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

PhD Thesis

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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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in affected Hungarian families

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List of Abbreviations

BAL	broncho-alveolar lavage
BE	Bergmeyer unit
bp	base pair
CAT	catalase
CYBB	cytochrome B-245 beta chain coding gene
cDNA	complementary deoxyribonucleic acid
Ct	threshold cycle number
2,4-DNPH	2,4-dinitrophenylhydrazine
DTNB	5,5′-dithiobis(2-nitrobenzoic acid)
EU	enzyme unit
EBC	exhaled breath condensate
FRAP	ferric reducing ability of plasma
FRET	fluorescence resonance energy transfer
GPx	glutathione peroxidase
GR	glutathione reductase
GSH	reduced glutathione
GSSG	oxidized glutathione
H_2O_2	hydrogen peroxide
HO	heme-oxygenase
HRM	high resolution melting
HRMC	high resolution melting curve
h-PBGD	human porphobilinogen deaminase
ICS	inhaled corticosteroid
IL-1β	interleukine-1β
LABA	long-acting β2-adrenoreceptor agonist
MDA	malondialdehyde
mRNA	messenger ribonucleic acid
NADPH	nicotinamide adenine dinucleotide phosphate (reduced)
NEM	N-ethylmaleimide
NF-κB	nuclear factor–κB
NOX	NADPH oxidase
$O_2^{\cdot-}$	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	reactive nitrogen species
SOD	superoxide dismutase
STR marker	short tandem repeat marker
TBA	tiobarbituric acid
TBARS	tiobarbituric acid reactive substances
TCA	trichloroacetic acid
TNF-α	tumor necrosis factor-α
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, identification of alterations in the human genome and the increasing knowledge of its influence on normal and pathological processes is a “hotspont” in medical sciences, as among 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 diganostics 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.

Study 1 - Redox imbalance and oxidative stress in pathological states

Electron transfer between molecules can be observed in many cellular processes and the generated reactive and non-reactive intermediates have an important role in normal cell function. Radical and non-radical intermediates are also essential for the normal function of the immune system. Superoxide anion radical is the primary reactive oxygen intermediate (ROI) molecule, formed by electron scavenging, and other reactive intermediates (RI) emerge with their ongoing 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 RI generation can be observed by the involvement of several isotypes of NADPH oxidase system and xanthine oxidase enzymes. Under pathological conditions cyclooxygenase, cytochrome P450, and unbound nitric oxide synthase also contribute to excessive radical generation. The excessive amount of free radicals can interfere with other biomolecules, and the emerging reactive intermediates can also be reactive. According to their atomic content, there are two main families: reactive oxygen intermediates (ROI) and reactive nitrogen intermediates (RNI). These various ROI/RNI participate in lipid and protein damage and also in nucleic acid impairment.

Redox homeostasis is maintained by cellular and extracellular antioxidant systems. Members of this very complex buffering system include the glutathione and thioredoxin system, the cysteine-cystine transformation with their enzymes involved in these cycles, the activity of CAT and SOD enzymes, which have a direct free radical scavenging effect, and other non-enzymatic antioxidants (such as α-tocopherol, β-carotene, ascorbate, flavonoids, and even serum bilirubin and albumin). The imbalance between pro-oxidants and antioxidants disrupts this redox homeostasis, so-called oxidative stress. Several complex cellular processes are altered as a consequence of molecular damage from mutagenesis through signal transduction to cell death. Oxidative stress has a well-known effect on the pathomechanisms of atherosclerosis, hypertension, diabetes mellitus, chronic renal failure, and on different inflammatory processes.

The major ROS-producing function of NOX in different pathological processes is widely studied. NOX is a membrane-bound multiprotein enzyme complex, and is present in inflammatory and non-inflammatory cells in seven isoforms. The membrane associated flavocytochrome b558 subunit (composed of $gp91^{phox}$ and $p22^{phox}$ proteins) of NOX2 isoform in activated phagocytic cells catalyses the electron transfer from NADPH to O_2, resulting in the production of $O_2^{\cdot -}$. Both membrane associated subunits are transcriptionally regulated by proinflammatory cytokines (TNFα and IL-1β) and by the ROI themselves, although other cytosolic subunits are also essential for the complete enzyme function. The elevated production of $O2^{\cdot -}$ radical and ROI during inflammation by activated mononuclear cells are derived especially from NADPH oxidase subunits, their activity is also regulated in a transcriptional manner and by signal transduction.

On the other side, the heme-oxygenase -1 (HO-1) plays an important role in the cytoprotection against oxidative overload by participating in antiinflammatory, antiproliferative and antiapoptotic processes. As HO-1 is activated in the early phase of oxidative stress, it is a frequently used marker of the adaptative mechanisms of redox imbalance. Environmental and pro-inflammatory stimuli, such as oxidative stress in the airways can modify the HO-1 gene's transcriptional activity and contribute to the antioxidant effects during inflammation.

The altered biomolecules, such as lipid derivates, proteins with modified side chains, and the altered activity of enzymatic and non-enzymatic antioxidants are widely studied for a better understanding of the role of oxidative stress in pathological states.

Oxidative stress in bronchial asthma

Airways inflammation and oxidative stress play a crucial role in the pathophysiology of bronchial asthma. Excessive ROI production is derived from epithelial cells, resident macrophages, endothelial cells in the lung tissue, as well as inflammatory cells, such as neutrophils, monocytes, macrophages, neutrophils, and eosinophils recruited to the site of tissue damage. The oxidative stress itself may initiate and augment the inflammation and may be the consequence of the inflammation as well. Structural and functional alterations can be observed in the small airways. Previous studies showed that a higher level of ROI, damaged biomolecules, and an altered antioxidant status correlate with the degree of airway hyperresponsiveness in animal models, as ROI were involved in smooth muscle cell contraction. Excessive mucus production, cilia dysfunction, altered β-adrenergic receptor function, damage of the epithelial layer, and airway remodelling were also observed. Most of the results were derived from animal models and adult asthmatic patients. Data from children remained restricted because examination of the airways required invasive or semi-invasive methods with their ethical doubts. Data from non-

invasive approaches (e.g. exhaled air condensate and induced sputum) to examine the inflamed airways are less reliable, because they could be influenced by patient cooperation and by pre-arrangements before the study. In certain diseases, including 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, available data are limited and conflicting.

Study 2 - Alport syndrome

Alport syndrome and thin basement membrane nephropathy are recognized as a specific disease 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 and dominant 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 a 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.

Thin basement nephropathy with its 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. 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.

Pediatric nephrologists must distinguish thin basement nephropathy 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 thin basement nephropathy can be determined by linkage analysis, but identifying the candidate mutation in these multi-exon-containing genes is demanding technically and financially. 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.

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-localized 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 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 localization of candidate point mutations as a prescreening 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 suffering from X-linked Alport syndrome.

Patients and methods

Study groups

In Study 1, 58 patients with the confirmed diagnosis of bronchial asthma, and 30 healthy controls were examined. The asthmatic patients were admitted to 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 before 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 those 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 adrenergic receprot 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.

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 the chromosome 2, 13, and X. 9 families were selected for further analysis with linkage to the COL4A5 locus with negative LOD scores for chromosome 2 markers and 0.43-4.2 LOD score range for the COL4A5 locus. Formerly, consent was given by the examined individuals.

Biochemical analysis

In Study 1 (Biochemical and gene expression alterations in the blood of childhood asthmatic patients) venous blood was obtained in EDTA-coated tubes. GSH and GSSG were measured in whole blood. FRAP was measured from the separated plasma after centrifugation. Other biochemical parameters (SOD, CAT, GPx, GR, TBARS, carbonylated protein) were determined in red blood cells after washing with phosphate-buffered saline (pH 7.4) three times. Whole blood levels of GSSG and GSH were assayed using a spectrophotometric enzymatic recycling method. Activities of erythrocyte SOD, CAT, GR, and GPx were measured with spectrophotometric methods, and results were provided relative to the protein concentration of the sample (determined by means of spectrophotometry). Enzyme activities are expressed in U/mg protein or in the case of CAT, in BU/mg protein. The concentration of TBARS derived from the red blood cells was measured with TBA reagent, which measures the level of total TBA-reactive substances. Results were reported in nmol/mg protein. Carbonylated protein concentrations in each sample were examined with 2,4-DNPH in a spectrophotometric assay. All samples had their own controls without 2,4–DNPH. The result was expressed in mmol/mg protein.

Relative quantification of gene expression by real-time PCR assay

In Study 1, CYBB and HMOX-1 gene expressions were determined by using the RT-PCR technique. After mRNA extraction from whole blood leukocytes according to the manufacturer's protocol (Roche), reverse transcription was performed by using a special synthesis kit (Fermentas) to set up cDNA molecules. 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. Sequence-specific primer pair was used to amplify the examined target

sequence and sequence-specific hybridisation probe pair was applied to detect PCR products from cycle to cycle via FRET. All the primers and labelled oligonucleotide probes were designed by LightCycler Probe Design Software 2.0. h-PBGD was used as housekeeping gene, marked by different dyes, in the same capillary, to normalize the difference between the samples' mRNA concentrations. Relative expressions of the candidate mRNAs were determined by using the Ct data obtained by real-time PCRs, with each sample normalized to its own h-PBGD housekeeping gene (ΔCt). After the reaction, amplicons were checked by electrophoresis on an agarose gel according to their size. 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)

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. 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.

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 depended 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
Biochemical and gene expression alterations in the blood of childhood asthmatic patients

The GSH and GSSG levels, the GSSG/2GSH ratio did not differ significantly in any of the examined subgroups from those measured in the healthy controls. There was no significant change in the activity of GPx and GR. 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. These data were not influenced by sex or asthma control either. The SOD activity was significantly higher in the asthmatic patients who did not receive ICS medication than in healthy children or in those who required ICS therapy. CAT activity exhibited a small, but not significant elevation in the asthmatic subgroup not treated with ICS. *(Table 1.)* Total antioxidant capacity of the plasma was reduced in asthmatic patients ((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). Lower FRAP was detected in patients who received ICS therapy than in healthy controls. Also lower FRAP values were obtained in patients without ICS maintenance therapy, but this reduction did not reach the level of significance ((median, 25^{th}-75^{th} percentile (µmol/mg protein) **healthy: 8.55 x 10^{-3}, 6.81 x 10^{-3} – 13.6 10^{-3}, *ICS-treated subgroup: 6.06 x 10^{-3}, 5.00 x 10^{-3} – 7.31 x 10^{-3}, ICS nontreated subgroup: 7.75 x 10^{-3}, 5.58 x 10^{-3} - 8.86 x 10-^{3}; * p<0.05).*

TBARS ((median, 25–75 pc) nmol/mg protein) : *healthy*: 0.56 (0.44–0.65) vs. *asthmatic*: 0.77 (0.66–0.90)) and carbonylated protein (mean ± SD (x10^{-5} mmol/mg protein): *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<0.03 and p<0.05, respectively). Both parameters were slightly higher in the subgroup which did not require steroids than in those who received ICS medication. ICS-treated group was analysed according to the dose of inhaled ICS, and these subgroups were also compared to each other. *(The Table 1 below, does not demonstrate these subgroups.)* The TBARS level was also high in all ICS-treated subgroups with different ICS doses and in asthmatic children not treated with ICS. The steroid use and its' dose did not influence widely the rate of lipid peroxidation. 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 parameters were influenced by sex or asthma control. *(Figures showing the results of TBARS and carbonylated proteins in relation to ICS doses are included in the dissertation.)*

Table 1. Biochemical oxidative stress and antioxidant parameters in blood of asthmatic children and healthy controls

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 prot.)	6.77±1.12	7.22±1.25	8.10±1.63*
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/2GSH (%)	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
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

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

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

Data are reported as mean±SD and in case of TBARS, median (25^{th}–75^{th} percentiles). One-way ANOVA and Kruskal-Wallis tests were performed to compare the groups, with Bonferroni's and Dunn's multiple comparsion test.

NADPH oxidase $gp91^{phox}$ subunit and HMOX-1 mRNA level in blood

We analysed the relative CYBB mRNA level in asthmatic subgroups according to ICS medication and determined the difference in the gene expression between the subgroups treated or not treated 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). Further analysis were performed in relation to the required ICS doses. The post-test following ANOVA analysis showed a significant difference between the subgroup not treated with ICS and the subgroup treated with a low daily dose of ICS (ΔΔCt=1.445, $p<0.05$), indicating a downward change of approximately two-fold (*Table 2*). Negative ΔΔCt values mean upregulation of the gene expression according to the comparative ΔCt method, contrarily the positive values indicate downregulation. No significant difference was observed between the two other ICS-treated subgroups, where the higher ICS doses did not significantly influence the CYBB gene expression as compared with the subgroup treated with a low daily dose of ICS. Higher ICS doses did not cause further changes in the transcription. *(Figures showing the CYBB and HMOX1 gene transcription profiles in relation to ICS doses are included in the dissertation.)*

There was no significant difference in HMOX-1 gene expression between asthmatic and healthy children, and the ICS therapy did not cause a noteworthy change in the expression profile of this gene.

Table 2. CYBB (NADPH oxidase gp91^{phox} subunit) gene mRNA expression 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.

∗ *$p<0.05$: compared with steroid nontreated patients.*

Study 2 - Mutation prescreening in families suffering from Alport nephropathy

During the analyses of 9 families for the COL4A5 gene, 6 different melting curve was detected. The differences in the melting curves were derived from the alterations in the base sequence of the screened amplicons. The sequencing method followed by HRMC analysis to characterize the base sequence of the detected alterations was performed by ABI Prism 3130 automated sequencer. The identified mutations were the following: 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 chain. We also found 2 intronic polymorphisms: IVS4+69T→C and IVS103+21T→C. In the case of 5 families, the causing mutation remained unclear with HRMC mutation prescreening. There was no false-positive results.

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 localized 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 the 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 administration. 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 the $gp91^{phox}$ 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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