adjusted[CI]; p <sup>b</sup> value
bb	76/107	referent	
aa	1/16	0,088[0,011;0,678]; p <sup>a</sup> value =0.020	0,076[0,010;0,596]; p <sup>b</sup> value =0.014

**Conclusion:** those who express allele b (ab+bb) have an increased risk almost 12 times (crude) and almost 14 times (adjusted) of having asthma when compared with control (OR: 11,972[1,563;91,683]; p<sup>a</sup>value =0,017 ;OR: 13,582[1,757;104,967]; p<sup>b</sup>value =0,012).

Those who are homozygous for the allele a (genotypes aa) have protection of having asthma when compared with control (crude: OR 0,088[0,011;0,678]; p<sup>a</sup>value =0.020); adjusted:OR: 0,076[0,010;0,596]; p<sup>b</sup>value =0.014).

## **B) NO ASSOCIATED SYSTEM**

NOS2 polymorphism (exon 16-14CT)

NOS2 polymorphism (intron 16 - 88GT)

NOS2 polymorphism (intron 20 - IVS20 + 524 GA)

Asthmatics were compared with a control group ; the polymorphisms were analyzed by PCR-RFLP. Control of asthma assessed by ACQ7 and PAQLQ. Statistical analysis with PASW version 18 ;a significance level of  $p < 0.05$ .

## **B) NO ASSOCIATED SYSTEM**

NOS2 polymorphism (exon 16-14CT)- iNOS: exon 16: + 14C> T (Ex16+14C>T, Ser608Leu; rs 2297518; antisense sequence.

We had studied 72 controls of healthy volunteers and 102 asthmatics. Polymorphisms analyzed by PCR-RFLP(Polymerase chain reaction- restriction fragment length polymorphism) . Control of asthma assessed by validated instrument (ACQ7 and PAQLQ). Statistical analysis was performed with PASW 18, establishing a significance level of  $p < 0.05$ . The control group is in HWE ( $\chi^2=0.061$ ;  $p=0.970$ ).

**Table IV.2.12:**Participant's demographic and clinical characteristics.

Ex16+14C>T polymorphism	Controls	Asthma	p value
N (%)	72 (41.4)	102 (58.6)	n.a.
Female	25(34.7)	67(65.7)	<b>&lt;0.001</b>
Male	47(65.3)	35 (34.3)	
Age (years)	(42,29±12,02 )	(38,10±19,02 )	0.060 <sup>†</sup>
< 15	0 (0.0)	12 (12.0)	
15-30	13(18.1)	30 (30.0)	<b>=0.001</b>
> 30	59 (81.9)	58 (58.0)	
Atopy/No Atopy	n.a.	84/16 (84.0/16.0)	n.a.
Asthma controlled / Asthma not controlled	n.a.	74/26 (74.0/26.0)	n.a.

Bolded results are less than 0.05 of statistical significance.

p\*  $\chi^2$  test values; the values represent absolute frequencies (relative frequencies, %).

p<sup>†</sup> non-parametric Mann-Whitney; the values represent means  $\pm$  standard deviation (SD).

n.a.: non applicable.

We had studied 72 (41.4) individuals in the control group and 102 (58.6) in the asthmatics

There are differences in the distribution of gender between asthmatics (F/M: 67(65.7); 35 (34.3)) and controls (F/M: 25(34.7); 47(65.3) )(p<**0.001**) being more females in the asthmatics and more males in the control group.

The mean age is no different across controls and asthma (p=0.060- Mann-Whitney non-parametric test). . There are also differences when we stratify by age being youngest the asthmatics and older the control group (**=0.001**).

In asthmatics the frequencies of allele C were 0.81 and allele T: 0.19 In control group, the frequencies of allele C were 0.92 and for allele T : 0.08 ; (OR: 2.858; 95%CI: [1.409-5.796]; p=0.004). There is statistical difference between these groups (p=0.004) with an increased risk of being asthmatic for those that express the Allele T of almost 3 times in asthmatics vs controls.

Genotypes in controls were: CC: 86.1%; CT:12.5 %; TT: 1.4% and for asthma group: CC: 65.7%; CT: 30.4%; TT: 3.9%. There is statistical difference between these groups (p=0.01). The genotypes who express allele C are more frequent in controls and those who express allele T in asthmatics.

## Genotype and allelic frequencies between groups (asthma and controls)

**Table IV.2.13:** Distribution of alleles and genotypes by groups in Ex16+14C>T-NOS 2 polymorphism

Ex16+14C>T-NOS 2 polymorphism	Controls	Asthma	p value	OR(95%CI) <sup>a</sup>
<b>C</b>	0.92	0.81	0.004	2.858 [1.409-5.796]
<b>T</b>	0.08	0.19		
<b>CC (%)</b>	9(86.1)	35(65.7)	0.010	
<b>CT n (%)</b>	84(12.5)	62(30.4)		
<b>TT n (%)</b>	78(1.4)	56(3.9)		

In asthmatics the frequencies of Allele C 0.81 and of Allele T 0.19; in controls: 0.92 and 0.08 respectively. There is statistical difference between these groups with a risk of being asthmatic of almost 3 for Allele T (OR:2.858;IC95%[1.409;5.796]; p<sup>a</sup> value =0.004; Genotypes in the asthmatics- CC: 65.7%; CT: 30.4%; TT: 3.9%; in control group- CC: 86.1%; CT:12.5%; TT: 1.4%. There is statistical difference between these groups (p=0.01).Being the genotypes who express allele T more frequent in asthmatics and those who express allele C in controls.

In the genetic models we had considered as Major allele: C and minor T.

In the Dominant model (CT+TT vs CC) those who are homozygous for the allele C have a protection of having asthma when compared with controls (crude: OR: 0.309; ;95%CI: [0.141;0.676]; p<sup>a</sup>value =0.003;adjusted values: OR: 0.326; 95%CI: [0.144;0.738]; p<sup>b</sup>value =0.007).

In the recessive model (TT vs CT+CC ) have no increased risk of having asthma when compared with controls (crude) and (adjusted) (OR: 0,345;95%CI: [0,038;3,153]; p<sup>a</sup>value =0,346;OR: 0,347; 95%CI: [0,034;3,582]; p<sup>b</sup>value =0,374.

In the additive model 1(CC vs CT) those who are heterozygous with genotype CT have an increased risk of asthma of 3 times when compared with controls (crude:OR: 3,187 ; 95%CI: [1,406;7,227]; p<sup>a</sup>value =0,006 ; adjusted: OR: 3,002; 95%CI [1,276;7,062]; p<sup>b</sup>value =0,012).

In the additive model 2 (CC vs TT) those who are homozygous for the allele T (genotypes TT) have not increased risk of having asthma when compared with controls (crude:OR :5,701; 95%CI :[0,403;34,025]; p<sup>a</sup>value =0,248); and 7 when adjusted for age and gender (adjusted:OR: 3,700; , 95%CI :[0,345;39,681]; p<sup>b</sup>value =0,280).



**Table IV.2.14:** Genetic models for Ex16+14C>T iNOS gene polymorphism in cases and controls and their association with the risk of asthma

Dominant model	N cases/controls	OR crude; [95%CI]; p <sup>a</sup> value	OR adjusted [95%CI]; p <sup>b</sup> value
CT+TT	35/10	referent	
CC	67/62	0.309[0.141;0.676]; p <sup>a</sup> value =0.003	0.326[0.144;0.738]; p <sup>b</sup> value =0.007

Recessive Model	N cases/controls	OR crude[95%CI CI]; p <sup>a</sup> value	OR adjusted[95%CI CI]; p <sup>b</sup> value
TT	4/1	referent	
CT+CC	98/71	0,345[0,038;3,153]; p <sup>a</sup> value =0,346	0,347[0,034;3,582]; p <sup>b</sup> value =0,374

Additive1	N cases/controls	OR crude[95%CI CI]; p <sup>a</sup> value	OR adjusted[95%CI CI]; p <sup>b</sup> value
CC	67/62	referent	
CT	31/9	3,187[1,406;7,227]; p <sup>a</sup> value =0,006	3,002[1,276;7,062]; p <sup>b</sup> value =0,012

Additive2	N cases/controls	OR crude[95%CI CI]; p <sup>a</sup> value	OR adjusted[95%CI CI]; p <sup>b</sup> value
CC	56/78	referent	
TT	35/9	3,701[0,403;34,025]; p <sup>a</sup> value =0,248	3,700[0,345;39,681]; p <sup>b</sup> value =0,280

**Results:** For this polymorphism in asthmatics the frequencies of Allele C 0.81 and of Allele T 0.19; in controls: 0.92 and 0.08 respectively. There is statistical difference between these groups with a risk of being asthmatic of almost 3 for Allele T(OR:2.858;IC95%[1.409;5.796]; p<sup>a</sup> value =0.004;

Genotypes in the asthmatics- CC: 65.7%; CT: 30.4%; TT: 3.9%; in control group- CC: 86.1%; CT:12.5%; TT: 1.4%. There is statistical difference between these groups (p=0.01). Being the genotypes who express allele T more frequent in asthmatics and those who express allele C in controls.

In the Dominant model (CT+TT vs CC) those who are homozygous for the allele C have a protection of having asthma when compared with controls (crude: OR: 0.309; 95%CI: [0.141;0.676]; p<sup>a</sup>value =0.003;adjusted values: OR: 0.326; 95%CI: [0.144;0.738]; p<sup>b</sup>value =0.007).

In the recessive model ( TT vs CT+CC ) have no increased risk of having asthma when compared with controls (crude) and (adjusted) (OR: 0,345;95%CI: [0,038;3,153]; p<sup>a</sup>value =0,346;OR: 0,347; 95%CI: [0,034;3,582]; p<sup>b</sup>value =0,374.

In the additive model 1(CC vs CT) those who are heterozygous with genotype CT have an increased risk of asthma of 3 times when compared with controls (crude: OR: 3,187 ; 95%CI: [1,406;7,227]; p<sup>a</sup>value =0,006 ; adjusted: OR: 3,002; 95%CI [1,276;7,062]; p<sup>b</sup>value =0,012).

In the additive model 2 (CC vs TT) those who are homozygous for the allele T (genotypes TT) have not increased risk of having asthma when compared with controls (crude: OR :5,701; 95%CI :[0,403;34,025]; p<sup>a</sup>value =0,248); and 7 when adjusted for age and gender (adjusted: OR: 3,700; , 95%CI :[0,345;39,681]; p<sup>b</sup>value =0,280).

## **B) NO ASSOCIATED SYSTEM**

### NOS2 polymorphism (intron 16 - 88GT) (rs9282801)

We had studied 72 controls and 97 asthmatics. Asthmatic patients were compared with a control group of healthy volunteers; polymorphisms analyzed by PCR-RFLP(Polymerase chain reaction- restriction fragment length polymorphism) . Control of asthma assessed by validated instrument (ACQ7 and PAQLQ). Statistical analysis was performed with PASW 18, establishing a significance level of  $p < 0.05$ . The control group is in HWE ( $\chi^2=2.485$ ;  $p=0.289$ ).

**Table IV.2.15:** Participant's clinical and demographic characteristics.

iNOSintron 16 + 88 G>T (rs9282801)	Controls	Asthma	p value
N (%)	72 (42.6)	97 (57.4)	n.a.
Female	25 (34.7)	60(61.9)	<b>0.001</b>
Male	47(65.3)	37 (38.1)	
Age (years)	(42,13±11,67 )	(36,78±19,39 )	<b>0.025<sup>†</sup></b>
< 15	0 (0.0)	15 (15.8)	
15-30	13 (18.1)	27 (28.4)	<b>&lt;0.001</b>
> 30	59 (81.9)	53 (55.8)	
Atopy/No Atopy	n.a.	89/19 (82.4/17.6)	n.a.
Asthma controlled / Asthma not controlled	n.a.	80/28(74.1/25.9)	n.a.

Bolded results are less than 0.05 of statistical significance.

p\*  $\chi^2$  test values; the values represent absolute frequencies (relative frequencies, %).

p<sup>†</sup> non-parametric Mann-Whitney; the values represent means  $\pm$  standard deviation (SD).

n.a.: non applicable.

We had studied 72 individuals in the control group and 97 in the asthmatics

There are differences in the distribution of gender between asthmatics (F/M:60(61.9%); 37(38.1)) and controls (F/M:25(34.7%); 47(65.3%)) (p=0,001) being more females in the asthmatics and more males in the control group.

The mean age is different across controls and asthma(p=0.025) (Mann-Whitney non-parametric test) , being the youngest in the asthma group. There are also differences when we stratify by age being youngest (<30 years) in the asthma group and older in the control group (<30 years) (**<0.001**).

**Table IV.2.16:**Distribution of alleles and genotypes by groups in iNOSintron 16 + 88 G>T (rs9282801)

iNOSintron 16 + 88 G>T (rs9282801)	Controls	Asthma	p value	OR(95%CI) <sup>a</sup>
<b>G</b>	0.72	0.64	0,175	1.418 [0.890-2.259]
<b>T</b>	0.28	0.36		
<b>GG (%)</b>	37(45.4)	55(56.9)	0,330	
<b>GT n (%)</b>	27(37.1)	28(29.2)		
<b>TT n (%)</b>	13(17.5)	14(13.9)		

In asthmatics the frequencies of allele G were 0.64 and allele T: 0.36 In control group, the frequencies of allele G were 0.72 and for allele T : 0.28 ; (OR: 1.418; 95%CI: [0.890-2.259]; p=0.175). There is no statistical difference between these groups (p=0.175) and no increased risk of being asthmatic for those that express the AlleleT in asthmatics vs controls. Genotypes in asthmatics were: GG: 56.9%; GT:29.2 %; TT: 13.9% and for control group: GG: 45.4%; GT: 37.1%; TT: 17.5%. There is no statistical difference between these groups (p=0.330)

In the Dominant model (TT+ GT vs GG) there is no increased risk of having asthma when compared with control (crude: OR: 0.628[.340;1.160]; p<sup>a</sup>value =0.137;adjusted values: OR: 0.665[0.346;1.278]; p<sup>b</sup>value =0.221).

In the recessive model (TT vs GT+GG) there is no increased risk of having asthma when compared with control (OR: 0,759[0,325;1,773]; p<sup>a</sup>value =0,524;OR: 0,715[0,291;1,766]; p<sup>b</sup>value =0,469).

In the additive model 1(GG vs GT) there is no increased risk of having asthma when compared with control (crude:OR 1,597[0,804;3,172]; p<sup>a</sup>value =0,181 ; adjusted: OR: 1,449[0,701;2,992]; p<sup>b</sup>value =0,317).

In the additive model 2(GGvsTT) there is no increased risk of having asthma when compared with control (crude:OR: 1,584[0,651;3,855]; p<sup>a</sup>value =0.311; adjusted:OR: 1,707[0,644;4,529]; p<sup>b</sup>value =0.282).

**Table IV.2.17:** Genetic models for iNOSintron 16 + 88 G>T (rs9282801) in cases and controls and their association with the risk of asthma

<b>Dominant model</b>	<b>N cases/controls</b>	<b>OR crude[CI]; p<sup>a</sup>value</b>	<b>OR adjusted[CI]; p<sup>b</sup>value</b>
<b>TT+GT</b>	53/31	referent	
<b>GG</b>	44/41	0.628[.340;1.160]; p <sup>a</sup> value =0.137	0.665[0.346;1.278]; p <sup>b</sup> value =0.221

<b>Recessive Model</b>	<b>N cases/controls</b>	<b>OR crude[CI]; p<sup>a</sup>value</b>	<b>OR adjusted[CI]; p<sup>b</sup>value</b>
<b>TT</b>	17/10	referent	
<b>GT+GG</b>	62/80	0,759[0,325;1,773]; p <sup>a</sup> value =0,524	0,715[0,291;1,766]; p <sup>b</sup> value =0,469

<b>Additive1</b>	<b>N cases/controls</b>	<b>OR crude[CI]; p<sup>a</sup>value</b>	<b>OR adjusted[CI]; p<sup>b</sup>value</b>
<b>GG</b>	44/41	referent	
<b>GT</b>	36/21	1,597[0,804;3,172]; p <sup>a</sup> value =0,181	1,449[0,701;2,992]; p <sup>b</sup> value =0,317

<b>Additive2</b>	<b>N cases/controls</b>	<b>OR crude[CI]; p<sup>a</sup>value</b>	<b>OR adjusted[CI]; p<sup>b</sup>value</b>
<b>GG</b>	44/41	referent	
<b>TT</b>	17/10	1,584[0,651;3,855]; p <sup>a</sup> value =0,311	1,707[0,644;4,529]; p <sup>b</sup> value =0,282

**Results:** For this polymorphism there is no increased risk of having asthma when compared with controls.

## B) NO ASSOCIATED SYSTEM

### NOS2 polymorphism (IVS20 + 524 G>A-rs944722)

Asthmatic patients were compared with a control group of healthy blood donors; polymorphisms analyzed by PCR-RFLP (Polymerase chain reaction- restriction fragment length polymorphism). Control of asthma assessed by validated instrument (ACQ7 and PAQLQ). Statistical analysis was performed with PASW 18, establishing a significance level of  $p < 0.05$ . The control group is in HWE ( $\chi^2=2.645$ ;  $p=0.266$ ).

**Table IV.2.18:** Participant's demographic and clinical characteristics.

IVS20 + 524 G>A-rs944722	Controls	Asthma	p value
N (%)	171 (52.8)	153 (47.3)	n.a.
Female	59(34.5)	101(66.0)	<b>&lt;0.001</b>
Male	112(65.5)	52 (34.0)	
Age (years)	(42,18±11,78 )	(38,30±18,52 )	<b>0.027<sup>†</sup></b>
< 15	0 (0.0)	18 (11.9)	
15-30	36 (21.1)	42 (27.8)	<b>&lt;0.001</b>
> 30	134 (78.9)	91 (60.3)	
Atopy/No Atopy	n.a.	128/23 (84.8/15.2)	n.a.
Asthma controlled / Asthma not controlled	n.a.	107/44(70.9/29.1)	n.a.

Bolded results are less than 0.05 of statistical significance.

$p^*$   $\chi^2$  test values; the values represent absolute frequencies (relative frequencies, %).

$p^†$  non-parametric Mann-Whitney; the values represent means  $\pm$  standard deviation (SD).

n.a.: non applicable.

There are differences in the distribution of gender between asthmatics (F/M:101(66.0%); 52(34.0)) and controls (F/M:59(34.5%); 112(65.5%)) ( $p < 0.001$ ) being more females in the asthmatics and more males in the control group.

The mean age is different across controls and asthma ( $p=0.027$ - Mann-Whitney non-parametric test). There are also differences when we stratify by age being youngest the asthmatics and older the control group ( $<0.001$ ).

**Table IV.2.19:** Distribution of alleles and genotypes by groups in IVS20 + 524 G>A-rs944722

IVS20 + 524 G>A-rs944722)	Controls	Asthma	p value	OR(95%CI) <sup>a</sup>
<b>G</b>	0.30	0.43	p<0.001	1.785 [1.291- 2.468]
<b>A</b>	0.70	0.57		
<b>GG (%)</b>	9(5.3)	35(22.9)	p<0.001	
<b>GA n (%)</b>	84(49.1)	62(40.5)		
<b>AA n (%)</b>	78(45.6)	56(36.6)		

In asthmatics the frequencies of allele G were 0.43 and allele A: 0.57. In control group, the frequencies of allele G were 0.30 and for allele A: 0.70; (OR: 1.785; 95%CI: [1.291-2.468]; p<0.001). There is statistical difference between these groups (p<0.001) with an increased risk of being asthmatic for those that express the Allele G of 1.8 times in asthmatics vs controls.

Genotypes in controls were: GG: 5.3%; GA:49.1%; AA: 45.6% and for asthma group: GG: 22.9%; GA: 40.5%; AA: 36.6%. There is statistical difference between these groups (p<0.001). GG genotypes are more frequent in asthmatics.

Genotype and allelic frequencies between groups (asthma and controls)

In asthmatics the frequencies of Allele G 0.43 and of Allele A 0.57; in controls: 0.30 and 0.70 respectively. There is statistical difference between these groups with a risk of being asthmatic of almost 2 (OR:1.785; IC95%[1.291;2.468]; p<sup>a</sup>value <0.001;

Genotypes in the asthmatics- GG: 22.9%; GA: 40.5%; AA: 36.6%; in control group- GG: 5.3%; GA:49.1%; AA: 45.6%. There is statistical difference between these groups (p<0.001).

In the genetic models we had considered as Major allele: A and minor G.

In the Dominant model (GG+AG vs AA) those who are homozygous for the allele A have no increased risk of having asthma (crude: OR: 0.688[.441;1.075]; p<sup>a</sup>value =0.101; adjusted values: OR: 0.709[.441;1.138]; p<sup>b</sup>value =0.155).

In the recessive model those who express allele A (AG+AA) have protection of having asthma when compared with controls (crude) and (adjusted) (OR: 0,187[0,087;0,405]; p<sup>a</sup>value <0,001; OR: 0,145[0,063;0,332]; p<sup>b</sup>value <0,001).

In the additive model 1(AA vs AG) those who are heterozygous with genotype AG have no increased risk of asthma when compared with controls (crude:OR 1,028[0,639;1,653]; p<sup>a</sup>value =0,909 ; adjusted: OR: 0,939[0,562;1,570]; p<sup>b</sup>value =0,810).

In the additive model 2(AA vs GG) those who are homozygous for the allele G (genotypes GG) have increased risk of having asthma when compared with controls 5 times (crude:OR 5,417[2,412;12,164]; p<sup>a</sup>value <0,001); and 7 when adjusted for age and gender (adjusted:OR: 6,873[2,832;16,680]; p<sup>b</sup>value <0,001).

**Table IV.2.20:** Genetic models for IVS20 + 524 G>A-rs944722 iNOS gene polymorphism in cases and controls and their association with the risk of asthma

Dominant model	N cases/controls	OR crude[95%CI]; p <sup>a</sup> value	OR adjusted [95%CI]; p <sup>b</sup> value
GG+AG	97/93	referent	
AA	56/78	0.688[.441;1.075]; p <sup>a</sup> value =0.101	0.709[.441;1.138]; p <sup>b</sup> value =0.155

Recessive Model	N cases/controls	OR crude[95%CI]; p <sup>a</sup> value	OR adjusted[95%CI]; p <sup>b</sup> value
GG	35/9	referent	
AG+AA	118/162	0,187[0,087;0,405]; p <sup>a</sup> value <0,001	0,145[0,063;0,332]; p <sup>b</sup> value <0,001

Additive1	N cases/controls	OR crude[95%CI]; p <sup>a</sup> value	OR adjusted[95%CI]; p <sup>b</sup> value
AA	56/78	referent	
AG	62/84	1,028[0,639;1,653]; p <sup>a</sup> value =0,909	0,939[0,562;1,570]; p <sup>b</sup> value =0,810

Additive2	N cases/controls	OR crude[95%CI]; p <sup>a</sup> value	OR adjusted[95%CI]; p <sup>b</sup> value
AA	56/78	referent	
GG	35/9	5,417[2,412;12,164]; p <sup>a</sup> value <0,001	6,873[2,832;16,680]; p <sup>b</sup> value <0,001



**Results:** We had 171 controls and 153 asthmatics. For this polymorphism: in asthmatics the frequencies of allele G were 0.43 and allele A: 0.57 In control group, the frequencies of allele G were 0.30 and for allele A : 0.70 ; (OR: 1.785; 95%CI: [1.291-2.468];  $p < 0.001$ ). There is statistical difference between these groups ( $p < 0.001$ ) with an increased risk of being asthmatic for those that express the Allele G of 1.8 times in asthmatics vs controls.

In the Dominant model (GG+AG vs AA) those who are homozygous for the allele A have no increased risk of having asthma (crude: OR: 0.688[.441;1.075];  $p^a$ value =0.101;adjusted values: OR: 0.709[.441;1.138];  $p^b$ value =0.155).

In the recessive model those who express allele A (AG+AA) have protection of having asthma when compared with controls (crude) and (adjusted) (OR: 0,187[0,087;0,405];  $p^a$ value  $< 0,001$ ;OR: 0,145[0,063;0,332];  $p^b$ value  $< 0,001$  ).

In the additive model 1(AA vs AG) those who are heterozygous with genotype AG have no increased risk of asthma when compared with controls ( crude:OR 1,028[0,639;1,653];  $p^a$ value =0,909 ; adjusted: OR: 0,939[0,562;1,570];  $p^b$ value =0,810).

In the additive model 2(AA vs GG) those who are homozygous for the allele G (genotypes GG) have increased risk of having asthma when compared with controls 5 times ( crude:OR 5,417[2,412;12,164];  $p^a$ value  $< 0,001$ ); and 7 when adjusted for age and gender (adjusted:OR: 6,873[2,832;16,680];  $p^b$ value  $< 0,001$ ).

## B) NO ASSOCIATED SYSTEM

### MPO Polymorphism (- 463 GA)- rs2333227

Asthmatic patients (n= 152) were compared with a control group (n=247); the polymorphisms were analyzed by PCR-RFLP (Polymerase chain reaction- restriction fragment length polymorphism). The control group is in HWE ( $\chi^2$  test=0,500 for control group; p value=0,779).

**Table IV.2.21:** Participant's demographic and clinical characteristics

<b>MPO (- 463 GA (rs2333227)</b>	<b>Controls</b>	<b>Asthma</b>	<b>p value</b>
N (%)	247 (61.9)	152 (38.1)	n.a.
Female	106 (42.95)	100(65.8)	<b>&lt;0.001</b>
Male	141 (57.1)	52 (34.2)	
Age (years)	(40,69±11,3 )	(38,27±18,6 )	0.096 <sup>†</sup>
< 15	0 (0.0)	18 (12.0)	
15-30	55 (22.3)	42 (28.0)	<b>&lt;0.001</b>
> 30	192 (77.7)	90 (60.0)	
Atopy/No Atopy	n.a.	127/23 (84.7/15.3)	n.a.
Asthma controlled /			
Asthma not	n.a.	106/44(70.7/29.3)	n.a.
controlled			

Bolded results are less than 0.05 of statistical significance.

p\*  $\chi^2$  test values; the values represent absolute frequencies (relative frequencies, %).

p<sup>†</sup> Non parametric Mann Whitney test; the values represent means ± standard deviation (SD). n.a.: non applicable.

We had studied 247 individuals in the control group and 152 in the asthma group.

The mean age in the control group for 247 individuals is 40,69±11,3 years and the mean age in the asthma group for 150 individuals is 38,27±18,6 years. There is no difference in the distribution of age across groups (p=0.096);(**Table IV.2.21**).

We had studied 106 females(42.95) and 141 males (57.1%) in the control group and 100 females (65.8%) and 52 males (34.2%). There are more women in the asthma group and more males in the control group (p<0.001); (**Table IV.2.21**).

We had studied in the control group: 0 (<15 years)(0,0%); 55(15-30 years)(22,3%); 192(>30 years)(77,7%). In the asthma group there are 18 (<15 years)(12,0%); 42(15-30 years)(28%); 90(>30 years)(60%). There are more younger than 30 years in the asthma group and more older than 30 years in the control group ( $p < 0.001$ ). In the asthmatics there are 127 allergic and 23 non allergic (84.7%/15.3%); and 106 with controlled asthma symptoms and 44 with uncontrolled asthma symptoms (70.7%/29.3%)-Table 1.

There are no differences in the distribution of genotypes by age( controls ( $p = 0,349$ ) and asthma( $p = 0,435$ )), allergy status( $p = 0,585$ ) (asthmatics) and controlled/uncontrolled asthma ( $p = 0,145$ ) (asthmatics).

**Table IV.2.22:** Distribution of alleles and genotypes by groups in **MPO (- 463 GA (rs2333227))**.

<b>MPO (- 463 GA (rs2333227))</b>	<b>Controls</b>	<b>Asthma</b>	<b>p value</b>	<b>OR(95%CI)<sup>a</sup></b>
Allele G	0.70	0.49	$p < 0,001$	2,423 [1,802-3,259]
Allele A	0.30	0.51		
GG n (%)	125(50.6)	29(19.1)	$p < 0,001$	
GA n (%)	97(39.3)	92(60.5)		
AA n (%)	25(10.1)	31(20.4)		

There are differences in the distribution of genotypes between asthmatics and controls ( $p < 0,001$ ) being the GG genotype more frequent in controls (50,6% vs 19,1%) and those who express allele A : GA (60,5% vs 39,3%) and AA (20,4% vs 10,1%) genotypes more frequent in the asthmatics.

The allelic frequencies are in controls: Allele G: 0.70; Allele A: 0.30; and in asthmatics: Allele G: 0.49; Allele A: 0.51. OR: 2,423; IC95%:[1,802-3,259];  $p < 0,001$ ; are significantly different being the Allele A more frequent in asthmatics and Allele G in controls. There is a risk of 2 times of being asthmatic if they express Allele A.

**Conclusion:** In the Dominant model( AA+GA vs GG) those who are homozygous for the allele G (higher enzyme activity) have protection of having asthma( crude: OR:0.230[0.143;0.370];  $p$  value  $< 0.001$ ; adjusted values: OR:0.252[0,155;0,409];  $p$  value  $< 0.001$  ). In the recessive model those who express allele G (GA+GG) have protection of having asthma (OR:0,440[0,248;0,778];  $p$  value =0,005; OR: 0,433[0,240;0,782];  $p$  value =0,005). In the additive model 1(GGvsGA) those who are heterozygous with genotype GA have an increased risk of asthma of almost 4 times when compared with control( crude:OR

4,088[2,493;6,703]; p<sup>a</sup>value <0.001; adjusted: OR:3,690[2,225;6,117]; p<sup>b</sup>value <0.001). In the additive model 2(GGvsAA) those who are homozygous for the allele A (genotypes AA) have an increased risk of asthma of 5 times when compared with control( crude:OR 5,345[2,752;10,382]; p<sup>a</sup>value <0.001; adjusted:OR5,065[2,571;9,975]; p<sup>b</sup>value <0.001) (Table V.1.23).

**Table IV.2.23:** Genetic models in **MPO** (- 463 GA (rs2333227 ) in cases and controls and their association with the risk of asthma: susceptibility of disease.

Dominant model	N cases/controls	OR crude[CI]; p <sup>a</sup> value	OR adjusted[CI]; p <sup>b</sup> value
AA+GA	123/122	referent	referent
GG	29/125	0.230[.143;0.370]; p <sup>a</sup> value <0.001	0.252[0,155;0,409]; p <sup>b</sup> value <0.001

Recessive Model	N cases/controls	OR crude[CI]; p <sup>a</sup> value	OR adjusted[CI]; p <sup>b</sup> value
AA	31/25	referent	referent
GA+GG	121/222	0,440[0,248;0,778]; p <sup>a</sup> value =0,005	0,433[0,240;0,782]; p <sup>b</sup> value =0,005

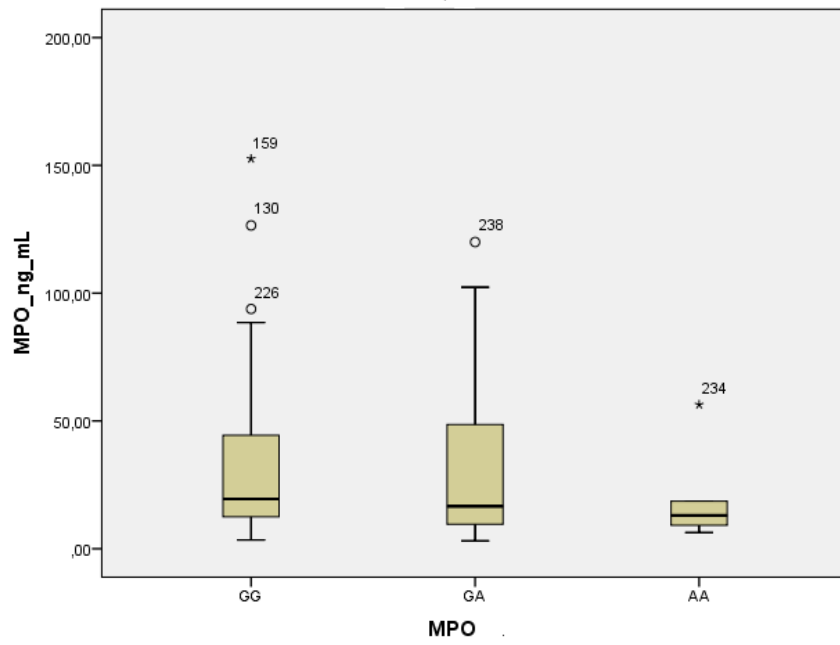
Additive1	N cases/controls	OR crude[CI]; p <sup>a</sup> value	OR adjusted[CI]; p <sup>b</sup> value
GG	29/125	referent	referent
GA	92/97	4,088[2,493;6,703]; p <sup>a</sup> value <0.001	3,690[2,225;6,117]; p <sup>b</sup> value <0.001

Additive2	N cases/controls	OR crude[CI]; p <sup>a</sup> value	OR adjusted[CI]; p <sup>b</sup> value
GG	29/125	referent	referent
AA	31/25	5,345[2,752;10,382]; p <sup>a</sup> value <0.001	5,065[2,571;9,975]; p <sup>b</sup> value <0.001

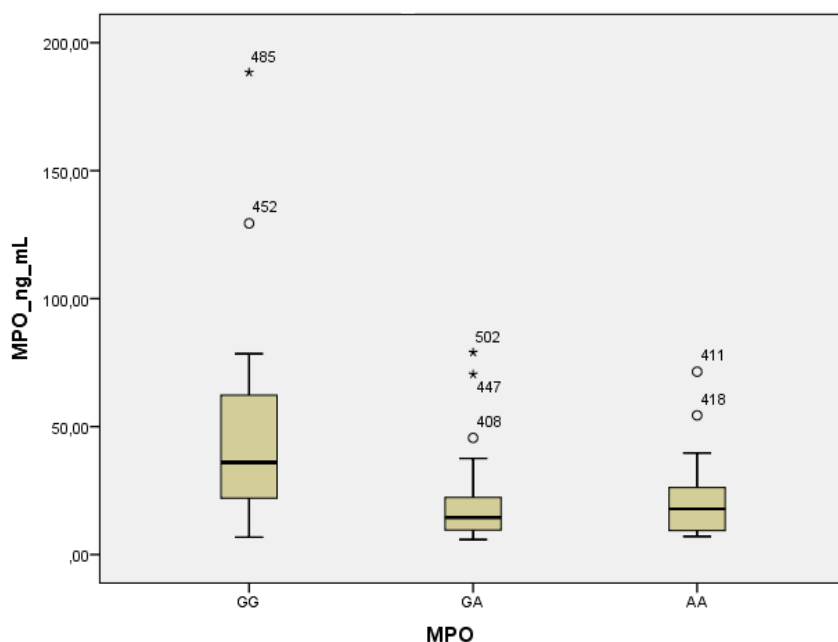
## **MPO LEVELS BY GENOTYPES IN CASES AND CONTROLS**

The role of myeloperoxidase gene promoter region polymorphism in asthma. Myeloperoxidase (MPO) is important in regulating oxidative stress through production of hypohalogenic oxidants that may further mediate oxidative modification of lipids, proteins and DNA, and also has a key role in innate immune system. MPO may also be involved in the pathophysiology of irreversible airflow obstruction in asthmatics. The purpose of this study is to analyze the association between single nucleotide polymorphism in the MPO promoter region of the gene, -463G>A (rs2333227) with asthma severity when compared with a control group of healthy blood donors and its relationship with MPO levels (determined by ELISA kit).

Material and Methods: Asthmatic patients (n= 90) were compared with a control group (n=65); the polymorphisms were analyzed by PCRRFLP (Polymerase chain reaction restriction fragment length polymorphism). MPO levels determined by ELISA kit. Control of asthma assessed by validated instrument (ACQ7 and PAQLQ). Statistical analysis was performed with PASW version 18 establishing a significance level of  $p < 0.05$ .



**Fig.IV.2.9:** MPO levels by genotype in control group (p=0.514).



**Fig.IV.2.10:**MPO levels by genotype in asthma group( $p<0.001$ ).

The mean MPO levels were no different in asthmatics ( $N=90$ ) ( $25.18 \pm 26.57$  ng/mL) when compared with control group ( $N=65$ ) ( $32.83 \pm 33.94$  ng/mL) ( $p=0.526$ ) ( $p>0.05$ )-Mann-Whitney non-parametric test.

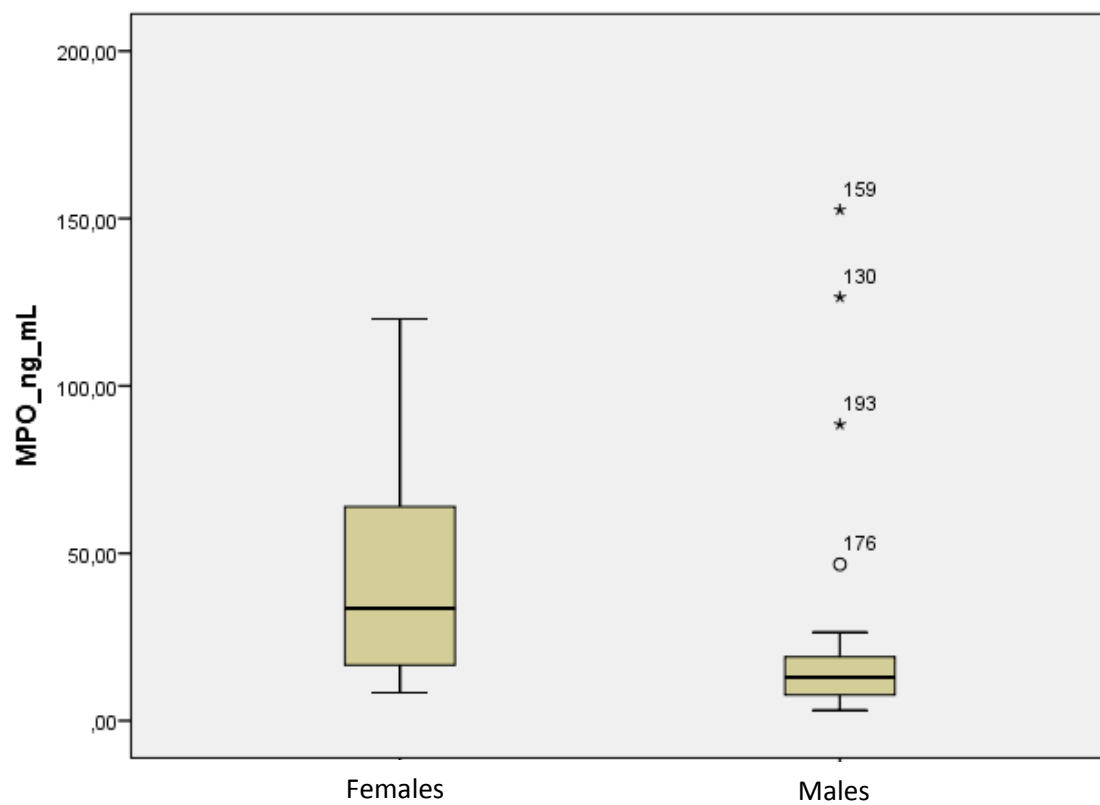
In the control group the distribution of MPO ng/mL by genotypes (mean $\pm$ SD; min,max): GG: ( $36,60 \pm 37.53$ ; 3.4; 152.6) GA: ( $31.53 \pm 32.30$ ; 3.1; 120) AA: ( $19.43 \pm 18.59$ ; 6.3; 56.4).

In the asthma group the distribution of MPO ng/mL by genotypes (mean $\pm$ SD; min,max): GG: ( $51.51 \pm 47.28$ ; 6.8; 188.4) GA: ( $18.55 \pm 13.82$ ; 5.85; 79) AA: ( $22.37 \pm 17.32$ ; 7.1; 71.45).

There are differences in MPO levels by genotypes ( $p<0.001$ ) (Kruskal-Wallis non parametric Test) in the asthmatics: GG: ( $51.51 \pm 47.28$ ; 6.8; 188.4) GA: ( $18.55 \pm 13.82$ ; 5.85; 79) AA: ( $22.37 \pm 17.32$ ; 7.1; 71.45). ; being the AA and GA with lower MPO levels than GG.

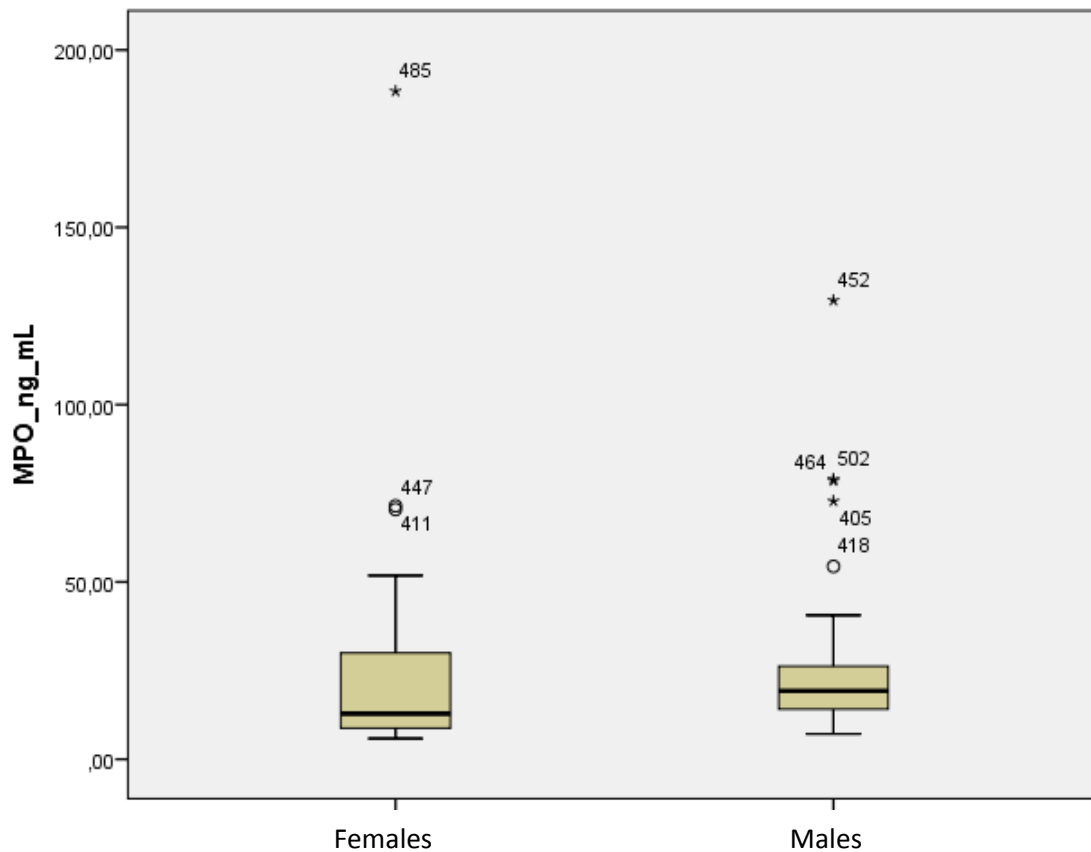
There are no differences in MPO levels by genotypes ( $p=0.514$ ) in the control group : GG: ( $36,60 \pm 37.53$ ; 3.4; 152.6) GA: ( $31.53 \pm 32.30$ ; 3.1; 120) AA: ( $19.43 \pm 18.59$ ; 6.3; 56.4);  $p=0.393$ ) (Kruskal-Wallis non parametric Test).

Distribution of MPO levels by gender in controls



**Fig.IV.2.11:** MPO levels by gender in controls (Mann-Whitney non parametric test ( $p < 0,001$ ))



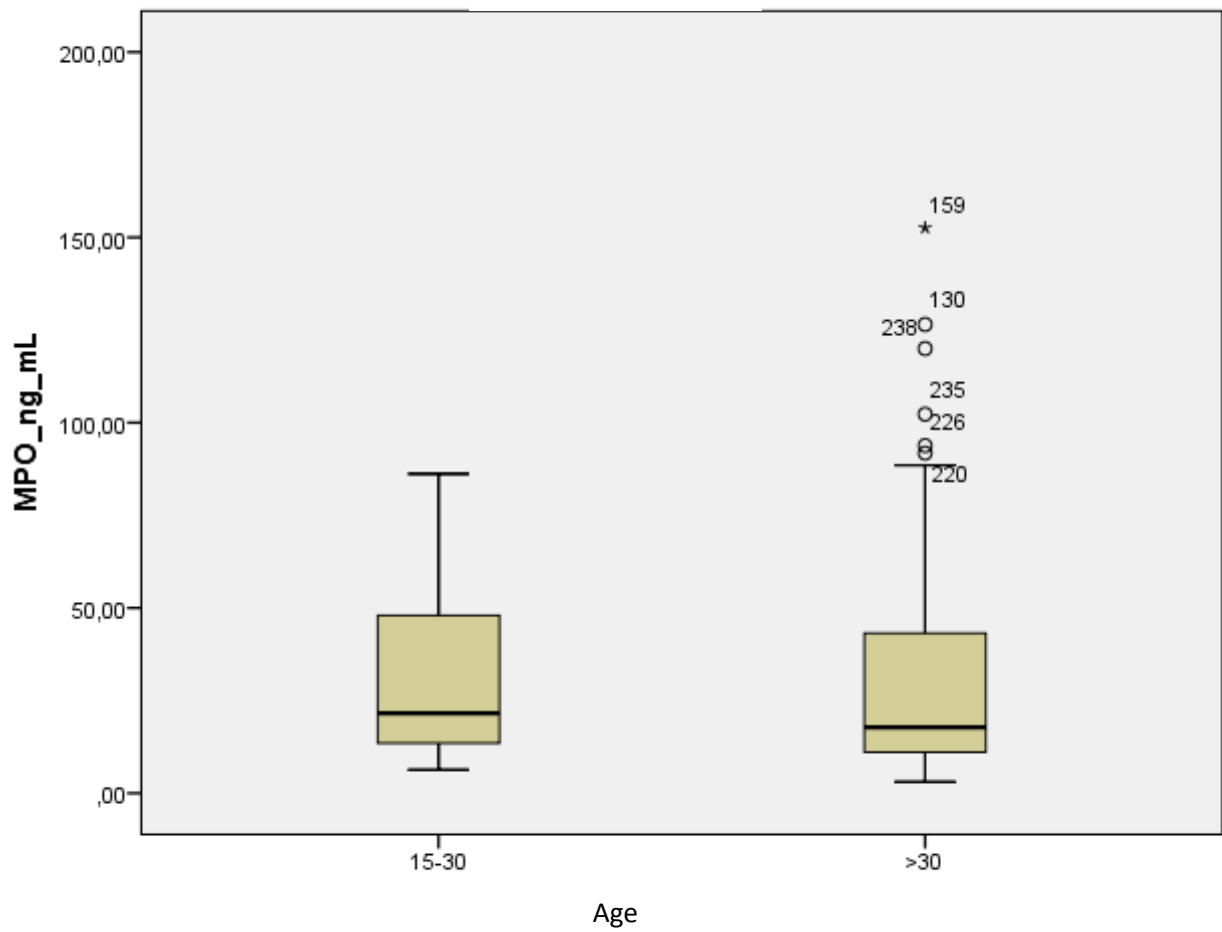


**Fig.IV.2.12:** MPO levels by gender in asthmatics (Mann-Whitney non parametric test ( $p=0.038$ ))

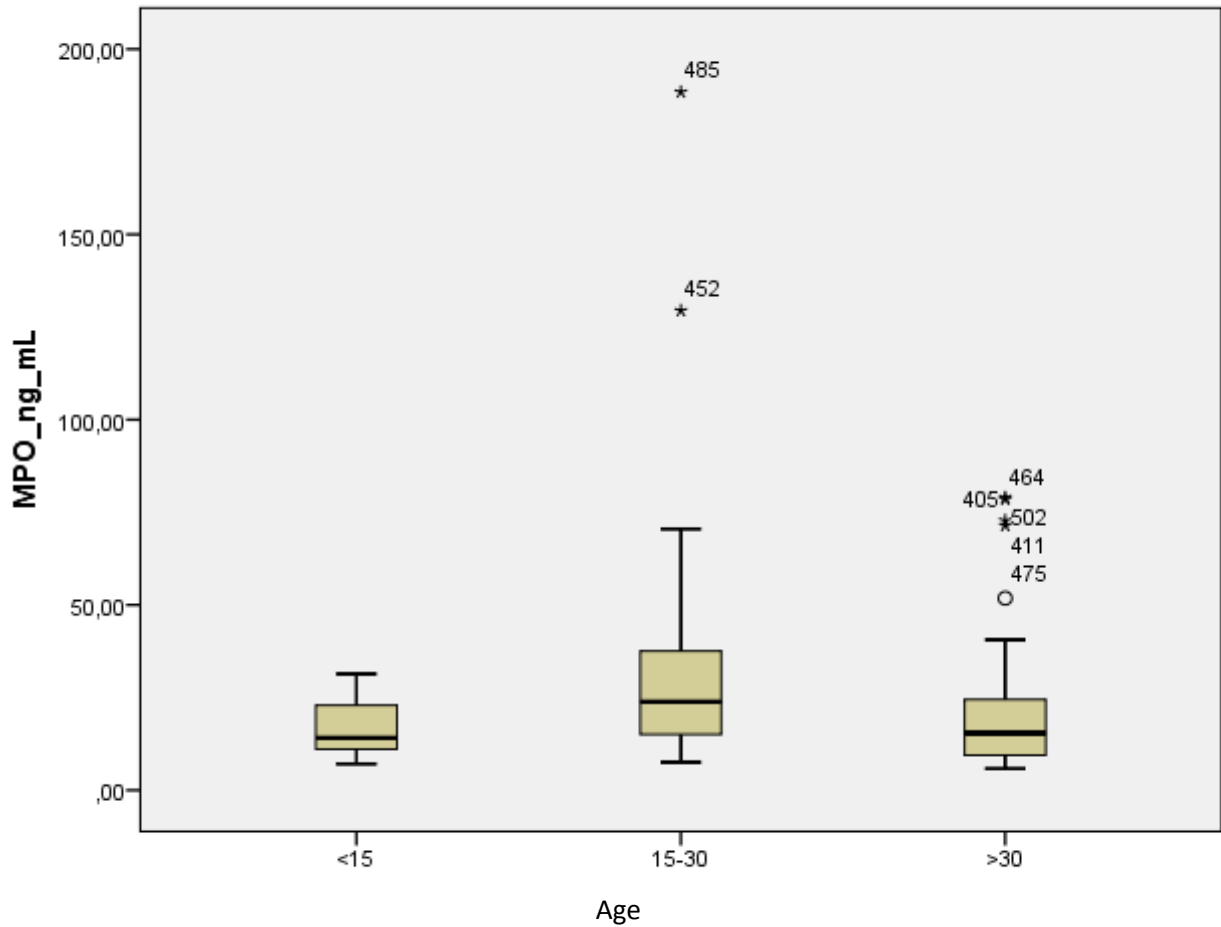
There are differences in MPO levels by gender in asthmatics ( $p=0.038$ ) and in control group ( $p=0.000$ ), having the women in controls higher levels and in the asthmatics lower levels.

Control Group: women (mean $\pm$ SD; min,max): (43.30 $\pm$ 31.87;8.4;120) ; men (mean $\pm$ SD; min,max): (23.29 $\pm$ 33.38;3.1;152.6).

Asthma group: women (mean $\pm$ SD; min,max): (23.84 $\pm$ 29.83;5.85;188.41) ; men (mean $\pm$ SD; min,max): (26.51 $\pm$ 23.11;7.1;129.40).



**Fig.IV.2.13:**MPO levels by age cutoff in control group (  $p=0,579$ ; Mann-Whitney non-parametric test).



**Fig.IV.2.14:**MPO levels by age cutoff in asthma group (p=0,080; Mann-Whitney non-parametric test).

There are no differences in MPO levels by age cutoff(<15; 15-30; >30 years) in asthmatics (p=0.080) and in control group (p=0.579). There are no differences in the distribution of MPO levels by, allergy status (p=0.946)(asthmatics)and controlled/uncontrolled asthma (p=0.693)(asthmatics).

**Conclusion:** The mean MPO levels were no different in asthmatics(N=90) (25.18 ±26.57 ng/mL) when compared with control group (N=65) (32.83 ±33.94 ng/mL)(p=0.526) (**p>0.05**)-Mann-Whitney non-parametric test.

There are differences in MPO levels by genotypes(p<0.001)( Kruskal-Wallis non parametric Test) in the asthmatics: GG: GG: (51.51±47.28;6.8;188.4) GA:(18.55±13.82;5.85;79) AA: (22.37±17.32;7.1;71.45). ; being the AA and GA with lower MPO levels than GG .

There are no differences in MPO levels by genotypes( $p=0.514$ ) in the control group : GG: (36,60±37.53;3.4;152.6) GA:(31.53±32.30;3.1;120 ) AA: (19.43±18.59;6.3;56.4);  $p=0.393$ (Kruskal-Wallis non parametric Test).

There are differences in MPO levels by gender in asthmatics ( $p=0.038$ ) and in control group ( $p=0.000$ ), having the women in controls higher levels and in the asthmatics lower levels

### C) BETA2ADRENERGIC RECEPTORS

#### Polymorphism of beta2adrenergic Receptor Arg16 Gly (rs1042713)

The Arg16Gly polymorphism determined with polymerase chain reaction- restriction fragment length polymorphism(PCR-RFLP). We had 91 individuals in the control group and 84 asthmatics. The control group is not in HWE ( $\chi^2$  test=7,134 for control group;  $p$  value=0,028).

**Table IV.2.24:**Demographic and clinical characteristics of the study population

<b>Beta2 adrenoreceptor polymorphisms: Arg16Gly (rs1042713)</b>	<b>Controls</b>	<b>Asthma</b>	<b>p value</b>
N (%)	91 (52.0)	84(48.0)	n.a.
Female	75 (82.4)	47(56.0)	<0.001
Male	16 (17.6)	37 (44.0)	
Age (years)	(52,31±14,22 )	(37,08±18,5 )	$p<0.001^\dagger$
< 15	0 (0.0)	11(13.1)	$p<0.001$
15-30	6(6.6)	24 (28.6)	
> 30	85 (93.4)	49 (58.3)	
Atopy/No Atopy	n.a.	75/9 (89.3/10.7)	n.a.
Asthma controlled / Asthma not controlled	n.a.	58/26(69.0/31.0)	n.a.

Bolded results are less than 0.05 of statistical significance.

$p^*$   $\chi^2$  test values; the values represent absolute frequencies (relative frequencies, %).

$p^\dagger$  T test for independent samples; the values represent means  $\pm$  standard deviation (SD).

n.a.: non applicable.

We had studied 91 (52%) individuals in the control group and 84 (48%) asthmatics.

Distribution of gender in asthmatics and controls that had been successfully genotyped to ADRB2 polymorphism Arg16 Gly(rs1042713): There are in the control group 75 ( 82,4% ) women and 16 (17,6%)men. There are in the asthma group 47 (56,0%) women and 37 (44,0%)men. There are more women in the control group( $p < 0.001$ ).

The distribution of age across both groups successfully genotyped to ADRB2 polymorphism Arg16 Gly(rs1042713).

There are differences in the age between groups ( $p < 0.001$ ). Being the asthmatics ( $\bar{x} = 37.08 \pm 18.488$ ) younger than the control group ( $\bar{x} = 52.31 \pm 14.219$ ) ( Student's t parametric test).

There are no differences in the distribution of gender among the different genotypes in the control group ( $p = 0,432$ ). There are no differences in the distribution of gender among the different genotypes in the control group ( $p = 0,520$ ). There are no differences in the distribution of controlled and uncontrolled asthma among the asthmatic group ( $p = 0,432$ ). There are no differences in the distribution of controlled and uncontrolled asthma among the genotypes (AA,AG,GG) in the asthmatic group ( $p = 0,592$ ). There are no differences in the distribution of allergic and nonallergic asthma among the genotypes (AA,AG,GG) in the asthmatic group ( $p = 0,592$ ).

The variables age and gender are not independent and being male and younger than 30 years (younger than 30 years :OR:10,119[3,974;25,767];  $p$  value  $< 0,001$  )increases per se the risk of asthma in this hospital based population (gender male.OR:3,690[1,850;7,361];  $p$  value  $< 0,001$  ).

**Table IV.2.25:**Distribution of alleles and genotypes by groups in **Beta2 adrenoreceptor polymorphisms :Arg16Gly (rs1042713)**

<b>Arg16Gly (rs1042713)</b>	<b>Controls</b>	<b>Asthma</b>	<b>p value</b>	<b>OR(95%CI)<sup>a</sup></b>
Allele A	0.59	0.61	0,710	1,111 [0,724-1,705]
Allele G	0.41	0.39		
AA n (%)	23(25.3)	21(25.0)	0.274	
AG n (%)	61(67.0)	61(72.6)		
GG n (%)	7(7.7)	2(2.4)		

We had studied in the control group AA (25,3%), AG (67,0%) and GG (7,7%) in the asthma group we had AA (25,0%), AG (72,6%) and GG (2,4%). There are no differences in genotype frequencies distribution between groups ( $p=0,274$ ) (**Table IV.2.25**).

The allelic frequencies between controls :Allele A (0.59); Allele G (0.41); and asthmatics: Allele A (0.61) and Allele G (0.39); OR: 1,111; IC95%:[0,724-1,705];  $p=0,710$ ; are not significantly different (**Table V.1.25**).

There is no increased risk of being asthmatics for those that are homozygous for AA: Dominant model(GG+AGvsAA): n.s.( OR: 0,986[0,497;1,952];  $p^a$ value=0,967)crude; n.s.(OR: 1,471[0,666;3,250];  $p^b$ value=0,340) adjusted (age and female gender) (**Table IV.2.25**).

**Genetic models (Table IV.2.26)**:There is no increased risk of being asthmatics for those that are homozygous and heterozygous for Allele A: Recessive model (GG vs AG+AA): n.s.( OR: 3,417[0,689;16,934];  $p^a$ value=0,132)crude; n.s. although there is a trend when adjusted for age and female gender(OR: 5,465[0,908;32,889];  $p^b$ value=0,064) .

There is no increased risk of being asthmatics for those that are homozygous for allele A vs heterozygous AG genotypes (Additive 1 model): n.s.(OR: 1,095[0,549;2,183];  $p$ value=0,796)crude; n.s.(OR: 0,745 [0,334;1,662];  $p$ value=0,472) adjusted(age and gender).

There is no increased risk of being asthmatics for those that are homozygous for allele A vs homozygous for allele G genotypes(Additive 2 model): n.s.( OR: 0,313[0,058;1,678];  $p$ value=0,175)crude.

Although when adjusted for age and gender it becomes significant: those who are homozygous for allele G genotypes have a protection of 90,8% of having asthma (OR: 0,092[0,2010;0,817];  $p$ value=0,032).

**Table IV.2.26:** Genetic models in **Beta2 adrenoreceptor polymorphisms :Arg16Gly (rs1042713)** and risk of asthma: susceptibility of disease.

Dominant model	N cases/controls	OR crude <sup>a</sup> [95%CI]; pvalue	OR adjusted <sup>b</sup> [96%CI]; pvalue
<b>GG+AG</b>	63/68	referent	referent
<b>AA</b>	21/23	0,986[0,497;1,952]; p <sup>a</sup> value=0,967	1,471[0,666;3,250]; p <sup>b</sup> value=0,340

Recessive Model	N cases/controls	OR crude[CI]; p <sup>a</sup> value	OR adjusted[CI]; p <sup>b</sup> value
<b>GG</b>	2/7	referent	referent
<b>AG+AA</b>	82/84	3,417[0,689;16,934]; p <sup>a</sup> value=0,132	5,465[0,908;32,889]; p <sup>b</sup> value=0,064

Additive1	N cases/controls	OR crude[CI]; p <sup>a</sup> value	OR adjusted[CI]; p <sup>b</sup> value
<b>AA</b>	21/23	referent	referent
<b>AG</b>	61/61	1,095[0,549;2,183]; pvalue=0,796	0,745[0,334;1,662]; pvalue=0,472

Additive2	N cases/controls	OR crude[CI]; p <sup>a</sup> value	OR adjusted[CI]; p <sup>b</sup> value
<b>AA</b>	21/23	referent	referent
<b>GG</b>	2/7	0,313[0,058;1,678]; pvalue=0,175	0,092[0,2010;0,817]; pvalue=0,032

**Conclusion:** There is no increased risk of being asthmatics for those that are homozygous for AA: Dominant model(GG+AGvsAA): n.s.(OR: 0,986[0,497;1,952]; p<sup>a</sup>value=0,967)crude; n.s. (OR: 1,471[0,666;3,250]; p<sup>b</sup>value=0,340) adjusted (age and female gender).

There is no increased risk of being asthmatics for those that are homozygous and heterozygous for Allele A: Recessive model (GG vs AG+AA): n.s.( OR: 3,417[0,689;16,934]; p<sup>a</sup>value=0,132)crude; n.s. although there is a trend when adjusted for age and female gender (OR: 5,465[0,908;32,889]; p<sup>b</sup>value=0,064) .

There is no increased risk of being asthmatics for those that are homozygous for allele A vs homozygous for allele G genotypes (Additive 2 model): n.s.( OR: 0,313[0,058;1,678]; pvalue=0,175)crude. Although when adjusted for age and gender it becomes significant: those who are homozygous for allele G genotypes have a protection of 90,8% of having asthma (OR: 0,092[0,2010;0,817]; pvalue=0,032).

## D) DETOXIFICATION

### GSTT1

Asthmatic patients (n= 62) were compared with a control group (n=90); the polymorphisms ( GSTT1) were analyzed by using the PCR-Multiplex technique. There are differences in the distribution of gender between asthmatics (F/M:40(64.5%); 22(35.5)) and controls (F/M:37(41.1%); 53(58.9%)) (p=0,005) being more females in the asthmatics and more males in the control group. The mean age is not different across controls and asthma(p=0.208). Although there are differences when we stratify by age being youngest the asthmatics and older the control group (<**0.001**).

**Table IV.2.27:**Participant's demographic and clinical characteristics

<i>GSTT1 non_null/hull polymorphisms</i>	<b>Controls</b>	<b>Asthma</b>	<b>p value</b>
N (%)	90 (59.2)	62 (40.8)	n.a.
Female	37 (41.1)	40(64.5)	<b>0.005</b>
Male	53(58.9)	22 (35.5)	
Age (years)	(40,49±10,96 )	(37,92±19,94 )	0.208 <sup>†</sup>
< 15	0 (0.0)	8 (13.3)	<b>&lt;0.001</b>
15-30	19 (21.1)	18 (30.0)	
> 30	71 (78.9)	34 (56.7)	
Atopy/No Atopy	n.a.	53/7 (88.3/11.7)	n.a.
Asthma controlled / Asthma not controlled	n.a.	42/18(70.0/30.0)	n.a.

Bolded results are less than 0.05 of statistical significance.

p\*  $\chi^2$  test values; the values represent absolute frequencies (relative frequencies, %).

p<sup>†</sup> non-parametric Mann-Whitney; the values represent means  $\pm$  standard deviation (SD).n.a.: non applicable.



**Table IV.2.28:** Distribution of alleles and genotypes by groups *GSTT1* null/ *non\_null* polymorphisms

<i>GSTT1</i> null/ <i>non_null</i> polymorphisms	Controls	Asthma	p value
<i>GSTT1</i> *0	28 (31.1%)	31(50%)	p=0.029
<i>GSTT1</i> +	62(68.9%)	31(50%);	

In asthmatics the genotype frequencies of *GSTT1*\*0 were: 31(50%) and *GSTT1*+ were: 31(50%); in the control group the genotype frequencies of *GSTT1*\*0 were: 28 (31.1%) and *GSTT1*+ were: 62(68.9%). There are differences in the frequencies of genotypes between asthmatics and controls(p=0.029). being the *GSTT1*\*0 more frequent in asthmatics and *GSTT1*+ in controls.

There is 12.5 times the risk of being allergic asthmatics if they are *GSTT1*\*0 and women (OR:12.449; [1.189-130.429]; p=0.035). There is no increased risk of having uncontrolled asthma symptoms for those that are *GSTT1*\*0, in this hospital based sample(p=0.084).

**Conclusion:** There are differences in the frequencies of genotypes between asthmatics and controls(p=0.029). being the *GSTT1*\*0 more frequent in asthmatics and *GSTT1*+ in controls.

There is 12.5 times the risk of being allergic asthmatics if they are *GSTT1*\*0 and women (OR:12.449; [1.189-130.429]; p=0.035). There is no increased risk of having uncontrolled asthma symptoms for those that are *GSTT1*\*0, in this hospital based sample(p=0.084).

## D) DETOXIFICATION

GSTM1 polymorphism: search for null genotypes (homozygous for the allele GSTM1 \* 0) (M-)

### GSTM1

Asthmatic patients (n= 62) were compared with a control group (n=93); the polymorphisms (GSTM1) were analyzed by using the PCR-Multiplex technique.

**Table IV.2.29:** Participant's demographic and clinical characteristics

<i>GSTM1</i> <i>non_null/null</i> polymorphisms	<b>Controls</b>	<b>Asthma</b>	<b>p value</b>
N (%)	93 (60.0)	62 (40.0)	n.a.
Female	40 (43)	40 (64.5)	<b>0.009</b>
Male	53 (57)	22 (35.5)	
Age (years)	(40,40±10,94 )	(37,92±19,94 )	0.217 <sup>†</sup>
< 15	0 (0.0)	8 (13.3)	
15-30	20 (21.5)	18 (30.0)	<b>&lt;0.001</b>
> 30	73 (78.5)	34 (56.7)	
Atopy/No Atopy	n.a.	53/7 (88.3/11.7)	n.a.
Asthma controlled / Asthma not controlled	n.a.	42/18 (70.0/30.0)	n.a.

Bolded results are less than 0.05 of statistical significance.

p\*  $\chi^2$  test values; the values represent absolute frequencies (relative frequencies, %).

p<sup>†</sup> non-parametric Mann-Whitney; the values represent means  $\pm$  standard deviation (SD).

n.a.: non applicable.

We had studied 90 individuals in the control group and 62 in the asthmatics.

There are differences in the distribution of gender between asthmatics (F/M:40(64.5%); 22(35.5)) and controls (F/M:40(43%); 53(57%)) (p=0,005) being more females in the asthmatics and more males in the control group.

The mean age is not different across controls and asthma( $p=0.217$ ). Although there are differences when we stratify by age being youngest the asthmatics and older the control group ( $<0.001$ ).

**Table IV.2.30:**Distribution of alleles and genotypes by groups *GSTM1* null/ *non\_null* polymorphisms

<i>GSTM1</i> null/ <i>non_null</i> polymorphisms	Controls	Asthma	p value
GSTM1*0	48 (51.6%)	34(54.8%)	p=0.693
GSTM1+	45(48.4%)	28(45.2%)	

In asthmatics the genotype frequencies of GSTM1\*0 were: 34(54.8%) and GSTM1+ were: 28(45.2%); in the control group the genotype frequencies of GSTM1\*0 were: 48 (51.6%) and GSTM1+ were: 45(48.4%). There are no differences in the frequencies of genotypes between asthmatics and controls( $p=0.693$ ).

There is no more susceptibility in the group of asthmatic allergics for the null homozygous GSTM1(  $p=0.998$ ), and to be uncontrolled asthmatics( $p=0.615$ ).

**Conclusion:** There are no differences in the frequencies of genotypes between asthmatics and controls( $p=0.693$ ). There is no more susceptibility in the group of asthmatic allergics for the null homozygous GSTM1( $p=0.998$ ), and to be uncontrolled asthmatics( $p=0.615$ ).

## E) ECZEMA AND ASTHMA

LELP-1 (late cornified envelope-like proline-rich 1) polymorphism [rs7534334] located within the EDC

Asthmatic patients (n= 131) were compared with a control group (n=110); the polymorphisms were analyzed by PCR-RFLP (Polymerase chain reaction- restriction fragment length polymorphism). Control of asthma assessed by validated instrument (ACQ7 and PAQLQ). Statistical analysis was performed with PASW version 18 establishing a significance level of  $p < 0.05$ . The control group is in HWE ( $\chi^2=0.044$ ;  $p=0.978$ ).

**Table IV.2.31:**Participant's demographic and clinical characteristics

LELP1 polymorphism [rs7534334]	Controls	Asthma	p value
N (%)	110 (54.4)	131 (45.6)	n.a.
Female	42 (38.2)	84(64.1)	<b>&lt;0.001</b>
Male	68(61.8)	47 (35.9)	
Age (years)	(42,82±10,88 )	(38,40±19,24 )	0.037 <sup>†</sup>
< 15	0 (0.0)	16 (12.4)	
15-30	15 (13.6)	37 (28.7)	<b>&lt;0.001</b>
> 30	95 (86.4)	76 (58.9)	
Atopy/No Atopy	n.a.	111/18 (86/14)	n.a.
Asthma controlled /			
Asthma not controlled	n.a.	92/37(71.3/28.7)	n.a.

Bolded results are less than 0.05 of statistical significance.

p\*  $\chi^2$  test values; the values represent absolute frequencies (relative frequencies, %).

p<sup>†</sup> non-parametric Mann-Whitney; the values represent means  $\pm$  standard deviation (SD).

n.a.: non applicable.

We had studied 110 individuals in the control group and 131 in the asthmatics.

There are differences in the distribution of gender between asthmatics (F/M:84(64.1%); 47(35.9)) and controls (F/M:42(38.2%); 68(61.8%)) ( **<0.001**).being more females in the asthmatics and more males in the control group.

The mean age is different across controls and asthma (p=0.037- non-parametric Mann-Whitney). There are also differences when we stratify by age being youngest the asthmatics and older the control group (**<0.001**).

**Table IV.2.32:**Distribution of alleles and genotypes by groups in LELP1 polymorphism [rs7534334]

LELP1 polymorphism [rs7534334]	Controls	Asthma	p value	OR(95%CI) <sup>a</sup>
Allele C	0.64	0.67	0,529	0.870 [0.597-1.267]
Allele T	0.36	0.33		
CC n (%)	45(40.9)	60(45.8)	0,745	
CT n (%)	50(45.5)	55(42.0)		
TT n (%)	15(13.6)	16(12.2)		

In asthmatics the frequencies of allele C were 0.67 and allele T: 0.33 In control group, the frequencies of allele C were 0.64 and for allele T : 0.36 ; (OR: 0.870; 95%CI: [0.597-1.267]; p=0.529). There is no statistical difference between these groups (p>0.05). Genotypes in asthmatics were: CC: 45.8%; CT:42 %; TT: 12.2% and for control group: CC: 40.9%; CT: 45.5%; TT: 13.6%. There is no statistical difference between these groups(p=0,745) (p>0.05).

**Genetic models :** Genetic models for LELP1 polymorphism [rs7534334].

In the Dominant model (TT+CT vs CC) those who are homozygous for the allele C have no increased risk of having asthma (crude: OR: 1.221; 95%IC:[0.731;2.038]; p<sup>a</sup>value =0.446;adjusted values: OR: 1.069; 95%IC:[0,624;1,831]; p<sup>b</sup>value =0.807). In the recessive model (TT vs CT+CC) there is no increased risk of having asthma (OR: 1,135; 95%IC:[0,533;2,415]; p<sup>a</sup>value e=0,743;OR: 1,025; 95%IC:[ [0,468;2,248]; p<sup>b</sup>value =0,950). In the additive model 1(CCvsCT) there is no increased risk of having asthma ( crude:OR 0,825; 95%IC:[ [0,479;1,422]; p<sup>a</sup>value =0,488; adjusted: OR: 0,938; 95%IC:[0,529;1,664]; p<sup>b</sup>value =0.828).In the additive model 2(CCvsTT) there is no increased risk of having asthma ( crude:OR 0,800[0,358;1,787]; p<sup>a</sup>value =0.586; adjusted:OR: 0,960; 95%IC :[0,411;2,240]; p<sup>b</sup>value =0.925).

There is no increased risk of being allergic/nonallergic asthmatics for those that are homozygous for CC(p<sup>a</sup>=0,578); (p<sup>b</sup>=0,377).

There is no increased ris of being uncontrolled asthmatics for those that are homozygous for CC(p<sup>a</sup>=0,523); (p<sup>b</sup>=0,486).

There is no increased ris of being allergic/nonallergic asthmatics for those that are homozygous for CC and hetrozygous CT(p<sup>a</sup>=0,556); (p<sup>b</sup>=0,424).

There is no increased risk of being uncontrolled asthmatics for those that are homozygous for CC and heterozygous CT ( $p^a=0,728$ ); ( $p^b=0,672$ ).

There is no increased risk of being uncontrolled asthmatics by genotypes ( $p=0,680$ ).

There is no increased risk of being allergic/non allergic by genotypes ( $p=0,781$ ).

**Conclusion:** In asthmatics the frequencies of allele C were 0.67 and allele T: 0.33. In control group, the frequencies of allele C were 0.64 and for allele T: 0.36; (OR: 0.870; 95%CI: [0.597-1.267];  $p=0.529$ ). There is no statistical difference between these groups ( $p>0.05$ ). Genotypes in asthmatics were: CC: 45.8%; CT: 42%; TT: 12.2% and for control group: CC: 40.9%; CT: 45.5%; TT: 13.6%. There is no statistical difference between these groups ( $p=0,745$ ) ( $p>0.05$ ). There is not a significant evidence, that LELP1 polymorphism (rs7534334) could be a genetic marker for atopic asthma ( $p>0.05$ ) in this hospital-based population.

## F) EPIGENETICS

### MTHFR C677T (rs1801133)

MTHFR C677T polymorphism evaluated by PCR-RFLP. We had 67 individuals in the control group and 139 asthmatics. The control group is in HWE ( $\chi^2$  test=0,796 for control group;  $p$  value=0,671).

**Table IV.2.33:** Demographic and clinical characteristics of the study population

(Polymorphism of the MTHFR C677T (rs1801133))	Controls	Asthma	p value
N (%)	67 (32.5)	139 (67.5)	n.a.
Female	45 (67.2)	90 (64.7)	0.853
Male	22 (32.8)	49 (35.3)	
Age (years)	(38,31±10,21)	(38,04±18,94)	0.636 <sup>†</sup>
< 15	0 (0.0)	17 (12.4)	
15-30	15 (22.4)	39 (28.5)	<b>0,004</b>
> 30	52 (77.6)	81 (59.1)	
Atopy/No Atopy	n.a.	116/21 (84.7/15.3)	n.a.
Asthma controlled / Asthma not controlled	n.a.	98/39 (71.5/28.5)	n.a.

Bolded results are less than 0.05 of statistical significance.

$p^*$   $\chi^2$  test values; the values represent absolute frequencies (relative frequencies, %).

$p^†$  non-parametric Mann-Whitney; the values represent means  $\pm$  standard deviation (SD).

n.a.: non applicable.

We had studied 67 individuals in the control group and 139 asthmatics.

There is no statistical difference in the mean age between groups ( $p=0,636$ ) . there are statistical differences by subgroups of age ( $p=0,004$ ), being youngest the asthmatics and older the control group.

We had 45 (67.2%) females and 22 males (32.8%) in the control group and 90 (64.7%) females and 49 males (35.3%) in the asthmatics. There are no statistical differences between these groups ( $p=0.853$ ).

We had for CC in the control group 20(44.4%) females and 10 males (45.5%) ; for CT in the control group 18(40.0%) females and 8 males (36.4%) ; for TT in the control group 7(15.6%) females and 4 males (18.2%) . There are no statistical differences between these groups ( $p=0.944$ ).

We had for CC in the asthma group 40(44.4%) females and 28 males (57.1%) ; for CT in the asthma group 39(43.3%) females and 16 males (32.7%) ; for TT in the asthma group 11(12.2%) females and 5 males (10.2%) . There are no statistical differences between these groups ( $p=0.355$ ).

For genotype CC: we had 50 (51%) asthmatic patients with controlled asthmatic symptoms and 17(43.6%) with uncontrolled asthmatic symptoms; for genotype CT: we had 38(38.8%) asthmatic patients with controlled asthmatic symptoms and 17(43.7%) with uncontrolled asthmatic symptoms; for genotype TT: we had 10 (10.2%) asthmatic patients with controlled asthmatic symptoms and 5(12.8%) with uncontrolled asthmatic symptoms. There are no statistical differences between these groups ( $p=0.722$ ). Control of asthma assessed by (ACQ7 and PAQLQ).

For genotype CC: we had 6 (28.6%) asthmatic allergic patients and 61(52.6%) with asthmatic non-allergic patients; for genotype CT: we had 13(61.9%) asthmatic allergic patients and 42(36.2%) with asthmatic non-allergic patients; for genotype TT: we had 2 (9.5%) asthmatic allergic patients and 13(11.2%) with asthmatic non-allergic patients. There is a **trend** to have statistical differences between these groups ( $p=0.079$ ), being the CC (52.6%) more frequent in asthmatic allergic patients and CT (61.9%) in asthmatic non-allergic patients.

**Table IV.2.34:** Distribution of alleles and genotypes by groups in Polymorphism of MTHFR C677T (rs1801133)

MTHFR C677T (rs1801133)	Controls	Asthma	p value	OR(95%CI) <sup>a</sup>
Allele C	0.64	0.69	0.421	0.816[0.528-1.260]
Allele T	0.36	0.31		
CC n (%)	30(44.8)	68(48.9)	0.606	
CT n (%)	26(38.8)	55(39.6)		
TT n (%)	11(16.4)	16(11.5)		

In asthmatics the frequencies of genotypes are: CC (48,9%); CT (39,6%); TT (11, 5%). In the controls the frequencies of genotypes are: CC (44,8%); CT (38,8%); TT (16,4%). There are no statistical differences between these groups ( $p=0.606$ ).

The Allele frequencies are: in control group: Allele C:0,64; Allele T: 0,36. In the asthmatics: Allele C:0,69; Allele T: 0,31. There is no increased risk of asthma: OR:0,816; CI 95%:[0,528;1,260];  $p^a$ value=0,421.

**Genetic models:** There is no increased risk of being asthmatics for those that are homozygous for CC: Dominant model(TT+CTvsCC): n.s. (OR: 0,847[0,472;1,520];  $p^a$ value=0,577)crude; n.s. (OR: 0,856[0,475;1,545];  $p^b$ value=0,607) adjusted(age and female gender).

There is no increased risk of being asthmatics for those that are heterozygous and homozygous for allele C: Recessive model (TTvs CT+CC ): n.s.( OR: 1,510; [95%CI ]:[0,658;3,464];  $p^a$ value=0,331)crude; n.s.(OR: 1,596; [95%CI ] [0,688;3,702];  $p^b$ value=0,276) adjusted (age and gender).

There is no increased risk of being asthmatics for those that are homozygous for allele C vs heterozygous CT genotypes(Additive 1 model): n.s.( OR: 0,933; [95%CI ]:[0,495;1,760];  $p$ value=0,831)crude; n.s.(OR: 0,962; [95%CI ]:[0,508;1,823];  $p$ value=0,906) adjusted(age and gender).

There is no increased risk of being asthmatics for those that are homozygous for allele C vs homozygous for allele T genotypes (Additive 2 model): n.s.( OR: 0,642[0,266;1,547];  $p$ value=0,323)crude; n.s.(OR: 0,637[0,260;1,561];  $p$ value=0,324) adjusted (age and gender).



**Conclusion:** There is a trend to have statistical differences between these groups ( $p=0.079$ ), being the CC (52.6%) more frequent in asthmatic allergic patients and CT(61.9%) in asthmatic non-allergic patients.

### IV.3. Clinical study: Haplotype and epistatic interactions

**In control vs asthma** after univariate analysis we considered for the model: GSTT1 (null), MPO (GA or AA) and (GG or GA), Nos2 exon 16 (CT or TT); Nos2 intron 20 (GG); eNOS (bb or ab); ACE (II).

**Statistical analysis with logistic regression: Haplotype analysis** (in the same gene) Exon 16 (CT or TT = 1) and others = 0 and also intron 20 (GG) = 1, all others = 0. Variable Haplotype1 = exa016 CTorTT and intron 20 GG; All others = 0. **Epistatic:** exon16 + intron20 + eNOS + ACE 1 = exa016 CTouTT and intron 20 GG and eNOS bb or ab and ACE II; All others = 0. **Epistatic:** GSTT1, MPO, exon16 + intron20 + eNOS + ACE: 1 = null GSTT1 and MPO GA or AA and exon16 CTorTT and intron 20 GG and eNOS bb or ab and ACE II; All others = 0.

**Controlled / uncontrolled asthma:** NOS2intron16 increase of GT in uncontrolled and ECA ID + II- protective and DD risk. **Epistatic:** NOS2intron16 + ECA = 1 (intron 16 GT and ECA DD) and remaining = 0

**Allergic-Non allergic asthma:** MTHFR: increased CC in asthmatics allergic; GSTT1 null; ECA ID

#### In the analysis of Asthma vs controls:

For the haplotype analysis at NOS 2 , the overall difference in haplotype frequencies between asthmatics and controls pointed to an increased risk of asthma: *there is a risk of being asthmatics when compared with controls for those that are (CT+TT) at Ex16 +14C>T and GG at (intron 20 + 524 G> A gene polymorphisms ( p=0,011), but because of the limited number of individuals due to low variant allele frequency for the haplotype analysis we have to be cautious about these results.*

The epistatic interaction between (CTorTT)at Ex16 +14C>T and GG at (intron 20 + 524 G> A and eNOS bb or ab and ACE II , gene polymorphisms are *n.s.*( Fisher test p=0,497 )

The epistatic interaction between (CTorTT)at Ex16 +14C>T and GG at (intron 20 + 524 G> A and eNOS bb or ab , gene polymorphisms are significant. ( Fisher test p=0,013). The epistatic frequencies between between (CTorTT)at Ex16 +14C>T and GG at (intron 20 + 524 G> A and eNOS bb or ab in asthmatics vs controls pointed to an increased risk of asthma : *there is a risk of being asthmatics when compared with controls for those that are (CT+TT)at Ex16 +14C>T and GG at (intron 20 + 524 G> A and eNOS bb or ab, gene polymorphisms( p=0,013), but because of the limited number of individuals due to low variant allele frequency for the epistatic analysis we have to be cautious about this results.*

The epistatic interaction between MPO GA or AA and GSTT1 null polymorphisms, and asthma risk vs controls. There is a risk of being asthmatic when compared with controls for those that are: MPO GA or AA and GSTT1 null polymorphisms) (Qui square; p=0,004). We obtained an OR crude<sup>a</sup>:3.125[1.489-6.558]; p<sup>a</sup>=0.003; OR adjusted ( for age and

gender)<sup>b</sup>:3.151[1.458-6.808];  $p^b=0.004$  it increases the risk of being asthmatic of approximately 3 times.

**In the analysis of Controlled vs uncontrolled asthma:**

The epistatic interaction between NOS2: IVS16+ 88T>G (GT) and ACE I/D(DD) polymorphisms, and uncontrolled asthma risk in asthmatics. There is a risk of being uncontrolled asthmatic when compared with controlled asthmatics for those that are: IVS16+ 88T>G (GT) and ACE I/D(DD) polymorphisms (Fisher exact test  $p=0,025$ ). We obtained an OR crude<sup>a</sup>:4.317[1.234-15.107];  $p^a=0.022$ ; OR adjusted (just for age because there are no statistical differences between gender distribution in controlled and uncontrolled asthma) <sup>b</sup>:4.739[1.184-18.968];  $p^b=0.028$  it increases the risk of being uncontrolled of approximately 5 times.

**In the analysis of Allergic vs Non allergic asthma:** (the epistatic interaction between MTHFR(CC) and GSTT1 null and ACE ID where 1; the others are 0).

For the epistatic interaction analysis at MTHFR(CC) and GSTT1 null and ACE ID, the overall difference in frequencies between allergic asthmatics and non allergics pointed to a trend of increased risk of allergic asthma when compared with non allergic asthma for those that are MTHFR(CC) and GSTT1 null and ACE ID gene polymorphisms( $p=0,085$ ), but because of the limited number of individuals in the non allergics group for the epistatic analysis we have to be cautious about these results.

For the epistatic interaction analysis at MTHFR(CC) and GSTT1 null, the overall difference in frequencies between allergic asthmatics and non allergics pointed to a trend of increased risk of allergic asthma when compared with non allergic asthma for those that are MTHFR(CC) and GSTT1 null gene polymorphisms( $p=0,052$ ), but because of the limited number of individuals in the non allergics group for the epistatic analysis we have to be cautious about these results.

#### IV.4. Clinical study: Endothelial dysfunction in asthma

##### Endothelial dysfunction in asthma

**Background:** Recent studies indicate that endothelial dysfunction is related to abnormalities in nitric oxide (NO) and in the activation of the renin-angiotensin system. Angiotensin converting enzyme (ACE) and nitric oxide (NOS) gene polymorphisms, are important in endothelial dysfunction and in the pathophysiology of asthma. The purpose of this study is to analyze the association between : cytokine-inducible (iNOS or NOS-2) NOS2 (exon 16 + 14C> T); NOS2 (intron 16 + 88 G> T); NOS2 (intron 20 + 524 G> A); constitutive NOS (cNOS): endothelial NOS (**eNOS**), also known as nitric oxide synthase 3 (**NOS3**) , eNOS 4b/4a (27 VNTRs) and ACE gene insertion/deletion (I/D) polymorphisms with asthma susceptibility and severity .

**Material and Methods:** Asthmatics (n= 47) were compared with a control group (n=45). The polymorphisms were analyzed by PCR and PCR-RFLP. Control of asthma assessed by (ACQ7 and PAQLQ). Statistical analysis with PASW version 18 establishing a significance level of  $p < 0.05$ .

**Results:** The mean age of the 47 asthmatics was  $39.04 \pm 18.72$  ; 29 females and 18 males; 39 atopics and 8 non-atopics; 34 with controlled and 13 with uncontrolled asthma. The mean age of the 45 individuals in the control group was  $42.87 \pm 11.01$ ; 14 females and 31 males; There are more women in the asthmatic group when compared with controls ( $p = 0.006$ ) and they are younger than controls ( $p = 0.011$ ) (Table IV.4.1a).

For the different SNPs that we had studied there are differences in the allelic frequencies distribution between controls and asthma for NOS2(Ex16 +14C>T), being the allele T more frequent in asthma. Those who express T allele have a risk of having asthma  $4.387[1.523-12.635]$  ;  $p^b = 0.006$  . For IVS20 + 524G>A there is no differences in allelic distribution of frequencies between controls and asthmatics ( $p^a = 0.094$ ). Although when adjusted for age and gender there is a risk of almost 2 for those who express allele G (  $1.903[1.011-3.583]$ ,  $p = 0.046$ ). (Table IV.4.2a).

For the different SNPs that we had studied there are differences in the allelic frequencies distribution between controlled and uncontrolled asthma: there is a trend (adjusted for age and female gender):OR $2.916[0.970-8.765]$ ;  $p^b = 0.057$ . For NOS2 (intron 16 + 88 G> T)), being a trend for the allele T to be more frequent in asthma. There are differences in the allelic frequencies distribution between controlled and uncontrolled asthma for ACE gene insertion/deletion (I/D) polymorphism: (crude and adjusted for age and female gender):OR:  $0.217[0.068-0.697]$ ;  $p^a = 0.010$ ;OR:  $0.132[0.034-0.517]$ ;  $p^b = 0.004$  ; being the Allele I protector to asthma (Table IV.4.2 ).

For each SNP, additive<sub>1</sub> , additive<sub>2</sub> and potential dominant effects were evaluated by combining homozygote and heterozygote variant carriers for comparison with reference : for NOS2 :Ex16 +14C>T: , there are differences between controls and asthmatics in the different genetic models (additive<sub>1</sub>;OR:7.259[1.736-30.350]; p=0.007) and (dominant model;OR:5.311[1.477-19.095]; p=0.011): there is a risk of being asthmatic of 7.3 for those who express genotype CT and 5.3 for those who express the allele T. For NOS2 (intron 20 + 524 G> A): For IVS20 + 524G>A, there are differences between controls and asthmatics in the different genetic models (additive<sub>2</sub>;OR: 4.654[1.165-18.601] ; p=0.030) , there is a risk of being asthmatic of almost 5 for those who express genotype GG. (Table IV.4.3). There is no statistical difference (p>0.05) for the other SNPs studied concerning allelic and genotype frequencies between asthmatics and controls. Trend tests assume ordinal steps to homozygous with major allele, heterozygous and homozygous with minor allele genotypes, respectively, the trend is statistically significant for NOS2:Ex16 +14C>T: 12.08/p=0.00051(Table IV.4.3a)

For each SNP, additive and potential dominant effects were evaluated also by combining homozygote and heterozygote variant carriers for comparison with reference, comparing controlled and not controlled asthma : For IVS16+ 88T>G, there are differences between Controlled and uncontrolled asthmatics in the different genetic models (additive<sub>1</sub>;OR: 12.406[1.576-97.620]; p=0.017) and (dominant ; OR: 7.917[1.389-45.122]; p=0.020): there is a risk of being uncontrolled asthmatic of 12 for those who express genotype GT and almost 8 for those who express the allele T. For ACE I/D there are differences between Controlled and uncontrolled asthmatics in the different genetic models (additive<sub>2</sub>: 0.064 [0.005-0.857]; p=0.038) and (dominant model; OR: 0.146 [0.025-0.845]; p=0.032): there is a protection of being uncontrolled asthmatic for those who express genotype II and for those who express the allele I . For IVS20 + 524G>A, 27-bp repeat in intron 4-eNOS, Ex16 +14C>T there are no differences between Controlled and uncontrolled asthmatics in the different genetic models (additive<sub>1</sub>; additive<sub>2</sub> and dominant model (Table IV.4.4a). Trend tests assume ordinal steps to homozygous with major allele, heterozygous and homozygous with minor allele genotypes, respectively, the trend is statistically significant for ACE I/D polymorphism : 5.67/p=0.0172( Table IV.4.4a).

For the haplotype analysis at NOS 2 , the overall difference in haplotype frequencies between asthmatics and controls pointed to an increased risk of asthma : there is a risk of being asthmatics when compared with controls for those that are (CT+TT)at Ex16 +14C>T and GG at (intron 20 + 524 G> A gene polymorphisms( p=0,012), but because of the limited number of individuals due to low variant allele frequency for the haplotype analysis we have to be cautious about this results.The epistatic interaction between NOS2: IVS16+ 88T>G (GT+TT ) and ACE I/D( ID+DD) polymorphisms, and uncontrolled asthma risk in

asthmatics we obtained an OR crude<sup>a</sup>:5.400[1.345-21.675];  $p^a=0.017$ ; OR adjusted<sup>b</sup>:9.582[1.524-60.227];  $p^b=0.016$  it increases the risk of being uncontrolled of approximately 10 times(*Data not showed*).

From logistic regression analysis including significant results from univariate analysis (Table IV.4.3a), we concluded that *the risk of having asthma for intron 20 + 524 G> A gene polymorphism and NOS2(Ex16 +14C>T, are not independent of each other and gender.*

For the next logistic regression evaluation of genetic risk score, we considered as models: the genotype homozygous for the major allele as 0 and the association of genotypes with minor allele as 1.

Stepwise multivariate logistic regression with backward elimination ( $p$ -value for retention =0.10) was conducted in significant SNPs in asthma vs controls and adjusted for gender and age. We constructed a genetic risk score for each participant by summing the coefficients for each of the resulting variables after stepwise regression analyses. The variables included in the model were: Ex16 +14C>T and gender. For this SNP, the risk genotypes (CT or TT) were coded as 1 and the non-risk genotype(CC) as 0. For gender female was considered as the risk and coded as 1 and male 0. We obtained 4 risk scores of being asthmatic for this model: high genetic risk score=3.187; intermediate genetic risk score:(1.045 or 1.150); low genetic risk score=-0,992.The individuals that has a high genetic risk score according to this model have an increased risk of 14.500 of having asthma comparing to those low genetic risk score.

From logistic regression analysis including significant results from univariate analysis (Table IV.4.4a) between controlled and uncontrolled asthma, we concluded that *the risk of having uncontrolled asthma for NOS2: IVS16+ 88T>G (GT+TT) and ACE I/D(ID+II) polymorphisms, are not independent of each other and age.*

Stepwise multivariate logistic regression with backward elimination ( $p$ -value for retention =0.10) was conducted in significant SNPs between controlled and uncontrolled asthma and adjusted for gender and age. We constructed a genetic risk score for each participant by summing the coefficients for each of the resulting variables after stepwise regression analyse. The variables included in the model were: IVS16+ 88T>G, ACE I/D and age. For these SNPs: the risk genotype for IVS16+ 88T>G (GT or TT) were coded as 1 and the non-risk genotype(GG) as 0; the risk genotype for ACE I/D (DD) were coded as 0 and the non-risk genotype(ID+II) as 1. Age considered as continuous variable. We divided the Genetic risk score in tertiles as T1:  $\leq -2,68$ (low genetic risk score) ; T2:  $>-2,68 \leq -0,98$  (intermediate genetic risk score); T3:  $>-0,98$ (high genetic risk score). The individuals that has a high genetic risk score according to this model have an increased risk of 7.222 of having asthma comparing to those with intermediate genetic risk score. In the uncontrolled group we had 0

individuals in the low grade Genetic risk score that is why it is not used as reference in this logistic regression model(Table IV.4.5a and Table IV.4.6a).

#### **IV.5.Clinical study: Genetic Risk Scores**

**Genetic Risk score of endothelial dysfunction:** ((ACE) and nitric oxide (NOS) gene polymorphisms)); endothelial dysfunction and epigenetics (MTHFR+(ACE) and nitric oxide (NOS) gene polymorphisms)); uncontrolled asthma (IVS16+ 88T>G , ACE I/D and age) and allergic asthma (MTHFR and GSTT1 and ACE and gender).

Stepwise multivariate logistic regression with backward elimination (p-value for retention =0.10) was conducted in significant SNPs in asthma vs controls and adjusted for gender and age. We constructed a genetic risk score for each participant by summing the coefficients for each of the resulting variables after stepwise regression analyses. The variables included in the model were: Ex16 +14C>T and gender. For this SNP, the risk genotypes (CT or TT) were coded as 1 and the non-risk genotype(CC) as 0. For gender female was considered as the risk and coded as 1 and male 0. We obtained 4 risk scores of being asthmatic for this model: high genetic risk score=3.187; intermediate genetic risk score:(1.045 or 1.150); low genetic risk score=-0,992.The individuals that has a high genetic risk score according to this model have an increased risk of 14.500 of having asthma comparing to those low genetic risk score.

From logistic regression analysis including significant results from univariate analysis between controlled and uncontrolled asthma, we concluded that *the risk of having uncontrolled asthma for NOS2: IVS16+ 88T>G (GT+TT ) and ACE I/D( ID+II) polymorphisms , are not independent of each other and age.*

Stepwise multivariate logistic regression with backward elimination (p-value for retention =0.10) was conducted in significant SNPs between controlled and uncontrolled asthma and adjusted for gender and age. We constructed a genetic risk score for each participant by summing the coefficients for each of the resulting variables after stepwise regression analyse. The variables included in the model were: IVS16+ 88T>G , ACE I/D and age. For these SNPs: the risk genotype for IVS16+ 88T>G (GT or TT) were coded as 1 and the non-risk genotype(GG) as 0; the risk genotype for ACE I/D (DD) were coded as 0 and the non-risk genotype(ID+II) as 1. Age considered as continuous variable. We divided the Genetic risk score in tertiles as T1:  $\leq -2,68$ (low genetic risk score) ; T2:  $>-2,68 \leq -0,98$  (intermediate genetic risk score); T3:  $>-0,98$ (high genetic risk score).The individuals that has a high genetic risk score according to this model have an increased risk of 7.222 of having asthma comparing to those with intermediate genetic risk score. In the uncontrolled group we had 0 individuals in the low grade Genetic risk score that is why it is not used as reference in this logistic regression model

**Genetic Risk score of endothelial dysfunction and epigenetics** (MTHFR+eNOs and iNOs and ACE polymorphisms



Stepwise multivariate logistic regression with backward elimination ( $p$ -value for retention =0.10) was conducted in significant SNPs between controlled and uncontrolled asthma and adjusted for gender and age. We constructed a genetic risk score for each participant by summing the coefficients for each of the resulting variables after stepwise regression analyse. The different SNPs: IVS16+ 88T>G and ACE I/D are not independent of MTHFR in logistic regression, but the variables included in the model after Stepwise multivariate logistic regression with backward elimination, were: IVS16+ 88T>G , ACE I/D and age. For these SNPs: the risk genotype for IVS16+ 88T>G (GT or TT) were coded as 1 and the non-risk genotype(GG) as 0; the risk genotype for ACE I/D (DD) were coded as 0 and the non-risk genotype(ID+II) as 1. Age considered as continuous variable. We divided the Genetic risk score in tertiles as T1:  $\leq -2,809$ (low genetic risk score) ; T2:  $>-2,80 \leq -0,99$  (intermediate genetic risk score); T3:  $>-0,98$ (high genetic risk score).

The individuals that has a high genetic risk score (T3) according to this model have an increased risk of 10.889 (OR: 10.889 [1.140-103.977];  $p=0.038$ ) of having uncontrolled asthma comparing to those with low genetic risk score(T1). There is no increased risk between T1 and T2(OR: 5.091 [0.496-52.285];  $p=0.171$ ) or between T2 and T3(OR: 2.139 [0.472-9.699];  $p=0.324$ ).

#### **Genetic Risk Score of uncontrolled asthma**

Stepwise multivariate logistic regression with backward elimination ( $p$ -value for retention =0.10) was conducted in significant SNPs between controlled and uncontrolled asthma and adjusted for age. We constructed a genetic risk score for each participant by summing the coefficients for each of the resulting variables after stepwise regression analyse. The variables included in the model were: IVS16+ 88T>G , ACE I/D and age. For these SNPs: the risk genotype for IVS16+ 88T>G (GT ) were coded as 1 and the non-risk genotype (GG an TT) as 0; the risk genotype for ACE I/D (DD) were coded as 1 and the non-risk genotype(ID+II) as 0. Age considered as continuous variable. We divided the Genetic risk score in tertiles as T1:  $\leq -1,78$  (low genetic risk score) ; T2:  $>-1,78 \leq -0,86$  (intermediate genetic risk score); T3:  $>-0,86$ (high genetic risk score). The individuals that has a high genetic risk score according to this model have an increased risk of almost 22 :OR: 21.818[2.495-190.825]( $p=0,005$ ) of having uncontrolled asthma comparing to those with low genetic risk score. There are differences in age distribution between controlled and uncontrolled asthma: younger the controlled asthmatics: mean $\pm$ SD(34,00 $\pm$ 19,96 years) and older the uncontrolled asthmatics: mean $\pm$ SD (56,46 $\pm$ 14,29 years).

#### **Genetic Risk Score of allergic asthma**

MTHFR and GSTT1 and ACE

**Allergic vs Non allergic asthma:** (the epistatic interaction between MTHFR(CC) and GSTT1 null and ACE ID where 1; the others are 0)

For the epistatic interaction analysis at MTHFR(CC) and GSTT1 null and ACE ID, the overall difference in frequencies between allergic asthmatics and non-allergics pointed to a trend of increased risk of allergic asthma when compared with non-allergic asthma for those that are MTHFR(CC) and GSTT1 null and ACE ID gene polymorphisms ( $p=0,085$ ), but because of the limited number of individuals in the non-allergics group for the epistatic analysis we have to be cautious about these results.

For the epistatic interaction analysis at MTHFR(CC) and GSTT1 null, the overall difference in frequencies between allergic asthmatics and non-allergics pointed to a trend of increased risk of allergic asthma when compared with non-allergic asthma for those that are MTHFR(CC) and GSTT1 null gene polymorphisms ( $p=0,052$ ), but because of the limited number of individuals in the non-allergics group for the epistatic analysis we have to be cautious about these results.

Genetic risk allergic asthma with GST and gender (the only that remain significant after Stepwise multivariate logistic regression with backward elimination)

Stepwise multivariate logistic regression with backward elimination ( $p$ -value for retention  $=0.10$ ) was conducted in significant SNPs in allergics vs non-allergics asthmatics and adjusted for gender and age. We constructed a genetic risk score for each participant by summing the coefficients for each of the resulting variables after stepwise regression analyses. The variables included in the model were: GSTT1 and gender. For this SNP, the risk genotype (GSTT1\*0 null genotype) were coded as 1 and the non-risk genotype (GSTT1\*1 non null genotype) as 0. For gender female was considered as 1. However in this case being female, confers protection of being allergic with OR:0,242 [0.068-0.857];  $p=0,028$ ; and male 0.

We obtained 4 risk scores of being allergics asthmatics for this model: high genetic risk score= $22.744$ ; intermediate genetic risk score:( $2.944$  or  $20.493$ ); low genetic risk score= $0.693$ .

There are differences between allergic and non-allergic asthmatics ( $p=0,007$ ) in the 4 risk scores of being allergics asthmatics for this model: high genetic risk score= $22.744$  (11/0); intermediate genetic risk score:(  $2.944$  (19/1) or  $20.493$  (11/0)); low genetic risk score= $0.693$  (12/6) respectively.

The individuals that has a intermediate genetic risk score according to this model have an increased risk of 15.000 of being allergic asthmatics comparing to those with low genetic risk score (OR: 15.000) [1.629-138.156];  $p=0,017$ . The individuals that has a high or intermediate genetic risk scores according to this model have an increased risk of 20.500 (OR: 20.500) [2.243-187.355];  $p=0,007$  of being allergic asthmatics comparing to those with low genetic risk score. We didn't compare high with low genetic risk scores because there is no individuals in the group of non-allergic with high genetic risk score.

**CHAPTER V. Discussion**

## CHAPTER V. Discussion

Many shared pathways (134-138) ,are common to CVD and asthma , and this approach could elucidate about risk genes and its polymorphisms as emerging risk factors, with disease developing processes with similar pathogenesis and pleiotropic effects.

**For the The I / D ACE polymorphism** that might be related to hypertension and the D allele predisposes to higher blood pressure values, as well as to four major groups of diseases (obesity, dyslipidemia, diabetes and hypertension ) and is associated with higher ACE levels (31) that is a risk factor for cardiovascular disease . Clinical trials reported a role for genes encoding components of the renin angiotensin system (*ACE* and *AT1R*) in influencing hypertension and other CVD , and provided a possible interaction between these two loci.

***In this literature review and meta-analysis for CVD in this thesis***, if the DD genotype is present and susceptibility to Heart condition (CAD; EAM; cardiomyopathy; HTA) The risk of having heart condition in those that are DD is almost 2, and if the II genotype is present and susceptibility to Heart condition (CAD; EAM; cardiomyopathy; HTA) there is a trend of having heart condition in those that are II and the risk is 53,5% decreased.

In asthma some authors found DD genotype of ACE polymorphism was involved in susceptibility to asthma(139),although not related with the degree of airway obstruction (140). But for others D allele of ACE polymorphism did not show an association with either asthma or asthma severity (141)(31).

***In this study for asthma patients***: we concluded that those who has an allele D have protection of having asthma (OR crude: 0,465[0,251;0,861]; pvalue=0,015; 53,5% decreased risk);(OR adjusted:0,496[0,259;0,949]; pvalue=0,034; 50,4% decreased risk) in this hospital - based population. Genotypes II are more prevalent in the asthmatics than controls.

In asthma and in a hospital-based population, the ACE SNP genotype II in intron 16 (287 bp on chromosome 17q23 (rs1799752)) was found to be the most frequent ( $p < 0.05$ ). So, patients with asthma in which genotype II is more prevalent could have a similar prevalence of genetic risk factors for cardiovascular disease as in the Portuguese population in general.

**In the polymorphism in type 1 receptor of Angiotensin II (AGTR1) 1166A / C (rs5186)** does not appear to be a risk factor for asthma ( $p > 0.05$ ) in the studied group although it may be for cardiovascular disease. Allele C of this polymorphism is associated with hypertension namely essential hypertension(142).

For some authors the Genotypes CC are associated with Pulmonary arterial hypertension (PAH) developed at later age in the 1166C polymorphism in AGTR1(142) .In sarcoidosis AT2R1 genotypes CC and AA possibly increase the risk (30) or have no interference with the prognosis of respiratory disease(31).

***In this literature review and meta-analysis for CVD in this thesis:*** The risk of having heart condition in those that express allele C there is a trend to be decreased although n.s. The risk of having heart condition in those that express CC genotype is non significant.

***In this study for asthma patients:*** In this study group there is not a significant evidence, that AGTR1 gene A1166C polymorphism could be a genetic marker for the pathophysiology of asthmatic disease.

**For Haptoglobin** there has been implication between carotid intima-media thickness, CVD mortality, and Type 2 Diabetes ( T2DM) and Hp2-2 polymorphism (143). Other studies found (Hp1-1) polymorphism as a risk factor for coronary heart disease (CHD) mortality (91).

Free Hb and Hp bound to Hb inactivate NO, Hp alone does not. An increase in the level of circulating Hp-Hb may inhibit NO formation and endothelium relaxation, enhancing the risk of CVD and endothelial dysfunction in asthma. The Hp1-1:Hb complex is removed from circulation more rapidly than the other Hp complexes and might have also a role in CVD and endothelial dysfunction in asthma and NO bioavailability interfering in oxidative stress, inflammation and immunomodulation in asthma (90).

***In this literature review and meta-analysis for CVD in this thesis:*** if the Hp2.2 genotype is present and susceptibility to Heart condition (CHD; CHD and DM;CAD mortality; CABG<45 years). The risk of having heart condition in those that are Hp 2.2 is almost 2. Hp1-1 is associated with non significant overall. If the Hp1.1 genotype is present and susceptibility to Heart condition (CHD; CHD and DM;CAD mortality; CABG<45 years). The risk of having heart condition in those that are Hp 1.1 has although an effect tendentially to be decreased although n.s..

***In this study for asthma patients:*** The distribution of Hp \* 1 and Hp \* 2 allelic frequencies and genotype frequencies (Hp 1-1, Hp 2-1, Hp 2-2) did not present statistically significant differences between the asthmatic patients and the control group ( $p > 0,05$ ). Hp 2-2 present lower circulating protein levels when compared to Hp 2-1 and Hp 1-1, and the difference is statistically significant ( $p = 0.000$ ). Lower levels of Hp in individuals who are homozygous for allele 2 may be associated with an increased risk of cardiovascular disease and other comorbidities that may compromise the prognosis of these patients.

**For the NO associated systems** there are 3 isoenzymes of the nitric oxide synthase (NOS): neuronal (nNOS or NOS I-chromosome 12); Inducible NOS (iNOS or NOS II) - on chromosome 17; Endothelial NOS (eNOS or NOS III) on chromosome 7.

Nitric oxide (NO) originated from NOS, in addition of being a signaling molecule, plays an important role in the defense mechanisms against infectious agents and participates in the inflammatory process, correlating with bronchial hyperreactivity and asthma severity. The amount of NO can be modulated by NOS (I, II, III) polymorphisms. NO either directly or through interference with homocysteine levels may interfere with CVD(44).

The **endothelial nitric oxide synthase (eNOS)** could be a candidate gene for coronary artery disease (CAD) (144) although there is some controversy about this subject (144). Endothelial cells produce nitric oxide (NO), that regulates blood pressure and vascular blood flow. Endothelial nitric oxide synthase (eNOS) gene polymorphisms are associated with coronary artery disease, and other CVD such as hypertension.

***In this literature review and meta-analysis for CVD in this thesis:*** Endothelial nitric oxide synthase (eNOS) is one of the most important candidate genes in coronary artery diseases and CVD in general namely the functional polymorphism (bb) within eNOS gene : a 27 bp VNTR on its intron 4.

These studies had high heterogeneity (68%) the values of fixed (OR:0.91)and random effect (OR:0.88)are very similar and are associated with decreased risk of Heart Disease(HTA) if the genotype b/b is present in eNOS polymorphism and susceptibility to Heart condition(HTA) although n.s.. For a/a: These studies had low heterogeneity (22%) the values of fixed (OR:1.85)and random effect (OR:1.63)are very similar and are associated with increased risk of Heart Disease(HTA) if the genotype aa is present in eNOS polymorphism and susceptibility Heart condition(HTA) although n.s..

***In this study for asthma patients:*** For some authors (145) the bb genotype is associated with pulmonary hypertension and hypoxemia in some patients with respiratory disease..

***In our study group:*** for the polymorphism in eNOS intron 4: Homozygous a / a; Heterozygotes ab; Homozygous b / b shows differences in the distribution of genotypes ( $p < 0.05$ ) between asthmatics and control group. Being the least frequent a /a on asthmatics. Those who express allele b (ab+bb) have an increased risk almost 12 times (crude) and almost 14 times (adjusted) of having asthma when compared with control (OR: 11,972[1,563;91,683];  $p^a$ value =0,017 ;OR: 13,582[1,757;104,967];  $p^b$ value =0,012). Those who are homozygous for the allele a (genotypes aa) have protection of having asthma when compared with control (crude:OR 0,088[0,011;0,678];  $p^a$ value =0.020); adjusted:OR: 0,076[0,010;0,596];  $p^b$ value =0.014).

***In this literature review and meta-analysis for CVD in this thesis*** NOS2 polymorphism (intron 16 - 88GT); NOS2 polymorphism (exon 16-14CT); NOS2 polymorphism (intron 20 - IVS20 + 524 GA): *Usually, there is no inducible nitric oxide synthase (NOS2) in the normal heart (44,104), however macrophages associated with repair following various forms of cardiac damage contain this isoform-NOS2 and its expression is induced by pro-inflammatory mediators.*

However, some authors refer that in some inflammatory diseases such as rheumatoid arthritis there is the potential contribution of inducible and endothelial nitric oxide synthase (iNOS/ NOS2) gene polymorphisms to cardiovascular (CV) events.

Several candidate genes (EGF, LTA, HIF1A, HIF1AN, MMP2, MMP9, iNOS, NOS3 and VEGF) play a role in angiogenesis and endothelial dysfunction. Polymorphisms in angiogenesis-related genes have been associated with CVD and respiratory diseases such as asthma. Inducible nitric oxide synthase (iNOS) catalyzes the synthesis of nitric oxide (NO), which can be proangiogenic and iNOS is overexpressed in some diseases (e.g.: asthma) and some inflammatory cells such as macrophages.

Accelerated atherosclerosis and CVD might be associated with the traditional CV risk factors such as age and hypertension as well as non-traditional risk factors comprising current inflammation associated with asthma and genetic polymorphisms that predisposes to different status of oxidative stress and inflammation. In this multiple risk factor assessment, the risk charts that are based only on traditional risk factors are insufficient to capture CV risk extent in CVD and bronchial asthma.

NOS2 polymorphism (Intron 16 + 88 G> T) (rs9282801) - ***In this study for asthma patients:*** One of the functional polymorphisms with relevance to iNOS enzyme activity constitutes a transition from T to G in intron 16 (rs9282801) . For this polymorphism there is no increased risk of having asthma when compared with controls.

NOS2 polymorphism (intron 20 - IVS20 + 524 GA) - ***In this study for asthma patients*** : One of the functional polymorphisms with relevance to iNOS enzyme activity constitutes a transition from G to A in intron 20 (rs944722). It might affect the splicing or protein expression of the gene increasing NOS2 activity and NO synthesis. We had 171 controls and 153 asthmatics. For this polymorphism: in asthmatics the frequencies of allele G were 0.43 and allele A: 0.57 In control group, the frequencies of allele G were 0.30 and for allele A : 0.70 ; (OR: 1.785; 95%CI: [1.291-2.468]; p<0.001). There are statistical differences between these groups (p<0.001) with an increased risk of being asthmatic for those that express the Allele G of 1.8 times in asthmatics vs controls. In the Dominant model( GG+AG vs AA) those who are homozygous for the allele A have no increased risk of having asthma( crude: OR: 0.688[.441;1.075]; p<sup>a</sup>value =0.101;adjusted values: OR: 0.709[.441;1.138]; p<sup>b</sup>value =0.155). In the recessive model those who express allele A (AG+AA) have protection of having asthma when compared with controls (crude) and (adjusted) (OR: 0,187[0,087;0,405]; p<sup>a</sup>value <0,001;OR: 0,145[0,063;0,332]; p<sup>b</sup>value <0,001). In the additive model 1(AA vs AG) those who are heterozygous with genotype AG have no increased risk of asthma when compared with controls ( crude:OR 1,028[0,639;1,653]; p<sup>a</sup>value =0,909 ; adjusted: OR: 0,939[0,562;1,570]; p<sup>b</sup>value =0,810). In the additive model 2(AA vs GG) those who are homozygous for the allele G (genotypes GG) have increased risk of having asthma when compared with controls 5 times ( crude:OR 5,417[2,412;12,164]; p<sup>a</sup>value <0,001); and 7 when adjusted for age and gender (adjusted:OR: 6,873[2,832;16,680]; p<sup>b</sup>value <0,001). In

relation to INOS intron 20 (IVS20 + 524 G> A (rs944722) the G allele and the GG genotype are more frequent in asthmatic patients when compared with controls.

NOS2 polymorphism (exon 16-14CT)- ***In this study for asthma patients:*** In this polymorphism the T allele is a risk factor for asthma, and the T allele is more frequent in asthmatic patients when compared with controls and is also associated with many diseases namely neoplastic diseases.

Being the genotypes who express allele T more frequent in asthmatics and those who express allele C in controls. This single-nucleotide polymorphisms in coding region, exon16 of iNOS causes non-synonymous amino acid substitution, that is also implicated in CVD. Induction of iNOS due to the different polymorphisms in this gene in cardiovascular tissues could contribute significantly to the depressed pressor response in anti-hypertensive response to vasoactive agents and potentially lead to endothelial dysfunction (44). For this polymorphism in asthmatics the frequencies of Allele C 0.81 and of Allele T 0.19; in controls: 0.92 and 0.08 respectively. There is statistical difference between these groups with a risk of being asthmatic of almost 3 for Allele T (OR:2.858;IC95%[1.409;5.796]; p<sup>a</sup> value =0.004.

Genotypes in the asthmatics- CC: 65.7%; CT: 30.4%; TT: 3.9%; in control group- CC: 86.1%; CT:12.5%; TT: 1.4%. There is statistical difference between these groups (p=0.01).Being the genotypes who express allele T more frequent in asthmatics and those who express allele C in controls.

In the Dominant model( CT+TT vs CC) those who are homozygous for the allele C have a protection of having asthma when compared with controls ( crude: OR: 0.309; ;95%CI: [0.141;0.676]; p<sup>a</sup>value =0.003;adjusted values: OR: 0.326; 95%CI: [0.144;0.738]; p<sup>b</sup>value =0.007).

In the recessive model ( TT vs CT+CC ) have no increased risk of having asthma when compared with controls (crude) and (adjusted) (OR: 0,345;95%CI: [0,038;3,153]; p<sup>a</sup>value =0,346;OR: 0,347; 95%CI: [0,034;3,582]; p<sup>b</sup>value =0,374.

In the additive model 1(CC vs CT) those who are heterozygous with genotype CT have an increased risk of asthma of 3 times when compared with controls ( crude:OR: 3,187 ; 95%CI: [1,406;7,227]; p<sup>a</sup>value =0,006 ; adjusted: OR: 3,002; 95%CI [1,276;7,062]; p<sup>b</sup>value =0,012).

In the additive model 2 (CC vs TT) those who are homozygous for the allele T (genotypes TT) have not increased risk of having asthma when compared with controls ( crude:OR :5,701; 95%CI :[0,403;34,025]; p<sup>a</sup>value =0,248); and 7 when adjusted for age and gender (adjusted:OR: 3,700; , 95%CI :[0,345;39,681]; p<sup>b</sup>value =0,280).

**Myeloperoxidase (MPO)** is an important enzyme (147,148), in the regulation of oxidative stress through the production of hypoalogenated compounds, which may also contribute to the oxidative modification of lipids, proteins and DNA and also play an important role in innate immunity. Still play an important role in irreversible airway obstruction in asthmatics.



MPO can accelerate the process of atherosclerosis through oxidative stress, modifying high and low density lipoproteins and production of other bioactive molecules. The polymorphism (MPO 463G> A, rs2333227) results in different expressions at the MPO level and the AA genotype may be protective against CVD. **Myeloperoxidase (MPO) -463G/A** gene polymorphism may be associated with an increased risk of developing coronary artery disease (CAD) and increased mortality associated with CVD.

***In this literature review and meta-analysis for CVD in this thesis:*** for the genetic model GG/total, this studies had medium heterogeneity (45%) the values of fixed and random effect are very similar and is associated with higher overall effect and a trend to be significant ( $p=0,0521$ ) if the GG genotype is present in ***MPO (-463G>A (rs2333227) polymorphism*** and susceptibility to Heart condition CAD; Nephrosclerosis hypertensive; HTA; Carotid atherosclerosis) in the Fixed effect model. The random overall effect is non significant (ns)( $p=0.1845$ ).

For the Genetic model AA/total( heart disease (CAD; Nephrosclerosis hypertensive; HTA; Carotid atherosclerosis), this studies had low heterogeneity (0%) the values of fixed and random effect are very similar and is associated with higher risk and significant ( if we consider the fixed effect model) if the AA genotype is present in ***MPO (-463G>A (rs2333227) polymorphism*** and susceptibility to Heart condition CAD; Nephrosclerosis hypertensive; HTA; Carotid atherosclerosis) in the Fixed effect model ( $p= 0.0065$ ). This CI95% shows that the overall effect is significant with a global risk effect (fixed effect) is 1,34 of having a heart condition (CAD; Nephrosclerosis hypertensive; HTA; Carotid atherosclerosis) if the AA genotype is present. The study of Nikpoor et al with great weight (28,2%) the CI95% shows that the effect is significant with OR 1,56 of having a heart condition.

***In this study for asthma patients:*** For MPO the polymorphism -463 GA (rs2333227) in the promoter region of the gene, genotypes carrying the allele A are more frequent in asthmatics and the genotypes bearing the G allele more frequent in the controls.

MPO levels: Asthma levels in MPO are higher than in the control group, genotypes expressing the A allele are related to lower levels (being the AA and GA with lower MPO levels than GG) as well as a tendency to have allergic asthma. There are differences in MPO levels by gender in asthmatics ( $p=0.038$ ) and in control group ( $p=0.000$ ), having the women in controls higher levels and in the asthmatics lower levels. Allele A appears to be associated with lower levels of MPO in asthmatic patients as well as a tendency to have allergic asthma and a lower prevalence of CVD, and may be protective (the AA genotype) for premature coronary disease.

**In the beta2-adrenergic receptor (ADRB2) gene**, the polymorphisms: Arg16 Gly (rs1042713) the Arg / Arg homozygotes for the polymorphisms: Arg16 Gly (rs1042713) have a reduced response to beta2 agonists being subject to tachyphylaxis which makes these

patients susceptible to novel therapeutic approaches. The Arg allele was associated with poor asthma control, a worst lung function and bronchial hyperreactivity (60,61).

In relation to hypertension and cardiovascular disease, the in vitro studies of agonist stimulation of the Gly receptors show an increase in downregulation.

***In this literature review and meta-analysis for CVD in this thesis:*** These studies had low heterogeneity (0%) the values of fixed and random effect are very similar and is associated with higher risk although not significant if the AA genotype is present in **Beta2 adrenoreceptor polymorphisms: Arg16Gly (rs1042713)** and susceptibility to Heart condition (MI; CHF and HF; HTA). This CI95% shows that the overall effect is non significant(ns). Even in the study of Leineweber et al with major weight (31,7%) the CI95% shows that the effect is non significant (ns).

These studies had moderately high heterogeneity (56%) the values of fixed and random effect are very similar and is associated with higher risk and significant (if we consider the fixed effect model) if the GG genotype is present in **Beta2 adrenoreceptor polymorphisms: Arg16Gly (rs1042713)** and susceptibility to Heart condition (MI; CHF and HF; HTA). This CI95% shows that the overall effect is significant with a global risk effect( fixed effect) is 1,33 of having a heart condition (MI; CHF and HF; HTA) if the GG genotype is present, and significant. Two studies of Leineweber et al with major weight(31,7%) and Xie et al (weight 9,9%) the CI95% shows that the effect is significant with OR 1,45 and 2.20 respectively of having a heart condition.

***In this study for asthma patients:*** In this study population those who have allele A as homozygous or heterozygous and younger than 30 years, have an increased risk: OR:7,134[1,064;47,842]; p value=0,043 of having asthma.

The major risk factors for cardiovascular disease (CVD) are related to changes in cholesterol metabolism , blood pressure regulation , diabetes, obesity among others(62).However like in respiratory diseases and cancer the exposure to environmental pollutants and tobacco exposure could also increase CVD risk and asthma susceptibility. The concept that the mechanisms of detoxification regulate disease susceptibility through detoxification mechanisms such as Glutathione S-transferases (**GSTs**) is very important in asthma and CVD.

***In this literature review and meta-analysis for CVD in this thesis;*** Genetic model): GSTM1 \* 0/total (heart diseases(HDs) (CAD associated or independent of smoking status; HTA); these studies had high heterogeneity (96%) the values of fixed and random effect are different and is associated with overall effect non significant n.s. (p=0,2789)if the null genotype is present in and susceptibility to Heart condition (CAD associated or independent of smoking status; HTA). Random effects model 1.4878 [0.7248; 3.0543] z=1.08 ;p= 0.2789.

Genetic model : GSTT1 \* 0/total( heart diseases(HDs) (CAD associated or independent of smoking status; HTA); these studies had high heterogeneity (95%) the values of fixed and random effect are different and is associated with overall effect significant ( $p=0,0495$ )if the null genotype is present in and susceptibility to Heart condition (CAD associated or independent of smoking status; HTA). Random effects model 2.3599 [1.0018; 5.5595]  $z=1.96$ ;  $p= 0.0495$ ; with OR of having heart condition of 2 if they are GSTT1 \* 0.

***In this study for asthma patients:*** In this study sample for GSTT1 : There are differences in the frequencies of genotypes between asthmatics and controls( $p=0.029$ ). being the GSTT1\*0 more frequent in asthmatics and for GSTM1 There are no differences in the frequencies of genotypes between asthmatics and controls.

The chromosomal region 1q21 that has been linked to allergy, atopic dermatitis and asthma in previous studies, with a peak linkage overlying the epidermal differentiation complex (EDC) and its genes polymorphisms are also related with an increased risk of CVD.

In this thesis we have studied **LELP1** polymorphism LELP-1(67) (late cornified envelope-like proline-rich 1) polymorphism [rs7534334] located within the EDC complex and There is not a significant evidence, that LELP1 polymorphism (rs7534334) could be a genetic marker for atopic asthma ( $p>0.05$ ) in this hospital-based population.

**MTHFR** could have a role on epigenetics and susceptibility to asthma and CVD and related with homocysteine metabolism representing another model of gene-environment interaction but its role in asthma and atopy is controversial (149).

Elevations in homocysteine may be caused by genetic defects in enzymes involved in its metabolism such as the polymorphism in the gene coding for the 5,10-methylene tetrahydrofolate reductase (MTHFR) (C677T, Ala --> Val) that is associated with a decreased activity of the enzyme due to its thermolability. In case of homozygosity (TT) for the Val allele, a relative deficiency in the remethylation process of homocysteine into methionine leads to a mild-to-moderate hyperhomocysteinemia, a condition recognized as an independent risk factor for atherosclerosis and CVD.

***In this literature review and meta-analysis for CVD in this thesis:*** Genetic model ): TT/total( heart diseases(HDs) (CHD; CAD;MI; CAD <45 years); this studies had low heterogeneity (1%) the values of fixed ( OR:1,21) and random effect (OR:1,20) are very similar and is associated with a non significant overall effect : 1.2114 [0.9756; 1.5042]  $z=1.74$  ;  $p=0.0824$ ,if the genotype TT is present and susceptibility to Heart condition (CHD; CAD;MI; CAD <45 years). Genetic model ): CC/total( heart diseases(HDs) (CHD; CAD;MI; CAD <45 years); this studies had high heterogeneity (85%) the values of fixed ( OR:0,72) and random effect (OR:0,66) are very similar and is associated with a significant overall effect : Random effects model : OR:0.6552 [0.4428; 0.9694]  $z=-2.12$  ;  $p= 0.0344$  ,if

the genotype CC is present and susceptibility to Heart condition (CHD; CAD;MI; CAD <45 years), there is a decreased risk of HDs.

***In this study for asthma patients:*** in this study sample for (MTHFR) (C677T, Ala --> Val) in asthmatics there is a trend to have statistical differences between these groups ( $p=0.079$ ), being the CC (52.6%) more frequent in asthmatic allergic patients and CT(61.9%) in asthmatic non-allergic patients. Epigenetics is likely to be involved in the biology and molecular pathways of the major nosologic cardiac syndromes, putting MTHFR in the pipeline of candidate genes in asthma and CVD.

There is a molecular, cellular and clinical heterogeneity among patients with asthma and patients with CVD. This heterogeneity might link some genetic risk profiles that could be helpful to decide target therapies and delineate prevention programs.

These assumptions define the need of profiling genetic risk scores and noninvasive biomarkers that could better engage against the epidemics of NCDs worldwide and facilitate the assessment of novel target therapies as in asthma as in CVD.

**CHAPTER VI. Conclusions**

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These polymorphisms may lead to different genotype-dependent therapeutic responses as well as different methylation pattern that could contribute to different endotypes / phenotypes in asthma with different probabilities of developing asthma and/or cardiovascular diseases and its most relevant risk factors such as obesity, dyslipidemia, diabetes and hypertension as well as more severe or/and atopic asthma.

The characterization of heterogeneity of asthma and CVD has promoted the concept that these diseases +«of *multiple phenotypes* or consistent groupings of characteristics.

These phenotypes were initially focused on combinations of clinical characteristics, but they are now evolving to *link biology to phenotype*.

The mapping of genetic susceptibility by candidate genes approach and the mechanistic approach of asthma and CVD is considered a hallmark of asthma and CVD, putting the emerging biomarkers and genetic susceptibility to disease in relation to asthma and CVD as the mainstream in its response to target therapy.

Co-morbidities such as Cardiac failure and lifestyle and environmental (like pollutants indoor and outdoor) and social factors not related with the disease-“treatable traits”-could influence its outcome (systemic inflammation non-type 2-alternate asthma paradigm) . Otherwise inflammatory subtypes of asthma (namely allergic inflammation type 2 high-current asthma paradigm) could have biomarkers that points to a better or worst response to corticosteroids and might differentiate among the heterogeneity of the asthma disease and be a better guide to precision medicine and target therapies.

**CHAPTER VII. Future perspectives**

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The association between different gene polymorphisms and the phenotypic expression of heart diseases and susceptibility to asthma and its severity is the main scope of this thesis. With this thesis I also wanted to investigate what could be the influence of genetic factors when compared with conventional risk factors and its additive effect on the outcome of the disease and co-morbidities.

Asthma and CVD has been considered as single diseases for years, but recent studies have increasingly focused on its *heterogeneity linking genetic predisposition to phenotypes*.

The genetic factors and biomarkers will be central and required in future studies when we intended to evaluate a particular outcome, or personalizing emerging therapies (although they might require validation and standardization).

Gene polymorphisms and expression, functionality of protein networks, complex interactions between genetic, epigenetic such as environmental factors, associated with the integration of multiple levels of information is nowadays the hallmark of bioinformatics and systems medicine and could update the progress in pharmacogenomics and pathophysiology of asthma and cardiovascular diseases.



## CHAPTER VIII. References

## CHAPTER VIII. References

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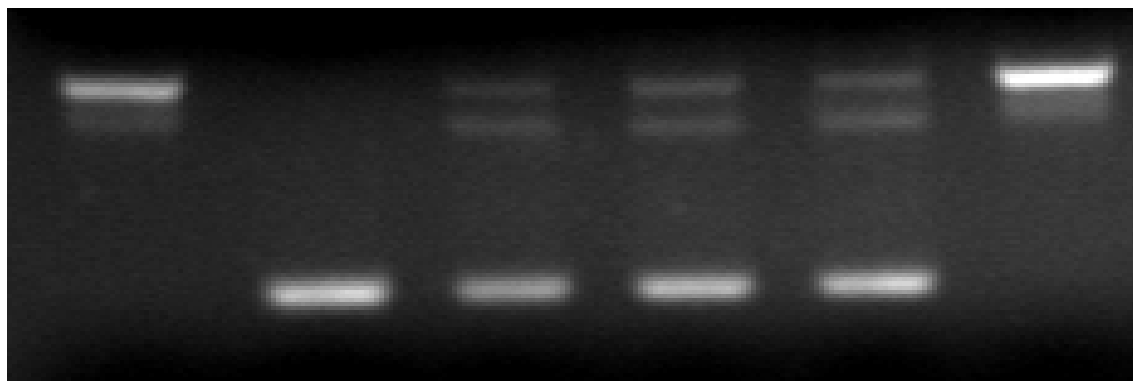
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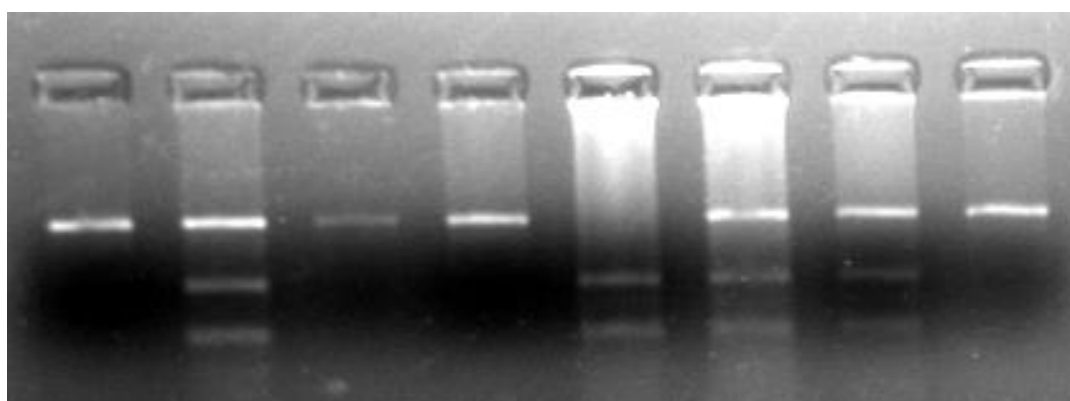
**CHAPTER IX. Annexes**

**CHAPTER IX. Annexes (Figs.)**

Gel photos

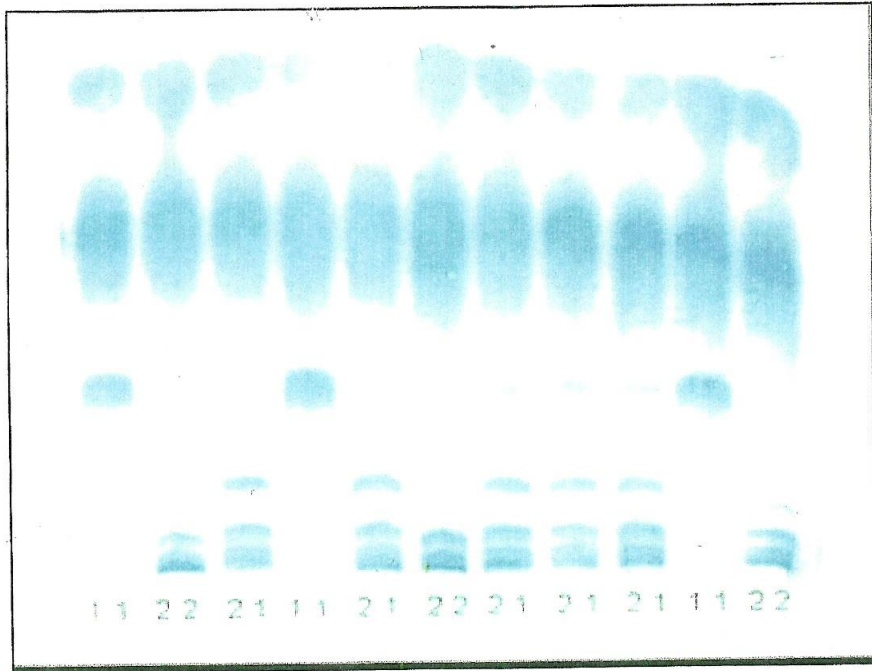


**Fig. 1a** : The insertion/deletion (I/D) polymorphism of ACE (rs4340).

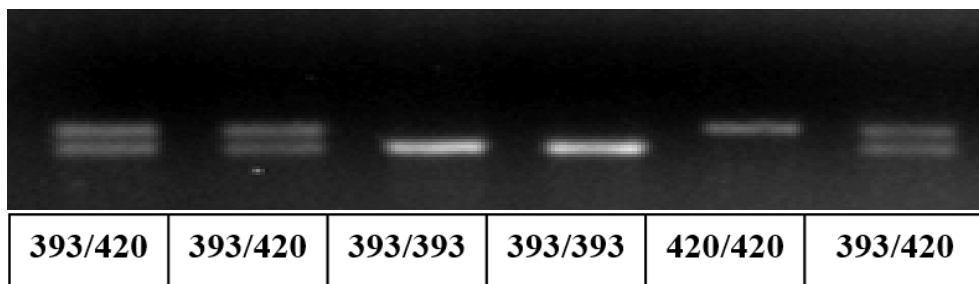


**Fig. 2a** : The polymorphism of AGTR1 1166A/C (rs5186) was determined by PCR-RFLP and, according with the fragment, we have: (lines 1, 3, 4 and 8: **AA** (350 bp); Line 5: **CC** (139 + 211 bp) and Line 2, 6 and 7: **AC** (350 + 139 + 211 bp).

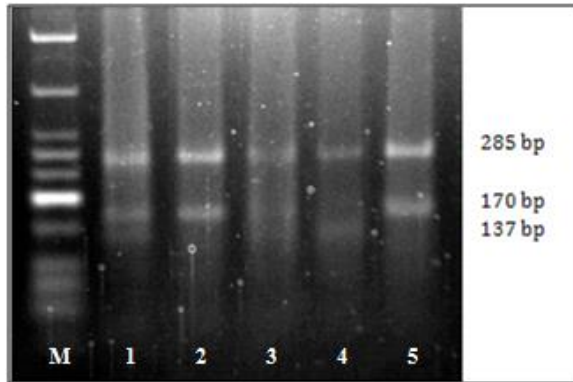




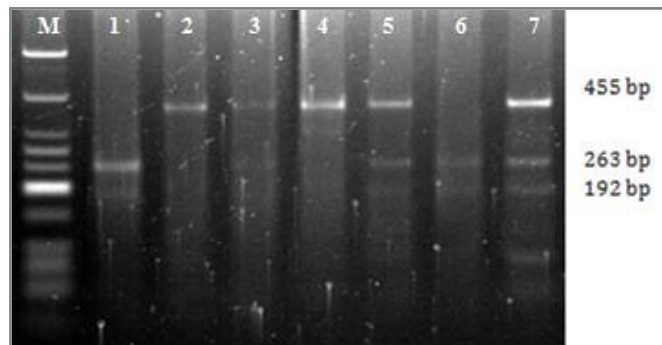
**Fig.3a:** Haptoglobin Genotypes in polyacrylamide gel



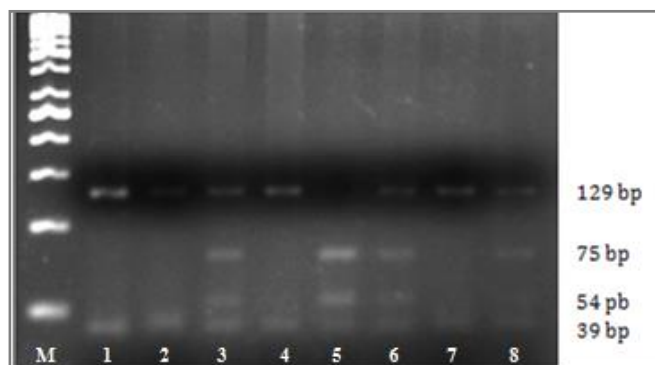
**Fig 4a:**eNos polymorphism(Alelo a/a - 393 bp (Homozygous); Alelo a/b – 393 bp and 420 bp (Heterozygous); Alelo b/b – 420 bp (Homozygous – wild type)



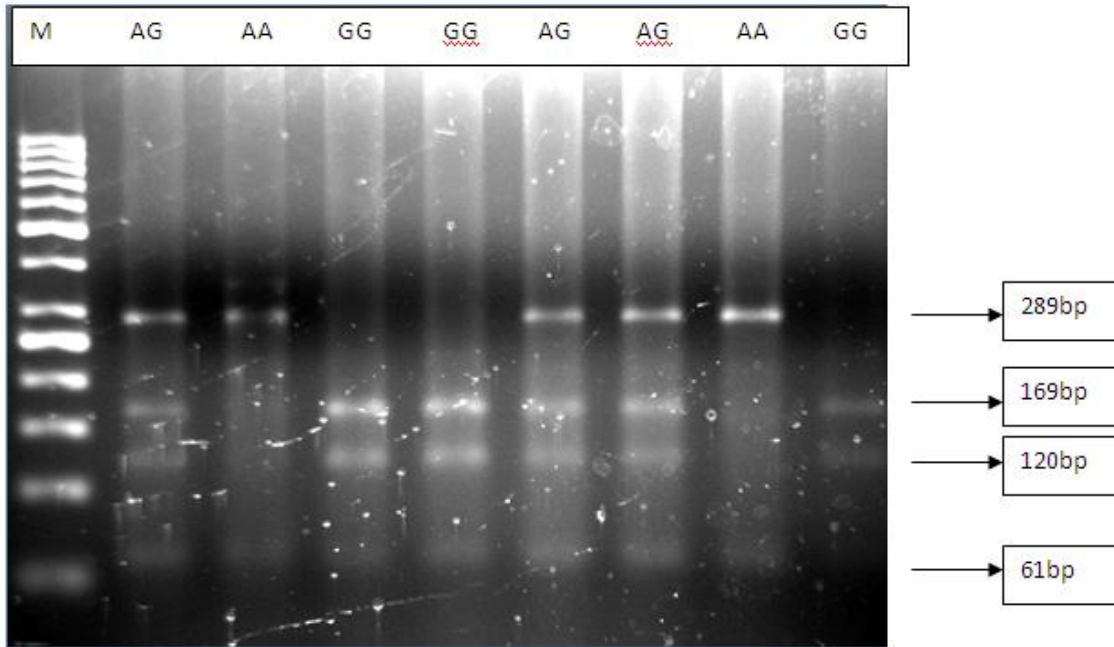
**Fig. 5a** - Electrophoretic profile of NOS2 gene fragments (exon-16) on agarose gel. M-DNA molecular weight marker (DNA Ladder 50 bp); 2 and 5 homozygous phenotype without CC mutation (285 bp + 170 bp); 1 and 3 heterozygous phenotype CT (285 bp + 170 bp + 137 bp + 33 bp); 4 - homozygous phenotype with TT mutation (285 bp + 137 bp).



**Fig. 6a** - Electrophoretic profile of fragments of the NOS2 gene (intron-16) on agarose gel. M-DNA molecular weight marker (DNA Ladder 50 bp); 2, 3 and 4 - homozygous phenotype without GG mutation (455 bp); 5 and 7-heterozygous phenotype GT (455 bp + 263 bp + 192 bp); 1 and 6 - homozygous phenotype with TT mutation (263 bp + 192 bp).



**Fig. 7a** - Electrophoretic profile of NOS2 gene fragments (intron 20) on agarose gel. M-DNA molecular weight marker (DNA Ladder 50 bp); 5 - homozygous phenotype without GG mutation (75 bp + 54 + 39 bp); 3, 6 and 8 - heterozygous phenotype GA (129 bp + 75 bp + 54 bp + 39 bp); 1, 2, 4 and 7 - homozygous phenotype with AA mutation (129 bp + 39 bp).

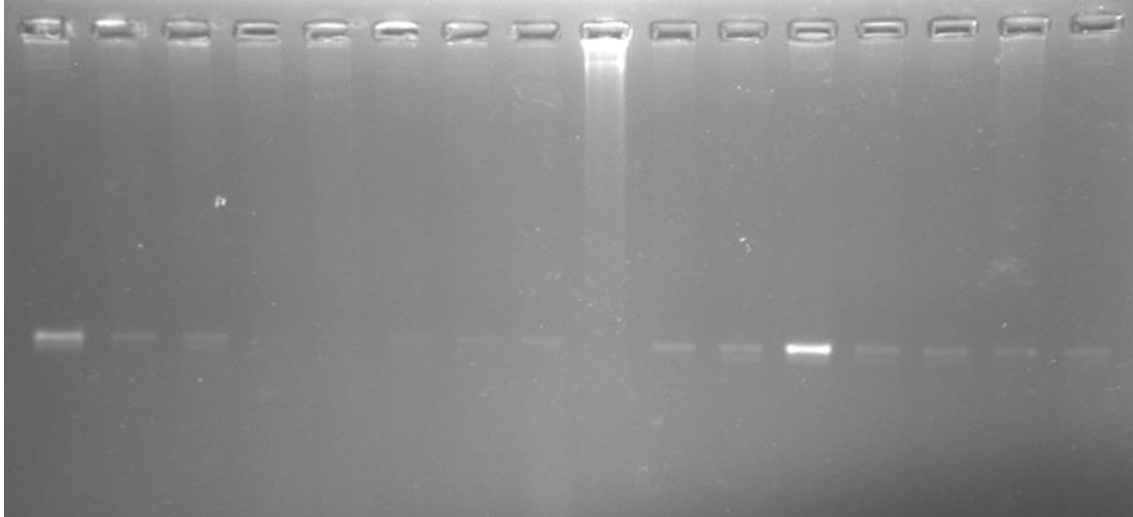


**Fig.8a:** The polymorphism of MPO (-463G>A (rs2333227)) was determined by PCR-RFLP: lines 3 and 8: AA (289 bp+ 61 pb); Lines 2, 6 and 7: AG (289 + 169 + 120 + 61 bp) and Line 4, 5 and 9: GG (169 + 120 + 61 bp). The M (line 1) is the DNA ladder (50 bp GeneRuler).

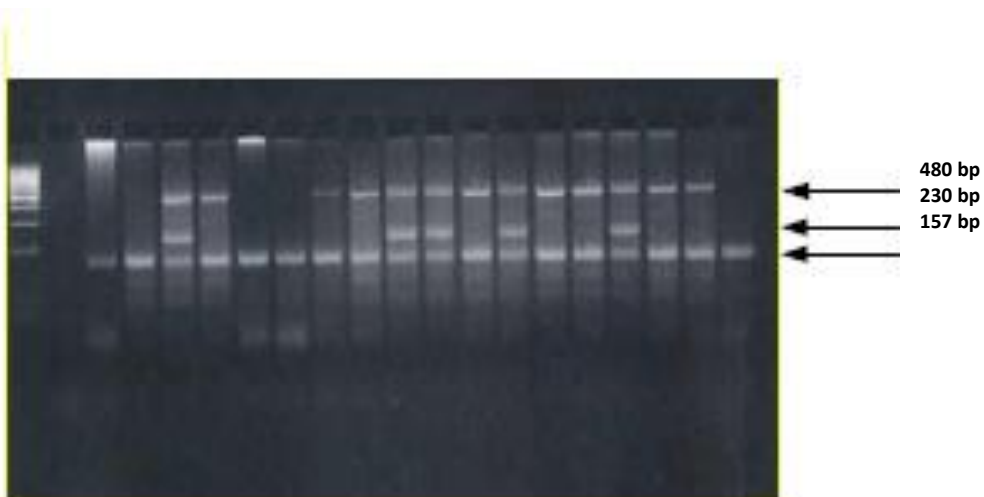
AG AA AG

AA AG AG AG AG AG AG

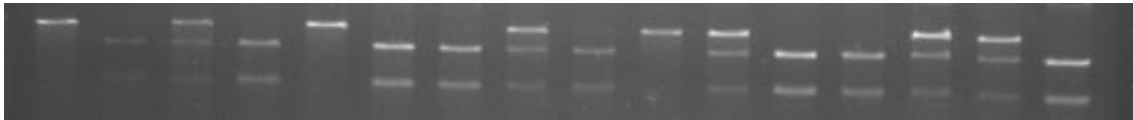
308  
291



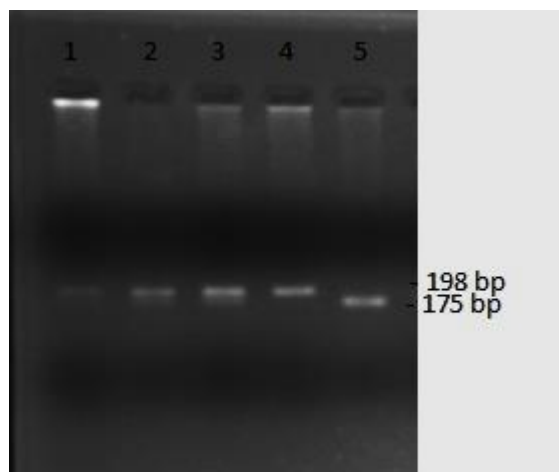
**Fig.9a:** Agarose 3 % with different genotype of Arg16Gly polymorphism determined with polymerase chain reaction- restriction fragment length polymorphism. There were one fragment of 308 bp for homozygous without mutation (ArgArg), three fragments of 308 bp, 291 bp and 17 bp for heterozygous ArgGly and two fragments of 291 bp and 17 bp for homozygous with mutation (GlyGly).



**Fig.10 a:** The presence of 230 bp fragment represent GSTM1 non-null genotypes; The presence of 480 bp fragment represent GSTT1 non-null genotypes; the 157 bp fragment corresponds to GSTM4 is used as a internal control for amplification



**Fig.11a:**LELP1 polymorphism: : the TT genotype gives rise to one single band of 506 bp; the CC genotype appears as two bands, one with 339 bp, and other with 167 bp; the CT genotype has all the three bands.



**Fig12a:** MTHFR gene polymorphism C677T (rs1801133) (PCR-RFLP MTHFR): There were one fragment of 198 bp for homozygous without mutation (CC), three fragments of 198 bp,175 bp and 23 bp for heterozygous CT and two fragments of 175 bp and 23 bp for homozygous with mutation (TT).

**Table IV.4.1a:**Participant's demographic and clinical characteristics

	<b>Controls</b>	<b>Asthma</b>	<b>p value</b>
N (%)	45 (48.9)	47 (51.1)	n.a.
Female	14 (31.1)	29(61.7)	<b>0.006*</b>
Male	31 (68.9)	18 (38.3)	
Age (years)	(42.87 ±11.014)	(39.04 ±18.72)	0.234†
< 15	0 (0.0)	5 (10.6)	
15-30	6 (13.3)	13 (27.7)	<b>0.011*</b>
> 30	39 (86.7)	29 (61.7)	
Atopy/No Atopy	n.a.	39/8 (83.0/17.0)	n.a.
Asthma controlled / Asthma not controlled	n.a.	34/13(72.3/27.7)	n.a.

Bolded results are less than 0.05 of statistical significance; p\*, p value of  $\chi^2$  test values, the values represent absolute frequencies (relative frequencies, %); p†, Independent sample T-test, the values represent means  $\pm$  standard deviation (SD); n.a., non applicable.

**Table IV.4.2a:** Distribution of allele frequencies between controls and asthmatics

	<b>Controls</b>	<b>Asthma</b>	<b>p value</b>	<b>OR(95%CI)<sup>a</sup></b>	<b>p<sup>a</sup></b>	<b>OR(95%CI)<sup>b</sup></b>	<b>p<sup>b</sup></b>
IVS16+ 88T>G							
Allele G	70 (0.78)	64 (0.68)	0.140	1.00(reference)	0.141	1.491[0.738-3.011]	0.265
Allele T	20 (0.22)	30 (0.32)		1.641[0.848-3.173]			
Ex16 +14C>T							
Allele C	<b>85 (0.94)</b>	<b>73 (0.78)</b>	0.002	1.00(reference)	0.002	<b>4.387[1.523-12.635]</b>	<b>0.006</b>
Allele T	<b>5 (0.06)</b>	<b>21 (0.22)</b>		<b>4.890[1.756-13.619]</b>			
IVS20 +524G>A							
Allele A	57 (0.63)	47 (0.50)	0.094	1.00(reference)	<b>0.069</b>	<b>1.903[1.011-3.583]</b>	<b>0.046</b>
Allele G	33 (0.37)	47 (0.50)		<b>1.727[0.958-3.114]</b>			
27-bp repeat in intron 4-eNOS							
Allele b	74 (0.82)	79(0.84)	0.894	1.00(reference)	0.742	0.770[0.332-1.784]	0.542
Allele a	16 (0.18)	15 (0.16)		0.878[0.406-1.901]			
ACE I/D							
Allele D	52 (0.58)	59 (0.63)	0.589	1.00(reference)	0.490	0.735[0.389-1.387]	0.342
Allele I	38(0.42)	35 (0.37)		0.812[0.449-1.467]			

Bolded results are less than 0.05 of statistical significance. The values for allele frequencies represent absolute frequencies (relative frequencies, %). OR<sup>a</sup>, odds ratio, crude values; OR<sup>b</sup>, odds ratio; values adjusted for age and gender; CI, confidence interval; p values for  $\chi^2$  test values; p<sup>a</sup>, crude values; p<sup>b</sup>, values adjusted for age and gender (regression binary logistic).



**Table IV.4.3a:** Distribution of genotype frequencies between asthmatics and controls

SNPs	Asthma/Controls	OR(95%CI)	p <sup>a</sup>	OR(95%CI) <sup>b</sup>	p <sup>b</sup>
<b>IVS16+ 88T&gt;G</b>					
GG	24/30	1.00(reference)			
GT	16/10	2.000[0.769-5.198]	0.233	1.506[0.519-4.374]	0.451
TT	7/5	1.750[0.493-6.213]	0.581	2.002[0.486-8.255]	0.337
GT/TT	23/15	1.917[0.825-4.455]	0.191	1.650[0.649-4.195]	0.293
<b>Ex16 +14C&gt;T</b>					
CC	29/41	1.00(reference)			
CT	16/3	7.540 [2.011-28.273]	0.002	7.259[1.736-30.350]	0.007
TT	2/1	2.828[0.245-32.672]	0.571	1.005[0.071-14.248]	0.997
CT/TT	18/4	6.363[1.949-20.771]	0.002	5.311[1.477-19.095]	0.011
<b>IVS20 + 524G&gt;A</b>					
AA	16/17	1.00(reference)			
GA	15/23	0.693[0.270-1.779]	0.446	0.681[0.242-1.919]	0.467
GG	16/5	3.400[1.010-11.451]	0.048	4.654[1.165-18.601]	0.030
GA/GG	31/28	1.176[0.501-2.760]	0.709	1.252[0.502-3.123]	0.630
<b>27-bp repeat in intron 4-eNOS</b>					
bb	33/32	1.00(reference)			
ab	13/10	1.261[.484-3.283]	0.817	1.422[0.481-4.210]	0.525
aa	1/3	0.323[.032-3.272]	0.627	0.142[0.010-2.082]	0.154
ab/aa	14/13	1.044[0.425-2.563]	1.000	1.036 [0.381-2.815]	0.945
<b>ACE I/D</b>					
DD	24/16	1.00(reference)			
ID	11/20	0.367[0.139-0.968]	0.070	0.360 [0.113-1.148]	0.084
II	12/9	0.889 [0.305-2.94]	1.000	0.689 [0.213-2.230]	0.534
ID/II	22/29	0.506[0.218-1.173]	0.166	0.435 [0.167-1.135]	0.089

Bolded results are less than 0.05 of statistical significance. The values for genotype frequencies represent absolute frequencies . OR a odds ratio, crude values; OR b odds ratio, values adjusted for age and gender; CI, confidence interval; p<sup>a</sup>, crude values; p<sup>b</sup>, values adjusted for age and gender (regression binary logistic). n.a., non applicable.

**Table IV.4.4a:** Distribution of genotype frequencies between controlled and uncontrolled asthma

SNPs	Controlled/uncontrolled asthma	OR(95%CI) <sup>a</sup>	p <sup>a</sup>	OR(95%CI) <sup>b</sup>	p <sup>b</sup>
<b>IVS16+ 88T&gt;G</b>					
GG	21/3	1.00(reference)			
GT	8/8	<b>7.000[1.476-33.207]</b>	<b>0.014</b>	<b>12.406[1.576-97.620]</b>	<b>0.017</b>
TT	5/2	2.000[0.321-12.463]	0.458	1.765[0.259-12.017]	0.562
GT/TT	13/10	<b>5.385[1.246-23.277]</b>	<b>0.024</b>	<b>7.917[1.389-45.122]</b>	<b>0.020</b>
<b>Ex16 +14C&gt;T</b>					
CC	21/8	1.00(reference)			
CT	11/5	1.193 [0.314-4.531]	0.795	1.161[0.247-5.454]	0.850
TT	2/0	n.a.	n.a.	n.a.	n.a.
CT/TT	13/5	1.010[0.271-3.757]	0.989	1.054[0.229-4.844]	0.946
<b>IVS20 + 524G&gt;A</b>					
AA	9/7	1.00(reference)			
GA	13/2	0.198[0.033-1.181]	0.075	0.185[0.019-1.783]	0.144
GG	12/4	0.429[0.095-1.925]	0.269	0.469[0.089-2.478]	0.373
GA/GG	25/6	0.309[0.082-1.167]	0.083	0.343[0.075-1.579]	0.170

27-bp repeat in intron 4-eNOS					
bb	23/10	1.00(reference)			
ab	11/2	0.418[0.078-2.243]	0.309	0.297[0.047-1.858]	0.194
aa	0/1	n.a.	n.a.	n.a.	n.a.
ab/aa	11/3	0.627[0.143-2.747]	0.536	0.440 [0.086-2.262]	0.326
ACE I/D					
DD	14/10	1.00(reference)			
ID	9/2	0.311[0.055-1.762]	0.187	0.272 [0.037-2.018]	0.203
II	11/1	0.127 [0.014-1.151]	0.067	<b>0.064 [0.005-0.857]</b>	<b>0.038</b>
ID/II	20/3	<b>0.221[0.051-0.955]</b>	<b>0.043</b>	<b>0.146 [0.025-0.845]</b>	<b>0.032</b>

Bolded results are less than 0.05 of statistical significance. The values for genotype frequencies represent absolute frequencies. OR a odds ratio, crude values; OR b odds ratio, values adjusted for age and gender; CI, confidence interval; pa, crude values; pb, values adjusted for age and gender (regression binary logistic). n.a., non applicable.

**Table IV.4.5a:** Differences in Genetic risk score between asthma and controls(Low; Intermediate; High)

Genetic risk score	Control/asthma	p <sup>a</sup> value	OR <sup>a</sup> (95%CI)	p <sup>a</sup> value
low risk score=-0,992	29/10	< <b>0,001</b>	1.00(reference)	
intermediate risk score=1.045	12/19		4.592[1.657-12.724]	<b>0,003</b>
intermediate risk score =1.150	2/8		11.600[2.102-64.013]	<b>0,005</b>
high risk score=3.187	2/10		14.500[2.703-77.779]	<b>0,002</b>

Bolded results are less than 0.05 of statistical significance. The values for Genetic risk score frequencies represent absolute frequencies . OR a odds ratio, crude values; CI, confidence interval; pa, crude values; (regression binary logistic).

**Table IV.4.6a:**Differences in Genetic risk score between Controlled/Uncontrolled asthma (Low; Intermediate; High)

Genetic risk score	Controlled/Uncontrolled asthma	p <sup>a</sup> value	OR <sup>a</sup> (95%CI)	p <sup>a</sup> value
low risk score ≤ -2,68	15/0	<b>&lt;0,001</b>	n.a.	n.a
intermediate risk score >-2,68 ≤-0,98	13/3		1.00(reference)	
high risk score >-0,98	6/10		7.222[1.440-36.224]	<b>0,016</b>

Bolded results are less than 0.05 of statistical significance. The values for Genetic risk score frequencies represent absolute frequencies . OR a odds ratio, crude values; CI, confidence interval; pa, crude values; (regression binary logistic).n.a.: because in the uncontrolled group we had 0 individuals in the low grade Genetic risk score.



Research Article

Open Access

## Lelp-1, Its Role in Atopic Dermatitis and Asthma: Poland and Portugal

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### Abstract

**Background:** Atopic dermatitis (AD) that begins in childhood and is the first step of the so-called 'atopic march'. The chromosome 1q21 region has been associated with AD and psoriasis, with a peak in Epidermal Differentiation Complex (EDC) in a region of 2.05 Mb. The aim of this work was to study LELP-1 (late cornified envelope-like proline-rich 1) polymorphism [rs7534334] located within the EDC, in AD and asthma in two European populations: Portugal and Poland.

**Methods:** We studied 110 individuals in the control group and 129 asthmatics in the Portuguese cohort; 100 controls and 45 patients with AD and asthma in the Poland cohort. Written informed consent was obtained from all participating individuals. LELP-1 genotypes were determined by the PCR-RFLP technique. All statistical analyses were carried out using SPSS 21.0 software.

**Results:** The results were considered statistically significant with  $p < 0.05$ . We found that the CC genotype was more frequent in Poland's cohort with AD and asthma when compared with controls ( $p = 0.004$ ), (OR: 2.80 [1.34-5.82]; adjusted  $p = 0.006$ ) and the C allele was also a risk factor (OR: 2.40 [1.35-4.28]; adjusted  $p = 0.003$ ) to both diseases in this group. When compared the cohort from Portugal with Poland, there was a trend for TT genotype to be a risk for asthma in the Portuguese cohort (OR=7.49 [0.92-60.91], adjusted  $p = 0.06$ ). C allele was more frequent in the cohort from Poland and T allele, in the cohort from Portugal ( $p = 0.047$ ).

**Conclusion:** These findings demonstrate that genetic variation of skin barrier genes like LELP-1 might contribute to allergic diseases.

**Keywords:** LELP-1; Atopy; Atopic dermatitis; Asthma; Portugal; Poland

## Introduction

Epidermal keratinocytes undergo a terminal differentiation and programmed cell death (physiological apoptosis) known as cornification [1-3]. Cornification leads to the cornified layer, and different genes proceed in an organized sequence to provide this outermost skin barrier in the spinous and granular layers that express proteins like keratins (namely: K1, K2 and K10) and non-keratin proteins like filaggrin (FLG), loricrin (LOR), involucrin (IVL) and small proline rich proteins (SPRRs) [4-7]. These proteins are cross-linked in the cornified cell envelope by transglutaminase enzymes, and this insoluble envelope associated with the keratin-containing macrofibrils fills corneocytes and with the lipids, forms the skin barrier that protect from dehydration and environment allergens [4].

Atopic dermatitis (AD), or eczema, is a skin disease often associated with other allergic diseases, such as, allergic rhinitis and asthma [8,9]. AD is very common in westernized societies, where it affects about 20% of children and 3% of adults [8-12]. In children with AD about 60% will develop asthma, being a strong predictor of subsequent asthma development and the natural history of atopic march. The biological approach of AD implies a defective barrier defect, and overexpression of inflammatory mediators associated with immune dysregulation [9].

FLG mutations predispose significantly to an increased risk to develop atopic eczema. Apart from FLG other proteins involved in skin barrier functions such as SPRR, lipids synthesis and metabolism, protease and protease inhibitor function, all seem to play a role. Besides skin barrier function, immune deviation versus a Th2 dominance and increased IgE production is also genetically determined. Polymorphisms have been found in genes encoding IL-4, IL-13 and STAT-6, and recently a polymorphism on the high-affinity IgE receptor gene has been found. Using genome-wide association studies, new genes with yet unknown functions have been determined to be associated with atopy and atopic eczema [13,14,15,16].

The molecular signature of AD is mainly associated with Th2 [8,17,19] IgE high (extrinsic) and IgE low (intrinsic) mediated by keratinocyte thymic stromal lymphopoietin (TSLP) regulating dendritic cells. This Th2 activation contributes to barrier dysfunction by impairing FLG and other skin barrier genes expression [20-23]. IL-22 and IL-33 play also its role, [24,25] in this Th2 driven inflammation by allergens, associated with FLG and other EDC gene polymorphisms, and are also important in other allergic diseases such as asthma, besides AD. Biphasic T cell response in the skin (Th2 cells in acute AD; Th1 cells in chronic AD) [8] and reduced skin innate immune response [26,27] are characteristics of this disease.

The epidermal and dermal AD transcriptomes and their respective contributions to abnormalities in respective immune and barrier phenotypes have been highlighted recently in lesional and nonlesional AD skin [28].

The upregulated genes in lesional epidermal transcriptome consisted of proliferation-related, EDC, inflammatory antimicrobial genes and the upregulated genes dermal transcriptome included T-cell activation, IL-2 receptor  $\alpha$ , Th2-related, Th22, Th17-related and collagen genes [24,25].

Studies of association of genes in AD put in evidence the cluster of the EDC [7,23] and other barrier candidates [29], but the most important associations were related to FLG (filaggrin) [13,30-32] and two null mutations (R510X and 2282del4) [33,34]. In this study we have studied the role of LELP1 (another EDC gene) polymorphism (late cornified envelope-like proline-rich 1) [rs7534334] (a polymorphism 258 bp downstream of the LELP1) using the HapMap database (HapMap data rel28 Phase II+III, August 10, NCBI B36 assembly) and its association with atopic dermatitis and asthma in a Portuguese and Poland's cohort.

LELP1 codes for a SPRR (cornifin) family protein, [35] assuming that many of those proteins (FLG, SPRR, loricrin, involucrin) are stored and released from keratohyalin granules in the granular layer. The cell membrane is then, covered with cross-linked intercellular proteins forming the cornified envelope [2,6,36,37]. Transglutaminases crosslink intercellular proteins and also link lipids to the cornified envelope, forming also the lipid envelope to provide a water barrier function [37].

The chromosome 1q21 region [38] has been associated with skin pathology like AD, ichthyosis vulgaris and psoriasis, in a region of about 2.05Mb (mega basis) in Epidermal Differentiation Complex (EDC).

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**Received** January 04, 2016; **Accepted** January 22, 2016; **Published** January 29, 2016

**Citation:** Cortez M, Matos A, Wesserling M, Pawelczyk T, Trzeciak M, et al. (2016) Lelp-1, Its Role in Atopic Dermatitis and Asthma: Poland and Portugal. *J Allergy Ther* 7: 229. doi:[10.4172/2155-6121.1000229](https://doi.org/10.4172/2155-6121.1000229)

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Bronchial epithelial cells and keratinocytes were found to have a high degree of overlap in gene expression [39]. Bronchial epithelial cells, similar to keratinocytes, express components that are able to form a cross-linked protein envelope that may contribute to an effective barrier against noxious stimuli and pathogens [39]. SPRRs are part of the portfolio of genes expressed by both bronchial epithelial cells and keratinocytes in response to pro-inflammatory cytokines, suggesting the importance of these proteins in host defense [40,41]. There is an epithelial-specific molecular signature of gene expression in bronchial epithelial cells and keratinocytes comprising a family member of keratins, small proline-rich proteins and proteinase inhibitors [39].

It has become clear that epithelia and also epithelial tissues [42,43] have three main mechanisms to protect the organism from pathogens [39], pollutants and allergens. First, the epithelial cells form an impermeable barrier which both prevents pathogen entry and minimizes dehydration (xerosis). Secondly, epithelial cells are capable of producing defense molecules such as antimicrobial peptides and proteinase inhibitors. Finally, these cells are able to produce signaling molecules such as cytokines and chemokines, playing an active role in innate and adaptive immunity.

The aim of this work was to study the role of LELP-1 (late cornified envelope-like proline-rich 1) polymorphism [rs7534334] located on EDC, in atopic dermatitis and asthma in two different European populations: Portugal and Poland.

## Material and Methods

The study population consisted of 110 individuals in the control group and 129 asthmatics from the Portuguese cohort and 100 controls and 45 AD with asthma from the Poland cohort.

Written informed consent was obtained from all participating individuals. The genetic study on EDC has been approved by the Independent Bioethics Commission for research.

Patients were diagnosed by physicians for asthma according to the guidelines of GINA, and as having atopy or not according to WAO/ EAACI guidelines, they were examined for a self-reported history of breathlessness, wheezing, atopic dermatitis and family history, atopic individuals have a positive skin prick test (SPT) for at least one of the common environmental allergens or the presence of specific IgE, associated with high serum IgE levels estimated using enzyme-linked immunosorbent assay and suffered from asthma, or AD and asthma. The SCORAD (SCORing Atopic Dermatitis) index was completed in all patients with AD. The demographic and clinical details of the study population are given in Table 1.

## Genomic DNA Isolation

Whole blood samples from patients and controls were stored with EDTA at -20°C. The genomic DNA was isolated through a nonenzymatic method (salting out method) adapted from Lahiri, D. K., & Nurnberger, J. I et al., 1991 [44].



## Genotyping of Lelp-1 [Rs7534334]

The LELP-1 genotypes were determined by the polymerase chain reaction and restriction fragment length polymorphism (PCR-RFLP) technique, the polymorphic region was amplified in a 50 µl reaction mixture: 10 mM of each primer (forward: 5'- CCTCCACCATGTACAACGCT-3'; and reverse: 5'- TTGCATTAACCCATGCAGCC-3'), 200 ng of genomic DNA and 0.2 mM of PCR nucleotide Mix Thermo Scientific® DreamTaq Green containing 10 mM dNTPs, 1.5 mM MgCl<sub>2</sub>, 1 U Taq polymerase. PCR conditions involved an initial denaturation of DNA at 94°C for 3 min,

Portugal	Controls	Asthma	p.
N (%)	110 (46.0)	129 (54.0)	n.a.
Female	42 (38.2)	82 (63.6)	<b>&lt;0.001</b>
Male	68 (61.8)	47 (36.4)	
Age (years) †	110 (42.82 ± 10.88)	129 (38.40 ± 19.24)	<b>0.027</b>
<15-15-30> 30	0 (0.0)	16 (12.4)	<b>&lt;0.001</b>
	15 (13.6)	37 (28.7)	
	95 (86.4)	76 (58.9)	
Atopy	n.a.	111 (86.0)	n.a.
Asthma controlled	n.a.	92 (71.3)	n.a.
Poland	Controls	AD and Asthma	p.
N (%)	100 (69.0)	45 (31.0)	n.a.
Female	64 (64.0)	27 (60.0)	0.712
Male	36 (36.0)	18 (40.0)	
Age (years) ††	100 (25) [18-61]	45 (23) [7-59]	<b>0.027</b>
<15 15-30> 30	0 (0.0)	8 (17.8)	<b>&lt;0.001</b>
	66 (66.0)	27 (60.0)	
	34 (34.0)	10 (22.2)	
Atopy	n.a.	38 (84.4)	n.a.
Asthma controlled	n.a.	45 (100.0)	n.a.
SCORAD †	n.a.	45 (54.7 ± 20.5)	n.a.

The values represent absolute frequencies (relative frequencies, %) for dichotomous dependent variables. Values statistically significant for p value <0.05; p,  $\chi^2$  test values. p, † Independent sample-test; and values are means ± standard deviation (SD). p, †† Mann-Whitney-test; and values are (median) and [range]. n.a., non applicable; AD, Atopic Dermatitis; SCORAD, SCORing AD index.

**Table 1:** Participant's characteristics of Portugal and Poland with asthma, AD and controls.

followed by 35 cycles of amplification at 94°C for 30 s, 53°C for 45 s, 72°C for 1 min and 30 s and one cycle at 72°C for 5 min. The amplified fragments of 506 bp were then digested by the restriction endonuclease MwoI at 60°C for 3 hr according to the manufacturer's recommendations. The digestion products were analyzed by electrophoresis in 3% agarose gel stained with ethidium bromide (10 µg/mL) for 60 minutes, with 80 volts. With this process we are able to differentiate genotypes: the TT genotype gives rise to one single band of 506 bp; the CC genotype appears as two bands, one with 339 bp, and other with 167 bp; the CT genotype has all the three bands. **Statistical Analysis**

Observed genotype frequencies were tested for deviation from HardyWeinberg equilibrium (HWE) with the Chi-square goodness-of-fit test. This test was also used to evaluate the significant differences between groups, in and within the two populations, in order to know if the odds ratio (OR) test was justifiable. In the two cohorts OR for patients risk and the corresponding 95% confidence intervals (95% CI) were calculated using logistic regression analysis. This test was applied to the polymorphism, to analyze its risk factor individually. The power of the sample

was verified every time there were statistical differences among genotype distribution. All statistical analyses were carried out using the SPSS 21.0 software. The results were considered statistically significant for  $p < 0.05$ .

## Results

LELP-1 polymorphism [rs7534334] was evaluated in the 2 cohorts: Portugal and Poland, within 2 atopic diseases: AD and asthma. Table 1 shows the characteristics of participants of these two cohorts compared with controls.

In the Portuguese cohort there are differences in gender, being the females more frequent in asthmatics and males in the control group ( $p < 0.001$ ) (Table 1). The asthmatics in the Portuguese cohort were younger than the control group ( $p = 0.027$ ) (Table 1) and this was more evident when we stratified the groups being the older than 30 years more frequent in the control group ( $p < 0.001$ ) (Table 1). The asthmatic patients were in the majority of them atopic (86%) and had their asthma symptoms controlled (71.3%) (Table 1).

In the Polish cohort there were no differences by gender ( $p = 0.712$ ) (Table 1). The patients (AD and asthma) from Poland were younger than the control group ( $p = 0.027$ ) (Table 1) and this was more evident when we stratified the groups by age being the older than 30 years more frequent in the control group ( $p < 0.001$ ) (Table 1). The majority of asthmatic patients are atopic (84.4%), all of them had the asthma symptoms controlled under anti-asthmatic treatment. The SCORAD index has been done in all patients with AD with a mean (mean  $\pm$  SD: 54.7 20.5) compatible with a more severe cutaneous disease (Table 1). There is a significant difference ( $p = 0.035$ ) being the value of SCORAD by genotype (mean  $\pm$  SD): CC (52.96  $\pm$  18.94); CT (59.72  $\pm$  19.9) and TT (8  $\pm$  0). We think that these findings might be indicative of a trend for those who express allele C to have higher values, but we think that we must increase the sample to have more robust results.

In the control group and between the two cohorts, there were statistical significances in gender and age; being the females more frequent among the controls of the Poland's cohort and the males among the Portuguese cohort ( $p < 0.001$ ) (data not showed); and the controls from Poland were younger than the control group from Portugal ( $p < 0.001$ ) (data not shown).

### LELP-1 polymorphism [rs7534334]

For LELP-1 polymorphism [rs7534334] in the Portuguese cohort with asthma comparing with controls, there were no differences in genotype and allele frequencies ( $p > 0.05$ ) (Table 2). The genotype distributions in asthma and controls were in HWE ( $p > 0.05$ ) (data not shown).

The CC genotype was more frequent in the cohort from Poland with AD and asthma ( $p = 0.004$ ) (power sample  $> 0.8$ ) (Table 2) being a risk (OR: 2.80 [1.34-5.82]; adjusted  $p = 0.006$ ) to both diseases in this cohort when compared to controls (Table 2). The genotype distributions in patients and controls were in HWE ( $p > 0.05$ ) (data not showed). The C allele ( $p = 0.001$ ) was more frequent in the cohort from Poland with asthma and atopic dermatitis being a risk factor to both diseases in this group (OR: 2.40 [1.35-4.28]; adjusted  $p = 0.003$ ) (Table 2).

			OR [95% CI]		OR adjusted b	p a	p b
			p	OR crude a			
<b>Portugal</b>	<b>Controls</b>	<b>Asthma</b>					
<i>LELP-1</i>	<i>n=110</i>	<i>n=129</i>					
rs7534334							
CC	45 (40.9)	58 (45.0)		1.18 [0.71-1.97]	1.07 [0.62-1.83]	0.528	0.807
CT	50 (45.5)	55(42.6)	0.817	0.89 [0.53-1.45]	0.95 [0.55-1.62]	0.662	0.841
TT	15 (13.6)	15 (13.6)		0.90 [0.42-1.91]	0.90 [0.42-1.91]	0.777	0.95
C	140 (0.64)	140 (0.64)	0.565	1.12 [0.77-1.64]	1.04 [0.70-1.55]	0.546	0.834
T	80 (0.36)	87 (0.35)		0.89 [0.61-1.30]	0.96 [0.65-1.42]	0.546	0.834
<b>Poland</b>	<b>Controls</b>	<b>AD and Asthma</b>					
<i>LELP-1</i>	<i>n=100</i>	<i>n=45</i>					
rs7534334							
CC	32 (32.0)	26 (57.8)		2.91 [1.41-6.00]	2.80 [1.34-5.82]	0.004	0.006
CT	53 (53.0)	18 (40.0)	0.004	0.59 [0.29-1.21]	0.60 [0.29-1.24]	0.149	0.167
TT	15 (15.0)	1 (2.2)		0.13 [0.02-1.00]	0.14 [0.02-1.07]	0.051	0.058
C	117 (0.59)	70 (0.78)	<b>0.001</b>	2.45 [1.39-4.39]	2.40 [1.35-4.28]	<b>0.002</b>	<b>0.003</b>
T	83 (0.41)	20 (0.22)		0.41 [0.23-0.71]	0.42 [0.23-0.74]	<b>0.002</b>	<b>0.003</b>

The values for the genotypes and respective allele frequencies represent absolute frequencies (relative frequencies, %). Values statistically significant for p value < 0.05; AD, Atopic Dermatitis; OR, odds ratio; CI, confidence interval; p,  $\chi^2$  test values; p a, crude values; p b, values adjusted for age and gender (binary logistic regression).

**Table 2:** Distribution of *LELP-1* [rs7534334] genotype in asthma, AD and controls in the two cohorts (Portugal and Poland).

There were significant differences in the mean age between the two cohorts, being the patients in the Portuguese cohort older than

the Poland's patients ( $p < 0.001$ ) (Table 3). Comparing the 2 cohorts of patients there were no significant differences in gender distribution or atopic status ( $p > 0.05$ ) (Table 3).

When comparing the two cohorts, the CC genotype was more evident in the cohort from Poland and the TT genotype in the cohort from Portugal although it didn't reach the significance level ( $p = 0.094$ ) (Table 4). When considering all possible models of genotype analysis within these two cohorts, we found a trend for TT genotype to be a risk in asthma in the Portuguese cohort when comparing with patients with AD and asthma from Poland cohort (adjusted values: OR=7.49 [0.9260.91],  $p = 0.06$ ) (Table 4).

For allele frequencies, there were significant differences in the 2 cohorts, being the C allele more frequent in Poland and the T allele in Portugal ( $p=0.047$ ) (Table 4). These results were reflected in risk analysis being, the C allele protector and the T allele a risk for asthma in in the Portuguese population when compared with the cohort from Poland (OR=0.53 [0.29-0.95] and OR=1.90 [1.06-3.42], adjusted  $p=0.033$ , respectively) (Table 4).

## Discussion

This study is part of a project which purpose is to identify novel polymorphisms in genes involved in skin barrier function and its association with atopic diseases.

LELP1, is a protein-coding gene located at chromosome 1q21 that belongs to the cornifin family (SPRR). The SPRR gene family, which includes the rs7534334- tag SNP of LELP1 is in the EDC complex that contains various other important genes such as IVL, LOR, FLG , trichohyalin (THH) and the S100 gene family [2,6,37].

Some authors [35], have found an association of this chromosome 1q21 tagged single nucleotide polymorphism (SNPs) within the LELP1 gene [rs7534334] with serum IgE levels. These results pointed to the need for research on LELP1 and other genes on EDC that could be related with many inflammatory diseases of the skin like AD and psoriasis.

However, most of the studies that have been done linking EDC with atopic disease involves the two null mutations in the FLG gene (R501X and 2282del4), that are associated with skin diseases like AD and asthma with AD [33,45]. As far as we know, this is the first paper studying LELP1 on the EDC, in 2 European cohorts with atopic disease (AD and asthma).

In the Portuguese cohort there were statistical differences within the control group by gender, being the females more frequent in asthmatics and males in the control group ( $p<0.001$ ) and the asthmatics in the Portuguese cohort were younger than the control group ( $p=0.027$ ).

In the Poland cohort there were no differences by gender ( $p=0.712$ ) and the patients (AD and asthma) from Poland were younger than the control group ( $p=0.027$ ).

According to authors that found differences in asthma and gender namely sex hormone estrogen and the physiopathology of asthma and increases in IL-4 and IL-13 production (46) we performed our analysis adjusted for gender and age between the controls and the patient groups.

In our study with LELP1 polymorphism [rs7534334] we found that the CC genotype was more frequent in Poland's cohort with AD and asthma when compared with controls ( $p=0.004$ ), (OR: 2.80 [1.345.82]; adjusted  $p=0.006$ ) and the C allele was also a risk factor (OR: 2.40 [1.35-4.28]; adjusted  $p=0.003$ ) to both diseases in this group. When compared the cohort from Portugal with Poland, there was a trend for

Poland & Portugal	AD and Asthma	Asthma	p
N (%)	45 (25.9)	129 (74.1)	76 (58.9)
Female	27 (60.0)	82 (63.6)	0.722
Male	18 (40.0)	47 (36.4)	
Age (years) ††	45 (23) [7-59]	129 (38.0) [7-86]	<b>&lt;0.001</b>
<15	8 (17.8)	16 (12.4)	
15-30	27 (60.0)	37 (28.7)	<b>&lt;0.001</b>
>30	10 (22.2)	76 (58.9)	
Atopy	38 (84.4)	111(86.0)	0.807

The values represent absolute frequencies (relative frequencies, %) for dichotomous dependent variables. Values statistically significant for p value <0.05; p,  $\chi^2$  test values. p, †† Mann-Whitney-test; and values are (median) and [range]. AD, Atopic Dermatitis; n.a., non applicable.

**Table 3:** Participant's characteristics of Poland and Portugal with asthma, AD.

TT genotype to be a risk for asthma in the Portuguese cohort (OR=7.49 [0.92-60.91], adjusted p=0.06). C allele was more frequent in the cohort from Poland and T allele, in the cohort from Portugal (p=0.047).

The molecular basis for the skin barrier deficiency could be a secondary phenomenon associated with the epidermal differentiation complex (EDC) and barrier candidate genes like FLG (filaggrin) and LELP1 (late cornified envelope-like proline-rich 1) as we found in our results.

Other authors [35], refer a correlation of log10 serum IgE levels and rs7534334 in a group of asthmatic patients being the mutant genotype (TT) in patients, those with higher levels of IgE comparing with controls (TT) and comparing with wild type genotype (CC) in patients ( $3.49 \pm 0.91$  vs  $2.43 \pm 0.52$  vs  $2.92 \pm 0.59$ ). This point to the works who

showed that when skin barrier function is compromised even without skin disease there is an increased incidence of atopic disease [28].

The clinical manifestations of atopic dermatitis in infancy are different from adults; first the lesions are on the cheeks and scalp, then the flexures, the posterior area of the scalp and popliteal region. In adults lichenified plaques of the flexures, head and neck are more frequent, with a chronic and relapsing skin inflammation, and a disturbance of epidermal-barrier function and IgE-mediated sensitization to allergens.

	AD and Asthma	Asthma	OR [95% CI]		OR adjusted <sup>b</sup>	a <sup>a</sup> p	b <sup>b</sup> p
			p	OR crude <sup>a</sup>			
Portugal and Poland							
LELP-1	n=45	n=129					
rs7534334							
CC	26 (57.8)	58 (45.0)		0.60 [0.30-1.19]	0.57 [0.28-1.17]	0.140	0.124
CT	18 (40.0)	55 (42.6)	0.094	1.12 [0.56-2.23]	1.12 [0.54-2.31]	0.758	0.766
TT	1 (2.2)	16 (12.4)		6.23 [0.80-48.40]	7.49 [0.92-60.91]	0.080	0.060
C	70 (0.78)	171 (0.66)	<b>0.047</b>	0.56 [0.32-0.98]	0.53 [0.29-0.95]	<b>0.043</b>	<b>0.033</b>
T	20 (0.22)	87 (0.34)		1.78 [1.02-3.12]	1.90 [1.06-3.42]	<b>0.043</b>	<b>0.033</b>

The values for the genotypes and respective allele frequencies represent absolute frequencies (relative frequencies, %). Values statistically significant for p value <0.05; AD, Atopic Dermatitis OR, odds ratio; CI, confidence interval; pa,  $\chi^2$  test values; p b, crude values; pc, values adjusted for age (regression binary logistic).

**Table 4:** Distribution of LELP-1 [rs7534334] genotype between asthma and AD in the two cohorts (Portugal and Poland).

AD might be the first step of the so-called “atopic march” [9] that include other allergic disorders later in life such as asthma and allergic rhinitis.

Some authors proposed that in a “dual” allergen exposure hypothesis the low dose exposure through the lesional skin in AD of the allergens might interfere with the uptake of the Langerhan’s cells that could polarize to a Th2 response and IgE diathesis. By other way: early high dose exposition could induce tolerance, being proposed that Th1 and Tregs might interfere with the gut-associated lymphoid tissue and develop tolerance.

In this hypothesis sensitization to allergens occur in the environmental exposure through the skin while the tolerance might occur when the allergens contact with the atopic patient via other route of absorption namely oral for food allergens [47].

This dual hypothesis point to the prioritization of the intensive treatment of AD in early infancy to decrease allergic sensitization and the atopic march with the emergence of asthma and allergic rhinitis [9,47-49].

LELP1 is a protein-coding gene located at chromosome 1q21 that belongs to the cornifin (SPRR) family and the allele T might be related with a poor prognosis of the disease, if we think that the families of small proline-rich proteins are present in epithelial cells of the airways and skin that utilize similar mechanisms in host defense [39]. The SPRR family are also induced in respiratory epithelia as a squamous cell marker metaplasia [41,50].

Being the skin barrier deficiency a secondary phenomenon associated with the EDC, FLG has demonstrated how the study of a monogenic trait could provide insight into a complex trait disease and the significance of *FLG* null mutations as a genetic risk factor for atopic dermatitis. This barrier defect could be present even in the absence of eczema [26,28,33,47], which could help us to understand the physiopathology of AD associated with LELP1 polymorphism [rs7534334] and other polymorphisms located on genes of EDC complex.

Different genes are expressed in a coordinated sequence to provide the structural component of cornification. Keratin intermediate filaments form a complex conglomerate in the cytoplasm and, after the removal of cell organelles, fill the cell interior. In addition, a number of proteins are cross-linked by transglutamination in the cell periphery to form the so-called cornified envelope where LELP1 as a member of epidermal differentiation complex (EDC) could be a barrier candidate gene.

As soon as keratinocytes are detached from the basement membrane of the epithelium, they change their gene profile under the control of many transcription factors [1,4].

In addition, keratins and the inflammatory profile can also regulate pathways involved in growth, proliferation, migration and apoptosis of epithelial cells [3]. The small proline-rich proteins are encoded by the EDC [2,37,40], where LELP1 play its role. The proteins that are encoded in this region share similarities, particularly in the glutamine- and lysine-rich regions that are involved in the action of the transglutaminases. Bronchial epithelial cells and keratinocytes not only share structural characteristics, but also share functional characteristics and that is why many barrier genes could be related with the “atopic march” and the pathophysiology of AD and respiratory diseases such allergic asthma.

The epithelial cells of the airways and the skin, utilize also similar defense mechanisms against infection [39], pollutants and allergens, despite the different structure of the epithelia.

Bronchial epithelial cells and keratinocytes have a high degree of overlap in gene expression and bronchial epithelial cells like keratinocytes, express proteins and other components that are able to form a cross-linked

protein envelope that may contribute to a barrier against allergens, pollutants and pathogens. That could be compromised in patients with LELP-1 polymorphism [rs7534334].

It has been demonstrated that peptides that could be related with defense mechanisms besides barrier function, like LELP1 that codifies a protein belonging to the cornifin (SPRR) family [16] could be lower in skin with AD, and that the Th2 cytokines could also play a role by interfering with the expression of these peptides [20,40,43]. IL13 (a Th2 cytokine), could induce the expression of small proline-rich proteins (SPRR) in airway epithelium during allergic inflammation in the animal models [40].

LELP 1 which codes for one of the small proline-rich proteins (SPRR), expressed in both bronchial epithelial cells and keratinocytes in response to pro-inflammatory cytokines, might be related with the pathophysiology of atopic dermatitis and also with host defense against allergens, pollutants and microbes and might interfere with respiratory disease and “allergic march”.

Our results point to the importance of the impaired skin barrier function on trans-epidermal entry of allergens and secondary development of allergic diseases like asthma and rhinitis. Some genome-wide association studies, point to the fact that FLG could partially tag some other mutations like those near LCE3E (rs61813875) [51,52] and according to this, we think that it might be important to study other polymorphisms in genes from the EDC complex namely FLG (loss-of-function mutations (R501X and 2282del4), in order to more accurately understand the importance of this complex in asthma, AD or other allergic diseases.

We also think that one of our limitations is the sample size, and we hope that in the future we could have larger cohorts to increase the robustness of our study.

These kind of studies are important because of the regulation of expression of epidermal barrier proteins and its clinical relevance on defense mechanisms in inflammatory disorders that affects epithelial surfaces [5,7,9] like AD and asthma and constitute an important therapeutic strategy for allergic diseases.

## **Conclusion**

Altogether, these findings demonstrate that as much as immune mechanisms and IgE hipersensitivity, genetic variation of skin barrier genes might contribute to major atopic diseases such as atopic dermatitis and bronchial asthma.

We could then infer that we should readily treat atopic dermatitis in early childhood, reducing inflammation in the skin, permeability to allergens and so preventing allergen sensitization. In the same way the “dual hypothesis” could help us to understand how helpful it could be to decrease the environmental exposure to allergens in the “allergic march” and the development of allergic diseases.

These kinds of studies are important because of the regulation in expression of epidermal barrier proteins and its clinical relevance on defense mechanisms in inflammatory disorders that could affect epithelial surfaces in atopy and might constitute an important therapeutic target in allergic diseases.

## **Acknowledgments**

The authors would like to thank the Instituto de Investigação Científica Bento da Rocha Cabral from Portugal, FCT-Portugal, National Center of Science to MT from Poland (grant number 2011/03/D/NZ5/00837) and Prof.J.W.Holloway-SO-UK.

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**Citation:** Cortez M, Matos A, Wesslering M, Pawelczyk T, Trzeciak M, et al. (2016) Lelp-1, Its Role in Atopic Dermatitis and Asthma: Poland and Portugal . J Allergy Ther 7: 229. doi:[10.4172/2155-6121.1000229](https://doi.org/10.4172/2155-6121.1000229)

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Available Online at <http://journalip.jcar.org> *International Journal of  
Current Advanced*

*International Journal of Current Advanced Research Research Vol 5, Issue 7, pp*

1047 -1052, July 2016

ISSN: 2319 - 6475

ISSN: 2319-6505

## RESEARCH ARTICLE

### HAPTOGLOBIN POLYMORPHISM AND BRONCHIAL ASTHMA

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#### ARTICLE INFO

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##### *Article History:*

Received 10<sup>th</sup> April, 2016

Received in revised form 09<sup>th</sup> May, 2016

Accepted 25<sup>th</sup> June, 2016

Published online 25<sup>th</sup> July, 2016

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##### *Key words:*

Haptoglobin Polymorphism; Immunity; Bronchial Asthma

## ABSTRACT

Asthma is considered a heterogeneous disease, characterized most of the times by a Th2 inflammatory response. Haptoglobin (Hp), is an alfa2-sialoglycoprotein known to bind free hemoglobin (Hb) and has been implicated in modulation of Th1/Th2 response, Intervening in innate and adaptive immune response. The Hp locus is situated at 16q22 chromosome, being in humans, polymorphic for the  $\alpha$  chain. The  $\alpha$  chain of Hp has 2 major co-dominant alleles Hp\*1 and Hp\*2, with 3 genotype variants, Hp1-1, Hp2-1, Hp2-2. The aim of the study is to establish a relation between Hp genotypes and Hp levels (intermediate phenotype), and the pathophysiology of asthma when compared with a control group of healthy blood donors. In a group of 114 asthmatic patients and 50 controls we studied the Hp levels that were determined by nephelometry and genotypes by polyacrylamide gel electrophoresis (PAGE). Statistical analysis was performed with statistical software PASW version 18, having established a level of significance of  $p < 0.05$ .

We found that Allelic (Hp\*1 e Hp\*2) and Hp genotypes (Hp 1-1, Hp 2-1, Hp 2-2) distribution in asthmatics, are not statistical different from control group ( $p > 0.05$ ). There is no statistical differences in the asthmatics between, gender, age-group, atopics and nonatopics, controlled and non-controlled asthma ( $p > 0, 05$ ). The different genotypes seem not to be related with an increased risk of having asthma when compared with the control group ( $p > 0, 05$ ). In control group there is no statistical differences in Hp levels by genotype and age- group ( $p > 0, 05$ ). When we compare asthmatics with control group we verified that in asthma , the levels of Hp are always lower than in the control group ( $125,13 \pm 50,95$  vs  $137,86 \pm 51,39$  mg/dL) and there was a statistical difference in Hp22 genotype ( $95,60 \pm 41,43$  vs  $128, 40 \pm 51,48$  mg/dL) ( $p < 0, 05$ ). In asthmatics Hp levels, are statistical different between ages  $> 30$  years and  $< 15$  years ( $135.6 \pm 50.05$  vs  $87.45 \pm 38.89$  mg/dL) ( $p < 0.05$ ). In asthmatics Hp levels, present statistical differences by genotype ( $p = 0,000$ ). Those who express Hp 2-2 had the lower levels of the circulating protein

when compared with Hp 2-1 and Hp 1-1 ( $95,6 \pm 41,93$  vs  $137,37 \pm 49,58$  vs

$146,09 \pm 47,37$  mg/dL) ,and it is statistical different ( $p = 0.000$  ). In those asthmatics with age  $\geq 15$  years Hp levels are different by genotype ( $p < 0.05$ ): 1-1 and 2-1 differ from 2-2. Those patients with age  $< 15$  years, Hp levels were no different between genotypes ( $p > 0, 05$ ). In a pos-Hoc analysis Hp 2-2 is an independent factor, as age  $< 15$  years, associated with lower levels of Hp.

Although no statistical differences were find between Hp genotype and allelic distribution in the group of asthmatics when compared to control group we verified that asthmatics had lower levels of the circulating Hp when compared to the control- group and that this difference is associated with Hp 2-2 genotype. In asthmatics, Hp levels are different between genotypes (with age  $\geq 15$  years) because Hp levels are lower in the Hp2-2 genotype when compared with the other genotypes. In the future, studies done with Hp should be controlled by age , because the Hp levels are lower in the pediatric group. These data point to differences among groups that could be related to Hp genotypes, and possibly with different immunological profiles.

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## INTRODUCTION

Haptoglobin (Hp), an alpha 2-sialoglycoprotein (acute phase protein), that is known by its ability to bind free hemoglobin (Hb) and to form an Hp-Hb complex that allows the recycling of globin and heme [1-4]. The Hp-Hb complex can be cleared receptor [5-7]. Hp consists of two different polypeptide chains, the  $\alpha$  and  $\beta$ -chain. The Hp  $\alpha$  gene, located in 16q22 chromosome, is highly polymorphic in humans, presenting 2 major co-dominant alleles: Hp\*1 and Hp\*2 that originate 3 from plasma by two different pathways, one in the hepatocyte (90%) and another in monocytes-macrophages by the CD163

different genotypes: Hp1-1, 2-1 and 2-2. The Hp gene has two major alleles: Hp\*1, (with five exons) and Hp\*2, (with seven exons) which probably arose from a duplication event involving exons 3 and 4, producing a 61 kDa protein. In its ancestral form, Hp is a dimer, however, the Hp 1–2 encoded protein exists as linear polymers containing 2–8 monomers, while the Hp 2–2 encoded protein exists as circular polymers of 3–10 Hp monomers [8].

These genetic variants present different affinities to bind hemoglobin [9] and so can modulate the toxicity and inflammatory nature of free iron, namely its capacity to consume nitric oxide [10,11] and to serve as a Fenton reagent [9]. Besides its antioxidant role, Hp also plays an immunoregulatory function, through the CD163 receptor in macrophages, modulating the cytokine profile released after endocytosis of the Hp-Hb complex [9].

One of the consequences of the allergic reaction is the increase of free hemoglobin. Hp\*1 has greater affinity for free Hb presenting higher antioxidant capacity; on the other hand, Hp\*2 has less affinity for Hb, being associated with more susceptibility to oxidative stress damage [9, 12-15]. As a response to tissue injury or infection, the target cells segregated IL-1 $\beta$  and TNF $\alpha$  that activate endothelial cells and neutrophils. The activated neutrophils, the first line of defense in immune response, help in the recruitment of other inflammatory cells, which promote reactive oxygen species (ROS) generation [16]. Hp is synthesized during neutrophils differentiation and is stored to be released when these are activated. Hp synthesis occurs mainly at hepatocyte level, and also in the alveolar macrophages and lung eosinophils with active inflammation, but not in the healthy lung [17]. The Hp binds to Apo-A1, protecting from free radical attack and preventing HDL to form complexes with other lipoproteins. The Hp has the ability to inhibit lipoxygenase and cicloxygenase activity, modulating the synthesis of prostaglandins and leukotrienes [13]. Hp is also an excellent suppressor of T cell proliferation [13]. The macrophages activated by the complex Hp2-2: Hb through the CD163 receptor deviates T helper response to a Th1 profile, while macrophages activated by complex Hp1-1: Hb phagocytosis produces Th2 cytokines. The balance between these T cell responses is particularly important in the extravascular space, since a localized expression of Hp minimizes tissue injury [9].

In extravascular space, dendritic cells respond to alert signals, like oxidative stress, and interact with antigens/allergens. These dendritic cells differentiate to mature cells and migrate to lymphatic nodes, where they interact with naïve T cells. Being the Hp a ligand to monocytes and macrophages, this protein may play an important role in the activation of these immune cells [9].

Allergic asthma is an inflammatory disease where predominates a Th2 response in the bronchial airways. Th2 response might be related to a more ancestral immune response. Asthma appears as a result from a failure in the immunoregulatory mechanisms of the respiratory epithelium. According to the “hygiene hypothesis”, a lack of Th1 response stimulation upon the adaptive immunity leads to a prevalence of Th2 response [19, 20]. Nowadays, there

has been an increase prevalence of autoimmune diseases (Th1 diseases) in the western societies, namely type I Diabetes and multiple sclerosis, suggesting that both diseases (Th1 and Th2) can coexist in the same patient. These data seem to oppose the bipolarization of an immunological environment Th1 or Th2, and points to a deficient regulation in both diseases (role of the Treg cells). Nevertheless, the defenders of this hypothesis assert that the absence of necessary stimulation could deprive the immune system from the necessary signals to the development of regulatory pathways able to control Th1 and Th2 response. Advances in the immunology field, came to focus the attention in the central role of innate immunity, as the “orchestrator” of immune response and maintenance of tolerance [21].

The activation of innate immunity through the antigen presenting cells (APCs), where are involved the Hp and its receptor, CD163 (marker of M2 macrophages), might be an additional factor in Th2 polarization of allergic response, similarly to the allergens [19]. Identically to the Th1/Th2 nomenclature, the polarized macrophages are reported as M1 and M2 [22, 23]. The M1, also designed as activated by classic pathway, can be induced by IFN $\gamma$ , LPS, TNF- $\alpha$  and GM-CSF. These macrophages act in the initial phase of the inflammation and produce great amounts of pro-inflammatory cytokines (IL1- $\beta$ , TNF $\alpha$ , IL-6), oxygen free radicals and nitrogen compounds, and participate as effectors in the polarized reactions Th1. The resolution phase is characterized by macrophages that produce anti-inflammatory cytokines with high phagocytic capacity and overexpression of the mannose receptor, CD206 and the Hb-Hp receptor, CD163. M2 macrophages are activated by the alternate pathway and can be induced by IL-4, IL-13, immune complexes, IL-10, glucocorticoids, activin-A (a member of the TGF- $\beta$  family) and IL- 21. In general, M1 macrophages are IL-12 high, IL-23 high and IL-10 low [22]. Despite participating in Th1 response, they are also responsible for the resistance against intracellular parasites and activity against the tumoral cells [22]. In contrast, M2 macrophages are IL-12 low, IL-23 low, IL-10 high, and have a variable capacity to produce inflammatory cytokines with a Th2 profile [22]. They are associated with atopic and allergic asthma, being able to promote the proliferation and tumor metastization.

The aim of this study is to establish a relation between Hp genotype and asthma susceptibility and to correlate the Hp genotype with plasma Hp levels (intermediate phenotype) establishing a possible, relationship between the modulation of Th1/Th2 immune response by Hp polymorphism and asthma pathophysiology.

## **MATERIAL AND METHODS**

To identify if there were differences in the two groups, we did a case-control study with a group of 114 asthmatic patients, (Immuno Allergy Department-CHLN/HSM-Director: Prof. Manuel Barbosa), 70 females and 44 males, mean age 41 $\pm$ 18 years; in the control group were 50 healthy blood donors, 45 females and 5 males, mean age 50 $\pm$ 13 years. Asthmatic patients were classified according to severity in intermittent and persistent (mild/moderate/severe) asthma as stated by GINA classification [24] (Global Initiative for Asthma) and according to levels of asthma control (controlled, partly controlled and uncontrolled) in compliance with ACQ7 (Asthma Control Questionnaire, Portuguese Version by Juniper) and PAQLQ (Pediatric Asthma Quality of Life Questionnaire- Portuguese Version by Juniper adjusted for patients between 7-17 years with at least 6 months of therapeutics for asthma) [25].

The sample consisted in 98 atopics and 16 non atopics (according to the definition of atopy by the WAO/EAACI [26]); 45 with uncontrolled asthma (evaluated by validated instrument ACQ7 (cutpoint: 0.75) and PAQLQ: global score is the average of all the answers, < 4 imply uncontrolled asthma) and 69 with controlled asthma. The exclusion criteria were non adherence to the anti-asthmatic therapy; existence of other co- morbidities that

could interfere with the severity of the respiratory disease; existence of a diagnosis of chronic obstructive pulmonary disease or another pulmonary disease; smoking habits and other co-infections, namely HIV, parasitosis or other type of infection, anemia or chronic liver disease. All participants gave their written informed consent for the study. Written informed consent was obtained from all participating individuals. The genetic study was done under the standards of the Bioethics Commission for research at Lisbon Medical School.

Patients were diagnosed by physicians for asthma according to the guidelines of GINA, and as having atopy or not according to WAO/EAACI guidelines, they were examined for a self-reported history of breathlessness, wheezing, atopic dermatitis and family history, atopic individuals have a positive skin prick test (SPT) for at least one of the common environmental allergens or the presence of specific IgE, associated with high serum IgE levels estimated using enzyme-linked immunosorbent assay and suffered from asthma.

Blood samples were collected after an overnight fast. The determination of the haptoglobin polymorphism was done from plasma samples (Hb-supplemented plasma) using a phenotyping method for Hp, based on polyacrylamide gel (4.7% in TRIS/HCl 0.504M, pH 8.9) electrophoresis (PAGE) followed by o-dianisidine staining, and assigned corresponding genotype. The Hb-supplemented samples for application into the gel (10 $\mu$ L) were previously prepared using a mixture of 40% saccharose (w/v), Hb 282 mg/mL and plasma in the 3:2:4 proportion for a final volume of 45 $\mu$ L. For identification of the Hp migratory bands we used a method of coloration by contact with o-dianisidine 16mM in 50% acetic acid and, subsequently, with 0.6% hydrogen peroxide (Fig.1). Samples underwent electrophoresis for 180 minutes (180 V, 0.05 A; Cleaver). Plasma haptoglobin concentration was measured by nephelometric method on a BN ProSpec (Siemens Healthcare Diagnostics) [27].

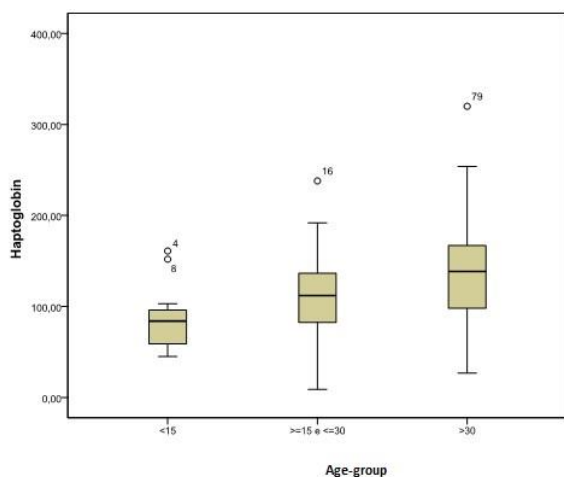
Statistical analysis was performed with Statistical software, and the continuous variables were summarized as means (standard deviation) or as medians according to their homogeneity. Categorical variables (allele and genotypes frequencies) were compared with the  $\chi^2$  test. Continuous variables among patients and controls were compared with Student t test. To compare the Hp levels in the different groups defined for genotype and age group, an ANOVA test was used, after verified the normality and the homogeneity of the variances, and post-Hoc tests. Associations are given as odds ratios with a confidence interval established at 95%. All statistical analysis was done using PASW version 18. A two-sided probability value of  $p < 0.05$  was considered significant.

## RESULTS

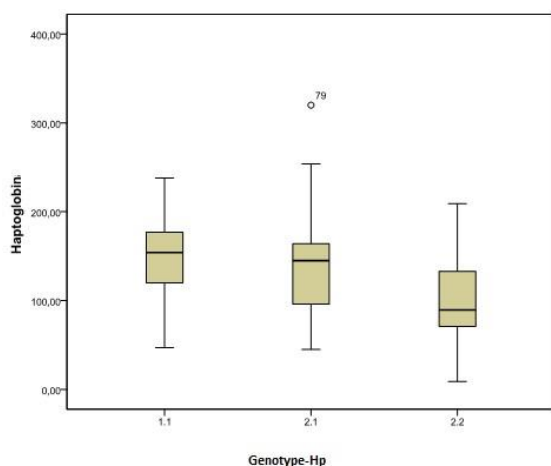
There were no statistically significant differences regarding the distribution of Hp genotypes between asthmatics and controls (Hp1-1: 19.3 vs 10.0, Hp 2-1: 47.4 vs 58.0, Hp 2-2: 33.3 vs 32.0,  $p > 0.05$ ), as well as in their allele frequencies (Hp 1\*: 0.43 vs 0.39, Hp 2\*: 0.57 vs 0.61,  $p > 0.05$ ) (Table 1). Our results concerning the frequency of allele 1 in the control group are consistent with those described by Carter and Worwood relatively to the European population (Fig.2) [28]. The Hp genotype frequencies among the asthmatic patients showed no statistically significant differences between males and females, uncontrolled and controlled asthma, atopic or non-atopic, age- group ( $p > 0.05$ ).

**Table 1** Haptoglobin genotype and allele frequencies in asthma and control group

GENOTYPE	ASTHMA		CONTROL		p- value
	N	Frequency (%)	N	Frequency (%)	
Hp 1-1	22	0.193	5	0.10	
Hp 2-1	54	0.474	29	0.58	>0.05
Hp 2-2	38	0.333	16	0.32	
Hp* 1		0.39		0.43	>0.05
Hp* 2		0.61		0.57	



**Figure 1** Plasma Hp levels (mg/d L) in asthmatics stratified by Age-group



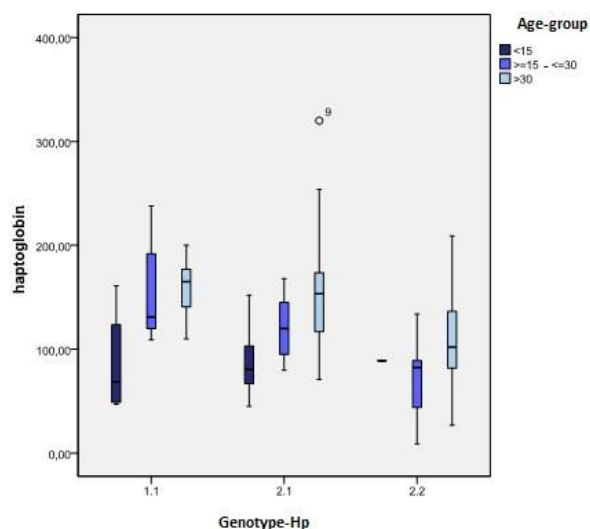
**Figure 2** Plasma Hp levels stratified by Hp genotypes (genotype-phenotype association) in asthmatics.

In patients with asthma (Table 2), the plasma concentration of Hp (intermediate phenotype) did not differ significantly between controlled and uncontrolled asthma, atopic and non atopic and between males and females, there is, however, a significant variation with age, having the patients with age > 30 years ( $135.60 \pm 50.05$  mg/dL); 15- 30 years ( $111.00 \pm 48.43$  mg/dL) and <15 years ( $87.45 \pm 38.89$  mg/dL) (ANOVA  $p=0,008$  between > 30 years and <15 years: pos- hoc test) (Fig. 3). The asthmatics with Hp 2-2 genotype presented lower concentrations of circulating protein when compared with patients Hp 2-1 and Hp 1-1 ( $95.60 \pm 41.93$  vs  $137.37 \pm 49.58$  vs  $146.09 \pm 47.36$  mg/dL), being this difference statistically significant ( $p = 0.000$ , ANOVA) (Fig. 4); this pattern was not observed among individuals in the control group. These differences in Hp concentration was significant only in asthmatics aged  $\geq 15$ ,

with no differences in patients <15 years (ANOVA) (Fig. 5). When analyzing the levels of circulating Hp between asthmatics and controls (Fig. 6), it was found that, overall, no statistically significant differences between mean values of this protein was found among both groups ( $125.13 \pm 50.95$  vs  $137.86 \pm 51.39$  mg/dL,  $p > 0.05$ ), despite the Hp 2-2 individuals ( $95.6 \pm 41.9$  vs  $128.4 \pm 51.5$  mg/dL) presented significantly differences in mean Hp values between asthma and control group ( $p=0,018$ ) (Fig. 6). Relatively to Hp 1-1 and Hp 2-1 genotypes, no significant differences were observed between asthmatics and controls ( $146.09 \pm 47.36$  vs  $175.8 \pm 15.78$  ;  $p > 0.05$ ;  $137.37 \pm 49.58$  vs  $136.55 \pm 53.46$ ;  $p > 0.05$  ) (Fig. 7). Thus, although not always significant, the asthma group had lower mean values of circulating Hp.

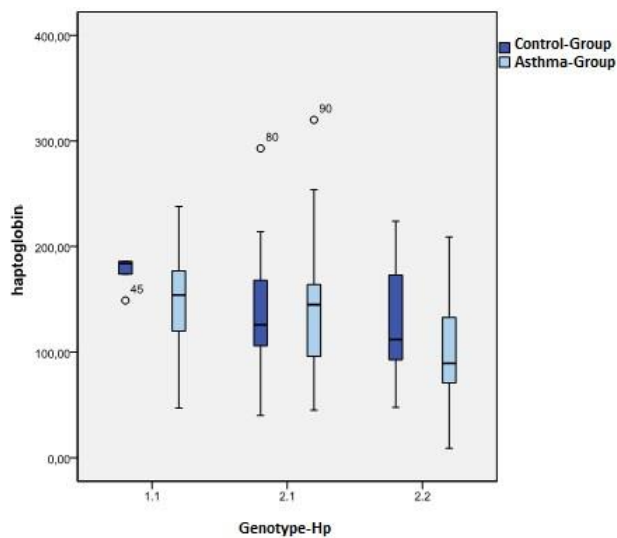
**Table 2** Plasma concentrations of Hp in asthmatics, stratified by: age; gender; clinical characteristics; and by Hp genotype

		N	Hp SD (mg/dL)	p-value
AGE	<15 years	11	87,45±37.8	0,008 among <15 and >30
	15-29 years	27	111.00±48,43	
	≥30 years	76	135,60±50,05	
GENDER	Female	70	129.17 ±53.36	0,288
	Male	46	118.70±46.73	
ASTHMA	Controlled	69	120.81±46.79	0.264
	Uncontrolled	47	131,75±56.64	
ATOPY	YES	98	126.81±51.83	0.388
	NO	18	114.87±45.27	
GENOTYPE	Hp 1-1	22	146.09±47.36	0.000
	Hp 2-1	54	137.37±49.58	
	Hp 2-2	38	95.60±41.93	



**Figure 3** Plasma Hp concentrations in asthmatic patients, stratified by Hp genotype and age-groups.





**Figure 4** Plasma Hp levels in asthmatics and controls, stratified by the haptoglobin genotypes.

In the control group there were no differences in the levels of Hp between individuals with age > 30 years ( $138.99 \pm 53.67$  mg/dL) and 15- 30 years ( $127.76 \pm 23.08$  mg/dL) ;( $p=0.648$ ), the same with their distribution by genotype (Hp 1-1:  $175.8 \pm 15.78$  mg/dL vs Hp 2-1:  $136.55 \pm 53.46$ mg/dL vs Hp2-2:  $128.40 \pm 51.48$ mg/dL,  $p = 0.196$  (ANOVA). The control group followed the Hardy-Weinberg equilibrium.

## DISCUSSION

With this study it was possible to put in evidence that the genetic polymorphism for the  $\alpha$  chain of Hp may be associated with differences in haptoglobin levels and that these differences are more pronounced in the asthmatics with longer disease evolution (the differences in relation to the Hp genotype are observed only in patients aged > 15 years). These observations could be derived from differences in the activation and polarization of macrophages (M1 and M2) in the innate immunity system, and therefore different immune response, Th1 (pro-inflammatory) or Th2 (anti-inflammatory) [22]. The genetic polymorphism of haptoglobin has a major role, by conditioning the nature and intensity of the response of macrophages to extravascular and extracorporeal hemoglobin, and potentially interfering with modulation of immunity that accompanies the allergic response and bronchial asthma [13, 18]. Haptoglobin also plays an important role by interacting with CD22 and the integrin CD11b/CD18, as recipients of the haptoglobin in the cells of the immunological system [29,30].

The toxicity and inflammatory nature of free hemoglobin is due to its ability to consume nitric oxide and to act as an oxidant, producing highly reactive radicals such as anion superoxide and hydroxyl. Haptoglobin binds to hemoglobin, inhibiting the ability of Hb to act as an oxidant and promoting its removal, despite the Hp-Hb complex is not completely inert and can also catalyze the formation of oxygen radicals. The removal of the Hp-Hb complex is done through hepatocytes and the CD163 receptor of monocytes/ macrophages. In the extravascular space there is only one way to remove the Hp-Hb, the CD163 receptor on macrophages [5, 9]. The allele 1 seems to generate a complex Hp1-Hb redox-inactive that binds to the CD163 receptor of the macrophage, inducing the secretion of anti-inflammatory cytokines such as IL-10 and TGF- $\beta$ . By contrast, the allele 2 potentiate the generation of a complex redox active Hp2-Hb, with release of pro-inflammatory cytokines, and consequent vascular injury and inflammation

[9, 13]. Atopy and allergic asthma may be due to an M2 polarization of innate immunity (with an important role for the Hp-CD163 receptor on macrophages) and consequent Th2 polarization in detriment of the adaptive immunity, Th1 [20, 22]. This polarization of the immune response may be related to genetic polymorphism of haptoglobin.

The highest values of circulating haptoglobin were observed in asthmatics with longer time of disease evolution (>15 years of age) , and this increase was dependent on Hp genotype (Hp1-1> Hp 2-1 > Hp 2-2). This fact might be related to the chronicity of the disease, reflecting a stress response and consequent induction of the release of glucocorticoids and catecholamines, which stimulate the production of haptoglobin and other acute phase proteins [31]. The agegroup under 15 years has lower levels of haptoglobin when compared with patients aged > 30 years ( $p < 0.05$ ). The difference in the Hp levels between genotypes occurs particularly in the age- group over 30 years ( $p < 0.05$ ). The asthmatics with genotype Hp 2-2 have lower levels of circulating haptoglobin when compared with Hp1-1 and 2-1 ( $p < 0.05$ ). Many studies point to the fact that the Hp1-1 genotype is associated with higher levels of Hp and a Th2 profile, whereas the Hp2-2 genotype was associated with lower levels of Hp and a Th1 profile [13, 28]. It is also described that asthma and respiratory allergy, are associated with the lowest levels of Hp [-32], and that this protein could act as a natural antagonist of the activation of the immune system when related to a series of stimulus. On the other hand, it was observed a lower expression of the CD163 receptor on macrophages of individuals who express Hp2-2 genotype, and that Hp1-1:Hb complex increases the activity of casein kinase II (CK II) associated with CD163 phosphorylation , leading to a distinct mechanism of activation as well as a different profile of cytokines after endocytosis of Hp1-1 complex (with increase of IL-10) versus Hp2-2 [33,34].

## CONCLUSIONS

Considering the results obtained, and the population samples studied, we believe it is essential to increase the number of patients studied in each subgroup, in order to increase the statistical power, leading to a better characterization of these subgroups, particularly, that under 15 years. We also think that future studies must be controlled by age-group, and that different polymorphisms could lead to different genotypespecific response to treatment and different asthma endotypes/phenotypes among patients. Our results suggest an important role of haptoglobin polymorphism in bronchial asthma, possibly associated with the polarization of the immune response, disease severity and response to antiasthmatic therapy.

### ACKNOWLEDGEMENTS

The authors are grateful to: Director of ImmunoAllergy Department-CHLN/HSM; Instituto de Investigação Científica Bento da Rocha Cabral from Portugal, FCT-Portugal, José Augusto Gamito Melo Cristino (MD PhD), Director of Clinical Pathology Service-CHLN and Helena Proença (MD) by the contribution for sample collection and plasma haptoglobin assay and Conceição Gonçalves (MLT) for her collaboration in haptoglobin phenotype determination.

Statement of Contribution of Each Author

All authors contributed equally.

### *Conflict of Interest*

The authors declare that they have no conflict of interests.

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Submitted to Annals of Allergy Asthma and Immunology

Manuscript Number:

Title: Endothelial dysfunction in asthma: eNOS, iNOS and ACE  
polymorphisms-Additive model and potential dominant effects

Article Type: Original Article

Keywords: Key words: NOS2 (exon 16 + 14C> T), NOS2 (intron 16 + 88 G> T),  
NOS2 (intron 20 + 524 G> A), eNOS 4a/b (27 VNTRs) and ACE gene  
insertion/deletion (I/D) polymorphisms; endothelial dysfunction; asthma  
susceptibility; asthma severity.

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Abstract: Background:Angiotensin converting enzyme (ACE) and nitric oxide  
(NOS) gene polymorphisms, are important in endothelial dysfunction and in  
the pathophysiology of asthma.

Objective:The purpose of this study is to analyze the association between  
: cytokine-inducible (iNOS or NOS-2) NOS2 (exon 16 + 14C> T); NOS2  
(intron 16 + 88 G> T); NOS2 (intron 20 + 524 G> A); endothelial NOS  
(eNOS-NOS3) , eNOS 4b/4a (27 VNTRs) and ACE gene insertion/deletion  
(I/D) polymorphisms with asthma susceptibility and severity .

Methods: Asthmatics (n= 47) were compared with a control group (n=45).The  
polymorphisms were analyzed by PCR and PCR-RFLP. Control of asthma  
assessed by (ACQ7 and PAQLQ). Statistical analysis with PASW version 18  
establishing a significance level of p< 0.05.

Results:For NOS2(Ex16 +14C>T), the allele T is more frequent in asthma.  
Those who express T allele have a risk of having asthma 4.387[1.523-  
12.635] ; pb=0.006 . For IVS20 + 524G>A when adjusted for age and  
gender there is a risk of asthma of almost 2 for those who express allele  
G ( 1.903[1.011-3.583], p=0.046). Between controlled and uncontrolled  
asthma for ACE gene insertion/deletion (I/D) polymorphism :OR:  
0.217[0.068-0.697]; pa=0.010;OR: 0.132[0.034-0.517]; pb=0.004 ; the  
Allele I is protector to have asthma . For each SNP, additive\_1 ,  
additive\_2 and potential dominant and recessive effects were evaluated  
by combining homozygote and heterozygote variant carriers for comparison  
with reference.Stepwise multivariate logistic regression with backward  
elimination was applied to construct a genetic risk score.

Conclusion: These single nucleotide polymorphisms could lead to  
different genotype-specific response to therapy and different  
endotypes/phenotypes among asthmatic patients.

## Introduction

The bioavailability of NO could be associated with endothelial dysfunction, and with bronchial hyperresponsiveness in asthma, it could interfere with pro-angiogenic or anti-angiogenic status, exerting diverse physiological actions related with vasodilation, inflammation, platelet physiology and vessel growth. In association ACE is related with the conversion of angiotensin I to angiotensin II that acts as a potent vasoconstrictor, and is also related with bronchoconstriction and bronchial hyperresponsiveness(49,53). Candidate gene-association studies put NOS and ACE enzymes as important regulators of oxidative stress, bronchial hyperresponsiveness, endothelial homeostasis and vascular remodeling through the up-regulation of angiogenic factors and the release of angiogenic mediators. The endothelium has emerged as a key regulator of vascular homeostasis, with its barrier and active signal/ cytokine transducer for circulating and tissue influences that could modify the endothelial phenotype from quiescent to activated endothelial phenotype and orchestrate remodeling and the physiopathology of asthma (50,150).

Angiotensin-converting enzyme (ACE) is an enzyme in the renin-angiotensin system and circulating ACE may proceed from the vascular endothelial as it is a membrane-bound enzyme of the cell and it catalyzes the conversion of angiotensin I to angiotensin II and inactivation of the vasodilatory and bronchoconstrictor bradykinin(137,151).

The ACE gene with an insertion/deletion (I/D) polymorphism, is associated with the presence or absence of a 287-bp fragment in intron 16. The D allele is associated with higher circulating and tissue ACE levels and has been associated with asthma and other respiratory and cardiovascular diseases(137,151-153).

Nitric oxide (NO) is synthesized after oxidation of L-arginine, by nitric oxide synthases (NOS), with three isoforms: two constitutive, the neuronal NOS (nNOS, NOS-1) and endothelial (eNOS, NOS-3), and one inducible NOS (iNOS, NOS-2). NOS2 (exon 16 + 14C> T), NOS2 (intron 16 + 88 G> T), NOS2 (intron 20 + 524 G> A), and NOS3 eNOS 4a/b (27 VNTRs) polymorphisms could be related with the levels of NO and in the context of inflammatory cytokines in asthma and other diseases results in changes in NO production, contributing to endothelial dysfunction, damage of tissues and modifying the course of asthma(44,154-162).

Patients with asthma and with ACE and NOS genetic polymorphisms, have different degrees of endothelial dysfunction, that could be involved in "angiogenic switch" at different levels of angiogenesis pathway, changes in NO bioavailability, different

activation of tissue renin-angiotensin system, decreased vasodilatory response and increased vascular remodeling that could be associated with the severity and progression of asthma.

The purpose of this study is to analyze the association between : NOS2 (exon 16 + 14C> T); NOS2 (intron 16 + 88 G> T); NOS2 (intron 20 + 524 G> A); eNOS 4a/b (27 VNTRs) and ACE gene insertion/deletion (I/D) polymorphisms with asthma susceptibility and severity.

## **Methods**

The study population consisted of 45 individuals in the control group and 47 asthmatics from a Portuguese cohort.

Written informed consent was obtained from all participating individuals. The genetic study has been approved by Ethics Commission for research.

Patients were diagnosed by physicians for asthma according to GINA guidelines(3,163), and as having atopy or not according to WAO/EAACI guidelines(164), they were examined for a history of breathlessness, wheezing, interval between daytime symptoms, night awaking, dose of reliever and controller, activity limitation due to asthma , family history, and comorbidities such as rhinitis, atopic dermatitis, among others. Atopic individuals have a positive skin prick test (SPT) for at least one of the common environmental allergens or the presence of specific IgE ,associated with high serum IgE levels estimated using enzyme-linked immunosorbent assay .The demographic and clinical details of the study population are given in Table-1.

Control of asthma assessed by (ACQ7 and PAQLQ). Statistical analysis with PASW version 18 establishing a significance level of  $p < 0.05$ .

Genomic DNA isolation and quantification: Whole blood samples from patients and controls were stored with EDTA at  $-20^{\circ}\text{C}$ . The genomic DNA was isolated through a non-enzymatic method (*salting out* method).

The polymorphisms -14 C/T in exon 16 and -88 G/T in intron 16 of NOS2 gene were detected using the forward primer 5'-TAAACCAACTTCCGTGGTGGG-3' and the reverse primer 5'-AGCTGGAGAATGGAGCTGGAC-3'. The PCR reaction was performed in a final volume of 50  $\mu\text{L}$  using 200 ng of genomic DNA, 10 pmol of both primers, 25  $\mu\text{L}$  of DreamTaq Green PCR Master Mix (2X) (Thermo Scientific®). The PCR reaction started with an initial denaturation at  $94^{\circ}\text{C}$  for 2 minutes, followed by 35 cycles for 45 seconds at  $94^{\circ}\text{C}$ , 45 seconds at  $61^{\circ}\text{C}$  and 45 seconds at  $72^{\circ}\text{C}$ , with a final extension of 5 minutes at  $72^{\circ}\text{C}$ . The PCR products were digested with 10U of



TaSI (Thermo Scientific®) for 16 hours at 65°C for exon 16 and with 10U of Adel (Thermo Scientific®) for 16 hours at 37°C for intron 16.

Fragments were separated by electrophoresis on a 2% agarose gel for 90 minutes at 85V and visualized by ethidium bromide staining.

For -14 C/T in exon 16, there were two fragments of 285 bp and 170 bp for homozygous without mutation (CC), four fragments of 285 bp, 170 bp, 137 bp and 33 bp for heterozygous CT and two fragments of 285 bp and 137 bp for homozygous with mutation (TT).

For -88 G/T in intron 16, there was an undigested fragment of 455 bp for homozygous without mutation (GG), three fragments of 455 bp, 263 bp and 192 bp for heterozygous GT and two fragments of 263 bp and 192 bp for homozygous with mutation (TT).

The polymorphism 524 G/A in intron 20 of NOS2 gene was detected using the forward primer 5'-TTATCCCAATCCCAGCCACTCG-3' and the reverse primer 5'-GCCAGGCTCTGTTTCTCTGATCC-3'. The PCR reaction was performed in a final volume of 50 µL using 200 ng of genomic DNA, 10 pmol of both primers, 25 µl of DreamTaq Green PCR Master Mix (2X) (Thermo Scientific®). The PCR reaction started with an initial denaturation at 94°C for 2 minutes, followed by 35 cycles for 45 seconds at 94°C, 45 seconds at 59°C and 45 seconds at 72°C, with a final extension of 5 minutes at 72°C. The PCR product was digested with 10U of HinfI (Thermo Scientific®) for 16 hours at 37°C.

Fragments were separated by electrophoresis on a 4% agarose gel for 90 minutes at 85V and visualized by ethidium bromide staining.

There were three fragments of 75 bp, 54 bp and 39 bp for homozygous without mutation (GG), four fragments of 129 bp, 75 bp, 54 bp and 39 bp for heterozygous GA and two fragments of 129 bp and 39 bp for homozygous with mutation (AA).

The polymorphism 4 b/a of NOS3 gene was detected using the forward primer 5'-AGGCCCTATGGTAGTGCCTTT-3' and the reverse primer 5'-TCTCTTAGTGCTGTGGTCAC-3'. The PCR reaction was performed in a final volume of 25 µL using 200 ng of genomic DNA, 10 pmol of both primers, 12.5 ml of DreamTaq Green PCR Master Mix (2X) (Thermo Scientific®). The PCR reaction started with an initial denaturation at 94°C for 2 minutes, followed by 35 cycles for 30 seconds at 94°C, 30 seconds at 53°C and 45 seconds at 72°C, with a final extension of 5 minutes at 72°C. Fragments were separated by electrophoresis on a 3% agarose gel for 120 minutes at 110V and visualized by ethidium bromide staining.

There were one fragment of 420 bp for homozygous b/b, two fragments of 393 bp and 420 bp for heterozygous a/b and one fragment of 393 bp for homozygous a/a.

The polymorphism I/D of ACE gene was detected using the forward primer 5'-CTGGAGACCACTCCCATCCTTTCT-3' and the reverse primer 5'-GATGTGGCCATCACATTCGTCAGAT-3'. The PCR reaction was performed in a final volume of 25  $\mu$ L using 200 ng of genomic DNA, 10 pmol of both primers, 12.5  $\mu$ L of DreamTaq Green PCR Master Mix (2X) (Thermo Scientific®). The PCR reaction started with an initial denaturation at 94°C for 2 minutes, followed by 35 cycles for 45 seconds at 94°C, 45 seconds at 58°C and 45 seconds at 72°C, with a final extension of 5 minutes at 72°C. Fragments were separated by electrophoresis on a 2% agarose gel for 60 minutes at 110V and visualized by ethidium bromide staining.

There were one fragment of 477 bp for homozygous I/I, two fragments of 477 bp and 190 bp for heterozygous I/D and one fragment of 190 bp for homozygous D/D.

## Results

The mean age of the 47 asthmatics was 39.04  $\pm$ 18.72 ; 29 females and 18 males; 39 atopics and 8 non-atopics; 34 with controlled and 13 with uncontrolled asthma. The mean age of the 45 individuals in the control group was 42.87  $\pm$ 11.01; 14 females and 31 males; There are more women in the asthmatic group when compared with controls ( $p=0.006$ ) and they are younger than controls ( $p=0.011$ ) (Table 1). For the different SNPs that we had studied there are differences in the allelic frequencies distribution between controls and asthma for NOS2(Ex16 +14C>T), being the allele T more frequent in asthma. Those who express T allele have a risk of having asthma 4.387[1.523-12.635] ;  $p^b=0.006$  . For IVS20 + 524G>A there is no differences in allelic distribution of frequencies between controls and asthmatics ( $p^a=0.094$ ). Although when adjusted for age and gender there is a risk of almost 2 for those who express allele G ( 1.903[1.011-3.583],  $p=0.046$ ). (Table 2).

For the different SNPs that we had studied there are differences in the allelic frequencies distribution between controlled and uncontrolled asthma: there is a trend (adjusted for age and female gender): OR 2.916[0.970-8.765];  $p^b=0.057$ , for NOS2 (intron 16 + 88 G> T)), being a trend for the allele T to be more frequent in asthma. There are differences in the allelic frequencies distribution between controlled and uncontrolled asthma for ACE gene insertion/deletion (I/D) polymorphism : (crude and adjusted for age and female gender): OR: 0.217[0.068-0.697];  $p^a=0.010$ ; OR: 0.132[0.034-0.517];  $p^b=0.004$  ; being the Allele I protector to asthma (Table 3).

For each SNP, additive\_1 , additive\_2 and potential dominant effects were evaluated by combining homozygote and heterozygote variant carriers for comparison with reference : for NOS2 :Ex16 +14C>T: , there are differences between controls and asthmatics in the different genetic models (additive\_1; OR: 7.259[1.736-30.350];

p=0.007) and (dominant model;OR:5.311[1.477-19.095]; p=0.011): there is a risk of being asthmatic of 7.3 for those who express genotype CT and 5.3 for those who express the allele T. For NOS2 (intron 20 + 524 G> A): For IVS20 + 524G>A, there are differences between controls and asthmatics in the different genetic models (additive\_2, OR: 4.654[1.165-18.601] ; p=0.030) , there is a risk of being asthmatic of almost 5 for those who express genotype GG. (Table 4). There is no statistical difference (p>0.05) for the other SNPs studied concerning allelic and genotype frequencies between asthmatics and controls. Trend tests assume ordinal steps to homozygous with major allele, heterozygous and homozygous with minor allele genotypes, respectively, the trend is statistically significant for NOS2:Ex16 +14C>T: 12.08/p=0.00051(Table 4).

For each SNP, additive and potential dominant effects were evaluated also by combining homozygote and heterozygote variant carriers for comparison with reference, comparing controlled and not controlled asthma : For IVS16+ 88T>G, there are differences between Controlled and uncontrolled asthmatics in the different genetic models (additive\_1;OR: 12.406[1.576-97.620]; p=0.017) and (dominant model; OR: 7.917[1.389-45.122]; p=0.020): there is a risk of being uncontrolled asthmatic of 12 for those who express genotype GT and almost 8 for those who express the allele T. For ACE I/D there are differences between Controlled and uncontrolled asthmatics in the different genetic models (additive\_2;OR: 0.064 [0.005-0.857]; p=0.038) and (dominant model\_;OR: 0.146 [0.025-0.845]; p=0.032): there is a protection of being uncontrolled asthmatic for those who express genotype II and for those who express the allele I . For IVS20 + 524G>A, 27-bp repeat in intron 4-eNOS, Ex16 +14C>T there are no differences between Controlled and uncontrolled asthmatics in the different genetic models (additive\_1; additive\_2 and dominant model ( Table 5). Trend tests assume ordinal steps to homozygous with major allele, heterozygous and homozygous with minor allele genotypes, respectively, the trend is statistically significant for ACE I/D polymorphism : 5.67/p=0.0172( Table 5). For the haplotype analysis at NOS 2 , the overall difference in haplotype frequencies between asthmatics and controls pointed to an increased risk of asthma : there is a risk of being asthmatics when compared with controls for those that are (CT+TT)at Ex16 +14C>T and GG at (intron 20 + 524 G> A gene polymorphisms( p=0,012), but because of the limited number of individuals due to low variant allele frequency for the haplotype analysis we have to be cautious about this results.The epistatic interaction between NOS2: IVS16+ 88T>G (GT+TT ) and ACE I/D( ID+DD) polymorphisms, and uncontrolled asthma risk in asthmatics we obtained an ORcrude<sup>a</sup>:5.400[1.345-21.675]; p<sup>a</sup>=0.017; ORadjusted<sup>b</sup>:9.582[1.524-

60.227];  $p^b=0.016$  it increases the risk of being uncontrolled of approximately 10 times(data not showed).

From logistic regression analysis including significant results from univariate analysis(Table4),we concluded that *the risk of having asthma for intron 20 + 524 G> A gene polymorphism and NOS2(Ex16 +14C>T, are not independent of each other and gender .*

For the next logistic regression evaluation of genetic risk score, we considered as models : the genotype homozygous for the major allele as 0 and the association of genotypes with minor allele as 1.

Stepwise multivariate logistic regression with backward elimination (p-value for retention =0.10) was conducted in significant SNPs in asthma vs controls and adjusted for gender and age. We constructed a genetic risk score for each participant by summing the coefficients for each of the resulting variables after stepwise regression analyses. The variables included in the model were: Ex16 +14C>T and gender. For this SNP, the risk genotypes(CT or TT) were coded as 1 and the non-risk genotype(CC) as 0. For gender female was considered as the risk and coded as 1 and male 0.We obtained 4 risk scores of being asthmatic for this model: high genetic risk score=3.187; intermediate genetic risk score:(1.045 or 1.150); low genetic risk score=-0,992.The individuals that has a high genetic risk score according to this model have an increased risk of 14.500 of having asthma comparing to those low genetic risk score(Table-6).

From logistic regression analysis including significant results from univariate analysis(Table5) between controlled and uncontrolled asthma ,we concluded that *the risk of having uncontrolled asthma for NOS2: IVS16+ 88T>G (GT+TT ) and ACE I/D( ID+II) polymorphisms , are not independent of each other and age.*

Stepwise multivariate logistic regression with backward elimination (p-value for retention =0.10) was conducted in significant SNPs between controlled and uncontrolled asthma and adjusted for gender and age. We constructed a genetic risk score for each participant by summing the coefficients for each of the resulting variables after stepwise regression analyses. The variables included in the model were: IVS16+ 88T>G , ACE I/D and age. For these SNPs: the risk genotype for IVS16+ 88T>G (GT or TT) were coded as 1 and the non-risk genotype(GG) as 0; the risk genotype for ACE I/D (DD) were coded as 0 and the non-risk genotype(ID+II) as 1. Age considered as continuous variable.We divided the Genetic risk score in tertiles as T1:  $\leq -2,68$ (low genetic risk score) ; T2:  $>-2,68 \leq -0,98$  (intermediate genetic risk score); T3:  $>-0,98$ (high genetic risk score).The individuals that has a high genetic risk score

according to this model have an increased risk of 7.222 of having asthma comparing to those with intermediate genetic risk score. In the uncontrolled group we had 0 individuals in the low grade Genetic risk score that is why it is not used as reference in this logistic regression model(Table-7).

### **Discussion**

Airway remodeling is a feature of Th2 inflammation in asthma and is linked to angiogenesis and endothelial dysfunction. The Th2 linked inflammation is associated with epithelial, stromal, dendritic and other immune cells derived cytokines like thymic stromal lymphopoietin (TSLP),IL-33,IL-25(epithelial cytokines) IL-4, IL-5 , IL-13 and IL-9 among others(165). Innate and adaptive immunity are crucial in Th2 inflammation in asthma, and endothelium could have a role in adaptive T cell response but its role in physiopathology of asthma remains unclear(166).

Otherwise endothelial cells have the potential to produce several key molecules involved in allergic inflammation and bronchial asthma such as periostin and other inflammatory cytokines.

IL-25 , a member of the IL-17 cytokine family(167), promotes responses similar to Th2(IL-4, IL-5, IL-13) and is associated with increased number of eosinophils , mucus, profibrogenic stroma, activation of alternative macrophage pathway, subepithelial layer thickening, airway smooth muscle hyperplasia and hypertrophy, angiogenesis and airway hyperreactivity(168,169).

IL-25 and other Th2-cytokines contribute to bronchial mucosal vascular remodeling through endothelial layer and vascular smooth muscle cells upregulating angiogenic factors, that includes , angiogenin, endothelin-1,epidermal growth factor (EGF), insulin-like growth factor (IGF-1) and vascular endothelial growth factor (VEGF). NO can be a positive or negative in modulating angiogenic factors depending on the amounts produced, by NOS (170).There is also an interference in the angiogenic course between VEGF and the renin-angiotensin system in vascular biology and pathophysiology of asthma(171,172).

Endothelial dysfunction is the result of disruption of the balance between vasoactive mediators and inflammation (173). It implies diminished availability of nitric oxide (NO)( mainly vasodilator) with an imbalance in the contribution of other endothelium-derived factors, such as endothelin-1 (ET-1)( vasoconstrictor ) and angiotensin among others. RAS and NOS play a key role in endothelial dysfunction and vascular remodeling, in part by its action on vascular tonus but also because they are involved in ROS signaling , interfering with senescence of endothelial cells, increased endothelial cell proliferation and inflammatory status.

Endothelium as an active integrant of the blood vessels, play a critical role in vascular homeostasis(174,175) and vascular endothelial cells could synthesize nitric oxide from L-arginine. RAS could act in a paracrine and autocrine way, renin has been identified in the smooth muscle layer, angiotensinogen in the adventitia and ACE is a membrane-bound enzyme that cleaves the carboxyterminal of Angiotensin I leading to Angiotensin II and is also involved in the metabolism of Bradykinin that could release NO and it is vasodilator and bronchoconstrictor(145,176).

There is also an age-related endothelial dysfunction with increased ROS production and downregulation of eNOS are mutually responsible for impaired bioavailability of NO and endothelial vasodilator dysfunction associated with an upregulation of TNF- $\alpha$ (177) associated with a sustained ROS signaling that induces senescence of endothelial cells with impairment of endothelial progenitor cells .

This imbalance between senescent/apoptotic endothelial cells and endothelial progenitor cells is also characteristic of asthma and for some authors could elicit Th2 response in asthmatics(165) angiogenesis and airway remodeling.

From the 3 isoforms of NOS (the enzyme that synthesizes NO in the cell.): neuronal NOS (NOS1), inducible NOS (NOS2) and endothelial NOS (NOS3); iNos can be expressed in many cells associated with inflammation(such allergic inflammation) and produces large amounts of NO; eNOS is mainly expressed in endothelial cells and is able to regulate the quiescent and activated endothelial phenotype contributing to endothelial homeostasis, The disturbance on redox vascular balance leads to uncoupling of eNOS and the generation of reactive oxygen species (ROS) instead of NO, ending up also in endothelial dysfunction(178,179) .

According to our results for NOS2(Ex16 +14C>T), those who express T allele have a risk of having asthma ; for NOS2 (intron 20 + 524 G> A): those who are heterozygous GA have a protection of having asthma and also when they express Allele A (GA+AA)have also a protection of having asthma. Comparing controlled and not controlled asthma For NOS2 : IVS16+ 88T>G : there is a risk of being not controlled when compared with controlled for those that are GT ; those who express Allele T (GT+TT)have also higher risk of being non controlled, and there is also a trend in the allelic frequencies for the allele T to be more frequent in the uncontrolled asthma . For ACE gene insertion/deletion (I/D) polymorphism those who express Allele I (ID/II)have protection of having asthma and uncontrolled asthma. For the haplotype analysis at NOS 2 , the overall difference in haplotype frequencies between asthmatics and controls pointed to an increased risk of asthma : (CT+TT)at Ex16 +14C>T and GG at (intron 20 + 524 G> A gene polymorphisms.The epistatic interaction between NOS2:

IVS16+ 88T>G (GT+TT ) and ACE I/D( ID+DD) polymorphisms, it increases the risk of being uncontrolled of approximately 10 times.

Angiogenesis and vascular leakage are prevalent in asthma and pharmacological control of bronchial vascular remodeling is an important issue in respiratory diseases , as vascular oxidative stress can be reduced and NO restored with drugs in the group of angiotensin-converting enzyme-inhibitors, angiotensin receptor blockers, statins and inhaled corticosteroids can positively affect all three main aspects of the vascular component of airway remodeling in asthma: vasodilatation, increased vascular permeability, and angiogenesis.

These single nucleotide polymorphisms could lead to different genotype-specific response to therapy and different endotypes/phenotypes.