Biochemical and genetic examinations in the diagnostics and treatment of childhood diseases

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The thesis is based on the following publications

I. Zsuzsanna Ökrös, Emoke Endreffy, Zoltan Novak, Zoltan Maroti, Peter Monostori, Ilona Sz. Varga, Agnes Király, Sandor Turi: Changes in NADPH oxidase mRNA level can be detected in blood at inhaled corticosteroid treated asthmatic children
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II. Emőke Endreffy, Zoltán Ondrik, Béla Iványi, Zoltán Maróti, Csaba Bereczki, Ibolya Haszon, Zsuzsanna Györke, Endre Worum, Krisztina Németh, Csaba Rikker, Zsuzsanna Ökrös, Sándor Túri: Collagen type IV nephropathy: Genetic heterogeneity examinations in affected Hungarian families
Molecular and Cellular Probes (2011) 25:28-34
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IF: 2.078

List of Abbreviations

AGEs	advanced glycation end-products
ALEs	advanced lipid peroxidation end-products
AP-1	activator protein-1
BAL	broncho-alveolar lavage
BALF	broncho-alveolar lavage fluid
BE	Bergmeyer unit
bp	base pair
CAT	catalase
CYBB	cytochrome B-245 beta chain coding gene
DNA	deoxyribonucleic acid
cDNA	complementary deoxyribonucleic acid
Ct	threshold cycle number
2,4-DNPH	2,4-dinitrophenylhydrazine
DTNB	5,5′-dithiobis(2-nitrobenzoic acid)
DUOX 1-2	dual-peroxidase 1 and 2 isoforms
DUOXA1 and 2	maturation factors of dual-peroxidase 1 and 2 isoforms
EU	enzyme unit
EBC	exhaled breath condensate
FAD	flavin adenine dinucleotide
FRAP	ferric reducing ability of plasma
FRET	fluorescence resonance energy transfer
GPx	glutathione peroxidase
GR	glutathione reductase
GSH	reduced glutathione
GSSG	oxidized glutathione
HO	heme oxygenase
H_2O_2	hydrogen peroxide
h-PBGD	human porphobilinogen deaminase
HRM	high resolution melting
HRMC	high resolution melting curve
ICS	inhaled corticosteroid
IFN-γ	interferon-γ
IGF-1	insulin-like growth factor 1
IL-1, IL-4, IL-5, IL-8, IL-12	interleukins
LABA	long acting β2-adrenoreceptor agonist
LOD	logarithm of the odds
MCO	metal-catalysed oxidation
MDA	malondialdehyde
mRNA	messenger ribonucleic acid
NADPH	nicotinamide adenine dinucleotide phosphate (reduced)
NEM	N-ethylmaleimide

NF-κB	nuclear factor–κB
NOX	NADPH oxidase
NOXA1	NADPH oxidase activator 1
NOXO1	NADPH oxidase organizer 1
Nrf2	nuclear factor erythroid 2-related factor 2
$O_2^{\cdot-}$	superoxide anion free radical
PCO	protein carbonyl derivate
RT-PCR	real-time polymerase chain reaction
ROI	reactive oxygen intermediates
ROS	reactive oxygen species
RNI	reactive nitrogen intermediates
RNS	ribonucleic acid
SNP	single nucleotid polymorhism
SOD	superoxid dismutase
STR marker	short tandem repeat marker
TBA	thiobarbituric acid
TBARS	thiobarbituric acid reactive substances
TBMN	thin basement nephropathy
TCA	trichloroacetic acid
Tm	melting temperature
TNF-α	tumor necrosis factor -α
TGF-β	transforming growth factor-β
2,4,6-TPTZ	2,4,6-tripyridyl-S-tryazin

Introduction

A better understanding of the pathogenesis and pathomechanism of diseases is essential in clinical practice. This is especially true in case of childhood diseases. Nowadays, the identification of the human genome and the increasing knowledge of its influence on normal and pathological processes is a "hotspot" in medical sciences, as among the diagnostic methods. Novel methods arose and developed rapidly in everyday laboratory work in the last twenty years and became part of diagnostic procedures, e.g. in newborn screening and DNA sequencing of rare diseases. In many cases, the final diagnosis can be determined solely with the help of genetic analysis or biochemical methods. However, it is important to know the possibilities and limits of these procedures in the practice.

Through developing laboratory methods, we get more and more information about the pathogenesis and pathomechanism of diseases. The growing knowledge helps us to understand not only the background of these diseases, but may be used in the care and follow-up of patients. It is particularly true in case of chronic pediatric diseases where the use of non-invasive or minimal-invasive, tolerable methods are crucial with the collaboration of the sick child and their parents.

In this thesis, I will discuss the possible use of a biochemical and genetic approach to facilitate the fast and reliable diagnostics of child patients with bronchial asthma and Alport syndrome. We examined the altered redox homeostasis and oxidative stress in childhood bronchial asthma accompanied by chronic airway inflammation, and we tested a prescreening method preceding mutation analysis in patients suffering from X-linked Alport syndrome. Our purpose was to find new methods in the diagnostic and follow-up steps, which did not cause extreme physical and psychological burdens for pediatric patients.

Scientific review

Free radicals and reactive intermediates

The existence of highly reactive molecular components during the organic and inorganic chemical reactions is supposed from the beginning of the 20^{th} century. The scientific work of Fridovich and colleagues, determining the role of SOD in the neutralization process of $O2^{\cdot-}$, has convinced researchers about the existence of free radicals. From this moment intensive attention

has emerged on the formation and role of free radicals both in physiological and pathological states. (*1*)

Free radicals are molecules or molecular fragments that contain one or two unpaired electrons in their external molecular orbits. Molecular fragments react with neighbouring molecules to stabilize their electron structure by obtaining another electron. There have been non-radical species that also have a highly positive redox potential due to being strong electron acceptors in chemical reactions, but do not contain unpaired electrons in their external electron orbits. They are formed by the reduction or excitation of oxygen. (*2*) Radical molecules and molecular fragments along with non-radical molecules have high redox potential, and all are defined as reactive intermediates. The two main families of reactive intermediates are reactive oxygen intermediates (ROI) and reactive nitrogen intermediates (RNI) according to their main atomic content. (*3*)

$O_2^{\cdot -}$ is the primary ROI molecule, formed by electron scavenging of molecular O_2. Secondary-ROI emerge by the uptake of the second and third electron in enzyme or metal catalysed reactions. There are several intracellular sources of $O_2^{\cdot -}$: continuous $O_2^{\cdot -}$ production occurs normally in the mitochondrial electron transport chain, in the endoplasmic reticulum during protein folding. Enzymatic members of ROI generation can be observed by the involvement of several isotypes of NADPH oxidase and xanthine oxidase enzymes. Under pathological conditions, cyclooxygenase, cytochrome P450, and unbound nitric oxide synthase also contribute to excessive ROI generation.

ROI is essential in the normal redox-homeostasis of the cells. They have a pivotal role in the regulation of normal cell function, and in the activity of the immune system due to participating in signal transduction pathways and microbial killing. The physiological process of aging accompanied by cell death is also linked to the regulated production of ROI. (*4,5,6*)

Antioxidant system

To maintain the controlled action of reactive intermediates, a complex antioxidant network developed during the evolution. Antioxidant molecules against the oxidant factors are available in small amounts, but their high efficiency can reduce or neutralize the harmful effects of free radicals. Enzymatic and non-enzymatic components are working synergistically to secure the

normal redox homeostasis. (*7*) Antioxidants can either be endogenous or derived from exogenous sources. These exogenous components do not always have direct free radical scavenging effect but can modulate and enhance the activity of other antioxidants. Enzymatic antioxidants include catalase (CAT), superoxid-dismutase (SOD), glutathione peroxidase (GPx) and glutathione reductase (GR). Non-enzymatic antioxidants include Vitamins E and C, thiol antioxidants (glutathione, thioredoxin, and lipoic acid), melatonin, carotenoids, natural flavonoids, uric acid, and bilirubin. Some elements of the plasma (ferritin, ceruloplasmin) are capable to form a chelate complex with metallic ions, inhibiting the initialization of an oxidative chain reaction. Heat-shock proteins, protease, and lipase enzymes, like the DNA repair enzymes are also members of the antioxidant family and represent the ultimate steps in the defensive mechanism, with a critical role in the repair of damaged biomolecules. Some antioxidants can interact with other antioxidants regenerating their original properties. All these mechanisms work together, forming the antioxidant network. (*8,3*)

Hereinafter the antioxidants measured in our study are detailed only.

Ferric reducing ability of plasma (FRAP)

According to the definition, antioxidants are present at low concentrations in contrast to oxidizable substrates, and can significantly delay or prevent the oxidation of those substrates. This definition covers all types of antioxidants in terms of preventing the generation of ROI or removing them enzymatically by a redox reaction, in which an antioxidant reduces the oxidant substance. In this context, in the plasma sample, where the non-enzymatic antioxidants serve as the primary mechanism, plasma antioxidant power can be analogously referred to as the stoichiometric reducing reaction in a colorimetric reaction. Major part of the antioxidant capacity of the plasma are composed of bilirubin, uric acid, ascorbic acid, α-tocopherol, and proteins, especially albumin. Ferric reducing ability of the plasma (FRAP) in colorimetric analysis is described by this method, in which at low pH, the FeIIITPTZ complex is reduced to FIITPTZ by the plasma-reducing ability. The complex has an intensive blue colour with maximum absorption at 593 nm. All the above-mentioned plasma components are measured together regardless of their distribution in the sample indicating the presence of non-enzymatic antioxidants in plasma. (*9*)

Superoxid-dismutase (SOD)

Dismutation of $O_2^{\cdot -}$ is a crucial step in the neutralization of $O_2^{\cdot -}$. SOD catalyses the following reaction:

$$O_2^{\cdot -} + O_2^{\cdot -} + 2H^+ \rightarrow H_2O_2 + O_2$$

This dismutation step is catalysed by metallic ions, localised in the active centre of the enzyme. There are three isotypes of superoxide dismutase in the human cells: Mn-SOD binds to the mitochondrion, the Cu/Zn-SOD is localised in the cytosol but also exists in the nucleus and mitochondrial intermembrane space. The third subtype is the extracellular SOD which is a glycolized form of Cu/Zn-SOD, and owing to its heparin-binding domain it remains in the extracellular matrix. Proteolytic cleavage of this form enables the appearance of SOD also in the extracellular fluids. (*1,10,11*)

Catalase (CAT)

CAT enzyme catalyses the transformation of H_2O_2 to H_2O and O_2 in the following manner:

$$H_2O_2 + H_2O_2 \rightarrow 2H_2O + O_2$$

CAT works in the tetramer structure with Fe^{3+} ion being in a porphyrin ring in its active site. The enzyme requires two molecules of H_2O_2 for its action but also participates in the degradation of other small-sized peroxides. The enzyme's substrate affinity is low and works only in higher H_2O_2 concentrations. Because of its structure, the CAT is highly resistant to thermal denaturation, proteolysis, and other structural changes. Elimination of H_2O_2 and other peroxides comes through cooperation with glutathione peroxidase (GPx): generally, where higher CAT activity can be seen, lower GPx activity is detectable, and vice versa, although the whole process is more complicated and not entirely understood. Accordingly, in mammalian tissues, CAT activity is the highest in liver and erythrocytes, relatively high in kidney and adipose tissue, moderate in lung and pancreas, and very low in heart and brain. (*12,13,14,15*)

Glutathione (GSH) and glutathione redox system

GSH is present in high abundance in the cells at millimolar concentrations. It is composed of glycine, cysteine, and glutamate in a three-amino acid structure. Glutathione is present in the cells in reduced and oxidized form, and the ratio of GSH/GSSG is typically 100:1, buffering the cells' redox homeostasis. The reduced GSH serves as a substrate for enzymes that scavenge ROI, inactivate electrophilic species, and restore reduced cysteine-thiol moieties of proteins,

with concomitant production of oxidized GSSG. GSH also participates in DNA repair, influences the signal transduction processes and gene regulation, and can regenerate the structure of other antioxidants such as Vitamin C and E. GSSG is depleted from cells or recycled back to GSH. (Figure 1.)

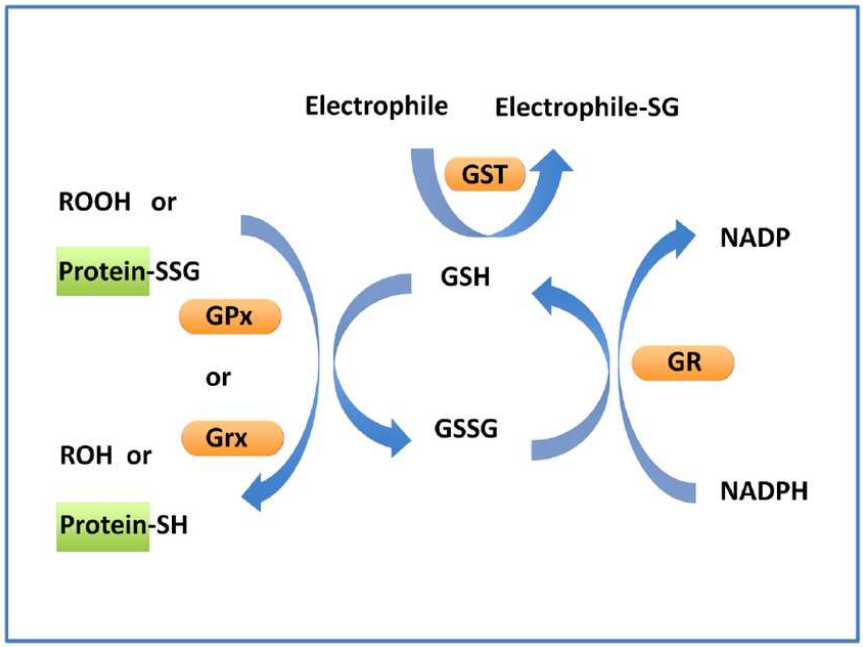


Fig. 1: The GSH redox cycle is maintained by the cooperation of glutathione peroxidase (GPx) and glutathione reductase (GR) enzymes. *(Fig.1 published: Johnson WM, Wilson-Delfosse AL, Mieyal JJ: Dysregulation of Glutathione Homeostasis in Neurodegenerative Diseases Nutrients (2012) 4: 1399-1440)(16)*

Substrates of the GPx enzyme are H_2O_2, organic hydroperoxides, and corresponding alcohols, catalysing reducing reactions with the conversion of GSH to GSSG. GPx is a member of the selenoprotein family with selenocysteine in its active centre. GPxs work synergistically with CAT and can be detected in a higher amount where lower CAT activity is measured. GR reduces oxidized glutathione to its reduced form, requiring NADPH as a cofactor. NADPH is derived from the hexose-monophosphate shunt, and the concentration of GSSG and NAPDH together determines the enzyme's activity. (*17,18,19,20*)

Heme-oxygenase (HO)

HO enzymes regulate cellular heme and iron levels by catalysing the rate-limiting step of heme catabolism. The oxidative cleavage of heme results in the equimolar amount of iron, CO, and biliverdin. The cytoprotective effect of HO is derived from the ability to eliminate the pro-oxidant heme, and also from the production of CO with its potent anti-inflammatory, antiapoptotic, and antiproliferative features. CO also has a putative neurotransmitter effect. In a physiologically narrow range, it can modify certain intracellular signaling pathways by activating guanylate cyclase and several gene regulation processes. The other end-product bilirubin, derived from biliverdin, has a potent antioxidant effect in the plasma in small ranges with its peroxyl-radical scavenging activity and reducing ability. Contrarily in a higher amount, it serves as a proinflammatory and toxic agent in several diseases. Three distinct isoforms of HO have been characterized: HO-1 which is an inducible form, and the constitutively expressed HO-2 and HO-3. HO-1 is expressed at a high level in certain tissues (liver, spleen) which are dedicated to eliminate senescent red blood cells as the only heme-degrading enzyme. In most other tissues HO-1 is typically expressed at a very low level in basal conditions. In response to various stimuli, which cause cell stress, the enzyme activity increases rapidly due to the marked increase of gene expression coding the HO-1 protein. The activity of this isotype is regulated solely transcriptionally, posttranscriptional effects do not influence the enzyme effect. HO-2 is localised mainly in the mitochondrial membrane and the coding gene expression is regulated constitutively. Novel results emerged about its role as an oxygen sensor in the cells, due to its cysteine motif and thiol-dithiol transformation. HO-3 is in 90% homologous to HO-2 and its catalytic activity is lower. (*21,22,23,24,25*)

Oxidative stress

Oxidative stress results from the imbalance of the pro-oxidant and antioxidant mechanisms of the body. This imbalance originate from the relative or absolute excess of the oxidizing agents or the deficiency of the antioxidant mechanisms. Pro-oxidants can be overproduced in inflammatory processes: either by an exogenic overload of reactive intermediates or by the overactivity of pro-oxidant enzymes. Antioxidant impairment can be derived from impaired antioxidant enzyme function or the altered level of extrinsic (molecules derived from the diet) or intrinsic (bilirubin, glutathione, etc.) molecules with antioxidant capacity.

The production of highly reactive ROI and RNI damages the biomolecules with structural and functional consequences, influencing gene expression, signal transduction pathways, enzyme functions, and several essential functions. In addition to the direct harmful effects, the accumulation of the damaged biomolecules can also alter the cell function. All these mechanisms are included in the chronic inflammatory processes and structural remodelling of tissues.

The endogen source of ROI and RNI are mitochondria, endoplasmic reticulum, and peroxisomes with various enzymes or enzyme complexes (cytochrome P450, xanthine oxidase, NADPH oxidase complex isotypes, nitric oxide synthase, heme proteins, Fenton reaction, etc.). Immune and non-immune cells (macrophages, neutrophils, alveolar macrophages, endothelial cells, smooth muscle cells, fibroblasts, etc.) also participate in the reactive intermediate generation. On the other hand, there are several external sources of reactive intermediates: ultraviolet radiation, tobacco smoke, ionizing radiation, metals, medical agents, etc. In pathological conditions both endogen and exogenous sources may implicate in the processes, often commonly influencing and exciting each other's effect. (*4,5*)

The damaged biomolecules (nucleic acid, proteins, lipids), the altered enzyme functions, or the by-products of the pathological processes are often detectable and may be useful markers of the oxidative stress accompanied by various diseases. (*3*)

Protein damage

Proteins are the most vulnerable by the oxidative damage because of their catalytic rather than their stoichiometric mediator function. Proteins can be modified and damaged in several ways in oxidative stress: by breakdown or oxidation of amino acid sidechains (mainly of the thiol groups), by amino-acid crosslinks, and by formation of reactive protein carbonyls. All these changes can induce alterations in the structure, resulting in modified or loss-of-function proteins. Cysteine and methionine are prone to oxidative attack by almost all ROS. Protein modifications elicited by direct oxidative attack on lysine, arginine, proline, and threonine, or by the secondary reaction of cysteine, histidine, and lysine residues with reactive carbonyl compounds can lead to the formation of protein carbonyl (PCO) derivatives (aldehydes and ketones). Studies examining PCOs could not differentiate whether these metabolites are produced by direct oxidation of side chains or by the addition of previously oxidized molecules, so the rate of PCOs is used as broad markers of the protein oxidative damage. Beyond that, PCOs are not only biomarkers of oxidative damage but also indicators of disease-derived

molecular dysfunction. As PCOs are relatively stable, they can circulate in the blood for a longer period than other parameters of oxidative stress (such as GSSG, and MDA), so many sensitive biochemical and immunological assays have been developed to quantify the amount of PCOs for indication of the oxidative damage. (*26,27,28,29*)

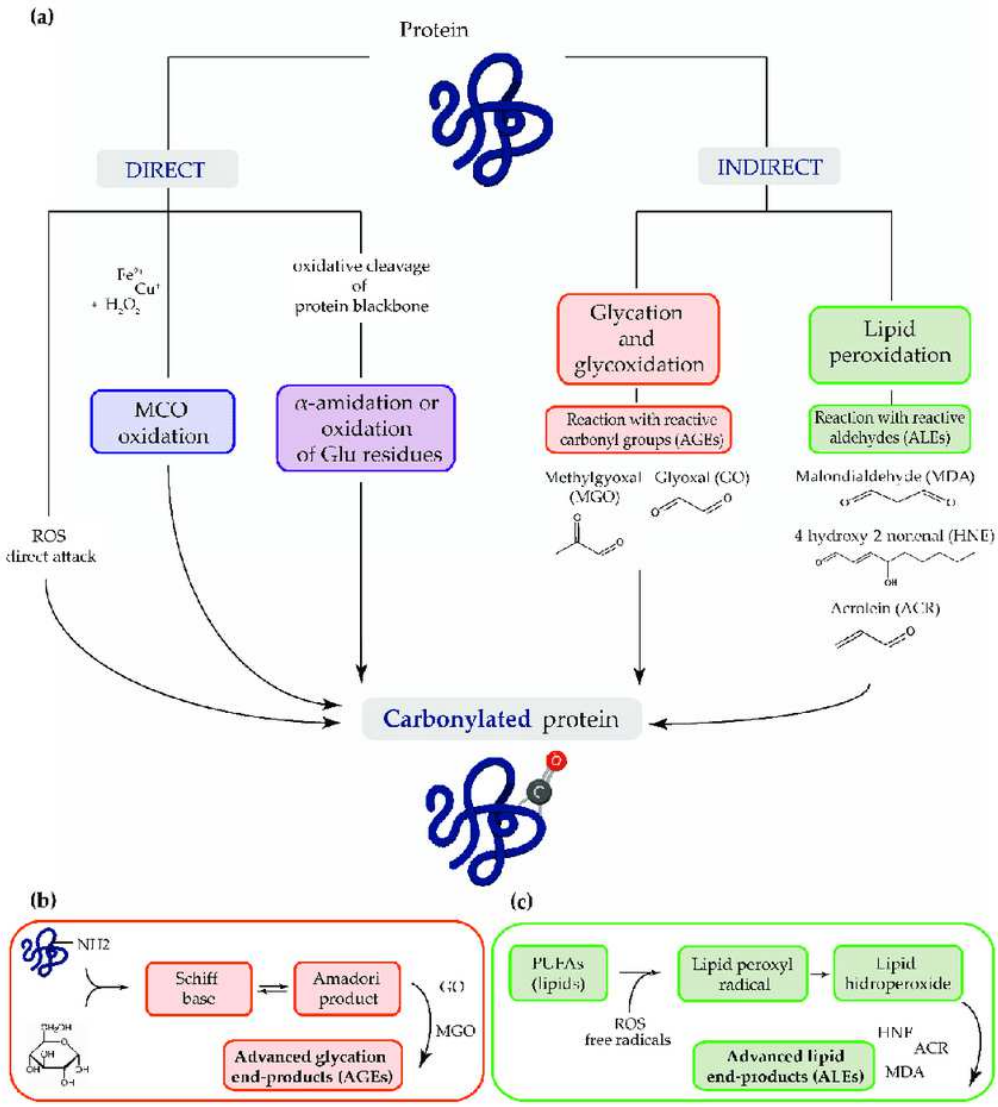


Fig. 2: (**a**) ***The most common mechanisms of protein carbonylation.*** Direct processes include reactive oxygen species (ROS) attack, metal-catalysed oxidation (MCO), and the oxidative cleavage of protein backbone (via the α-amidation pathway or through oxidation of glutamine side chains). The indirect mechanisms involve reaction with (**b**) advanced glycation end-products (AGEs) and (**c**) advanced lipid peroxidation end-products (ALEs).

(*Fig. 2, published: Rodríguez-García A., García-Vicente R, Morales ML, et al.: Protein Carbonylation and Lipid Peroxidation in Hematological Malignancies (Review) Antioxidants (2020) 9 1212)(30)*

Lipid peroxidation

ROI can interact with lipids in the process named lipid peroxidation. Normally, as oxygen has a weak oxidizing ability, lipid peroxidation proceeds slowly. However, electron distraction from the lipid generates lipid radical which is much more reactive to oxygen, forming lipid hydroperoxides. This chain reaction has three steps: initiation, propagation, and termination:

$$RH + \cdot OH \rightarrow R\cdot + H_2O$$

$$R\cdot + O_2 \rightarrow ROO\cdot$$

The generated peroxyl radical (ROO·) distracts electrons from the neighbouring molecules.

$$ROO\cdot + RH \rightarrow ROOH + R\cdot$$

During degradation of lipid peroxides, several highly cytotoxic elements are produced.

$$RO\cdot + RH \rightarrow ROH + R\cdot$$

Termination of lipid peroxidation due to assistance of the antioxidant system:

$$ROOH + 2\,GSH \rightarrow ROH + GSSG + H_2O$$

Polyunsaturated fatty acids are more affected by peroxidation because their weak double C-H bonds are more sensitive to electron distraction as the initiating step of oxidation. Lipid peroxidation does not only damage lipids but also affects the neighbouring carbohydrates and protein moieties, so its high reactive spectrum accounts for a broad harmful effect on membranes and cell particles. The loss of membrane fluidity, the reduction of membrane potential, and damage to protein and nucleic acid components all contribute to the consequences of lipid peroxidation. The major but not the sole end-product of lipid peroxidation is malondialdehyde (MDA). The thiobarbituric assay determines the concentration of thiobarbituric reactive substrates (TBARS), which method covers a broad range of substrates derived from lipid peroxidation. However, the major lipid oxidation product in biological samples such as serum/plasma, and tissue homogenates, is MDA, so TBARS methods determine mainly MDA. (*31,32,33*)

NADPH oxidase complex

The NADPH oxidase enzyme family contains homologous catalytic subunits, abbreviated as NOX. Seven NOX members exist. Their structures are characterized by at least six transmembrane helices containing two iron-heme prosthetic groups, as well as a flavin adenine dinucleotide (FAD) and a NAPDH-binding domain in the cytosolic C-terminus. Dual-

peroxidase 1 and 2 isoforms (DUOX 1-2) have an additional cytosolic N-terminal peroxidase-like domain, without real peroxidase activity in the human enzyme subtypes. All NOX isoforms catalyse a two-electron transfer from NADPH via their FAD domain and two iron heme prosthetic groups to molecular oxygen. While NOX1, NOX2, NOX3, and NOX5 produce superoxide, NOX4, DUOX1, and DUOX2 mainly release hydrogen peroxide.

NOX enzymes also differ in their enzymatic complex composition, mode of activation, and in the products of their enzymatic activity. Most NOX isoforms require at least one cytosolic or membrane-bound binding partner for their activity. In case of NOX1-4, the associated $p22^{phox}$ stabilizes the membrane protein, while DUOXA1 and DUOXA2 subunits are membrane-bound maturation factors for DUOX1 and DUOX2. All other proteins in this complex play activator or organizer roles in the activation or promotion of ROI generation. These are $p67^{phox}$ and $p40^{phox}$ for NOX2 and the functional homolog of $p67^{phox}$ named NOXA1 for NOX1. The organizer proteins set-up the structural requirements for the activation or binding of the activator proteins. $p47^{phox}$ stabilizes the complex formation for NOX2. NOXO1 enables the active complex formation for NOX1 and NOX3. Besides, NOX1-3 seems to require small GTPase, Rac, for their activity. Because of their tissue and function heterogeneity, NOX complex components also have many other regulatory elements (e.g. heat-shock proteins, tyrosine kinases) in vivo, influencing protein folding, tissue-specific expression, and also activity regulation in physiological and pathological states.

As mentioned above, NADPH oxidases are widely distributed through different tissues and cell types. NOX1 is mainly expressed in the colon, NOX2 in phagocytes and B lymphocytes, NOX3 in the inner ear and some fetal tissues, NOX4 in the kidney and the blood vessels, and NOX5 in lymphoid tissue and testis. DUOX1 and DUOX2 are most abundantly expressed in thyroid and lung tissue. Several studies have already investigated different diseases to elucidate the role of oxidative damage in the pathogenesis and pathomechanisms of NOX isotypes taking into consideration of their role, regulation, and components. However, several questions have remained unclear. (*34,35,36,37*)

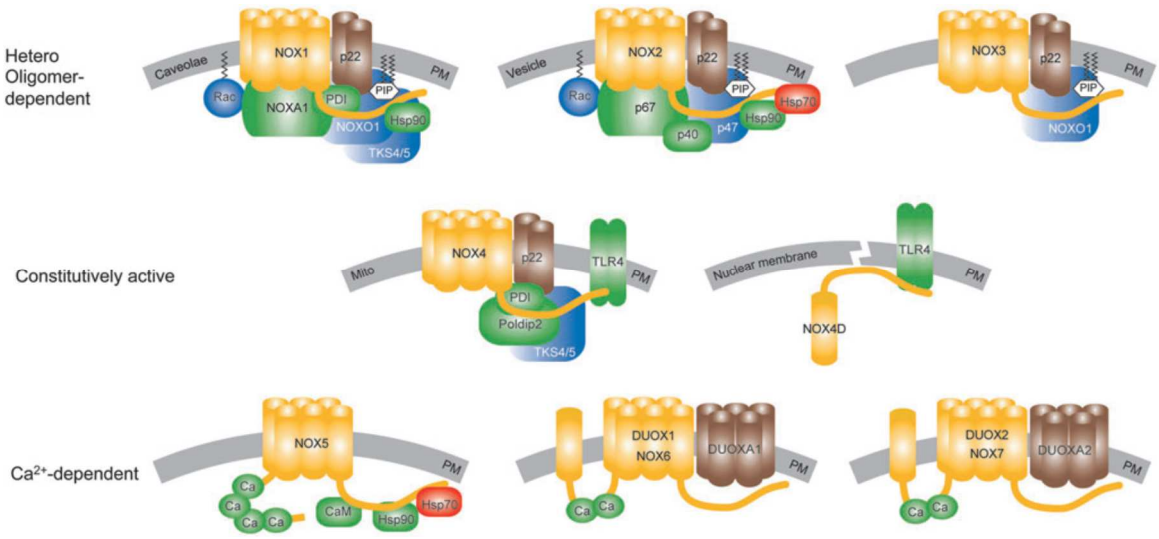


Fig. 3: The NADPH oxidase enzyme family.

All NOX isoforms (yellow) are membrane proteins that are localised in the plasma membrane or cellular compartments' membranes (grey). Stabilizing or maturation factors of NOX are presented in brown, activator binding partners are in green, complex organizer binding partners are in blue, and destabilizer binding partners are in red. *(Fig.3 published: Altenhöfer S. et al: Evolution of NADPH Oxidase Inhibitors: Selectivity and mechanisms for Target Engagement ANTIOXIDANTS & REDOX SIGNALING (2015) 23: 427-448) (38)*

In our study, we have focused on the NOX2 isotype, mainly expressed in mononuclear cells. NOX2 isoform's membrane-associated component, the flavocytochrome b558, has two main membrane-bound, electron transfer catalysing subunits ($gp91^{phox}$ and $p22^{phox}$) mediating the electron transmission from NADPH to O_2, producing $O_2^{\cdot-}$. Both subunits are highly regulated by proinflammatory cytokines (TNFα, IL-1) and by the ROI themselves, although other cytosolic subunits are also essential for the complete enzyme function. The upregulation of NOX2 subunits and increase of their activity contribute to the elevated $O_2^{\cdot-}$ and ROI production during inflammation by activated mononuclear cells. (*39,40*)

Oxidative stress in respiratory diseases and bronchial asthma

Because of its function, the respiratory tract is highly exposed to atmospheric oxygen. The large surface and intensive blood flow, as well as the exposure to environmental agents make the respiratory tract susceptible to oxidative damage. All the pro-and antioxidant components and processes in the lung are subtly determined, and the imbalance between them can lead to

lung injury. In physiological conditions, ROI/RNI inflicts their effect on mucus production and on the alveolar repair mechanism, affecting the airway and connective tissue remodelling and regulating the local immune response. All these subcellular actions are associated with chronic inflammatory processes, leading to a variety of diseases, such as bronchial asthma, chronic obstructive pulmonary disease, and idiopathic pulmonary fibrosis, and have a secondary role in other conditions affecting the lung, e.g. respiratory distress syndrome, cystic fibrosis, sepsis. (*41,42*)

At the molecular level, increased ROI/RNI levels have been implicated in the initiation of inflammatory responses in the lungs through the activation of transcription factors such as nuclear factor-κB (NF-κB) and activator protein-1 (AP-1), signal transduction, chromatin remodelling and gene expression of proinflammatory cytokines. Cytokines involved in inflammation and fibrogenesis are TNF-α, TGF-β, IGF-1, endothelin-1, IL-1, IL-8, and IL-4, while IL-12 and IFN-γ promote antifibrotic processes. Activated eosinophilic and neutrophilic granulocytes, macrophages, and also constitutive respiratory cells, such as epithelial cells, are also considered to participate in ROS/RNS overproduction and antioxidant defence. (*43,44,45,46,47,48,49*)

Because of the complexity and subtility of oxidative stress, several reports were published in the past decades examining components of the redox imbalance. Several ongoing studies still exist with the aim to understand and monitor objectively the pathological changes and to find therapeutic targets to control oxidative injury. Not only in vitro studies have been completed recently, but also data from animal models have been obtained and human clinical trials have been performed, investigating bronchoalveolar lavage (BAL) fluid, sputum, exhaled breath condensate (EBC), systemic circulation, or urine. (*50,51,52*)

Many biomarkers of inflammation and oxidative stress have been detected in exhaled breath condensate (EBC) samples. In asthmatic inflammation higher levels of hydrogen ion and lower pH have been detected in asthmatics as compared with controls, accompanied by elevated levels of NO, H_2O_2, and 8-isoprostane, a lipid peroxidation by-product. Leukotriene B4, C4, and interleukins (IL-4, IL-5) were also detectable in higher amounts in the EBC of asthmatics. Lower glutathione levels and lower catalase activity were measured in BAL and EBC as well in asthmatic children with an increase after oral steroid treatment. Acute asthma exacerbation may lead to a more significant decrease in the glutathione levels in asthmatic patients compared to healthy controls. The steroid treatment with oral formulas augments the GSH level compared to the pretreatment state. (*53,54,55,56*)

Despite the availability of results from human studies, data from children are still limited. Examination of the airways requires invasive or semi-invasive methods implicating ethical doubts. Data from non-invasive approaches (e.g. exhaled air condensate and induced sputum) to examine the inflamed airways are less reliable and could be influenced by the cooperation of the patient and by the pre-arrangements before the study. In bronchial asthma, the pathological events and symptoms originate from the primarily affected organs; however, the systemic effects have to be considered too. In accordance, studies using blood as specimen type, have gained attention in the past few years. However, the available data from blood samples are limited and conflicting. Monitoring of inflammatory processes from blood could be a useful and tolerable way to follow the extent of inflammation in pediatric patients. (*45,46,47,48,49*) In certain patient groups, such as asthmatic children, it would be a great advantage to develop protocols to monitor the oxidative stress before and during treatment using peripheral blood samples rather than collecting BALF or tissues, or to analyse exhaled breath condensates. (*53,54,55,56*)

Alport syndrome

Alport syndrome and thin basement membrane nephropathy are recognized as specific diseases of the type IV collagen chains. As a consequence of mutations in any of collagen type IV coding genes, the development switch from embryonic-type (heterotrimeric α1:α1:α2) collagen to adult-type (heterotrimeric α3:α4:α5) collagen is arrested. The autosomal recessive forms affect the COL4A3 and COL4A4 genes (2q35-37), while in the X-linked form, the mutation is located in the COL4A5 gene (Xq22). Most (~85%) of the families with Alport syndrome exhibit the X-linked form, while 10-15% of the remaining cases have an autosomal mutation in recessive, and less frequently in dominant way. The most severe clinical outcome is seen in the X-linked Alport syndrome in the affected males with persistent microscopic hematuria, hearing loss, and ocular abnormalities. The altered structure of the glomerular basal membrane is worsening and during the early 20s end-stage renal failure develops. Females tend to be less severely affected. Individuals homozygous or compound heterozygous for 2 mutations of COL4A3 or COL4A4 genes have been identified in autosomal recessive Alport syndrome with less severe clinical symptoms. With very few exceptions, each family carries unique mutations. Approximately 10-15% of the COL4A5 mutations are *de novo*, having occurred in the gamete of a parent. (*57,58,59,60,61,62,63,64*)

Thin basement membrane nephropathy (TBMN) with its reported prevalence between 1 to 14 % also has a big difficulty in the differential diagnosis. In this case, the persistent or recurrent hematuria, mild proteinuria, and the absence of renal failure or extrarenal symptoms are attributed to mutations usually in the COL4A3 or COL4A4 genes in heterozygous form. The hematuria usually remains isolated and only a minority of the cases progress toward end-stage renal disease. The rare occurrence of hearing loss in TBMN may also complicate the above mentioned differential diagnosis. The pattern of inheritance of TBMN is autosomal dominant: approximately two-thirds of patients with TBMN have an autosomal dominant familial form of the disease. In some cases, linkage to these genes could not be detected, so the role of other candidate genes and modifier factors have been postulated. The pattern of inheritance is autosomal dominant. (*65,66*)

Pediatric nephrologists must distinguish TBMN from the early stage of Alport syndrome, and give a prognosis to the parent and children. As there are overlaps between the symptoms and their appearance during the progression, the clinical pedigree analysis is not always sufficient to distinguish the mode of inheritance.

The inheritance of Alport syndrome and TBMN can be determined by linkage analysis, but identifying the candidate mutation in these multi-exon-containing genes is demanding technically and financially, because a large amount of candidate gene loci should be examined. ***In this study***, we have focused on COL4A5 gene mutation analysis after a former linkage analysis. We carried out the mutation prescreening method of point mutations by high resolution melting (HRM) curve analysis in all 56 amplicons of the 51 exons in the Col4A5 gene.

In HRM analysis, the region of interest is amplified by polymerase chain reaction (PCR) in the presence of a fluorescent double-strand DNA-binding dye. Following PCR, the product is gradually melted, and the emitted fluorescence is measured on a specialized instrument to generate a characteristic curve. The melting profile reflects the mix of amplicons present. The length of the amplicon, GC content, sequence, and heterozygosity will add to the melt curve characteristics for each amplicon. Melt curves that are similar in shape but that are distinguishable from each other by differences in melting temperature (Tm) of the amplicon, may represent homozygous variants, that are being compared to a wild-type sample. The Tm difference between samples is due to sequence variation from the wild type. Melt curves displaying a curve shape distinct from homozygote melt curves derive usually from the presence of base-pairing mismatches (heteroduplexes) present in the PCR product mix. (*67,68,69,70,71*)

Aims

1. In certain chronic diseases, including asthma, the pathological events and symptoms originate from primarily affected organs; however, the systemic effects are also considerable. We aimed to examine blood as a candidate sample, to monitor the airways-localised inflammation in the circulation as an easy-obtainable possibility.

2. To examine the supposed systemic effects of oxidative stress and antioxidant response in childhood asthmatic patients and age-matched controls, we set out to measure the biochemical parameters of oxidative stress. As antioxidants, we have chosen ferric reducing ability of the plasma, the elements of glutathione redox-cycle (GSH, GSSG, and enzymatic components by measuring GR and GPx activity), SOD, and CAT activities. To estimate the direct consequences of oxidative damage, we have measured the total of the damaged protein and lipid products from the blood.

3. We were interested in the expression profile of the NOX2 coding CYBB gene, one of the main $O2^{\cdot-}$ sources of ROS in inflammatory processes, and the transcriptional activity of the inducible HO-1 enzyme coding HMOX1 gene, representing the antioxidant system. We also proposed the optimization of our equipment to be suitable for real-time methods as well as gene quantification for further analysis.

4. We aimed to compare the oxidative status of blood and the clinical state of asthmatic patients, mainly focusing on asthma control.

5. In our second study, we decided to apply high-resolution melting curve analysis to high number of exon-containing genes to facilitate the localisation of candidate point mutations as a pre-screening method. Thus, we also planned to indroduce and test a new RT-PCR-based technology among our molecular biological diagnostic tests. For this purpose, we used the X-linked COL4A5 gene in our previously examined patients and their families.

Patients and Methods

Study group

In Study 1, 58 patients with the confirmed diagnosis of bronchial asthma, and 30 healthy controls were examined. The asthmatic patients were admitted to the inpatient and outpatient wards of the Department of Pediatrics and Pediatric Health Center, Albert Szent-Györgyi Clinical Center, University of Szeged. Healthy controls were enrolled from the ward of surgery waiting for elective intervention and from the outpatient department of our institution invited for medical check-ups after recovery. No controls had acute disease one month prior to sample collection. No controls had positive atopic status, respiratory disease, and chronic disease in their anamnesis, which could have influenced their oxidative status. There were 36 boys and 22 girls among patients (mean age: 14.55 years vs. 16.29 years) and 16 boys and 14 girls among healthy controls (mean age: 14.94 years vs. 16.03 years). The level of asthma control was assessed by the Global Initiative for Asthma references, and the medication was adjusted according to their complaints and the parameters were reported as per the actual guidelines at that time. The asthmatic patients were classified into 4 subgroups: those who did not require inhaled corticosteroid (ICS) (n=22), those who received a low daily dose of ICS (below 200 μg/day) (n=20), those who received a medium daily dose of ICS (200–400 μg/day) (n=6), and those who received a medium/high daily dose of ICS (≥200–400 μg/day) combined with long acting β2-adrenergic receptor agonist (LABA) (n=10). The steroid dose was interpreted in terms of the budesonide equivalent dose. The medication had been administered for at least six months before sample collection. All the patients had the possibility to use short-acting β2-adrenergic receptor agonists as rescue therapy. In some cases, histamine 2-receptor antagonists and leukotriene receptor antagonists were also administered as maintenance therapy when required. The study was approved in advance by The Institutional Research Ethics Committee of the University of Szeged (No. 2466/2008.), and written parental informed consent was obtained in all cases before the study.

Sample collection

Six ml of blood was taken from a peripheral vein in both groups. For the gene expression experiments, blood was stabilized (RNA/DNA Stabilization Reagent for Blood/Bone Marrow, Roche), and EDTA-coated tubes were used for the collection and analyses of oxidant and antioxidant parameters.

Blood antioxidant components and markers of oxidative damage

FRAP assay

According to the method of Benzie and Strain, the plasma total antioxidant capacity was determined by the FRAP assay. This test measures the ferric reducing ability of the plasma, using 2,4,6-tripyridyl-s-triazine (FeIII 2,4,6-TTPZ). At low pH, the reduction of ferric ion (Fe^{3+}) to ferrous ion (Fe^{2+}) results in the formation of a coloured ferrous-tripyridyltirazine complex, which is detectable at 593 nm. FRAP values were obtained by comparing the absorbance change in test reaction mixtures with those containing ferrous ions at a known concentration. (*9*)

Protein content

The protein content of the samples was determined by the method of Lowry and colleagues. To each 100 µL of the sample 1 mL „C" solution was added. „C" solution contained 20 g/L Na_2CO_3, 0.2 g/L K-Na-tartarate. Later solution A (0.1M NaOH) and solution B (5 g/L $CuSO_4$ x 5 H_2O) were added to this compound mixture in the ratio of 50:1. After 10 minutes of incubation, 100 µL of two-fold diluted Folin-Ciocalteu-reagent was added. Following a 30 minutes incubation, the extinction was measured at 675 nm wavelength spectrophotometrically. Albumin derived from bovine serum was used as a calibrator before the measurements. (*72*)

Whole blood GSH-GSSG content

Whole blood levels of GSSG and GSH were assayed employing a spectrophotometric enzymatic recycling method. In the presence of 5,5′-dithio-bis(2-nitrobenzoic acid), glutathione reductase and NADPH, the coloured product 5-thionitrobenzoate is formed upon the progressive reduction of GSSG to GSH. The rate of colour change at 412 nm over a period of 3 min, is proportional to the total glutathione (i.e. GSSG plus GSH) concentration. GSSG was determined via the blocking of GSH with the thiol-masking agent N-ethylmaleimide in a separate sample. (*73*)

Determination of GPx activity

To determine GPx activity we measured the GSH consumption in each sample and in its own control, with cumene hydroperoxide and GSH as substrates. The determination was performed spectrophotometrically at 412 nm with measurement of the residual GSH with 5,5′-dithio-bis(2-nitrobenzoic acid). All samples contained 0.1 mL from the 10-fold hemolysate, 0.7 mL of 0.05 M TRIS-HCl buffer, pH 7.6, and were preincubated at 37°C for 10 min. To the control sample, 0.1 mL of GSH solution was given, and the experimental samples were also supplemented with both 0.1 mL GSH solution and 0.1 mL 0.05% buffered cumene hydroperoxide solution. The samples were then incubated at 37°C for 10 min. After incubation, 15% TCA solution was added to all samples. 0.1 mL cumene hydroperoxide solution was also added to the control sample. The precipitated protein was spun down and 1mL of supernatant was used for the determination of the remaining GSH. This happened with the addition of 2 mL 0.4M TRIS-HCl buffer and 5,5′-dithio-bis(2 nitrobenzoic acid) (DTNB). Results were expressed in U/mg protein. (*74,75,76*)

GR activity

During determination of GR activity, GSSG was added as substrate and NADPH as a cofactor. The GR enzyme activity was determined at 373 nm wavelength. The experimental cuvette contained 0.5 mL 0.25M KH_2PO_4 buffer at pH 7.4, 0.1 mL of 25mM EDTA, 0.1 mL of 7.5mM NADPH, 0.25mL of 0.25mM GSSG, 0.05 mL of hemolysed blood as sample and 0.25 mL distilled water. A control phosphate buffer was used. Results were expressed in U/mg protein. (*74,76*)

Activity of SOD

According to the method of Matkovits et al., SOD activity was measured from the supernatant of the red blood cells via the inhibition of the epinephrine-adrenochrome spontaneous transformation. Before determination of SOD activity, hemolysates were treated with ethanol-chloroform (2:1) to remove hemoglobin from the samples and centrifuged at 3000 g. The supernatants were used for the determination of SOD activity via inhibition of the epinephrine-adrenochrome transformation. The control sample contained 2.9 mL of 0.05 M carbonate buffer, pH 10.2, warmed to 37°C and 0.1 mL epinephrine (16.5 mg/10 mL 0.1 N HCl). Absorbance was measured at 480 nm for 3 min after a 1 min delay. This way we got the

rate of epinephrine autooxidation. To determine the inhibition of autooxidation by SOD in the sample, we used the mixture of 0.02 mL supernatant, 2880 mL of carbonate buffer, and 0.1 mL of epinephrine. Spectrophotometric measurement was performed at 480 nm wavelength. Results were expressed in the inhibition degree of the sample, which was presented in U/mg protein. (According to the definition, one unit of SOD enzyme leads to 50% inhibition in the epinephrine-adrenochrome transformation.) (*1,77,78*)

Determination of CAT activity

The activity of CAT was measured according to the method of Beers and Sizer, in which the enzyme activity was determined by decomposition of H_2O_2. 100-fold diluted erythrocyte hemolysate was used. The blank sample was 3 mL of phosphate buffer, pH 7.0 in a quartz cuvette. The incubation mixture contained 2 mL of phosphate buffer (warmed at 37°C), 5 µL from the hemolysate sample, and 1 mL of 30 mM H_2O_2 solution. CAT activity was measured spectrophotometrically at 240 nm. Results were expressed in Bergmeyer unit/mg protein (BU). (79)

Determination of thiobarbituric acid active elements (lipid peroxidation) of blood

The determination of MDA level, derived from red blood cells, was performed by using thiobarbituric acid (TBA), which reveals the total amount of TBA-reactive substances. 200 µL from the hemolysed blood was added to 1.8 mL of TBA reagent solution (0.375% TBA, 0.25 M HCl, 15% TCA). After mixing components, it was heated for 20 minutes in a boiling water bath. Subsequently, samples were cooled in ice-cold water and centrifuged at 10000 rpm for 10 minutes. The absorbance of the supernatants was measured at 532 nm spectrophotometrically to calculate the concentration. Results are reported in nmol/mg protein. (*80*)

Protein damage

Carbonyl proteins are oxidatively modified proteins. Protein damage was determined using 2,4-dinitrophenylhydrazine (DNPH). All samples had their own control. Samples were incubated with DNPH, controls were incubated with 2M HCl at 37ºC for 1 h. After the addition of 0.5 mL TCA, samples were centrifuged at 13000 rpm for 10 min. Pellets were washed twice with methanol : ethylacetate (1:1). Samples were resuspended in 6M guanidine solution with

KH_2PO_4, and incubated at 37ºC for 20 min. The absorbance was read at 370 nm. Results are expressed in mmol/mg protein. (*81*)

Gene expression analysis

The $gp91^{phox}$ (CYBB) and HMOX-1 gene expressions were determined by using a real-time polymerase chain reaction (RT-PCR) technique. Briefly, after the extraction of mRNA from whole blood as described by the manufacturer (mRNA isolation Kit for Blood/Bone Marrow, Roche), reverse transcription was performed by using a cDNA Synthesis Kit (Fermentas). The RT-PCR process was carried out with a LightCycler Carousel-based System 1.5 (Roche) and LightCycler Software Version 3.5, with a FastStart HybProbe Kit (Roche). The following primers and hybridization probes were designed by the LightCycler Probe Design Software 2.0: *forward primer-CYBB*: 5'-AACACCCTAATACCAGA-3', *reverse primer-CYBB*: 5'-CATGGAAGAGACAAGT-3', *P1-CYBB*: 5'-CTCTGTGGACCTGAAGCC-Fluo3', *P2-CYBB*: 5'LcRed705-GGCTGAAACCCTGAGTAAAC-Pho3', *forward primer-HMOX-1*: 5'-GTTCCTGCTCAACATCCA-3', *reverse primer HMOX-1: 5'*-GCTTCCCTCTGGGAGTCT-3', *P1-HMOX-1*: 5'-GCTGACCCATGACACC-Fluo3', *P2-HMOX-1*: 5'-LcRed705-GGACCAGAGCCCCTCA-Pho3'. The annealing temperature was set at 55ºC. Human porphobilinogen deaminase (h-PBGD, HPBGD Gene Set, Roche) was used as the housekeeping gene, marked by different dyes, in the same capillary, to normalize the differences between the samples' mRNA concentrations. Relative expressions of candidate mRNAs were determined by using the threshold cycle number (Ct) data obtained by real-time PCRs, with each sample normalized to its own h-PBGD housekeeping gene *(*ΔCt). We have used the $2^{-\Delta\Delta Ct}$ method as relative quantification strategy for quantitative real-time polymerase chain reaction (qPCR) data analysis. The ΔCt data of the overall group and the individual subgroups were compared with each other or those for the healthy controls *(*ΔΔCt). (*82*, *83*)

Alport syndrome Col4A5 gene mutation prescreening

In Study 2, 20 families affected with hematuria and their family members were examined. Each family had at least 2 hematuric and 2 or more unaffected individuals. After the clinical diagnosis together with histological, and immunohistochemical examinations, segregation analysis was performed with STR markers for COL4A3, COL4A4, and Col4A5 genes to analyse the linkage to chromosomes 2, 13, and X. In our study, 9 families were selected for

further analysis with linkage to the COL4A5 locus with negative LOD scores for chromosome 2 markers and a 0.43-4.2 LOD score range for the COL4A5 locus. Formerly, consent was given by the examined individuals.

High Resolution Melting Curve Analysis

In Study 2 (mutation screening for X-linked Alport syndrome) DNA was extracted from blood leukocytes of previously selected, affected, and non-affected patients. In HRM analysis, the melt curve products are analysed following a RT-PCR by monitoring the melting of a saturating DNA-binding dye. Mutation screening was carried out for 51 exons with the surrounding splice sites on the COL4A5 gene. 56 primer pairs were designed by Primer 3 Software with the common annealing temperature of 60°C. The average amplicon size was 250 bp. Primers were checked with BLAST and RepeatMaskers. HRM was carried out after PCR reaction on the temperature range of 65°C to 95°C with temperature increase of 1°C per second. This method was carried out by LightCycler 480 (Roche) System with LC High-Resolution Melting Master containing Resoligth dye. Sanger-sequencing was used to confirm and characterize the screened amplicons identified by HRMC. The study protocol was performed in 2011 for the aim to obtain exact diagnosis and was considered as part of the diagnostic procedure. As per Hungarian regulations, written informed consent was obtained from each patient in the framework of a genetic counselling session, the result was then discussed with patients in the presence of a clinical geneticist and their nephrologist. (*67*,71)

Statistical analysis

Results are reported as means ± SD and median (25^{th}-75^{th} percentile) according to the normality of the parameters for Study 1. Statistical analyses were performed with GraphPad Prism 4.00 software (GraphPad Software Inc., La Jolla, CA, USA). Statistical comparisons included the unpaired *t*-probe, the nonparametric Mann-Withney test, the one-way analysis of variances followed by Bonferroni's *post hoc* test and the nonparametric Kruskal-Wallis test followed by Dunn's multiple comparison test. The employed analysis also depends on the distribution and normality of the examined parameter. For both studies, a *p*-value <0.05 was considered significant.

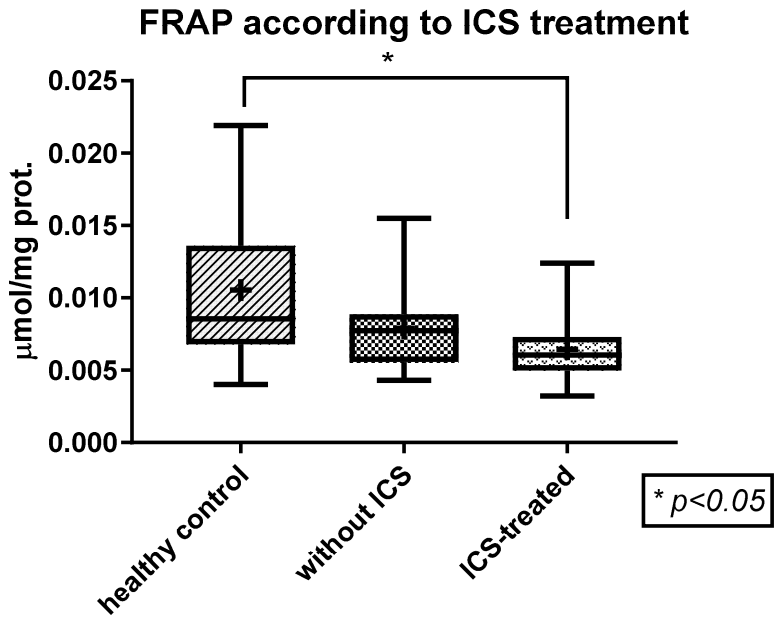
Results

Study 1- Oxidative stress in children with bronchial asthma

FRAP (unpublished results)

FRAP results showed significant differences between asthmatics and healthy controls. In asthmatics, lower FRAP levels were measured (median, 25^{th}-75^{th} percentile (µmol/mg protein): *asthmatic:* 6.29 x 10^{-3}, 5.14 x10^{-3}- 8.15 x 10^{-3} vs. *healthy*: 8.55 x 10^{-3}, 6.81 x 10^{-3} - 13.6 x 10^{-3}*, *p<0.05). The clinical control of asthma did not significantly influence the results: lower levels were detected in both controlled and partially controlled groups (median, 25^{th}-75^{th} percentile (µmol/mg protein): *controlled*: 6.61 x 10^{-3}, 5.45 x 10^{-3} – 8.18 x 10^{-3} vs. *partially controlled*: 5.62 x 10^{-3}, 4.93 x 10^{-3} – 7.84 x 10^{-3}), and according to the control state of asthma, there was no significant difference between the two subgroups. In our results, ICS use had a significant effect on the total non-enzymatic antioxidant system of the plasma: asthmatics not using ICS had lower FRAP results compared to healthy children, but this was not statistically significant. Also lower FRAP were obtained among ICS users, but the difference was only significant compared to healthy controls. *(see Fig. 4).* Further analysis of the ICS doses showed, that the treatment with low daily dose of ICS or ICS combined with LABA caused significantly lower FRAP levels compared to healthy controls (*p<0.05) (*Fig. 5).* Although not statistically significant, FRAP tended to be lower in all the asthmatic groups: also among them who treated with ICS and those, who did not get ICS because of their actual asthma control.

Fig. 4*: *Plasma Ferric Reducing Ability reflects the total, non-enzymatic activity of plasma

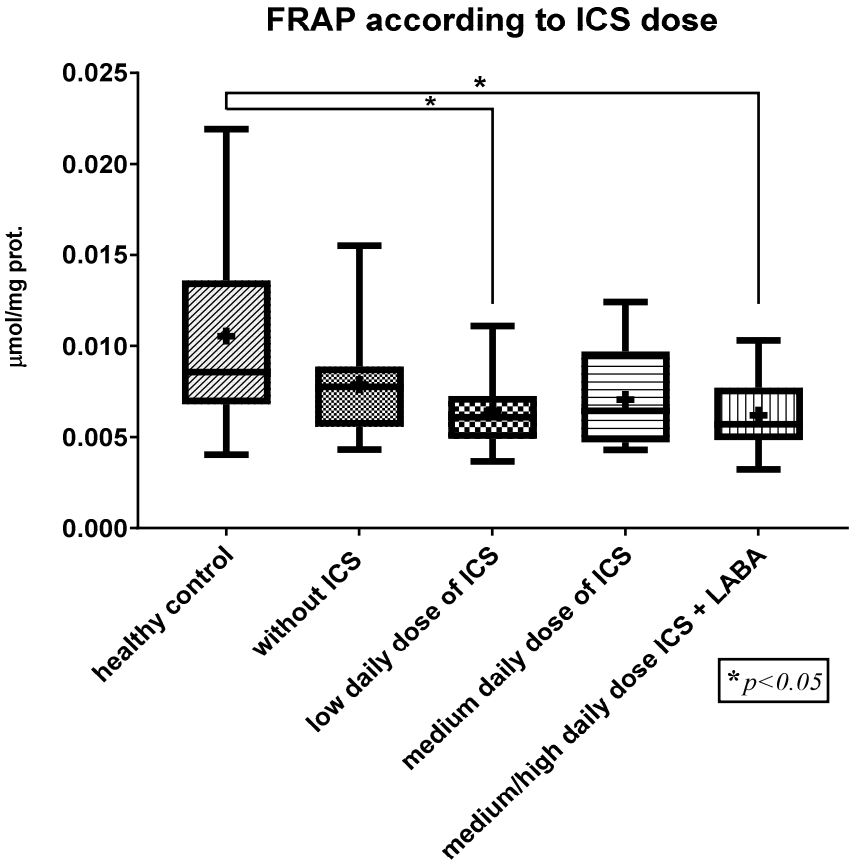


*FRAP levels (*median, 25th-75th percentile*; µmol/mg protein) were not statistically different between healthy controls and asthmatics who did not require ICS, however, ICS use significantly reduced FRAP levels in patients requiring ICS ($p<0.05$).*

"+" sign shows median in columns with minimum and maximum values.

Statistical analyses were performed by non-parametric Kruskal-Wallis probe, with Dunn's multiple comparison.

Fig.5: FRAP values in the context of the necessary ICS dose



Low daily dose of ICS or the ICS-LABA combination caused significantly lower FRAP levels compared to healthy controls. Also, lower FRAP results were measured in case of medium daily dose of ICS, though it did not reach the level of significance ($p<0.05$).
"+" sign shows median in columns with minimum and maximum values.
Statistical analyses were performed by Kruskal-Wallis probe with Dunn's correction.

GSH and GSH redox system

The GSH and GSSG concentrations, and the GSSG/2GSH ratio were similar in healthy and asthmatic patients. The ICS use, the dose of ICS, and the level of asthma control did not influence significantly the GSSG and GSH levels. The GPx or GR activities were also not significantly different in the asthmatic children from those in healthy children. It should be noted, that the ICS-treated asthmatic subgroup displayed higher GPx activities than those who did not receive ICS treatment, but the difference did not reach the level of significance. There

was no difference between the activities of these enzymes regarding asthma control and sex (*data not shown*). In summary, in our study, we could not detect significant changes in glutathione homeostasis between asthmatics and healthy individuals, and neither the asthma control nor the therapeutic actions caused remarkable changes (*Table 1).*

Table 1: Components of the GSH redox system in healthy controls and as per ICS treatment.

Parameters	***Healthy controls***	***ICS treated asthmatics***	***ICS non treated asthmatics***
GSH (µmol/g Hb)	8.69±2.32	9.31±1.51	9.19±1.36
GSSG (nmol/g Hb)	18.50±4.35	19.63±5.28	18.95±3.51
GSSG/ 2 GSH (%)	0.22±0.06	0.21±0.04	0.21±0.03
GPx ($x10^{-3}$U/mg prot.)	2.41±0.45	3.18±1.57	2.63±0.45
GR ($x10^{-4}$U/mg prot.)	7.68±1.84	7.44±2.28	8.28±2.78

Data are reported as mean ± SD.
The ICS dose did not influence the GSSG/2GSH ratio and enzyme activities (data not shown).
Statistical analyses were performed by one-way ANOVA, with Bonferroni's multiple comparison test.

SOD and CAT activity

SOD activity in asthmatic and healthy children was similar (mean ± SD (U/mg prot.): *healthy*: 2.54 ± 1.07 vs. *asthmatic*: 2.74 ± 0.95). There was also no change in the activity of SOD regarding asthma control and sex (*data not shown*). SOD activity was significantly higher in those asthmatics who did not receive ICS than in the healthy controls and all ICS-treated asthmatics (*Table 2*). SOD activities analysed by ICS doses were not different among the subgroups, except for the steroid nontreated patients ((mean ± SD (U/mg prot.*): asthmatic patients without ICS: 3.13 ± 0.97, with low daily dose of ICS: 2.53 ± 0.93, with medium dose of ICS: 2.72 ± 1.12, with high/medium dose of ICS + LABA: 2.25 ± 0.59*).

CAT activity was not altered significantly in asthmatics (mean ± SD (BU/mg prot.): *healthy*: $8.98 \times 10^{-6} \pm 2.23 \times 10^{-6}$ *vs. asthmatic:* $9.50 \times 10^{-6} \pm 3.36 \times 10^{-6}$). CAT activity exhibited a small, but not statistically significant elevation in the asthmatic subgroup without ICS treatment (*Table 2*), but there was no noteworthy difference in enzyme activity influenced by sex or asthma control, and also the required ICS dose did not influence enzyme activity measured.

Table 2: SOD and CAT activity in healthy controls and as per ICS treatment

Parameters	***Healthy controls***	***ICS treated asthmatics***	***ICS non treated asthmatics***
SOD (U/mg prot.)	2.54 ± 1.07	2.49 ± 0.86	3.13 ± 0.97*+
CAT (x10^{-5} BU/mg prot.)	0.89 ± 0.22	0.86 ± 0.29	1.04 ± 0.36

Data are reported as mean ± SD

*+p<0.05: *significant difference relative to healthy controls and to steroid treated patients*

Statistical analyses were performed by one-way ANOVA, with Bonferroni's multiple comparison test.

Lipid peroxidation and protein carbonylation

The lipid-damage indicator TBARS ((median, 25-75 pc (nmol/mg prot.): *healthy*: 0.56, 0.44–0.65 vs. *asthmatic*: 0.77, 0.66–0.90) and the carbonylated protein (mean ± SD (x10^{-5} mmol/mg prot.): *healthy*: 6.77 ± 1.12 vs. *asthmatic*: 7.42 ± 1.45) concentrations in the red blood cells were significantly higher in the asthmatic children than in the healthy controls (p<0.05). Both parameters were slightly higher in the subgroup which did not require ICS than in those who received ICS medication (*Table 3*), but in case of protein damage, significant difference could be detected compared only to the healthy controls, while the TBARS concentrations were also high in both asthmatic subgroup without the influence of ICS treatment.

Table 3: Oxidatively modified lipid and protein products from blood samples in healthy controls and as per ICS treatment

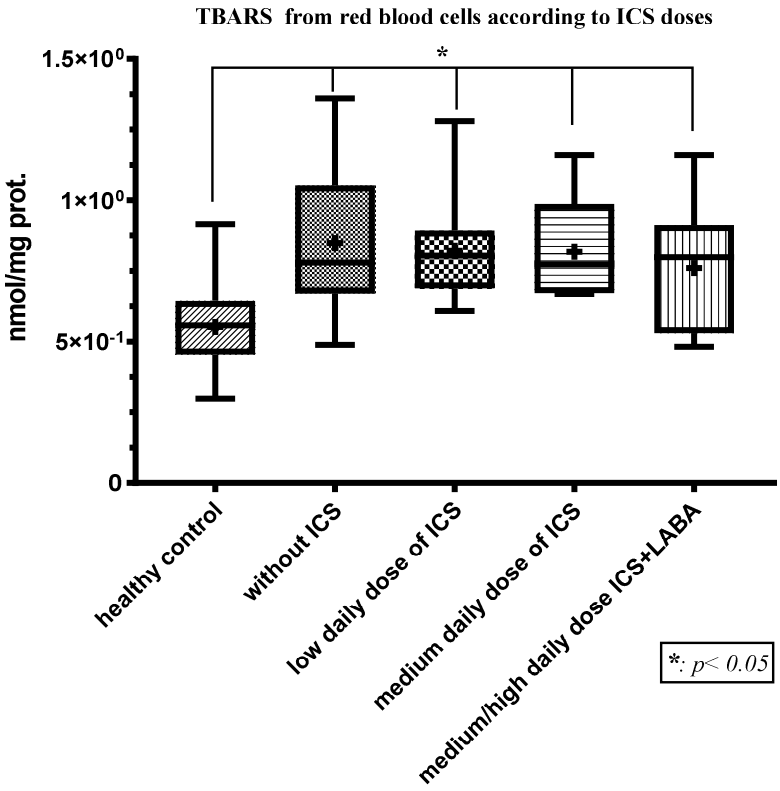
Parameters	***Healthy controls***	***ICS treated asthmatics***	***ICS nontreated asthmatics***
TBARS (nmol/mg prot.)	0.56 (0.44-0.65)	0.77 (0.66-0.89) *	0.78 (0.66-1.05) *
Carbonylated protein (x10^{-5} mmol/mg protein)	6.77±1.12	7.22±1.25	8.10±1.63 *

Protein carbonylation data are reported as mean ± SD, and in case of TBARS, median (25th–75th percentile). Statistical analyses were performed by one-way ANOVA, with Bonferroni's multiple comparison test in case of protein carbonylation, and by Kruskal-Wallis test in comparison TBARS data.

**p<0.05 statistically significant difference relative to healthy controls.*

Further analysis showed that TBARS levels were also significantly higher in all ICS treated and nontreated asthmatic subgroups as compared to healthy controls, but according to our results, the required ICS doses did not cause large-scale distinction in the lipid peroxidation (*Fig. 6*).

Fig. 6: TBARS (lipid peroxidation) level in the context of required ICS treatment



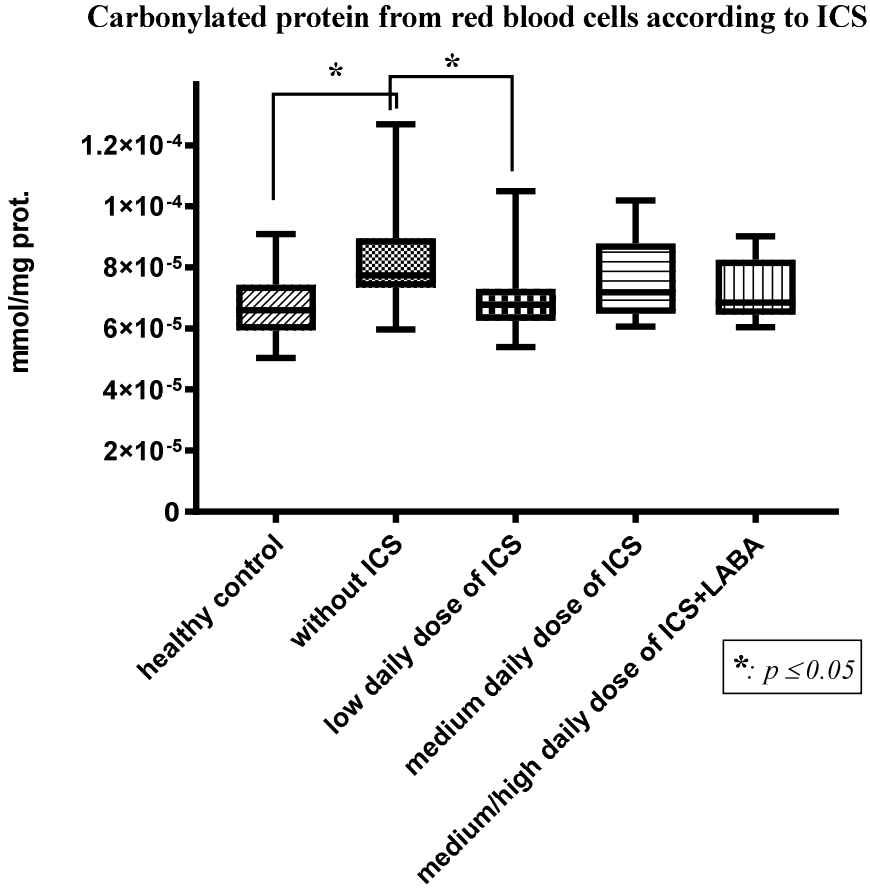
TBARS levels were significantly higher in all ICS treated subgroups as compared to healthy individuals. The ICS dose did not cause a distinction in the extent of lipid peroxidation.

"+" sign shows median in columns with minimum and maximum values.

Statistical analyses were performed by the Kruskal-Wallis test with Dunn's correction.

The highest carbonylated protein level was measured in the subgroup that did not require ICS, but the difference was significant only relative to those who received low daily dose of ICS and to healthy controls (*Fig.7* Figure was not published previously). The extent of oxidative damage to proteins was not significantly increased at higher doses of ICS used.

Fig. 7: Protein oxidation biomarker carbonylated protein level in the context of ICS doses



The highest carbonylated protein level was measured in the subgroup that did not require ICS, but the difference was significant only relative to those who received low daily dose of ICS and to healthy controls. ($p<0.05$). Statistical analysis were performed by one-way ANOVA with Bonferroni's multiple comparison test

Neither the lipid peroxidation parameters nor the oxidatively modified protein content were influenced by sex and asthma control.

NADPH oxidase gp91^{phox} subunit mRNA in blood

We analysed the relative gp91^{phox} subunit (CYBB) mRNA level in asthmatic subgroups according to ICS medication and determined the difference in the gene expression between subgroups treated and untreated with ICS (ICS treated asthmatics: ΔΔCt=0.819 vs. without ICS treatment group: ΔΔCt=-0.363, respectively; $p<0.05$; ΔCt values relative to the healthy controls). This result suggested a difference due to medical treatment: asthmatics, who need ICS, had lower CYBB gene expression profiles, than those, who did not need ICS for their adequate asthma control. As seen in each ICS treated subgroup, different ICS doses (low-medium-high/medium + LABA) were administered, reflecting the actual clinical control of asthma with its drug demand. In this context, the *post*-test after ANOVA analysis revealed a significant difference between the subgroup treated with a low daily dose of ICS and those who were not treated with ICS (ΔΔCt=1.445, $p<0.05$): approximately two-fold downregulation difference in patients treated with low daily dose of ICS was measured. These ΔCt values in each subgroup, which can be seen in *Table 4*, were compared with steroid nontreated asthmatics. Negative ΔΔCt values mean upregulation of the gene expression according to the comparative ΔCt method, contrarily the positive values, indicate downregulation of the candidate gene.

Table 4: ΔΔCt data derived from asthmatic patients treated with different ICS doses compared with ICS nontreated ones

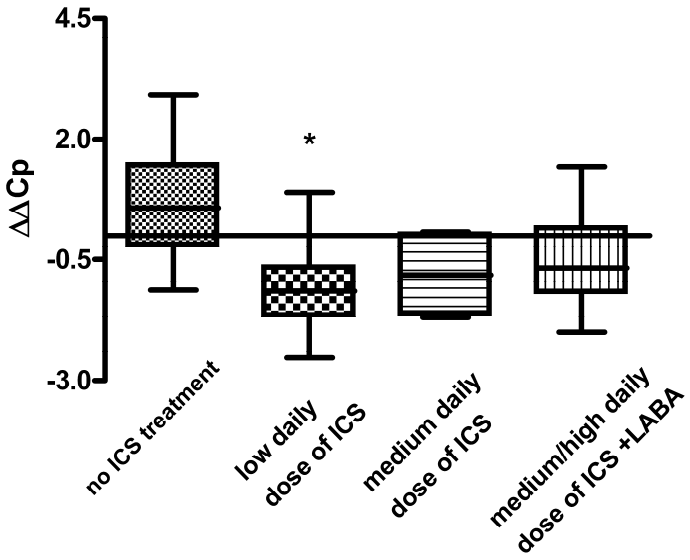
treatment	**ΔΔCt**
low daily dose of ICS	1.445*
medium daily dose of ICS	1.168
medium/high daily dose of ICS + LABA	1.034

Statistical analysis was performed by one-way ANOVA test with Bonferroni's multiple comparison test

**p<0.05: compared with steroid nontreated patients*

No significant difference was observed between the two other ICS treated subgroups, who had taken higher ICS doses. In our study, the higher ICS doses did not significantly influence the coding CYBB gene expression profile compared to asthmatics treated with lower daily doses. For a better presentation in *Fig.8*, the expression profiles of the CYBB gene were represented relative to healthy controls.

Fig.8: Relative expressions of CYBB in blood samples from the three subgroups of asthmatic patients



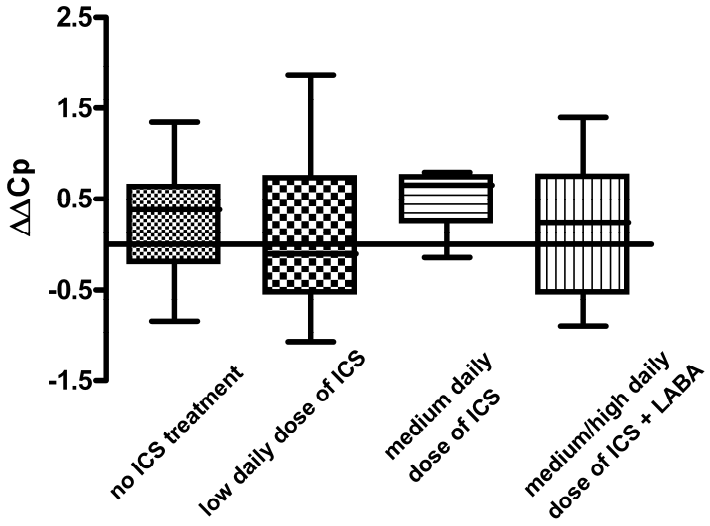
Relative mRNA levels of gp91^{phox} coding CYBB gene were determined by using the threshold (Ct) values of the CYBB gene by RT- PCR. Each sample was normalized to its own h-PBGD housekeeping gene (ΔCt). ΔCt data on groups and subgroups were compared (ΔΔCt). Values are compared and presented to healthy controls for a better presentation.

** Significantly lower mRNA levels $p<0.05$ were measured in the subgroup treated with a low daily dose of ICS than in those who were not treated with ICS. The downregulatory effect was lower (measurable higher mRNA levels) in the patients who received medium/high daily doses of ICS alone or in combination with LABA, but this difference did not reach the level of significance. Statistical analysis was performed by one-way ANOVA test with Bonferroni's multiple comparison.*

HMOX-1 gene expression in blood

There was no significant difference in HMOX-1 gene expression between the asthmatic patients and the healthy controls, and neither the ICS therapy itself nor the administered ICS dose influenced the expression of this gene *(Fig. 9)*

Fig. 9: Relative expressions of HMOX-1 in blood samples from the three subgroups of asthmatic patients



Relative mRNA levels of the HMOX-1 gene were determined by using the threshold (Ct) values of the HMOX-1 gene by real-time PCR. Each sample was normalized to its own h-PBGD housekeeping gene (ΔCt). ΔCt data on groups and subgroups were determined (ΔΔCt). Values are compared to healthy controls for better presentation. No significant difference was observed between the individual subgroups.

Statistical analysis was performed by one-way ANOVA test with Bonferroni's multiple comparison.

Study 2 – Mutation prescreening in families suffering with Alport nephropathy

In the 9 families with a mutation in the COL4A5 gene (analysis of a patient and a heterozygote in each family), prescreening of 56 Col4A5 amplicons by HRM revealed 6 different melting curves from the others. The differences in the melting curves are derived from the alterations in the base sequence of the screened amplicons (*Fig. 10*). The following Sanger-sequencing of the screened amplicons identified 4 mutations: Gly273Arg (1061G/C), 1620 ins C (frameshift from codon 458), Gly624Asp (2115G/A) and Gly1406Arg (4460G/A). We also found 2 intronic polymorphic variations: IVS4 +69T/C and IVS10 +21T/C. There was no false-positive results.

Fig. 10: High Resolution Melting Curve analysis in one of the amplicons from patients suffering from X-linked Alport syndrome

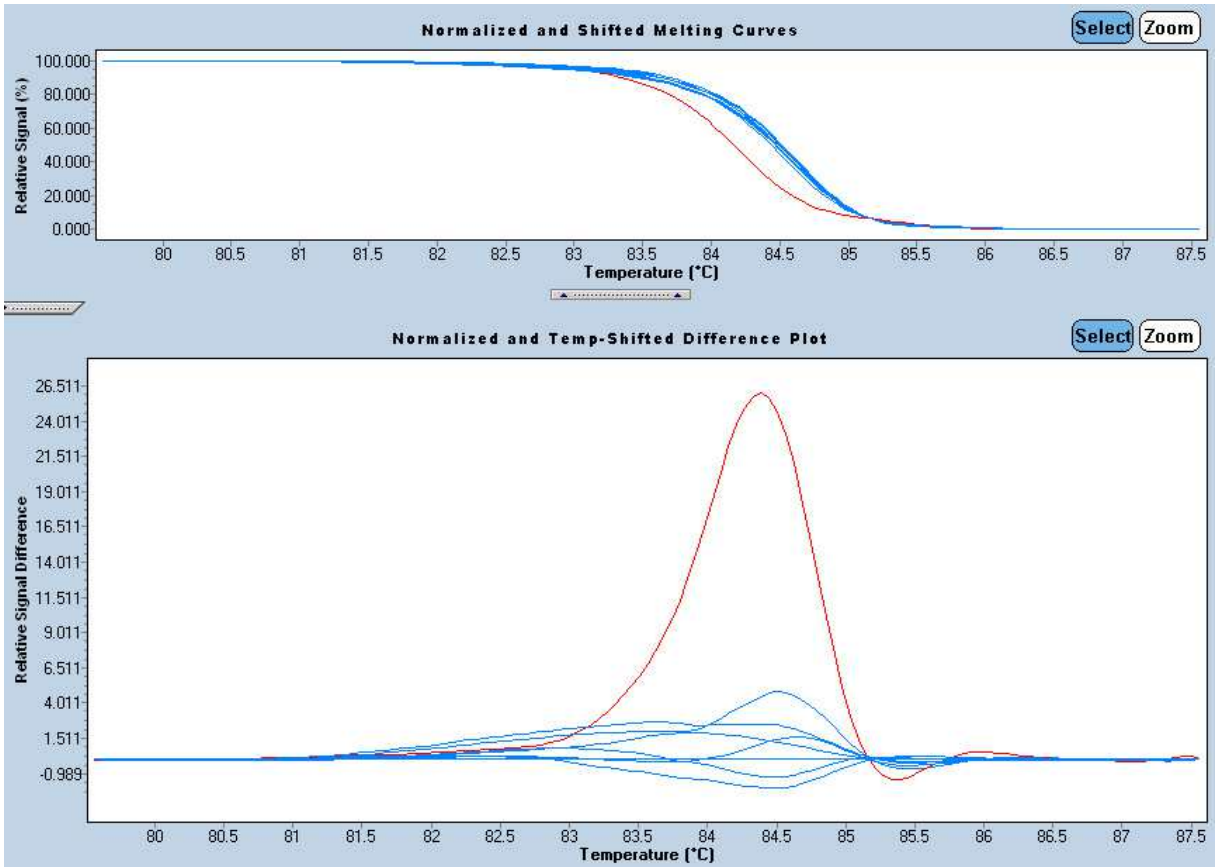


Figure represents a heterozygous allelic variant. The above picture is from our own study.

Discussion

In the first part of our study, we focused on the possible use of peripheral blood, which is the most easily accessible medium for the examination of systemic oxidative damage in children. We have determined the levels and activities of several antioxidants, the blood levels of lipid peroxidation products, and the extent of protein damage with the aim to describe as accurately as possible the presence of oxidative stress in young asthmatic patients. In addititon, we have measured the transcriptional activity of two genes representing the oxidative imbalance from the aspect of pro-oxidant stimuli and antioxidant defence. We have focused on the mRNA level of the NADPH oxidase enzyme complex $gp91^{phox}$ subunit (coding gene marked by CYBB), one of the main free radical sources, and the antioxidant Hmox-1 protein. All these parameters also have been analysed in the context of medical treatment.

In asthmatics, the non-enzymatic total antioxidant capacity in plasma was shown to be lower than that in the healthy controls. The lower FRAP levels both in controlled and partially controlled subgroups suggest, that this difference could be derived from the asthma morbidity itself accompanied by the ongoing inflammation so that symptomatic control of asthma did not influence FRAP levels. It has to be noted that the asthma control subgroups were inhomogeneous in their therapeutic regimen, as they received different therapy to reach adequate symptom control. Detectable differences could be seen with reduced FRAP values in all subgroups treated with ICS compared to those who did not require ICS. ICS did not influence notably the detected FRAP levels: no significant changes were measured depending on the different ICS doses. FRAP is a cumulative indicator comprising uric acid, plasma proteins, mainly albumin, bilirubin, α-tocopherol, ascorbic acid, and in small part other non-enzymatic components. (The enzymatic antioxidants were examined separately, derived mainly from the red blood cells.) Surprisingly, in biological terms, uric acid represents nearly 60% of the FRAP, while plasma albumin is contributing much less (~15%) to FRAP activity, despite its high amount. (*9*) It should be also mentioned, that all components of FRAP are strictly regulated, with their own antioxidant, free-radical scavenging features, and the excess amount of some of these components is also implicated in several pathological states (hyperbilirubinemia, gout, etc.). So the physiological range between the normal antioxidant

response and the pathological processes, which may also lead to chronic inflammation and oxidative stress, may be very narrow.

Regarding non-enzymatic components of the antioxidant defence, the role of FRAP, remains unclear, as the reaction conditions *in vitro* are far from the physiological: the metabolism of each FRAP component is strictly regulated, and the lung-blood distance can also influence the detectable changes. Therefore, further analyses are required to examine the individual non-enzymatic antioxidant elements of the plasma, mainly the generic effects of ICS on the absorption and production, metabolism of each component, and also the possible side effects. Even if the plasma antioxidant capacity is an indirect marker, it remains in the scope of scientific research with its inexpensive, highly reproducible, and simple-to-prepare nature.

As concerns, the glutathione redox cycle, GSH, and GSSG/GSH ratio, as sensitive markers of the inflammation accompanied by oxidative damage, were not altered in the red blood cells in asthmatics compared to healthy adolescents. The glutathione redox cycle was not assessed in the airways samples which could have provided additional information. The somewhat elevated GPx activity (statistically not significant) in the subgroup receiving ICS therapy, draws attention to the presence of oxidative insults even in the clinically stable, symptom-poor, or symptom-free asthma, although the results were just showing a tendency because of the relatively small number of patients. The lack of significant changes in the components of the glutathione redox cycle in the circulation does not exclude the possibility that alterations in the glutathione system would be present locally in the inflamed lung. In earlier studies, a lower level of GSH in EBC and BAL of asthmatic children has been demonstrated, indicating a higher free radical exposure of the lung epithelium, as well as the effect of the systemic–yield steroid treatment on its' level during an acute asthma exacerbation. (*84,85,86*) By contrast, in adults, controversial data have been published in studies using lung samples, suggesting that the results might be influenced by factors including the examined population, age, acute or chronic pulmonary insults, administered therapy, etc. (*86,87*) Differences between the literature and our results could be explained by the fact, that in sputum or BAL samples, most of the glutathione is found in the cell-free supernatant, indicating its principal role in protecting the lung from extracellular free radicals. It is also worth knowing that glutathione, measured in the supernatant of airways samples, is derived from intracellular sources, bronchial epithelial cells, as well as inflammatory cells, recruited to the site of inflammation. In contrast, most of the glutathione in circulation is found inside the red blood cell to preserve its role in oxygen physiology. The percentage of GSSG is typically lower in blood when compared to lung lining fluids, suggesting

either more effective recycling of GSH or a lower level of direct free radical exposure in the red blood cells. It is also known that changes in GSH/GSSG ratio can usually be seen during a short period after acute oxidative exposure, and as an adaptive response to chronic oxidative damage - as seen in the asthmatic lung - the glutathione redox cycle enzymes may adapt to the permanent free radical exposure. Bronchial asthma is a chronic disorder; thus, it is more likely that the glutathione system adapted to the permanent higher pro-oxidant stimuli, with excessive glutathione redox system activity locally in the lung. (*86*) It must be also noted, that our patients were mainly from the controlled asthmatics, and according to their age, they were mainly adolescents, which also might account for the controversial data contrary to the literature's data mentioned above.

Previous studies in both adults and children measured lower total Cu/Zn-SOD activity in the blood of controlled asthmatic patients, in contrast with *Suzy et al.* reporting marked elevation. (*47,88*) Since there was no difference in Cu/Zn-SOD activity of red blood cells between asthmatics and healthy controls in our results, we have decided on a more detailed analysis as the asthmatic group in our study was relatively heterogeneous in their clinical and therapeutic approach. We have found significantly higher SOD activity among ICS nontreated patients compared to the healthy and ICS-treated subgroups, suggesting the presence of free radical-mediated inflammation: it was represented in moderate extent in those who have had mild but clinically stable asthma compared with those who have required permanent maintenance therapy. The inhaled corticosteroid treatment might have an inhibitory effect on enzyme activity, reaching almost the same enzyme activity in ICS-treated asthmatics as in healthies, and the subgroup without ICS treatment could reflect the asthmatic inflammation accompanied by oxidative overload. Of note, the enzyme protein levels were not measured in the present study, just the activity of the Cu/Zn SOD enzyme.

Also, a detectable, but not statistically significant elevation could be seen in the CAT activity in the asthmatic subgroup not requiring ICS, similar to the trend as SOD, but in contrast to GPX activity. According to the extensive scientific literature about oxidative stress, the interpretation of the antioxidant system is a complex and difficult challenge, and the individual components can not be evaluated on their own, but in the context of the others. This is also the case for GPx and CAT, where the enzyme's synergetic effect is well-known. *H. J. Pennings* and colleagues in the 1990s published results about the dominant role of GPx and CAT in the antioxidant defence mechanisms in enzymatic and gene regulation levels, and about the potential influencing role of inhaled corticosteroids (beclomethasone) with their proposed detectable

systemic effects. (*89,90*) But contrary to them, our results demonstrated a not significant, but somewhat lower CAT activity, near to the normal range during ICS treatment. The explanation, however, became more complex, as CAT is the main antioxidant in red blood cells. Adding to all of these, not only the GPx works synergistically with CAT and can be detected in a higher amount where lower CAT activity can be measured, but in the last few years, further GPx isotypes were discovered with more complex roles and regulatory mechanisms. The activities of the GPx isoforms are connected to oxidative overload but may vary depending on the examined organelles, cell type, and organs. According to *Johnson and colleagues*, the erythrocyte GPx is capable of removing the endogenously produced H_2O_2, thus the additional role of CAT is thought to remove the exogenous H_2O_2. As erythrocytes circulate in the entire body system exposed mainly to harmful effects derived from their milieu, the importance of CAT in the free-radical neutralization in red blood cells is not surprising. However, its regulation and precise role among other antioxidant enzymes, like GPx and other thioredoxins, is yet to be explored. (*91*) These observations have implications concerning our results about the inverse alteration in CAT and GPX activity. All these cited further studies reveal, that the regulation of the antioxidant system is very complex, and despite the increasing knowledge, the precise physiological and pathological roles remain unclear. As we have mentioned previously, besides the complex co-work between elements of the antioxidant network, most of the data and information about accurate enzyme regulation were derived from *in vitro* studies and animal models. However, the human mechanisms remain unclear because of the limited possibilities to explore them. Coloring the whole picture, the dominant role of antioxidant elements may vary from tissue to tissue. Another problem to get a comprehensive picture is the difficulty to compare the results from the different studies due to different disease severity, sample type (whole blood, red blood cells, plasma), sensibility, sensitivity of the applied analytical method, and the interpreting measurement unit. (*12,19, 88*)

Elevated TBARS and PCO levels were detected in our asthmatic patients relative to healthy adolescents, confirming earlier data about the presence of permanent oxidative stress, detected in the circulation.(*51,45,46,92,93*) The level of TBARS is indicative of lipid damage, which is an early marker of ROS-mediated injury, while the carbonylated protein elevation due to protein damage is a later marker of oxidative stress. The TBARS, so as PCO levels were higher in that subgroup who did not require ICS treatment than in those treated with ICS, although the lipid peroxidation products were also elevated in all ICS-treated subgroups. In our opinion, the higher TBARS concentration reflects the ongoing oxidative stress, while the PCO level is

indicative of its chronic presentation. Results monitoring the direct effects of free radical processes and inhaled steroid treatment on the biomolecule level, were consistent with our above-mentioned data in connection with SOD and CAT activity, reflecting the chronic feature of asthmatic inflammation, and the possible effects of ICS treatment on the different antioxidant markers.

Besides studying some of the components of the complex antioxidant network and some of the direct signs of molecular damage, we were also interested in the gene expression alterations, which might have importance in the redox imbalance accompanying asthmatic inflammation.

The respiratory tract is a unique organ in terms of its physiological function by oxygen delivery and exposure to environmental toxic agents. It is highly exposed to airborne microbes, viruses, other irritants, and allergens. Therefore, the airway's normal function relies on a local, potent defence system that enables its successful combat against these environmental dangers without compromising lung function. Various NOX isoenzymes are represented in the airways in several different cell types, and analogous to the phagocyte's NADPH oxidase system, having critical roles in the local host defence. Besides the NOX-dependent oxidative microbial killing mechanisms, these isotypes may also contribute to cell signalling mechanisms regulating airway's responses to injury, by influencing cell proliferation, migration, and/or differentiation. It is suggested that altered expression or activation of these NOX isoforms may also have a role in the development of several lung illnesses by participating in tissue repair and/ or remodelling.(*34,39,94,95*) NOX isoforms are thought to have a Janus-faced role: upregulating the inflammatory process, having a harmful effect by mediating the ROI overproduction, and at the same time, inducing tissue remodelling with dual characteristics: the tissue remodelling not only means the healing process, but the connective tissue redevelopment make the bronchial and bronchiolar structure more rigid and hyperreactive. (*41,42*) To the best of our knowledge, most investigations about NOX isoforms are derived from cell- or tissue cultures, and ethical doubts also emerge in the human context, so we have focused on the easily obtainable sample, the blood to approach the inflammatory processes accompanied by oxidative stress in the lung. Not only the pro-oxidants but also the antioxidant components are very important to maintain the equilibrium in the radical-mediated processes. The role and detectable level in the blood of the inducible heme-oxygenase was well known in acute lung injury, but its function in chronic oxidative damage such as in asthmatic inflammation was not fully elucidated.

For the reason mentioned above, we aimed to find targets, which had possible roles in the development of oxidative stress in connection with asthmatic inflammation and had detectable

transcription activity in blood. We have measured the mRNA levels of the NADPH-oxidase enzyme complex gp91phox subunit (CYBB) from the pro-oxidant side, and the antioxidant inducible Hmox-1 transcription from the perspective of defence mechanisms.

The major role of the NADPH oxidase complex in the free radical production, and the importance of the NF-κB transcriptional factor and pro-inflammatory cytokines in the transcriptional regulation of the $gp91^{phox}$, $p67^{phox}$, and $p47^{phox}$ subunits were demonstrated earlier in the MonoMac1 cell line *in vitro*, but the mRNA level of CYBB has not been measured previously in blood leukocytes of childhood asthmatic patients. (*94,40,95*) Our results showed, that CYBB mRNA level proved to be significantly lower in patients treated with a low daily dose of ICS than in those who did not receive ICS therapy. There was also a tendency to lower expression in patients treated with a medium/high daily dose of ICS or with an ICS-LABA combination, relative to asthmatics without ICS treatment, but these differences did not reach the level of significance. Although it is widely accepted that inhaled corticosteroids in normal therapeutic doses exhibit effects only locally in the airways (96), our results demonstrated that alteration in the CYBB transcription could also be detected in the circulation. This systemic effect might be due to the activated inflammatory cells which appeared in the circulation. The alterations in CYBB expression may be the consequence of the inhibitory effects of ICS on NF-κB-regulated genes, similarly to the $gp91^{phox}$ subunit coding gene. (*35,40,95,97*). The present findings in the subgroups that received a higher dose of ICS, or ICS combined with LABA - known to improve the symptoms to reach the proper asthma control - may be associated with a more excessive inflammation or relative steroid resistance, but further examinations are needed. (*98*) Despite the administration of higher steroid doses, a lower level of CYBB gene transcription was found in steroid-treated asthmatic children than in healthy subjects, even when the serum cortisol concentrations were similar in the two groups (data not shown). The lack of the expected upregulation of the CYBB gene in the blood from asthmatics due to the inflammation does not exclude the important role of the NADPH oxidase complex in free radical production at the protein level. As demonstrated in other studies, the activity of the NADPH oxidase complex was determined by several posttranscriptional modifications (*34,94*), and a precise co-work of membrane-bound and cytosolic protein subunits was required for the superoxide production. It was also discovered, that cytosolic storage of the $gp91^{phox}$ protein existed in cells next to the presence of the membrane-bound protein forms, so the transcriptional modulation of the $gp91^{phox}$ subunit might not certainly be represented at the protein level or by enzyme activity. It is worth to be mentioned, that during the analysis of the CYBB mRNA levels and ICS doses, CYBB downregulation was not enhanced with higher doses of ICS: the

main downregulatory effect in CYBB transcription was reached at lower ICS doses. Thus, the main impact on gene expression was already detectable with low ICS doses, and the administration of higher amounts did not induce lower transcription inhibition.

The HMOX-1 gene and the coded protein's concentration are exclusively regulated transcriptionally. It is activated in the early phase of oxidative stress, so it is used as an early phase marker of the antioxidant system. (*100,101*) Our results did not indicate any change in the HMOX-1 mRNA levels in the analysed subgroups, by following the supposed chronic feature of oxidative stress in bronchial asthma. The difference in expression profile between CYBB and HMOX-1 genes in our samples in addition to these may originate from the different signalling pathways: the CYBB gene is mainly regulated by NF-κB, while HMOX-1 is regulated by Nrf2 during airway inflammation. (*95,99,102*)

In asthmatics' blood samples, some extent of oxidative stress could be confirmed, especially in those patients who were not treated with ICS. It should also be noted, that measurable alteration could already be observed using the smallest ICS dose, and an increase in ICS dose seemed not to have a further advantage. Our findings on changes after inhaled steroid treatment in asthmatics could be very important in terms of alterations in asthma treatment. According to our present knowledge, the inhaled corticosteroid treatment does not have systemic side effects. Although our patients did not report side effects in connection with ICS treatment, moderate effects of the ICS treatment are measurable in the circulation. In addition, in 2019 Global Initiative for Asthma guideline published new and important recommendations for treating adolescents/adults. In contrast with the earlier practice of using short-acting β2-mimetic in mild/moderate asthma with its undesirable side effects, ICS can now be used in the reliever therapy combined with LABA as well, as in the first and second step of the controller therapy with the required smallest effective doses. Our results detailed above, also highlight the importance of using low-dose ICS even in the presence of mild symptoms, as low-activity but persistent airway inflammation can be detected even when the asthma bronchiale is clinically controlled (103)

In the second part of our study, we have focused on a novel technique based on the real-time PCR method for mutation prescreening. At the time of making the dissertation, sequencing DNA for detecting mutations or single nucleotide polymorphism, was already an increasingly widespread technique, but still fairly expensive. In the case of TBMN or Alport syndrome, the final diagnosis could only be obtained by mutation analysis. With the post-PCR HRMC analysis, it became possible to accurately detect smaller gene loci where previously unknown mutations or SNPs existed. It was also possible to use it as a prescreening method to reduce the number of sequencing reactions required to detect a new variant. The previously performed genetic analysis of the family members and the linkage analysis was not part of this study, we have focused only on HRM analysis.

During the analyses of 9 families for the COL4A5 gene, 6 different melting curve was detected. HRMC analysis was followed by the Sanger sequencing method to characterize the base order of the detected differences: 1061G→C with an amino acid change Gly→Arg, a cytosine base insertion at position 1620 (1620insC), which causes a frameshift mutation from codon 458, 2115G→A nucleotide change causing Gly→Asp amino acid change and 4460G→A alteration with a Gly→Arg change in type IV α5 collagen chain. We also found 2 intronic polymorphisms: IVS4+69T→C and IVS10+21T→C. In the other 5 families, the causing mutation remained unclear with HRMC mutation prescreening.

This method proved to be useful to localise the possible site of SNPs or mutations. Its efficiency depends on the exact primer design, and the optimal, more-reaction-involving PCR condition, which allows performing a large number of investigations at the same time. Of note, the HRMC analysis identifies only regions in the DNS where genetic variants may exist, but their precise nature needs to be determined by DNA sequencing. In the meantime, new generations of sequencing methods have become more widespread and, under certain conditions, available to a wider range of researchers, so that HRM analysis is nowadays in decline.

Conclusions and original findings

1. The markers of oxidative stress in bronchial asthma were detectable also in peripheral blood, despite the fact that the asthmatic inflammation is mainly localised to the airways. We focused on the adolescent population, as recruiting younger patients was not possible because the parents and patients often refused blood sample collection.

2. Higher TBARS levels, which appear in the earlier phase of redox disequilibrium, and elevated carbonylated protein levels - used as a marker of protein damage in the later phase of oxidative overload - reflect the chronic oxidative stress in bronchial asthma in adolescents.

The lack of large-scale alterations in the glutathione system and no change in the mRNA level of the inducible HMOX-1 gene were in line with the chronic character of asthmatic inflammation in the examined patients. These parameters were in accordance with the fact that our patients had controlled asthma without acute exacerbation.

3. The use of ICS had a marked effect on some biomarkers of oxidative disequilibrium. Some components of the antioxidant defence system, such as SOD and CAT activity, and total antioxidant capacity of the plasma were dependent on the ICS dose. Higher TBARS, higher SOD, and marked CAT enzyme activities were detectable in the asthmatic subgroup without the need for ICS treatment as compared with those in healthy controls. ICS required to achieve clinically controlled symptoms reduced the levels of biochemical parameters altered by oxidative stress to levels close to those of healthy controls.

As a result of inhaled corticosteroid therapy, the expression of $gp91^{phox}$ subunit coding gene of NOX2 isoform proved to be significantly changed. The CYBB gene's expression altered in a dose-dependent manner: the ICS treatment used in a low daily dose caused a significant reduction in the CYBB gene's transcription activity regulated by the NF-κB pathway.

The altered biochemical parameters were shown to be influenced by the ICS therapy: the use of ICS had a measurable effect on CYBB gene expression and biochemical parameters, but the higher ICS doses did not cause further alterations on the examined parameters, so even low ICS dose caused significant alterations in the components of oxidative disequilibrium.

The effect of ICS administration was detectable in the circulation as well, demonstrating the systemic effect of ICS therapy.

4. Components of oxidative stress can only be assessed in the context of each other. The effect of disequilibrium between pro- and antioxidant stimuli may vary according to the examined

pathological state, the examined organism, and the age, but several other effects may also influence the results of the specific study.

5. Although blood is an easily obtainable medium for studying several markers of oxidative damage, in the case of bronchial asthma, large-scale alterations could only be examined during an acute exacerbation. In line with the chronic inflammatory feature of clinically stable asthma, only markers of chronic oxidative stress can be detected in the blood. The prominent alterations during acute exacerbation can probably be detected in the bronchial structure itself, and properly examined from lung samples. Results from blood only give information about tendencies.

6. The High-Resolution Melting Curve analysis could be a suitable prescreening method for mutation analysis, especially in the case of larger genes with numerous exons. In our study, during the examination of the COL4A5 gene, we found six alterations in the base sequence in families with X-linked Alport nephropathy. None of the detected alterations indicated false positivity.

7. HRMC method could be optimal in case of a large number of examinations in the diagnostic procedure, to reduce the cost of Sanger-sequencing.

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Co-author certification

I, myself as a corresponding author of the following publication(s) declare that the authors have no conflict of interest, and ***Zsuzsanna Ökrös MD.*** Ph.D. candidate had significant contribution to the jointly published research(es). The results discussed in her thesis were not used and not intended to be used in any other qualification process for obtaining a PhD degree.

Szeged, 2023.06.08. .....

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Co-author certification

I, myself as a corresponding author of the following publication(s) declare that the authors have no conflict of interest, and ***Zsuzsanna Ökrös MD.*** Ph.D. candidate had significant contribution to the jointly published research(es). The results discussed in her thesis were not used and not intended to be used in any other qualification process for obtaining a PhD degree.

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Changes in NADPH oxidase mRNA level can be detected in blood at inhaled corticosteroid treated asthmatic children

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ABSTRACT

Aim: Oxidative stress, observed in the asthmatic airways, is not localized only to the bronchial system. It would be a great advantage to monitor the oxidative stress markers from blood especially in childhood asthma following the inflammation. Our aim was to measure the levels of antioxidants and the oxidatively damaged biomolecules. We were also interested in the gene expression alterations of the free radical source $gp91^{phox}$ subunit (CYBB) of the NADPH oxidase system, and the antioxidant heme oxygenase-1 (HMOX-1) isoenzyme in the blood. Our findings were also examined in the context of medical treatment.
Main methods: Oxidative stress parameters via photometric methods, CYBB and HMOX-1 expressions via real-time PCR were measured in 58 asthmatic and 30 healthy children.
Key findings: Higher blood thiobarbituric acid reactive substances (TBARS) ($p<0.03$) and carbonylated protein ($p<0.05$) levels were found in the asthmatic children than in the controls. The relative expression of CYBB was significantly lower ($p<0.05$) in patients treated with a low daily dose of inhaled corticosteroid (ICS), than in asthmatics not receiving ICS therapy. Higher ICS doses alone or combined with long acting β2-receptor agonists did not influence the expression significantly. No similar tendency was found as regards to HMOX-1 expression.
Significance: Elevated levels of damaged lipid (TBARS) and protein (carbonylated) products corroborate the presence of oxidative stress in the blood during bronchial asthma and suggest the presence of chronic oxidative overload. Our findings also suggest that ICS treatment can influence the relative CYBB mRNA expression in circulating leukocytes in a dose dependent manner.

Introduction

Airway inflammation and oxidative stress play major roles in the pathophysiology of bronchial asthma (Rahman et al., 2006; Mak and Chan-Yeung, 2006). Structural and functional alterations can be observed as a result of the excessive accumulation of free radicals and reactive oxygen or nitrogen species (ROS/RNS), produced by activated eosinophilic and neutrophilic granulocytes and macrophages. Constitutive airway cells, such as epithelial cells, are also considered to participate in ROS overproduction and in the antioxidant defense (Rochelle et al., 1998; Dworsky, 2000). Even if the inflammation is not localized only to the respiratory system, but also involves the systemic circulation, the available data concerning oxidative markers from the blood are limited and conflicting (Shanmugasundaram et al., 2001; Perišić et al., 2007; Comhair et al., 2005; Nadeem et al., 2003; Vural et al., 2005). So far numerous data have emerged from studies using the bronchoalveolar lavage fluid (BALF) in animal or human experiments (Park et al., 2009; Novak et al., 2006; Schock et al., 2003; Ozaras et al., 2000). In certain patient groups, such as asthmatic children, it would be a great advantage to develop protocols to monitor the oxidative stress before and during treatment using peripheral blood samples rather than collecting BALF or tissues, or to analyze exhaled breath condensates (Grob et al., 2008; Baraldi and Carraro, 2006; Busse et al., 2005).

Phagocytic NADPH oxidase, a multiprotein enzyme complex, is one of the main sources of free radicals and ROS. The plasma membrane-associated flavocytochrome b558 (composed of $gp91^{phox}$ and $p22^{phox}$ proteins) catalyzes the electron transfer from NADPH to O_2, resulting in the superoxide anion ($O_2^{\cdot-}$). Both subunits are transcriptionally regulated by pro-inflammatory cytokines (TNFα and IL-1) and by the ROS themselves, although other cytosolic subunits are also essential for a complete enzyme function (Almeida et al., 2005; van der Vliet, 2008). The upregulation of NADPH oxidase subunits and increase in NADPH oxidase activity contribute to the elevated $O_2^{\cdot-}$ radical and ROS generation during inflammation by activated mononuclear cells.

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Heme oxygenase-1 (HMOX-1) plays an important role in the cytoprotection against oxidative stress by participating in the anti-inflammatory, anti-proliferative and anti-apoptotic processes. As HMOX-1 is activated in the early phase of oxidative stress, it is a frequently used marker of the antioxidant system via which the adaptation mechanisms follow. Different HMOX-1 levels have been observed in various pathological conditions and inflammatory diseases. Environmental and pro-inflammatory stimuli, such as oxidative stress, hypoxia and certain cytokines (TNFα and IL-1α), may regulate the HMOX-1 transcriptionally (Slebos et al., 2003; Lee et al., 2009; Taillé et al., 2004). It has been already known that inflammation and oxidative stress in the airways modify the expression of HMOX-1 (Slebos et al., 2003; Farkas et al., 2008), but there are few data about the changes of the enzyme level from blood leukocytes and about the events in HMOX-1 regulation in persistent stable asthma.

In certain chronic diseases, including asthma, the pathological events and symptoms originate from primarily affected organs; however, the systemic effects are also considerable. The aim of the present study was therefore to compare the systemic oxidative damage and antioxidant status in the blood of asthmatic children and age-matched healthy controls. Blood is suitable for the study of the systemic effects of inflammation, and is readily obtained in pediatric practice. We measured the levels of both enzymatic and non-enzymatic components of the antioxidant system such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione reductase (GR), reduced and oxidized glutathione (GSH vs. GSSG); and the concentrations of some damaged biomolecules indicating the extent of the direct molecular damage caused by free radicals. We were also interested in the expressions of a free radical source, the gp91^{phox} subunit of NADPH oxidase (the NOX2 isoform, which is highly expressed in leukocytes), and the expression of the antioxidant HMOX-1 isoenzyme (both are important in the bronchial system) in the leukocytes, and investigated whether these correlate with the biochemical parameters in the blood. Since all asthmatic patients were treated medically, the effects of the medication were compared with the biochemical parameters and the gene expression profiles.

Patients and methods

The study groups consisted of children with a confirmed diagnosis of bronchial asthma ($n=58$), and healthy children or young adults as controls ($n=30$). All of the asthmatic patients were admitted to inpatient and outpatient wards of the Department of Pediatric and Health Care Center Faculty of Medicine at University of Szeged in Hungary. Healthy controls were enrolled from ward of surgery waiting for elective intervention and from the outpatient department of our institution recalled for control medical check-up after recovery. No controls had acute disease one month prior to sample collection. No controls had positive atopic status, respiratory disease and chronic disease in their anamnesis, which could have influenced their oxidative status. There were 36 boys (mean age: 14.55 years) and 22 girls (mean age 16.29 years) among the patients, and 16 boys (mean age: 14.94 years) and 14 girls (mean age: 16.03 years) among the healthy controls. The level of asthma control was assessed via the GINA (Global Initiative for Asthma) references, and the medication was modulated according to the complaints and the parameters reported in the protocol. The asthmatic patients were classified in 4 subgroups: those who did not require inhaled corticosteroid (ICS) ($n=22$), those who received a low daily dose of ICS (below 200 μg/day) ($n=20$), those who received a medium daily dose of ICS (200–400 μg/day) ($n=6$), and those who received a medium or high dose ICS (≥200–400 μg/day ICS) combined with a long-acting β2-receptor agonist (LABA) ($n=10$). The medication had been applied for at least six months before sample collection. For all these patients, the possibility was given for the use of short-acting β_2-agonists (SABA) as rescue therapy. In some cases, histamine-2 receptor antagonists and leukotriene receptor antagonist were also administered as maintenance therapy when required. The study was approved in advance by The Scientific Committee and Ethical Council of the University of Szeged, and written informed parental consent was obtained in all cases before the study.

Sample collection

Six milliliters of blood was taken from a peripheral vein in both groups. For the gene expression experiments, blood was stabilized (RNA/DNA Stabilization Reagent for Blood/Bone Marrow, Roche), and EDTA-coated tubes were used for the collection and analyses of oxidant and antioxidant parameters.

Gene expression analysis

The gp91^{phox} (CYBB) and HMOX-1 gene expression was determined by using a real-time polymerase chain reaction (RT-PCR) technique. Briefly, after the extraction of mRNA from whole blood as prescribed by the manufacturer (mRNA isolation Kit for Blood/Bone Marrow, Roche), reverse transcription was performed by using a cDNA Synthesis Kit (Fermentas). The RT-PCR process was carried out with a LightCycler Carousel-based System 1.5 (Roche) and LightCycler Software Version 3.5, with a FastStart HybProbe Kit (Roche). The following primers and hybridization probes were designed by the LightCycler Probe Design Software 2.0: *forward primer-CYBB*: 5′-AACACCCTAATACCAGA-3′, *reverse primer-CYBB*: 5′-CATGGAAGAGACAAGT-3′, *P1-CYBB*: 5′-CTCTGTGGACCTGAAGCC-Fluo3′, *P2-CYBB*: 5′LcRed705-GGCTGAAACCCTGAGTAAAC-Pho3′, f*orward primer-HMOX-1*: 5′-GTTCCTGCTCAACATCCA-3′, *reverse primer-HMOX-1: 5*′-GCTTCCCTCTGGGAGTCT-3′, *P1-HMOX-1*: 5′-GCTGACCCATGACACC-Fluo3′, *P2-HMOX-1*:5′-LcRed705-GGACCAGAGCCCCTCA-Pho3′. The annealing temperature was set at 55 °C. Human porphobilinogen deaminase (h-PBGD, HPBGD Gene Set, Roche) was used as housekeeping gene, marked by different dyes, in the same capillary, to normalize the differences between the sample's mRNA concentrations. Relative expressions of candidate mRNAs were determined by using the threshold cycle number (Ct) data obtained by real-time PCRs, with each sample normalized to its own h-PBGD housekeeping gene (ΔCt). The ΔCt data for the overall group and the individual subgroups were compared with those for the healthy controls (ΔΔCt).

Biochemical analysis

We measured the levels of GSH vs. GSSG, thiobarbituric acid reactive substances (TBARS, an indicator of lipid peroxidation) and protein carbonylation (an oxidized protein end-product), and the activities of SOD, CAT, GPx and GR enzymes in the red blood cells. The plasma cortisol level was also examined. GSH and GSSG were measured in whole blood. All other parameters were determined on red blood cells after a period of washing with phosphate-buffered saline (pH 7.4) three times. For all analyses, 10- and 100-fold diluted hemolyzates were used.

GSH concentrations were determined in specific reactions with GR. To determine GSSG concentrations, thiol scavenging was carried out with N-ethylmaleimide (Németh and Boda, 1994). The total quantity of proteins was determined with the Folin–Ciocalteu phenol reagent, using bovine serum albumin as standard. Results are expressed in mg/ml (Lowry et al., 1951). After removal of the hemoglobin from hemolyzed samples, SOD activity was measured in the supernatant via the inhibition of the spontaneous epinephrine–adrenochrome transformation (McCord and Fridovich, 1969; Misra and Fridovich, 1972; Matkovics et al., 1982). CAT activity was measured spectrophotometrically, following the decomposition of H_2O_2, at 240 nm (Beers and Sizer, 1952). To determine GPx activity, we measured the GSH consumption in each sample and its own control, with cumene hydroperoxide and GSH as substrates. Determination was performed spectrophotometrically with measurement of the residual GSH with 5,5′-dithiobis-2-nitrobenzoic acid (Novák et al., 1990; Chiu et al., 1976; Pinto and Bartley, 1969). In

the determination of GR activity, GSSG was applied as substrate and NADPH as cofactor (Novák et al., 1990; Pinto and Bartley, 1969). Enzyme activities are expressed in U/mg protein, or in the case of CAT in Bergmeyer units (BU)/mg protein (Beers and Sizer, 1952). The concentration of TBARS derived from the red blood cells was measured with thiobarbituric acid (TBA), which gives the level of total TBA-reactive substances. Results are reported in nmol/mg protein (Placer et al., 1966). Carbonylated proteins are oxidatively modified proteins. The level of protein damage was examined with 2,4-dinitrophenylhydrazine (2,4-DNPH). All samples had their own controls without 2,4-DNPH. Results are expressed in mmol/mg protein (Levine et al., 1996).

Statistical analysis

Comparisons were performed by using one-way ANOVA, followed by Tukey's post-test; or for TBARS, the Kruskal–Wallis test. All values have had normal distribution, and means ± SD were calculated for all groups except for MDA, where median values are shown with the 25th and 75th percentiles. In latter case the variances were different and non-parametric test was applied. The p values <0.05 were considered indicative of a significant difference.

Results

NADPH oxidase gp91phox subunit mRNA in blood

We analyzed the relative mRNA level in each subgroup and determined the difference in expression between the subgroups treated or not treated with ICS ($\Delta\Delta Ct = 0.819$ vs. $\Delta\Delta Ct = -0.363$, respectively; $p<0.03$; ΔCt values referred to the healthy controls). This result suggested a difference due to medical treatment. The post test after ANOVA analysis revealed a significant difference between the subgroup not treated with ICS and that treated with a low daily dose of ICS ($\Delta\Delta Ct = 1.445$, $p<0.05$) indicating an approximately two-fold difference. These ΔCt values in each subgroup were compared with non steroid treated asthmatics in Table 1. Negative $\Delta\Delta Ct$ values mean upregulation of the gene expression according to the comparative ΔCt method, contrarily to the positive values which indicate downregulation. No significant difference was observed between the three other ICS-treated subgroups, where the taken higher ICS doses did not influence the CYBB gene expression significantly as compared with the subgroup treated with a low daily dose of ICS. For a better presentation in Fig. 1, the relative expression profile of the NADPH oxidase subunit coding CYBB gene was shown relative to healthy controls (Fig. 1).

HMOX-1 gene expression in blood

There was no significant difference in HMOX-1 gene expression between the asthmatic patients and the healthy controls, and the ICS therapy did not influence the expression of this gene either (Fig. 2).

Biochemical analyses in blood

The TBARS and carbonylated protein concentrations in the red blood cells were significantly higher in the asthmatic children than in the healthy controls ($p<0.05$ and $p<0.03$, respectively). Both parameters were somewhat higher in the subgroup which did not require steroids than in those who received ICS medication (Table 2). The TBARS level was also significantly higher in all the ICS-treated subgroups as compared the asthmatic children not treated with ICS. The highest carbonylated protein level was measured in the subgroup that did not require ICS, but the difference was significant only relative to those who received a low daily dose of ICS. Neither parameter was influenced by sex or asthma control (*data not shown*).

The GSH and GSSG levels and the ratio GSSG/2GSH did not differ significantly in any of the subgroups from those in the healthy controls. Nor were the GPx or GR activities in the asthmatic children significantly different from those in the healthy children. The ICS-treated asthmatic subgroup displayed higher GPx activities than those who did not receive ICS treatment, but the difference did not reach the

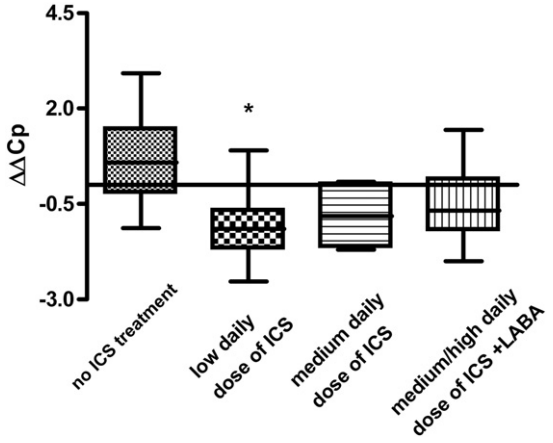

Fig. 1. Relative expressions of CYBB in blood samples from the three subgroups of asthmatic patients. Relative mRNA levels of $gp91^{phox}$ coding CYBB gene were determined by using the threshold (Ct) values of the CYBB gene by real-time PCR. Each sample was normalized to its own h-PBGD housekeeping gene (ΔCt). ΔCt data on groups and subgroups were compared (ΔΔCT). Values are referred to healthy controls for the better presentation. *Significantly lower mRNA levels $p<0.05$ were measured in the subgroup treated with a low daily dose of ICS than in those who were not treated with ICS. The blocking effect was lower (higher mRNA levels) in the patients who received medium/high daily dose of ICS alone or in combination with LABA, but this difference did not reach the level of significance. Statistical analysis was performed by one-way ANOVA test.

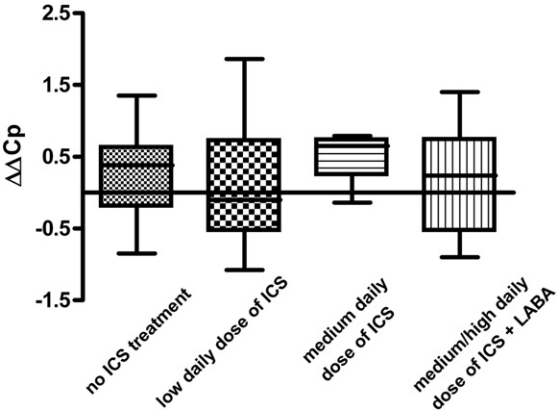

Fig. 2. Relative expressions of HMOX-1 in blood samples from the three subgroups of asthmatic patients. Relative mRNA levels of the HMOX-1 gene were determined by using the threshold (Ct) values of the HMOX-1 gene by real-time PCR. Each sample was normalized to its own h-PBGD housekeeping gene (ΔCt). ΔCt data on groups and subgroups were determined (ΔΔCt). Values are referred to the healthy controls. No significant difference was observed between the individual subgroups. Statistical analysis was performed by one-way ANOVA test.

Table 1
ΔΔCt data derived from asthmatic patients treated with different ICS doses compared with non ICS treated ones.

Treatment	ΔΔCt
Low daily dose of ICS	1.445*
Medium daily dose of ICS	1.168
Medium/high daily dose of ICS + LABA	1.034

Statistical analysis was performed by one-way ANOVA test.
* $p<0.05$: compared with non steroid treated patients.

Table 2
Biochemical oxidative stress and antioxidant parameters in blood of asthmatic children and healthy controls.

Parameters	Healthy	Steroid treated	Steroid non treated
TBARS (nmol/mg prot.)	0.56 (0.44–0.65)	0.77 (0.66–0.89)*	0.78 (0.66–1.05)*
Carbonylated protein ($\times 10^{-5}$ μmol/mg prot.)	6.77 ± 1.12	7.22 ± 1.25	8.10 ± 1.63*
GSH (μmol/g Hb)	8.69 ± 2.31	9.31 ± 1.51	9.19 ± 1.36
GSSG (μmol/g Hb)	18.50 ± 4.35	19.63 ± 5.28	18.95 ± 3.51
GSSG/2GSH	0.22 ± 0.06	0.21 ± 0.04	0.21 ± 0.03
SOD (U/mg prot.)	2.54 ± 1.07	2.49 ± 0.86	3.13 ± 0.97*+
CAT ($\times 10^{-5}$ BU/mg prot.)	0.89 ± 0.22	0.86 ± 0.29	1.04 ± 0.36
GPx ($\times 10^{-3}$ U/mg prot.)	2.41 ± 0.45	3.18 ± 1.57	2.63 ± 0.45
GR (($\times 10^{-4}$ U/mg prot.)	7.68 ± 1.84	7.44 ± 2.28	8.28 ± 2.78

Data are reported as mean ± SD and in case of TBARS median (25th–75th percentiles). One-way ANOVA and Kruskal–Wallis tests were performed to compare the groups.
* $p<0.05$ significant difference relative to healthy controls.
*+ $p<0.05$ significant difference relative to healthy controls and to steroid treated patients.

level of significance. These data too were not influenced by sex or asthma control (*data not shown*).

The SOD activity was significantly higher in the asthmatic patients who did not receive ICS medication than in the healthy children or in those who required ICS therapy (Table 1).

The CAT activity exhibited a small, but not significant elevation in the asthmatic subgroup not treated with ICS, but there was no noteworthy difference in enzyme level as a function of sex or asthma control.

Discussion

This study focused on the possible use of peripheral blood, which is the most performable medium for examination of the systemic oxidative damage in children. We measured the mRNA levels of the NADPH oxidase enzyme complex $gp91^{phox}$ subunit (CYBB), one of the main free radical sources, and the antioxidant HMOX-1 protein. Besides the transcriptional activities of the above genes, we determined the level of a number of antioxidant enzymes and the blood levels of lipid peroxidation and protein damage. All the results were considered in the context of the medical treatment too.

The major role of the NADPH oxidase complex in free radical generation and the importance of the NF-κB transcriptional factor and pro-inflammatory cytokines in the transcriptional regulation of the $gp91^{phox}$, $p67^{phox}$ and $p47^{phox}$ subunits were demonstrated earlier in the MonoMac1 cell line in vitro, but the mRNA level of CYBB has previously not been measured in blood leukocytes of childhood asthmatic patients (Bedard and Krause, 2007; Gauss et al., 2007; Yao et al., 2007). The CYBB mRNA level proved to be significantly lower in the patients treated with a low daily dose of ICS than in those who did not receive ICS therapy. There was also a tendency to a lower expression in the patients treated with a medium/high daily dose of ICS or with an ICS–LABA combination, relative to asthmatics without ICS treatment, but these differences did not reach the level of significance. Although it is widely accepted that inhaled corticosteroids in normal therapeutic doses exhibit effects only locally in the airways (Chung et al., 2009), our results clearly demonstrated that the CYBB expression was altered in the blood. This systemic effect may be due to the activated inflammatory cells which appear in the circulation, and the alterations in CYBB expression may be a consequence of the inhibitory effects of ICS on NF-κB-regulated genes as in the case of the NADPH oxidase $gp91^{phox}$ subunit (Almeida et al., 2005; Gauss et al., 2007; Yao et al., 2007; Condino-Neto et al., 1998). The present findings in the subgroups that received a higher dose of steroid or ICS combined with LABA, which are known to improve the symptoms (Hew et al., 2006), may be linked with relative steroid resistance, which demands further examinations. Despite the administration of higher steroid doses, a lower level of CYBB gene transcription was found in steroid treated asthmatic children than in healthy children, even when the serum cortisol concentration was similar to that in the healthy controls. The activity of the NADPH oxidase complex is also determined by several posttranscriptional modifications (van der Vliet, 2008; Bedard and Krause, 2007). The lack of transcriptional difference in the CYBB gene in the blood from asthmatics and healthy children does not exclude a possible role of the NADPH oxidase complex in free radical generation at the protein level, as demonstrated in other studies.

The HMOX-1 gene, transcriptionally activated in the early phase of the oxidative stress, is used as an acute marker in studies of the antioxidant system (Slebos et al., 2003; Jin and Choi, 2005). Our results did not indicate any change in the HMOX-1 mRNA levels in the analyzed groups, in accord with the chronic oxidative stress previously supposed in bronchial asthma. The difference in expression profile between CYBB and HMOX-1 genes in our samples may originate from the different signaling pathways: the CYBB gene is mainly regulated by NF-κB, while HMOX-1 is regulated by Nrf2 during airway inflammation (Yao et al., 2007; Lacy et al., 2003; Lee et al., 2000).

Elevated TBARS and carbonylated protein levels were detected in our asthmatic patients relative to healthy children, confirming earlier data (Schock et al., 2003; Shanmugasundaram et al., 2001; Perišić et al., 2007; Sugiura and Ichinose, 2008; Novák et al., 1991). The level of TBARS is indicative of lipid damage, which is an early marker of the ROS-mediated injury, whereas the carbonylated protein elevation due to the protein damage is a later marker of oxidative stress. The TBARS and carbonylated protein levels in the subgroup that did not participate in corticosteroid treatment were higher than in those treated with inhalative steroid. Higher SOD activity was also measured in the subgroup that did not require steroid, indirectly indicating the high $O_2^{\bullet-}$ generation. The enzymatic antioxidant CAT, GR and GPX concentrations in the red blood cells were not changed significantly.

The clinical status and therapy requirement of patients with asthma are influenced by various factors, including genetic, environmental and nutritional ones (Wong et al., 2001; Matsumoto et al., 2004). Recently, there have been many reports suggesting the presence of vitamin D insufficiency in immune-regulated inflammatory diseases such as rheumatoid arthritis or in bronchial asthma in which the balance between the Th1 and Th2 immune response is disturbed (Cutolo, 2009; Sandhu and Casale, 2010). It was also reported that vitamin D supplementation may be favorable on immune response of cells obtained from patients who required ICS–LABA combination therapy or oral steroid treatment in vitro (Sandhu and Casale, 2010; Searing et al., 2010). The immunmodulatory role, the possible effect of vitamin D on the corticosteroid pathway and results in adult asthmatics suggest that vitamin D may have positive effects in asthmatic children too. This hypothesis, as well as the possible relationship of vitamin D levels and oxidative stress requires further examinations.

To the best of our knowledge, there have been no reports of monocyte/myeloid cell lines expressing the $gp91^{phox}$ subunit coding gene in the blood of asthmatic patients, and nor of the modifying effects of ICS on this pro-oxidant gene from blood leukocytes. Our results were measured on native leukocytes from asthmatic children: cells after isolation were not treated in vitro. Besides the local inflammation in the airways, we could detect the systemic effects of chronic oxidative stress in childhood asthma.

Conclusions

In conclusion, we confirmed the presence of oxidative stress in the circulation of pediatric asthmatic patients: higher damaged lipid and carbonylated protein concentrations reflected a chronic oxidative overload. We also detected the effects of inhaled corticosteroid medication on the gene expression also in the blood, and examined for the first time the transcriptional activity of the main, electron transfer-catalyzing $gp91^{phox}$ subunit of the NADPH–oxidase complex on native leukocytes gained from asthmatic children. The examination of

blood samples proved suitable for the following oxidative processes which mainly occur locally in the lungs, but further investigations are needed to identify the best blood parameters and methods in pediatric practice and asthma management. It is also essential to determine reference values for oxidative stress in different pathological conditions.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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Collagen type IV nephropathy: Genetic heterogeneity examinations in affected Hungarian families

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ABSTRACT

The Col4A3, Col4A4 and Col4A5 collagen type IV genes are found to be mutated in Col IV nephropathy. In males with a mutation in the Col4A5 gene (X-linked Alport syndrome: XL-AS), progressive renal disease always develops. Female carriers with a mutation in the Col4A5 gene can develop thin basement membrane nephropathy (TBMN). Males and females who carry 1 Col4A3 or Col4A4 mutation usually manifest TBMN with nonprogressive hematuria. In the event of 2 Col4A3 or Col4A4 gene mutations, the autosomal recessive AS will develop. We examined the cosegregation pattern of hematuria in 20 families. The renal biopsies led to diagnoses of AS in 7 families, and of TBMN in 6 families. In 7 others, the diagnosis of familial hematuria (FHU) was based on the clinical symptoms. Markers of the ColA3/Col4A4 and Col4A5 loci (Col4A3: CA11 and D2S401; Col4A4: HaeIII/RFLP; and Col4A5: DXS456, 2B6 and 2B20) were used to assess their linkage to the clinical symptoms and morphological alterations. Maximum likelihood and the FASTLINK version of the linkage program were applied to compute logarithm of the odds (LOD) scores. A linkage to the Col4A3/Col4A4 genes was identified in 5 families (FHU in 3, AS in 2 families, 25%, LOD score range: 0.20–3.51). The XL-AS pattern of inheritance seemed likely with Col4A5 in 9 families (45%, LOD: 0.43–4.20); we found 4 disease-causative mutations by high-resolution melting curve analysis (LC480) and sequencing in this group. In 2 FHU families, the linkage to chromosomes 2 and X was precluded. Knowledge of the genetic background of Col IV nephropathy is essential to avoid the misdiagnosis of FHU and early AS. The allele frequencies, heterozygosity content and polymorphism information content of the applied STR markers on unrelated Hungarian normal and affected chromosomes 2 and X were also calculated.

1. Introduction

Type IV collagen (Col IV), a major constituent of basement membranes, consists of a family of 6 genetically distinct alpha chains, designated Col4A1 to Col4A6. Three alpha chains assemble into triple-helical molecules. These chains are encoded by pairs of genes located on 3 different chromosomes. The genes are unique in having characteristic pairwise, head-to-head chromosomal arrangements on 3 chromosomes, 13, 2 and X [1], theoretically affording 56 different triple-helical molecules [2].

During normal human glomerular development, the Col4A1/Col4A1/Col4A2 network is assembled first in the embryonic glomerulus, but there is then a developmental switch to the synthesis of the Col4A3/Col4A4/Col4A5 network, which forms the glomerular basement membranes (GBMs) of the mature glomerulus (and in the inner ear and eye). The Col4A3/Col4A4/Col4A5 chains, which have similar functions, are present in the same heterotrimers, and mutations in any of them therefore produce very similar phenotypes [3]. The GBM of the kidney (between the endothelial and epithelial cell layers of the glomerular capillary wall) is an essential component of the blood filtration barrier. The Alport syndrome (AS) and thin basement membrane nephropathy (TBMN) are recognized as specific diseases of the Col IV chains [4].

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The developmental switch is arrested in the AS by mutations in any of the genes encoding the Col4A3, Col4A4 (autosomal recessive, AR, loci: 2q35–37) or Col4A5 (X-linked dominant, XL-AS, locus: Xq22) chains. As a result, the GBM in AS patients is composed of the embryonic Col4A1/Col4A1/Col4A2 network rather than the mature Col4A3/Col4A4/Col4A5 network. This situation leads to a deterioration of the GBM and to progressive loss of the renal function over a period of 10–20 years, possibly by rendering the GBM susceptible to proteolysis. In the majority of AS cases, the Col4A3, Col4A4 and Col4A5 chains are all absent within the GBMs, although only one of these chains is actually mutated. Genetic heterogeneity is a well-known feature of the AS. Most (~85%) of the families with the AS exhibit an XL-AS pattern of inheritance (Col4A5 gene) with an estimated incidence of 1/5000–1/10,000. Classically, males develop persistent microscopic hematuria, hearing loss, ocular abnormalities at a young age, and splitting, thickening and thinning of the GBMs, which may lead to a renal impairment, progressing to end-stage renal disease (ESRD) in their early 20s. Females tend to be less severely affected. Individuals homozygous (or compound heterozygous) for 2 mutations in the Col4A3 or Col4A4 genes have been identified in AR-AS [5,6]. With merely a few exceptions, each family carries unique mutations [7]. Approximately 10–15% of the A5 mutations are *de novo*, having occurred in the gamete of a parent. Clinicians are usually familiar only with this dominant, XL-AS manner of inheritance. Although about 15% of the families display autosomal inheritance of the disease, such families may be missed without this knowledge. The AR-AS may account for 14% of the total number of cases. In some cases (~1%), autosomal dominant (AD) inheritance of the AS (male to male transmission) has been recorded [8]. The ability to distinguish TBMN from the early stages of the AS is critical.

TBMN is characterized by persistent or recurrent microscopic hematuria, usually detected in childhood, a thin GBM, the absence of or at most only mild proteinuria, and the absence of renal failure or extrarenal symptoms. The hematuria usually remains isolated and only a minority of the cases progress toward ESRD. The rare occurrence of a hearing loss in TBMN illustrates the difficulties in the differential diagnosis [9]. The pattern of inheritance of TBMN is AD. Its reported prevalence may range from <1% to 14% [10]. Approximately two-thirds of patients with TBMN have an AD familial form of the disease [11]. In at least 40% of the cases of familial hematuria (FHU), this symptom cosegregates with the Col4A3 or Col4A4 loci [12]. Thus, on the loss of one allele, the resulting lamina densa appears thin, but is seemingly resistant to proteolysis.

Pediatric nephrologists must give a prognosis to the parents of children with hematuria and must distinguish TBMN from adult types of the AS. Misdiagnosis of the AS and TBMN may be quite common, because microhematuria is not always present in TBMN, sporadic hematuria in females can obscure the picture, and clinical pedigree analysis rarely suffices to distinguish the mode of inheritance. The ultimate solution of this problem lies in a genetic diagnosis, but this is not readily available at present. The inheritance of TBMN and the AS can be determined by linkage analysis, which does not require the identification of a particular mutation. The complete sequencing of the Col4A3, Col4A4 or Col4A5 genes is a sensitive method that is capable of detecting over 80% of the mutations, but this technique is expensive and not yet generally available. The low rate of detection of mutations limits its usefulness. In the future, automation and the miniaturization of DNA analytical techniques are likely to result in relative simple, rapid and inexpensive assays of mutations at the genetic loci of TBMN and the AS.

We decided to compare the clinical, morphological and molecular genetic data on 20 families affected by inherited hematuria with their haplotype analysis data at the Col4A3, Col4A4 and Col4A5 loci. We carried out mutation screening in all 56 amplicons of the 51 exons in the Col4A5 gene by high resolution melting (HRM) curve analysis, a new approach to the screening of point mutations. The amplicons with altered melting profiles were consecutively sequenced.

2. Subjects and methods

2.1. Subjects

Members of 20 families (60 healthy and 90 hematuric persons) underwent clinical examinations and family investigations. Each family had at least 2 hematuric and 2 or more unaffected individuals. Renal biopsies were performed in cases of a progressive form of glomerulonephritis (frequently associated with sensorineural deafness). Ophthalmological examinations were carried out on the affected children, but no ocular abnormalities were found. Together with electron microscopy, immunohistochemical studies of renal biopsy specimens for the Col4A3 and Col4A5 chains were also performed in some of the patients. The clinical and morphological data are presented in Tables 1a and 1b. Pedigree reconstruction and molecular investigations of the genes encoding the Col IV chains were carried out with the informed consent of the patients and their families. The AS diagnosis was confirmed by renal biopsy in 7 families (5 families with male patients and 2 families with female patients). In 6 families, uniformly thinned GBMs were seen in the patients. The clinical diagnosis was FHU in an other 7 families. The FHU was set up on the basis of dysmorphic hematuria (erythrocytes with an irregular shape and size indicative of hematuria of glomerular origin) with minimal or no proteinuria, and a normal renal function.

2.2. Methods

The morphological evaluations of the renal biopsies were carried out by standard light microscopic, electron microscopic and immunofluorescence microscopic methods. In the cases of TBMN (and early AS), uniformly thinned GBMs were seen. The AS-diagnosed patients displayed the typical ultrastructural changes. The protein expression of the Col4A3 and Col4A5 chains was examined via the specific monoclonal antibody immunofluorescent staining of renal biopsy specimens (Alport Kit, Wislab AB, Sweden).

To determine the genetic linkage to the hematuria, segregation analysis was performed in 20 families. DNAs from affected and nonaffected family members were extracted from peripheral blood leukocytes [13]. Six fluorescent polymerase chain reactions (PCRs) were set up for amplifications of the Col4A3/Col4A4 and Col4A5 loci. We examined their polymorphic short tandem repeat (STR) markers, where the forward primers were labeled fluorescently with Cy5. Electrophoresis of PCRs was carried out with the Phast-System and ALFexpress™ (Amersham Pharmacia Biotech) system to investigate the length variants of the microsatellites. The amplification conditions were as described previously [4,14,15] and the following tightly linked, highly polymorphic markers were used.

The CA11 and D2S401 microsatellite markers were linked to the Col4A3 locus (2q35–37). The CA11 alleles were denoted D1–D5, where D1 had 18 CA repeats, and D5 had 14 CA repeats. The D2S401 alleles were denoted E1–E10, where E1 had 20 CA repeats, and E10 had 11 CA repeats. HaeIII intragenic restriction fragment length polymorphism (RFLP) [8] was used at the Col4A4 locus, where the alleles were denotes by 1 (HaeIII site absent) and 2 (HaeIII site present). Haplotypes were constructed at the Col4A3/Col4A4 loci for the family members. Linkage to the Col4A5 locus was analyzed by using the following X chromosome markers: 2B6 (GDB: 196580,

Table 1a
Families including members who exhibited symptoms of TBMN or Alport sy[**].

Family	Affected member/ gender/age++	Urinalysis			Hypertension	Renal biopsy				Hearing loss	Results of marker analysis
		Glomerular hematuria[++]	Proteinuria g/day	Creatinine clearance		Light microscopy	Electron microscopy	Col4A3*	Col4A5*		
I	1/F/46y	Yes	None	Norm.	None	No change	Diffusely thin GBM	Norm.	Norm.	Hypacusis	Carrier
	2/F/47y	Yes	None	Norm.	None	Glomerular scarring 18%	Diffusely thin GBM	Norm.	Norm.	Hypacusis	Carrier
	3/F/42y	Yes	0.8	70	Yes	Glomerular scarring 20%	Diffusely thin GBM	Norm.	Norm.	Hypacusis	Carrier
	4/M/16y	Yes	1.0−3.0	75	None	Glomerular scarring 10%	Diffusely thin GBM + mild basket weaving	Norm.	Discontinuous	Hypacusis	XLAS
	5/M/24y	Yes	1.0−3.0	Norm.	None	Glomerular scarring 3%	Diffusely thin GBM + mild basket weaving	Discontinuous	Discontinuous	Hypacusis	XLAS
	6/M/15y	Yes	0.5-0.8	56	None	Glomerular scarring 12%	Diffusely thin GBM	Discontinuous	Discontinuous	Mild hypacusis	XLAS
II	1/F/38y	Yes	None	Norm.	None	n.d.	n.d.	n.d.	n.d.	Hypacusis	Carrier
	2/F/18y	Yes	5	Norm.	None	Glomerular scarring 16%, foam cells in abundance	Diffusely thin GBM, basket weaving in several loops	Absent	Very focal expression	Normal	ARAS
	3/F/16y	Yes	None	Norm.	None	n.d.	n.d.	n.d.	n.d.	Normal	Carrier
	4/M/15y	Yes	None	Norm.	None	n.d.	n.d.	n.d.	n.d.	Hypacusis	Carrier
III	1/M/47y•	Yes	0.5	Norm.	Yes	n.d.	n.d.	n.d.	n.d.	n.d.	XLAS
	2/F/34y	Yes	None	63	None	n.d.	n.d.	n.d.	n.d.	n.d.	Carrier
	3//M/14y	Yes	None	57	None	n.d.	n.d.	n.d.	n.d.	n.d.	XLAS
	4/F/57y	Yes	0.5	Norm.	Yes	n.d.	n.d.	n.d.	n.d.	n.d.	Carrier
IV	1/M/39y	Yes	0.5	75	None	No change	Diffusely thin GBM	n.d.	n.d.	n.d.	TBMN
	2/M/15y	Yes	None	58	None	n.d.	n.d.	n.d.	n.d.	n.d.	TBMN
	3/M/18y	Yes	None	62	None	n.d.	n.d.	n.d.	n.d.	n.d.	TBMN
V	1/M/55y	Yes	None	Norm.	Yes	n.d.	n.d.	n.d.	n.d.	n.d.	Carrier
	2/F/53y	Yes	None	Norm.	None	n.d.	n.d.	n.d.	n.d.	n.d.	Carrier
	3/F/27y	Yes	None	Norm.	None	n.d.	n.d.	n.d.	n.d.	n.d.	Carrier
	4/F/20y	Yes	0.5-2.5	66	Yes	Glomerular scarring 6%	Thin GBM, basket weaving in several loops	n.d.	n.d.	n.d.	ARAS
VI	1/M/3y	Yes	0.5	38	None	No change	GBM thin	n.d.	n.d.	n.d.	?
	2/F/36y	Yes	None	Norm.	None	n.d.	n.d.	n.d.	n.d.	n.d.	?
VII	1/F/10y	Yes	None	Norm.	None	n.d.	n.d.	n.d.	n.d.	n.d.	Carrier?
	2/F/40y	Yes	None	Norm.	None	n.d.	n.d.	n.d.	n.d.	n.d.	Carrier?
VIII	1/M/9y	Yes	<0.5	Norm.	None	n.d.	n.d.	n.d.	n.d.	n.d.	XLAS
	2/F/31y	Yes	None	Norm.	None	n.d.	n.d.	n.d.	n.d.	n.d.	Carrier?
IX	1/M/11y	Yes	None	Norm.	None	No change	Diffusely thin GBM	n.d.	n.d.	n.d.	?
	1/F/50y	Yes	None	Norm.	Yes	n.d.	n.d.	n.d.	n.d.	n.d.	?

Abbreviations are explained in the footnotes to Table 1b.

Table 1b
Families including members who exhibited symptoms of TBMN or Alport sy**.

Family	Affected member/ gender/age+	Urinalysis			Hypertension	Renal biopsy				Hearing loss	Results of marker analysis
		Glomerular hematuria++	Proteinuria g/day	Creatinine clearance		Light microscopy	Electron microscopy	Col4A3*	Col4A5*		
X	1/M/43y	Yes	<0.5	Norm.	None	n.d.	n.d.	n.d.	n.d.	n.d.	TBMN
	2/F/17y	Yes	<0.5	Norm.	None	n.d.	n.d.	n.d.	n.d.	n.d.	TBMN
	3/M/10y	Yes	<0.5	Norm.	None	n.d.	n.d.	n.d.	n.d.	n.d.	TBMN
XI	1/M/12y	Yes	0.5-1.0	Norm.	Yes	Glomerular scarring 13%	Diffusely thin GBM	n.d.	n.d.	None	XLAS
	2/F/44y	Yes	<0.5	15	Yes	n.d.	n.d.	n.d.	n.d.	hypacusis	Carrier
XII	1/M/6y	Yes	<0.5	Norm.	None	n.d.	n.d.	n.d.	n.d.	n.d.	XLAS
	2/F/39y	Yes	None	Norm.	None	n.d.	n.d.	n.d.	n.d.	n.d.	Carrier?
XIII	1/F/71y	Yes	n.d.	ESRD	Yes	n.d.	n.d.	n.d.	n.d.	n.d.	Carrier?
	2/F/45y	Yes	n.d.	ESRD	Yes	n.d.	n.d.	n.d.	n.d.	n.d.	Carrier?
	3/F/1y	Yes	0.5	Norm.	None	no change	GBM thin	n.d.	n.d.	n.d.	XLAS carrier?
XIV	1/F/40y	Yes	None	Norm.	None	n.d.	n.d.	n.d.	n.d.	n.d.	Carrier
	2/M/17y	Yes	0.5	Norm.	Yes	Glomerular scarring 6%	Thin GBM, basket weaving in several loops	n.d.	n.d.	n.d.	XLAS
	3/M/15y	Yes	None	Norm.	Yes	Glomerular scarring 5%	Similar to his brother	n.d.	n.d.	n.d.	XLAS
	4/F/7y	Yes	None	Norm.	None	n.d.	n.d.	n.d.	n.d.	n.d.	Carrier
XV	1/M/72y	Yes	1.5	38	None	n.d.	n.d.	n.d.	n.d.	n.d.	Carrier?
	2/M/43y	Yes	1.2	Norm.	None	n.d.	n.d.	n.d.	n.d.	n.d.	Carrier?
	3/F/40y	Yes	2.0	28	Yes	Glomerular scarring 29%, foam cells	Diffusely thin GBM	Norm.	Norm.	None	TBMN?
XVI	1/M12y•	Yes	4.0	ESRD (19y)	Yes	glomerular scarring 20%	Thin GBM, basket weaving in several loops	n.d.	n.d.	hypacusis	XLAS
	2/F/35y	Yes	None	Norm.	None	n.d.	n.d.	n.d.	n.d.	n.d.	Carrier
XVII	1/M/17y•	Yes	3.0	ESRD (22y)	Yes	Glomerular scarring 29%	Thin GBM, basket weaving in several loops	n.d.	n.d.	Hypacusis	XLAS
	2/F/40y	Yes	0.5	n.d.	Yes	n.d.	n.d.	n.d.	n.d.	n.d.	Carrier?
XVIII	1/M/11y	Yes	2.5	ESRD (23y)	Yes	n.d.	n.d.	n.d.	n.d.	Hypacusis	XLAS?
	2/F/33y	Yes	None	Norm.	None	n.d.	n.d.	n.d.	n.d.	n.d.	Carrier?
XIX	1/M/7y	Yes	None	Norm.	None	Glomerular scarring 15%, foam cells	Typical Alport finding	n.d.	n.d.	n.d.	XLAS?
	2/F/28y	Yes	None	Norm.	None	n.d.	n.d.	n.d.	n.d.	n.d.	carrier?
XX	1/F/6y	Yes	None	Norm.	None	n.d.	n.d.	n.d.	n.d.	n.d.	?
	2/F/40y	Yes	None	Norm.	None	n.d.	n.d.	n.d.	n.d.	n.d.	?

**Not all affected members feature in this table, +age at diagnosis, F = female, M = male family member, ++glomerular hematuria: dysmorphic red blood cells, creatinine clearance normal: >80 ml/min/1.73 m^2, *immunofluorescence (with MAB-3 and MAB-5 antibodies, Alport kit, Wieslab), GBM = glomerular basement membrane, n.a. = not available, n.d. = not done, XLAS = X-linked Alport sy, ARAS = autosomal recessive Alport sy, TBMN = thin basement membrane nephropathy, •renal transplantation was performed, ESRD = end-stage renal disease.

Table 2
CA11 (92 normal, and 12 affected), D2S401 (120 normal, and 12 affected) Col4A3 and Col4A4/HaeIII RFLP (114 normal and 12 affected) allele frequencies in unrelated Hungarian chromosomes 2.

CA11 (intragenic)			D2S401 (extragenic, approx. 80 kb from 3' end)		
No. of CA repeats (allele name and length)	No. of normal alleles (frequency)	No. of affected alleles (frequency)	No. of CA repeats (allele name and length)	No. of normal alleles (frequency)	No. of affected alleles (frequency)
18 (D1, 83 bp)	–	1 (0.08)	20 (E1, 121 bp)	–	–
17 (D2, 81 bp)	5 (0.05)	2 (0.17)	19 (E2, 119 bp)	1 (0.01)	–
16 (D3, 79 bp)	27 (0.29)	2 (0.17)	18 (E3, 117 bp)	–	–
15 (D4, 77 bp)	17 (0.18)	–	17 (E4, 115 bp)	1 (0.01)	–
14 (D5, 75 bp)	**43 (0.47)**	**7 (0.58)**	16 (E5, 113 bp)	9 (0.08)	–
			15 (E6, 111 bp)	3 (0.03)	–
			14 (E7, 109 bp)	**59 (0.49)**	**6 (0.50)**
Col4A4/Hae III RFLP			13 (E8, 107 bp)	17 (0.14)	–
Cleavage site: no	52 (0.46)	3 (0.25)	12 (E9, 105 bp)	6 (0.05)	2 (0.17)
Cleavage site: yes	62 (0.54)	9 (**0.75**)	11 (E10, 103 bp)	24 (0.20)	4 (0.33)

Most frequent alleles are highlighted.

intragenic marker, alleles: A0–A6, CA repeats: 25-19, respectively), IVS48 (2B20, GDB: 96581, intragenic marker, alleles: B1-B4, CA repeats: 15-12, respectively) and DXS456 (alleles: C0-C9, CA repeats: 26-17, respectively) [16].

56 primer pairs were designed by PRIMER3 software to amplify the 51 exons and the splice sites of the COL4A5 gene to be annealed at the same temperature of 60 °C. Our aim was to obtain amplicons with a size of 250 bp on average (min 198, max 300); the large exons were amplified by overlapping primer pairs. The primers were checked with BLAST and RepeatMaskers to avoid repeats and conserved domains. The HRM was performed in a single run on two parallels on a LightCycler 480 (LC 480, Roche Diagnostic, Penzberg, Germany) in a reaction mix containing 20 ng of genomic DNA, 0.4 uM of each primer, and 3 mM $MgCl_2$ using the LC High Resolution Melting (HRM) Master containing ResoLight dye (Roche Diagnostic) with PCR-grade water adjusted to a total volume of 15 μl. The reaction conditions included an activation step at 95 °C for 10 min followed by 45 cycles of 95 °C for 15 s, 60 °C for 15 s and 72 °C for 25 s. Before the HRM step, the products were heated to 95 °C 1 min and held at 40 °C for 1 min. HRM was carried out over the range from 65 °C to 95 °C at a temperature increase of 1 °C per second. Sequencing was used to confirm and characterize the screened amplicons identified by HRM with an ABI Prism 3130 automated sequencer.

2.3. Statistics

We determined the repeat number of microsatellites (mostly CA repeats) on the alleles of both affected and healthy family members by gene sequencing of a few samples of each marker. These samples were used for the repeat calculation in all other gel electrophorese. The numbers of alleles and their frequencies with different microsatellite repeat numbers (sizes) were characterized in all individuals. Heterozygosity levels (HETs) and polymorphism information content (PIC) values were also calculated. Linkage analysis results were evaluated by using the method of maximum likelihood [17], and the FASTLINK version [18,19] of the LINKAGE program was applied to compute the logarithm of the odds (LOD) scores. The exact sequence and distance of the STR marker and disease loci were obtained from the Ensemble genome database. Allele frequencies were estimated from our data. The incidence and penetrance values were estimated from data found in OMIM (#120070, #120131 and #303630). ILINK or MLINK (when the ILINK output indicated a too small theta (Θ) value) was used to calculate the maximum likelihood of Θs. Two-point LOD scores were calculated for the corresponding Θs.

3. Results

The results of genetic linkage analysis with the CA11 (92 normal and 12 affected) and D2S401 (120 normal and 12 affected) Col4A3 and Col4A4/HaeIII RFLP (114 normal and 12 affected) allele frequencies on unrelated Hungarian chromosomes 2 are listed in Table 2. The most frequent alleles are highlighted. In 5 families (only FHU: 3, ARAS: 2), a linkage to the Col4A3/A4 genes was identified (LOD: 0.20–3.51) (Table 6), and the X chromosome markers (2B6, 2B20 and DXS456,) excluded the linkage of the hematuria to the Col4A5 locus (negative LOD scores).

The results of segregation analysis with the Col4A5 markers (XL-AS families) are presented in Tables 3 and 4. The allele frequencies with the 2B20 intragenic (IVS 48) and 2B6 (near the 3′ end) markers (79 normal and 14 affected unrelated Hungarian chromosome X) can be seen. The most frequent alleles are highlighted.

In 9 families (45%), a linkage to the Col4A5 gene was identified (LOD: 0.43–4.20), with negative LOD scores for the chromosome 2

Table 3
IVS48 intragenic (2B20) and 2B6 (3'-UTR) allele frequencies on 79 normal and 14 affected unrelated Hungarian chromosomes X.

IVS48 (2B20) (intragenic marker)			2B6 (extragenic, 1.64 kb from 3' end)		
No. of CA repeats (allele name and length)	No. of normal alleles (frequency)	No. of affected alleles (frequency)	No. of CA repeats (allele name and length)	No. of normal alleles (frequency)	No. of affected alleles (frequency)
15 (B1, 124 bp)	–	1 (0.07)	25 (A0, 167 bp)	–	1 (0.07
14 (B2, 122 bp)	**65 (0.82)**	**12 (0.86)**	24 (A1, 165 bp)	4 (0.05)	–
13 (B3, 120 bp)	13 (0.16)	–	23 (A2, 163 bp)	**33 (0.42)**	**7 (0.50)**
12 (B4, 118 bp)	1 (0.01)	1 (0.07)	22 (A3, 161 bp)	16 (0.20)	3 (0.21)
			21 (A4, 159 bp)	3 (0.04)	1 (0.07)
			20 (A5, 157 bp)	18 (0.23)	1 (0.07)
			19 (A6, 155 bp)	5 (0.06)	1 (0.07)

Most frequent alleles are highlighted.

Table 4
DXS456 extragenic allele frequencies on 79 normal and 14 affected unrelated Hungarian chromosomes X.

DXS456		
No. of CA repeats (allele name and length)	No. of normal alleles (frequency)	No. of affected alleles (frequency)
26 (C0, 166 bp)	2 (0.03)	–
25 (C1, 164 bp)	22 (0.28)	3 (0.21)
24 (C2, 162 bp)	4 (0.05)	2 (0.14)
23 (C3, 160 bp)	6 (0.08)	3 (0.21)
22 (C4, 158 bp)	1 (0.01)	–
21 (C5, 156 bp)	13 (0.16)	1 (0.07)
20 (C6, 154 bp)	**26 (0.33)**	**4 (0.29)**
19 (C7, 152 bp)	2 (0.03)	1 (0.07)
18 (C8, 150 bp)	1 (0.01)	–
17 (C9, 148 bp)	2 (0.03)	–

Most frequent alleles are highlighted.

markers (Table 6). Prescreening by HRM in these 9 families (analysis of a patient and a heterozygote in each family) for the 56 Col4A5 amplicons, and sequencing of the screened amplicons, identified 4 mutations: Gly273Arg (1061G → C), 1620 ins C (frame shift from codon 458), Gly624Asp (2115G → A) and Gly1406Arg (4460G → A). We also found 2 intronic polymorphic variations: IVS4 + 69 T → C and IVS10 + 21 T → C.

We precluded a linkage to chromosomes 2 and X in 2 FHU families (negative LODs for the markers) and the LOD score calculation was impossible in 2 FHU and 2 AS-affected families (19%) because of the low numbers of analyzed subjects (4–5) or the fact that some markers were only partially or noninformative.

As concerns the informative nature of the markers used, their calculated HET and PIC values are reported in Table 5.

4. Discussion

TBMN differs from the AS. In familial TBMN cases, the disease is transmitted as an AD trait, which is rarely observed in the AS. Genetically, TBMN is a heterogeneous disease: mutations in either of the Col4A3/Col4A4 chain genes produce very similar phenotypes, indicating their similar functions in the heterotrimer.

An accurate familial study is mandatory in patients with FHU or the AS. Correct identification of the different patterns of inheritance may result in great differences in genetic counseling.

TBMN may have clinical features that overlap with those of other causes of hematuria; genetic analysis may help in the differential diagnosis and promote a better understanding of the disease process. The thinned GBM may reflect a carrier state for AR-AS (a heterozygous male/female) or XL-AS (a female carrier), which is identical to that seen in TBMN. The AR-AS disease seems very similar to XL-AS, but in these cases females are just as affected as males. Hearing loss is not a constant feature in this entity.

A thinner female GBM relative to that in males is questionable [20,21], but a genetic mutation that causes further attenuation may increase the likelihood of hematuria. TBMN could be considered an intermediate phenotype of the AS in that a gene-dosage effect is present.

Linkage analysis is currently the only effective molecular diagnostic procedure that can be performed in routine molecular genetic laboratories for the examination of FHU. A molecular diagnosis of TBMN is still not readily available, as the coding sequences of the Col4A3/Col4A4/Col4A5 genes are very large and difficult to screen for mutations (no hot spots seem to exist and genetic heterogeneity is possible). The identification of other genes involved in TBMN will be of particular interest as concerns a clarification of the pathogenesis of this disorder [6].

The existence of XL inheritance was ruled out in 3 FHU and 2 AS-affected families by pedigree analysis and X chromosome marker examinations (DXS456, 2B6 and 2B20), and genetic linkage analysis with the CA11 and D2S401 Col4A3 gene markers demonstrated cosegregation with the hematuria in our investigations. These genetic results, the pedigree structure, and in some cases the 2 generations of male-to-male transmission of the isolated microscopic hematuria (clinical picture) pointed to a diagnosis of TBMN; the AS cases were all females.

In the 9 XL-AS families, the mode of inheritance and transmission of the disease phenotype for 2 or more generations are seen to affect the affected males more severely than the carrier females. Linkage to the Col4A3/Col4A4 genes was excluded (negative LODs).

The lack of linkage to the Col4A3, Col4A4 and Col4A5 loci in 2 families may be explained by the high rate of *de novo* mutations shown in the Col4A5 gene in the XL-AS and the Col4A3 and Col4A4 genes [7] or by coincidental hematuria without proteinuria in some family members. Candidate genes encoding other structural and regulatory proteins in the glomerular filtration barrier may also play roles, e.g. laminin-5, perlecan and fibronectin [6], but there are as yet no data indicating any genetic linkage with them [22]. Other causes of glomerular bleeding, such as IgA nephropathy and post-infectious glomerulonephritis, should also be considered.

A good pedigree analysis, renal biopsy, haplotype analysis and LOD score calculation together may facilitate the correct diagnosis of TBMN and the AS so as to enable correct genetic counseling. Early identification of carrier females is crucial for the genetic counseling.

The differential diagnosis of TBMN and early AS or “AS variants” is difficult. There are patients who exhibit an expression of Col IV that is indistinguishable from the control, despite the presence of pathogenic mutations in the Col4A3, Col4A4 or Col4A5 gene.

On analysis of the numbers and frequencies of alleles and HET values in our Col4A5 2B6 and 2B20 markers, our findings were closely similar to those reported earlier in the USA [23], with only minor differences. The DXS456 X chromosome marker displayed the highest HET and PIC values in our examinations. In the event of a reliable indirect diagnosis in an AS-affected family, direct diagnosis (mutation screening) is needed for requested prenatal/preimplantation diagnosis [24]. At the moment, however, sequence analysis is still very expensive and there is a real claim to avoid the sequencing of wild-type amplicons. Accordingly, there continues to be a demand for a rapid and low-cost prescreening method. At the same time, the conventional sequencing cannot identify all mutations (notably the large gene rearrangements, where the primer binding site is involved). Thus, linkage analysis still has some uses in carrier detection and indirect mutation analysis. The next generation of sequencing methods (SOLID and pyrosequencing)

Table 5
Informativeness of the markers used: heterozygosity content (HET) and polymorphism information content (PIC) on normal and affected Hungarian chromosomes.

Microsatellites	HET on chromosomes		PIC on chromosomes	
	Normal	Affected	Normal	Affected
CA11	0.66	0.60	0.60	0.55
D2S401	0.69	0.61	0.65	0.54
Col4A4/HaeIII	0.50	0.375	0.37	0.30
DXS456	0.78	0.80	0.75	0.77
IVS48 (2B20)	0.30	0.26	0.26	0.24
2B6	0.72	0.68	0.68	0.65

Table 6
Evaluation of clinical and genetic data on familial hematuria (FHU) or Alport syndrome (AS)-affected 20 families.[a]

Clinical and genetic data		Number of families (%)	LOD score range
Clinical and ultrastructural changes, haplotype analysis and LOD scores together	Identified linkage to Col4A3/A4 genes on chromosome 2 (only FHU: 3, AS: 2)	5 (25%)	0.20–3.51
	Identified linkage to Col4A5 gene on chromosome X (AS)	9 (45%)	0.43–4.20
	Identified gene mutations[b]: Gly273Arg (1061G → C), 1620 ins C (frame shift from codon 458), Gly624Asp (2115G → A), Gly1406Arg (4460G → A), Intronic polymorphic variations: IVS4 + 69T → C, IVS10 + 21T → C		
	Precluded linkage to chromosome 2 and chromosome X	2 (10%)	−0.8 and −1.4 −2.3 and −2.65

[a] The LOD score calculation was impossible in 2 FHU, and 2 AS-affected families (19%), because of the low number of analyzed subjects (4–5) or the fact that some markers were only partially or noninformative.
[b] Identified by HRM (high resolution melting) prescreening to avoid the sequencing of wild-type by HRM.

will overcome these problems, but these methods (including sequence capturing, which is necessary instead of long-range PCR) have not yet been validated for diagnostic purposes.

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