PATHOGENETIC STUDIES IN JUVENILE ESSENTIAL HYPERTENSION AND UREMIA

Role of oxidative stress and
gene polymorphisms of the renin-angiotensin system

Ph.D. thesis

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List of publications

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I Haszon, AL Friedman, **F Papp**, Cs Bereczki, S Baji, T Bodrogi, É Károly, E Endreffy, S Túri. ACE gene polymorphism and renal scarring in primary vesicoureteric reflux. *Pediatric Nephrology* 2002; 17:1027–1031. [IF: 1.420]

I Haszon, **F Papp**, M Bors, Cs Bereczki, S Túri. Platelet aggregation, blood viscosity and serum lipids in hypertensive and obese children. *European Journal of Pediatrics* 2003; 162:385–390. [IF: 1.157]

Túri S, Haszon I, **Papp F**, Bereczki Cs, Endreffy E, Németh I. A juvenilis esszenciális hypertonia patomechanizmusa. *Hypertonia és Nephrologia* 2001; 5:131–136.

Papp F, Haszon I, Kovács J, Bors M, Németh I, Bereczki Cs, Túri S. Thrombocyta aggregáció, vér viszkozitás és szérum lipidek alakulása hipertóniás és obes gyermekekben. *Transzfúzió* 2004; 37:3–12.

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Abbreviations

Ang II	angiotensin II
ACE	angiotensin converting enzyme
ADPKD	autosomal dominant polycystic kidney disease
APH	acetylphenylhydrazine
AT1R	angiotensin II type 1 receptor
AGT	angiotensinogen gene
AGTR1	angiotensin II type 1 receptor gene
BH_4	tetrahydrobiopterin
bp	base pair
BP	blood pressure
BMI	body mass index
CAT	catalase
CRF	chronic renal failure
CV	coefficient of variation
DBP	diastolic blood pressure
DNA	deoxyribonucleic acid
ECM	extracellular matrix
EH	essential hypertension
eNOS	endothelial nitric oxide synthase
ESRD	end stage renal disease
ET-1	endothelin-1
FSGS	focal segmental glomerulosclerosis
GPx	glutathione peroxidase
GSH	reduced glutathione
GSSG	oxidized glutathione
H_2O_2	hydrogene peroxide

Hb	hemoglobin
LPO	lipid peroxidation
LVH	left ventricular hypertrophy
MDA	malondialdehyde
NADPH	nicotinamide adenine dinucleotide phosphate
NO	nitric oxide
NOS	nitric oxide synthase
NO_x	sum of nitrite and nitrate
O_2^-	superoxide
OH	obesity-induced hypertension
OR	odds ratio
PCR	polymerase chain reaction
RAS	renin-angiotensin system
RBC	red blood cells
ROS	reactive oxygen species
SBP	systolic blood pressure
SOD	superoxide dismutase
SNS	sympathetic nervous system
TGF-β	transforming growth factor-β
TxA_2	thromboxane A_2
VSMC	vascular smooth muscle cells
XO	xanthine oxidase

1. INTRODUCTION

1.1. Epidemiology of essential hypertension and obesity in children

Essential hypertension (EH) is one of the most common disorders in adulthood and is the leading cause of premature death among adults [1]. Previously it was believed that secondary hypertension is the major form of childhood hypertension, but the clinical importance of primary or EH in children, and especially in adolescents, has significantly increased in the last decade which can be observed in the epidemiologic data. The increasing prevalence of childhood high blood pressure (BP) has been reported in several studies and national surveys. In the past, the estimated prevalence of childhood hypertension was 1–2%. Recent reports indicate that hypertension now affects 3–5% of the whole pediatric population, and at least the same percentage of children is in pre-hypertensive stage [2–6]. In a Hungarian population of adolescents the prevalence of hypertension was 2.53% [7]. The global public health significance of high BP in childhood as well as adolescence is based on observations that confirm a strong tracking of BP levels from childhood to adulthood. Children with high normal BP during adolescence have a greater tendency to develop hypertension during adulthood [8]. Over the last decade many studies have provided supporting evidence that hypertension associated vascular damage also develops in childhood, and many of the hypertensive children do in fact present with hypertensive end organ damage such as left ventricular hypertrophy (LVH) [9].

The increasing prevalence of high BP in children and adolescents is largely due to the increase in prevalence of obesity all over the world. In the United States childhood obesity has more than doubled in children and tripled in adolescents in the past 30 years, and now affects nearly 20% of the whole pediatric population. In 2010, more than one third of the children and adolescents were overweight or obese [10]. According to the WHO European Childhood Obesity Surveillance Initiative 2008 report the same trend can be seen in Europe. Data of 6–9-year-old children from 12 European countries were collected. The prevalence of overweight (including obesity) ranged from 18.4% to 49%, and 4.6 to 26.6% of children were actually obese [11].

In a large cross sectional survey performed in the period 2005–2006 the overall prevalence of overweight and obesity among the Hungarian schoolchildren aged 11–16 years was found to be 23.4% and 6.6% of the subjects were obese. Between the years 2001 and 2006, the prevalence of overweight and obesity nearly doubled from 13% to 23.4% in this age group of Hungarian adolescents [12].

Obesity is the primary risk for EH in children. The relationship between obesity and hypertension in children has been reported in numerous studies targeting a variety of ethnic and racial groups. All of these studies found higher BP and/or higher prevalence of hypertension in obese compared with lean controls [13]. Sorof et al. reported that more than one third of obese school children have abnormally elevated BP [5]. Apart from hypertension obesity is associated with other cardiovascular risk factors, such as dyslipidemia, insulin resistance, glucose intolerance, type 2 diabetes mellitus, LVH, and pulmonary hypertension. Many of these outcomes of obesity may begin in childhood and adolescence [13].

1.2. Pathogenetic factors in essential hypertension and obesity-induced hypertension

Human EH is a multifactorial disorder, and many pathophysiological factors have been implicated in its genesis (**Table 1**) [8, 14].

Obesity-induced hypertension or obesity hypertension (OH) is often considered a special form of hypertension, but considerable evidence indicates that obesity is the most common cause of EH. Hypertension and other obesity-related cardiovascular complications are associated with visceral/abdominal adiposity. OH is a complex and multifactorial condition. The pathophysiologic mechanisms whereby obesity causes hypertension have not been fully elucidated. Abnormal kidney function is an important cause as well as consequence of OH. Increased renal tubular sodium reabsorption and impaired pressure natriuresis play key roles in the initiation of hypertension. Several mechanisms contribute to altered kidney function and hypertension in obesity, but three of these mechanisms are especially important: 1) increased activity of the SNS, 2) the activation of the RAS, and 3) physical compression of the kidneys by fat accumulation within and around the kidneys and by increased abdominal pressure due to visceral fat. The activation of SNS appears to be mediated partly by the increased levels of the adipocyte-derived hormone leptin, and the activation of the

hypothalamic leptin – pro-opiomelanocortin – melanocortin 4 receptor pathway. Inflammatory cytokines and free fatty acids released from adipocytes, angiotensin II (Ang II), and other factors have also been suggested as mediators of SNS activation in obesity. In addition, hyperinsulinemia and/or insulin resistance and dyslipidemia, impaired endothelial function, genetic and lifestyle factors may also be of significance in the pathophysiology of OH [15–18].

Table 1. Pathogenetic factors of human essential hypertension

- increased sympathetic nervous system activity
- overproduction of sodium-retaining hormones and vasoconstrictors
- long-term high sodium intake
- increased or inappropriate renin secretion with resultant increased production of angiotensin II and aldosterone
- deficiencies of vasodilators, such as nitric oxide, prostacyclin, and the natriuretic peptides and/or overproduction of vasoconstrictor endothelin-1
- alterations in expression of the kallikrein-kinin system
- abnormalities of resistance vessels
- increased activity of vascular growth factors
- alterations in adrenergic receptors
- altered cellular ion transport
- structural and functional abnormalities in the vasculature, including endothelial dysfunction, and vascular remodeling
- oxidative stress
- hyperuricemia
- insulin resistance and diabetes mellitus
- obesity
- genetic factors
- fetal programming

Hereinafter, the pathogenetic factors of EH and OH related to our investigations will be discussed, namely oxidative stress, endothelial dysfunction and genetic predisposing factors.

1.2.1. Oxidative stress

In the course of normal aerobe cellular metabolism several highly reactive molecules are generated. These reactive species are generally reactive oxygen species (ROS), such as superoxide (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical, as well as reactive nitrogen species, e.g. peroxynitrite, which result from the cellular redox process and many of them are free radicals. The primary sources of ROS include mitochondrial electron transport system and various oxidase enzymes in the cell, and uncoupled nitric oxide synthase (NOS) among others. Moreover, free radicals can be derived from exogenous sources such as environmental toxins and cigarette smoke. It is important to note that while the excessive production of ROS causes injury and dysfunction, the normal rate of ROS production is essential for various physiological processes, such as gene expression, signal transduction, regulation of cell growth and apoptosis, fetal development, and innate immunity [19–21]. Under normal conditions, ROS and the byproducts of their reactions with various biomolecules are converted to harmless molecules by the natural antioxidant system [20]. The antioxidant defense system is a highly complex biochemical organization which consists of numerous enzymes, such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and a large number of scavenger molecules, e.g. ascorbic acid (Vitamin C), alpha-tocopherol (Vitamin E), glutathione, flavonoids, urate, and the like [21].

Oxidative stress is characterized by the imbalance between ROS production and antioxidant defense capacity of the body. This can be the result of either increased ROS generation, impaired antioxidant system, or the combination of both [20]. ROS are capable of damaging all types of biomolecules, including nucleic acids, proteins, and lipids. DNA modification by ROS may be the initial step in mutagenesis, carcinogenesis and aging. Proteins may also be damaged by ROS thus leading to structural changes and loss of enzyme activity. Oxidative degradation of lipids, called lipid peroxidation (LPO) affects many cellular components, but the primary action-sites involve membrane associated polyunsaturated fatty acids. The peroxidation of membrane associated fatty acids and cholesterol can alter cell membrane fluidity and permeability, and may eventually induce membrane damage. LPO also changes the low density lipoprotein to proatherogenic and proinflammatory forms [19, 22, 23].

Further decomposition of peroxidized lipids results in a wide variety of harmful end-products, such as malondialdehyde (MDA) and F_2-isoprostanes. These products of LPO have commonly been used to assess oxidative stress *in vivo* [23, 24].

Oxidative stress and ROS attack modify and denature functional and structural molecules thus leading to tissue injury and dysfunction. Oxidative stress plays a role in inflammation, accelerates aging and contributes to a variety of chronic and degenerative conditions, such as cancer, diabetes, autoimmune disorders, inflammatory diseases, rheumatoid arthritis, respiratory and kidney diseases, atherosclerosis, cardiovascular and neurodegenerative diseases [21, 22].

1.2.2. Oxidative stress in hypertension and obesity

Oxidative stress in hypertension

The association between oxidative stress and hypertension has been extensively studied. Most of the reports in animals as well as in humans demonstrate an increased oxidative stress in hypertension. Vascular oxidative stress has been observed in spontaneously hypertensive rats and various experimental animal models of hypertension. Increased ROS release from isolated vessels of hypertensive animals has also been detected [25–27]. There is extensive evidence concerning the increase of oxidative stress in human EH. Most of the studies using non-specific markers of oxidative damage demonstrate an obvious connection between oxidative stress and hypertension. Higher O_2^- and H_2O_2 production have been observed in hypertensive subjects, which returned to levels observed in control subjects after BP reduction. Increased LPO, and an imbalance in antioxidant status was reported in hypertensive patients, suggesting that oxidative stress is important in the pathogenesis of EH. Moreover, decrease in SOD and GPx activities have been demonstrated in newly diagnosed and untreated hypertensive subjects, and SOD activity was inversely correlated with BP within the hypertensive group [26]. In a study by Rodrigo et al., daytime systolic BP (SBP) and diastolic BP (DBP) of hypertensive patients negatively correlated with plasma antioxidant capacity, plasma vitamin C levels, erythrocyte activity of antioxidant enzymes (SOD, CAT, GPx), and erythrocyte reduced/oxidized glutathione ratio, showing a higher level of oxidative stress in hypertensive subjects. They also reported that BP positively correlated with both

plasma and urine 8-isoprostane (8-iso-prostaglandin $F_{2\alpha}$) [28]. F_2-isoprostanes are considered the best available biomarkers of oxidative stress status and LPO *in vivo* [24]. In studies using these highly specific markers the relationship between oxidative stress and hypertension is not convincing. The urinary concentrations of F_2-isoprostane were found to be the same in subjects with mild to moderate untreated hypertension as well as in the normotensive control group. In another study no difference has been observed in plasma and 24-h urinary F_2-isoprostanes in treated or untreated hypertensive subjects compared with normotensive controls [26].

The casual role of oxidative stress in hypertension is supported by several observations. Some studies in animal models of hypertension showed that the administration of pharmacological doses of antioxidants may reduce BP [20]. However, the data from clinical trials using antioxidants are less convincing, and most of them failed to show that the supplementation of antioxidants reduces BP [26, 27]. Induction of oxidative stress has been shown to cause hypertension in normal animals. Binding of Ang II to its type 1 receptor (AT1R), results in ROS production via activation of NADPH oxidase in the kidney and vasculature. The ROS production and hypertensive response to Ang II infusion is attenuated by pharmacological inhibition of NADPH oxidase. These observations demonstrate the role of ROS as a major mediator of the pressor action of Ang II [20]. Oxidative stress may contribute to the generation and/or maintenance of hypertension by several mechanisms, including damage to endothelial cells and endothelial dysfunction, damage to vascular smooth muscle cells (VSMC), generation of vasoconstrictor LPO products, stimulation of inflammation and growth signaling events [26].

Oxidative stress may not only be the cause, but also the consequence of hypertension. Animal experiments clearly illustrate the role of high BP and shear stress in the generation of vascular oxidative stress [20].

Although excessive production of ROS is the most common cause of oxidative stress in hypertension, it is occasionally caused by the primary impairment of the antioxidant system as it is seen in SOD deficient, therefore hypertensive mice. Moreover, the consumption of antioxidant molecules and the inactivation of the antioxidant enzymes caused by permanent oxidative stress can impair the antioxidant defense system and can enhance oxidative stress itself [20].

Oxidative stress in obesity

Several studies provide evidence that obesity *per se* is associated with enhanced oxidative stress. Keaney et al. reported that a relation between increasing BMI and increasing systemic oxidant stress may be observed. Using the quantification of urinary F_2-isoprostanes, it is shown in nearly 3000 patients involved in the Framingham Heart Study that enhanced isoprostane formation is strongly associated with increasing body mass index (BMI) [29]. In a study by Furukawa et al., fat accumulation closely correlated with the plasma and urinary LPO end-products. In addition, it was demonstrated that plasma adiponectin levels correlated inversely with oxidative stress markers. These results suggest that fat accumulation itself could increase systemic oxidative stress, and that increased oxidative stress in obesity might relate to the dysregulated production of adipocytokines [30].

Multiple mechanisms may contribute to increased oxidative stress in obesity. A number of ROS producing pathways are known to be perturbed in obesity. For example, RAS is activated, and Ang II induces ROS production via the activation of NADPH oxidase, and it also enhances lipoprotein oxidation. Moreover, obesity is also associated with reduced antioxidant defense mechanisms, elevated systemic inflammation and activation of coagulation cascades [31, 32]. The chronic over-nutrition in obesity itself can induce oxidative stress. High caloric intake composed of glucose, lipid or protein causes an increase in the generation of ROS by leukocytes. Lipid intake causes a prolonged increase in LPO [26]. On the other hand, caloric restriction in the obese and fasting in normal subjects leads to a marked reduction in ROS generation by leukocytes and other indexes of oxidative stress [33].

1.2.3. Endothelial dysfunction

The endothelium plays a fundamental role in the regulation of vascular function as it produces a large number of biologically active agents that participate in the regulation of vascular tone, cell growth, inflammation, and thrombosis/hemostasis [34]. Vasoactive substances derived from endothelium are vasodilators, among others nitric oxide (NO), and prostacyclin, or vasoconstrictors, such as endothelin-1 (ET-1), Ang II, thromboxane A_2 (TxA_2), and ROS.

Other inflammatory modulators, adhesion molecules, chemokines, and factors with hemostatic properties are also released by endothelium [35].

Primarily, endothelial dysfunction is characterized by impaired vasodilation to specific stimuli or an imbalance between endothelium derived vasodilation and vasoconstriction, resulting in reduced vasodilation [35–37]. In addition, it is also associated with a proinflammatory and prothrombic state. The dysfunction of the vascular endothelium has been implicated in the pathophysiology of various cardiovascular diseases, including hypertension, coronary artery disease, chronic heart failure, peripheral artery disease, diabetes, and chronic renal failure [35].

Multiple pathways are involved in the development of endothelial dysfunction; the main are: reduced NO, oxidative stress, vasoactive peptides, and inflammation. NO is the key endothelium-derived vasodilator that plays a pivotal role in the maintenance of vascular tone and reactivity, and also opposes the actions of endothelium-derived vasoconstrictors. Furthermore, it inhibits growth, inflammation, and aggregation of platelets [35, 36]. Not surprisingly, that reduced NO in the vessel wall has a central role in the complex mechanism of endothelial dysfunction. The reduction in NO may result from reduced endothelial NOS activity (eNOS; the enzyme, which synthesizes NO from L-arginine, with the cofactor tetrahydrobiopterin [BH_4] in the endothelium) and/or decreased NO bioavailability [35]. Oxidative excess may cause NO reduction by different mechanisms. Probably the most important is the inactivation of NO by ROS, which diminishes the amount of available NO, while a cytotoxic oxidant, peroxynitrite is formed. Furthermore, in oxidative states, the reduction in BH_4 caused by ROS and peroxynitrite results in uncoupling of eNOS, which leads to further ROS formation [35, 37]. The main sources of ROS implicated in the genesis of endothelial dysfunction are NADPH oxidase, uncoupled eNOS, and xanthine oxidase (XO) [38]. Moreover, oxidative stress induces proinflammatory and prothrombic state, and it is also involved in the detachment and apoptosis of endothelial cells [35]. A relation between oxidative stress and endothelial dysfunction, characterized by decreased NO has been demonstrated in animal models as well as humans with hypertension and chronic renal failure [39]. The oxidized end-products of NO are nitrite and nitrate, and the sum of their plasma levels (NO_x) are frequently used markers of NO bioavailability and endothelial function *in vivo* [40, 41].

Vasoconstrictors, such as Ang II and ET-1 also have been implicated in the development of hypertension and endothelial dysfunction. Pathogenic effects of these substances are partially mediated by ROS production due to the activation of NADPH oxidase [34, 42]. Our previous studies allude to the importance of vasoactive components in childhood EH. Increased *in vitro* platelet aggregation and thromboxane B_2 (stable degradation product of TxA_2) levels were observed in hypertensive children [43]. In a second study, the increased platelet aggregability was also confirmed in non-obese and obese hypertensive children, and simultaneously, decreased NO and depletion of plasma free thiols, – as the sign of oxidant injury – were demonstrated in both groups of hypertensives [44]. Increase in platelet aggregation releases TxA_2, serotonin, and other mediators causing local vasoconstriction and further aggregation.

The major pathways of endothelial dysfunction focusing on the role of oxidative stress and NO are summarized in **Figure 1**. The investigated markers of oxidative stress and endothelial dysfunction in our study are also illustrated. O_2^- is generated by different enzymes in the endothelial cells; the main are the NADPH oxidase, uncoupled eNOS and XO. NO is synthetized from L-arginine by eNOS. The action of different vasoconstrictive factors, such as Ang II, ET-1, cytokines, mechanical stretch is mainly mediated by the activation of NADPH oxidase leading to O_2^- production, which can causes the constriction of VSMC, leads to further vasoconstrictive peptide production (Ang II, ET-1) and inactivates NO resulting in peroxynitrite formation. Peroxynitrite and oxidative excess lead to the uncoupling of eNOS. The uncoupled eNOS has reductase function and produces ROS instead of NO, and aggravates the oxidative damage. Reduction in NO caused by decreased eNOS activity (due to ET-1 among others) and inactivation of NO by ROS results in impaired vasodilation, increased platelet aggregation, and amplifies the inflammatory process. From aggregated platelets TxA_2, and other mediators are released causing vasoconstriction and further aggregation. Oxidative stress induces proinflammatory and prothrombic state by the upregulation of different endothelium-derived cytokines, chemokines and hemostatic factors, leads to LPO and MDA production, and also enhances platelet aggregation. The imbalance between vasoconstrictors and vasodilators, mainly between ROS as vasoconstrictors and NO as vasodilator is a fundamental feature of endothelial dysfunction. Antioxidant mechanisms are activated in the presence of oxidative stress; oxidized glutathione (GSSG) is formed from reduced glutathione (GSH), O_2^- is transformed to H_2O_2 by SOD, which is neutralized by CAT

or GPx, and reduced plasma thiols (-SH) will be oxidized (-S-S-). Red blood cells (RBC) circulate in the direct vicinity of the endothelial surface. Their membranes are permeable to oxidants, therefore, antioxidants of the RBC are also activated. Decrease in end-products of NO metabolism (NO_x) is correlated to NO reduction in endothelial dysfunction.

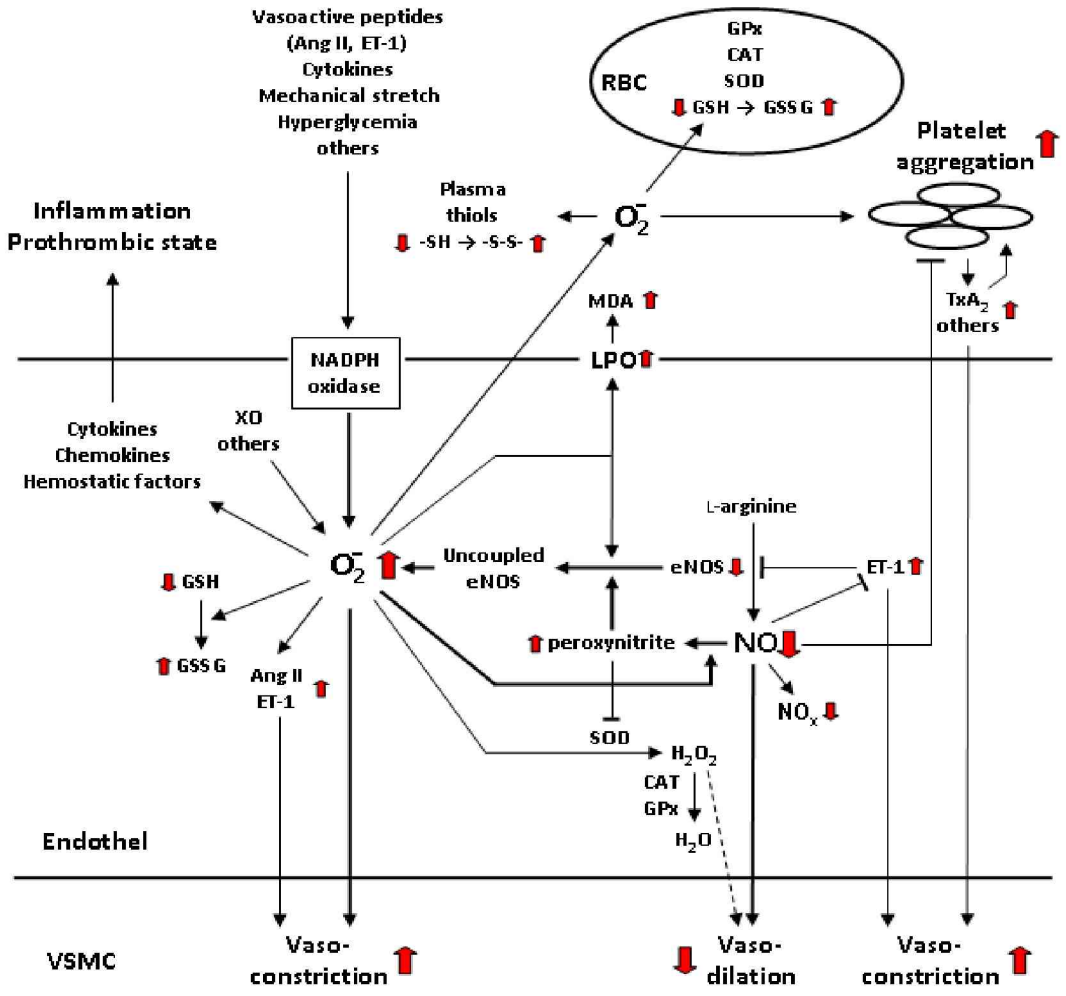

Figure 1. Schematic figure of the major pathways of endothelial dysfunction. See the text for the details. NO, nitric oxide; O_2^-, superoxide; eNOS, endothelial nitric oxide synthase; XO, xanthine oxidase; Ang II, angiotensin II; ET-1, endothelin-1; VSMC, vascular smooth muscle cells; ROS, reactive oxygen species; TxA_2, thromboxane A_2; LPO, lipid peroxidation; MDA, malondialdehyde; GSSG, oxidized glutathione; GSH, reduced glutathione; H_2O_2, hydrogen-peroxide; SOD, superoxide dismutase; CAT, catalase; GPx, glutathione peroxidase; -SH, reduced plasma thiols; -S-S-, oxidized plasma thiols; RBC, red blood cells; NO_x, NO end-products.

1.2.4. Genetic aspects of essential hypertension, gene polymorphisms of the renin-angiotensin system

BP level is a complex trait resulting from both genetic and environmental factors. It is widely accepted, that approximately 30–60% of the phenotypic variation in BP is determined by genetic factors [45]. The common form of EH is a polygenic disorder. It means that the development of hypertensive phenotype results from the effects of multiple genes and is modulated by multiple environmental factors. There are two approaches for the genetic dissections of complex and quantitative traits, such as EH: genome-wide scanning and candidate gene approach, and both have specific advantages and disadvantages [46]. Most published data on human EH arise from candidate gene studies. Many candidate genes have been tested for association with BP and hypertension in case-control and sib pair linkage studies with conflicting results.

Gene polymorphisms of the renin-angiotensin system

Polymorphisms in genes of the RAS have been the most commonly studied as potential genetic risk factors for hypertension and other cardiovascular diseases. The RAS is a multienzyme, multilocale hormone system, which regulates blood pressure, as well as the fluid and electrolyte balance, and also plays essential roles in several physiological and pathological processes. Two forms of the RAS exist, the systemic/endocrine and local/tissue RAS. The cascade of the RAS consists of two enzymatic steps: angiotensin I is cleaved from angiotensinogen by the action of renin, which undergoes a second cleavage, mainly by tissue angiotensin converting enzyme (ACE) to generate Ang II, the main effector hormone of the system, which acts primarily on AT1R resulting in aldosterone secretion from the adrenal cortex and many other systemic and/or locally effects. Because the RAS is intimately involved in the regulation of BP, a genetic variability in the degree of expression of its proteins may account for variability in the BP or may play a role in mediating hypertension [47].

The angiotensinogen gene (AGT) is located on 1q42, and consists of 5 exons. Molecular variant of AGT gene encoding threonine (T) instead of methionine (M) at position 235 (M235T) in exon 2 has an influence on the angiotensinogen level. The T235 allele is

associated with higher angiotensinogen levels, which could theoretically translate into higher Ang II levels and may lead to hypertension and other cardiovascular complications [48, 49].

The ACE gene is located on 17q23, and comprises 26 exons. The ACE gene insertion/deletion (I/D) polymorphism is characterized by the presence (insertion) or absence (deletion) of a 287-bp *Alu*I-repeat sequence inside intron 16. I/D polymorphism is strongly associated with variations in circulating and tissue ACE levels [47, 50–52]. The DD genotype with higher tissue ACE levels would correspond to higher Ang II generation in the vascular tissue leading to hypertension, and pathological cardiovascular states.

Most of the known actions of Ang II are mediated by the AT1R. The AT1R gene (AGTR1) maps to 3q24, containing five exons. Several polymorphisms of the AT1R gene have been identified. Polymorphism substituting cytosine (C) for adenine (A) at position +1166 in the 3'-untranslated region (A1166C) was the only, which had significantly increased frequency in hypertensive individuals. The physiological significance of this polymorphism is uncertain [53].

The possible pathogenetic effects of gene polymorphisms of the RAS in hypertension are presented and discussed in the Discussion chapter.

Other candidate genes for association with essential hypertension

Aldosterone, a major effector hormone of the systemic RAS, regulates the sodium and potassium balance in the kidney, thus plays a fundamental role in the regulation of extracellular volume. Gene encoding aldosterone synthase (CYP11B2) has also emerged as a candidate gene for hypertension [54]. Variations and mutations in a huge number of other candidate genes, such as α-adducin, eNOS, ET-1, atrial natriuretic factor, β_2-adrenergic receptor, insulin receptor, and many others, have also been reported to be associated with EH [8, 54]. For example, in a study published by our group, polymorphism of the ET-1 gene (G5665T) was significantly associated with EH and particularly OH. The same association was not detected in case of eNOS polymorphisms (T-786C promoter polymorphism and 4th intron 27-bp repeat polymorphism) [55].

1.2.5. Possible age-related differences in the pathogenesis of essential hypertension

The pathogenesis of EH in children and adolescents bears a close resemblance to that of the adults. The minor variations observed could mostly be due to the evolving nature of this condition [8]. On the other hand, most of the pathogenetic studies, including genetic association studies, have been performed in different hypertensive groups of adults. Therefore, studies in the pediatric and adolescent age groups may help to point out the possible age-related differences in the pathogenesis of essential hypertension. According to our supposition the effects of certain pathogenetic factors of EH compared to adults may be stronger or weaker in children. Children are relatively free from the common environmental and other risk factors (alcohol consumption, smoking, diabetes mellitus, dyslipidemia) contributing to hypertension. Therefore, we presume that the role of oxidative stress, endothelial dysfunction, and genetic predisposing factors may be more important in the occurrence of hypertension in children than in adults. In addition, lack of environmental and cardiovascular risk factors which are mainly associated with hypertension in adulthood allows a more precise investigation of oxidative stress and endothelial dysfunction in the case of hypertension of children and the alterations reflect hypertension only.

1.3. Renal fibrosis, the role of angiotensin II

Renal fibrosis or scarring, characterized by glomerulosclerosis and tubulointerstitial fibrosis, is the final common manifestation of a wide variety of chronic kidney diseases, irrespectively of the initial causes. The pathogenesis of renal fibrosis is a progressive process that clinically leads to chronic renal failure (CRF), and ultimately to end stage renal disease (ESRD), a devastating condition that requires dialysis or kidney transplantation [56].

Renal fibrosis represents a failed wound-healing response of the kidney tissue after chronic, sustained injury, which leads to the excessive deposition of the extracellular matrix (ECM) components. Briefly, activated kidney resident cells produce proinflammatory cytokines, and for this signal inflammatory monocytes/macrophages and T cells infiltrate the injured sites. Glomerular or interstitial infiltrated inflammatory cells become activated and produce injurious molecules, such as ROS, as well as fibrogenic and inflammatory cytokines.

Then, fibrogenic or fibrosis-promoting factors activate the matrix producing effector cells (glomerular mesangial cells, interstitial fibroblasts, and tubular epithelial cells, depending on the nature and sites of injury) to produce interstitial matrix components. Several different fibrogenic factors have been documented, but primary roles suggested transforming growth factor-β (TGF-β), connective tissue growth factor, Ang II and ET-1. Among them TGF-β has the highest significance, its induction appears to be a convergent pathway, that integrates directly or indirectly the effects of many other fibrogenic factors. The activated cells produce a large amount of ECM components; they are deposited in the extracellular compartment, and are often crosslinked and become resistant to degradation. Defective matrix degradation processes also contribute to ECM accumulation. Continuous deposition of ECM proteins begins to have destructive effects on the kidney structure, fibrous scars are generated, leading to the collapse of renal parenchyma and the loss of kidney function [56–58].

Angiotensin II in renal fibrosis

The role of Ang II in the pathogenesis of progressive kidney diseases is well established. In experimental models of kidney damage renal RAS activation, cell proliferation, growth factor upregulation and matrix production have been observed. Treatment with blockers of Ang II actions prevents proteinuria, inflammatory cell infiltration and fibrosis, and retards disease progression. Ang II could be involved in the fibrotic process in different ways. Ang II is not only a vasoactive agent, it is also a renal growth factor that activates ECM producing cells, increasing the expression and synthesis of ECM proteins, leading to glomerulosclerosis and tubulointerstitial fibrosis. These effects seem to be mediated mainly by TGF-β. Ang II has been shown to stimulate the TGF-β production of various cells, including renal tubular cells and fibroblasts. The use of ACE inhibitors or AT1R antagonists in experimental models of renal diseases reduces TGF-β production and attenuates renal fibrosis. In addition, Ang II activates mononuclear cells and increases the production of proinflammatory mediators (cytokines, chemokines, adhesion molecules), decreases matrix degradation, and may have direct effect on collagen gene expression. Ang II is also capable of affecting renal hemodynamics, increasing glomerular pressure and proteinuria, and enhancing tubulointerstitial ischemia, which may contribute to fibrotic process [59, 60].

Gene polymorphisms of the RAS affect the tissue Ang II level in the kidney, and therefore may have an influence on renal fibrogenesis [61]. This presumption has been studied in different types of chronic kidney diseases, mainly in adults, but also in children. Some of them showed a significant relation between genetic variants of the RAS and progressive loss of renal function. Genetic association studies of the RAS gene polymorphisms in chronic renal diseases will be reviewed in the Discussion chapter.

2. AIMS AND QUESTIONS OF THE STUDIES

The aims of our investigations were 1) to recognize the presence of oxidative stress and the related endothelial dysfunction in EH of adolescents (juvenile EH), BMI being taken into consideration as a confounding factor, and 2) to determine the polymorphisms of the RAS genes in adolescents with EH and patients with uremia. Thus, assessments were made of the relationship between NO production via its end-products in the plasma (nitrite + nitrate, NO_x) and the usual indicators of oxidative stress: the levels of plasma peroxidation end-products and free thiols. As a new parameter, the redox status of glutathione, and the glutathione recycling capacity of the RBC were also measured in patients with juvenile EH compared with normotensive controls with a similar BMI. In the genetic association study adolescents with EH, and also pediatric and adult patients with ESRD were genotyped for the M235T polymorphism of AGT gene, the I/D polymorphism of ACE gene and the A1166C polymorphism of AT1R gene.

The main questions of the studies were the following:

Question 1.

Is there any relationship between blood pressure and body mass index?

Question 2.

Does nitric oxide production, which is characterized by plasma concentration of its end-products (nitrite + nitrate), decrease in juvenile essential hypertension? Does it depend on body mass index?

Question 3.

Can we observe an increased oxidative stress state in juvenile essential hypertension? Is there any correlation between oxidative stress and blood pressure? Does the oxidative stress correlate with body mass index in normotensive and hypertensive adolescents?

Question 4.

May the polymorphisms of the renin-angiotensin system genes have any influence on the development of juvenile essential hypertension?

Question 5.

Is there any role of the renin-angiotensin system gene polymorphisms in the development of end stage renal disease in children and adults?

3. PATIENTS

3.1. Clinical characteristics of the oxidative stress study population

Fifty-two hypertensive patients (mean age 14.4 ± 3.1 years, male/female 37/15) and simultaneously, 48 age-matched control subjects (mean age 14.3 ± 4.3 years, male/female 20/28) with normal BP were studied for the biochemical analysis of oxidative stress.

Hypertension was defined as a BP greater than the 95th percentile for age, gender and height on three separate occasions at 5-min intervals at the time of enrolment [62]. Thereafter, the diagnosis of hypertension was confirmed with ambulatory BP monitoring as 24-h SBP and/or DBP mean values are equal to or greater than the 95th percentile of the age, height, and sex-matched normal values [63]. An oscillometric ambulatory BP monitor (ABPM-04; Meditech Kft., Budapest, Hungary) was used to record 24-h BP in hypertensive patients. BP was registered automatically at 15-min intervals during the day and 30-min intervals during the night. The measured values were analyzed by a computer (Medibase software) and shown as 24-h systolic and diastolic means. Cases of endocrine, cardiological, neurological, renal, and renovascular origin were excluded. Neither proteinuria (defined as > 300 mg/24-h urine) nor any impairment in renal function (creatinine clearance < 80 ml/min per 1.73 m^2) was observed. The patients had not yet received any treatment. They kept a regular diet, with a similar caloric intake and physical activity as the controls. None of them were smokers, as controlled via the carbon monoxide hemoglobin concentrations. Blood samples were always collected at the same time of the day (at 9:00 h). Twenty children were the offspring of treated hypertensive parents.

The BMI (weight in kilograms divided by the square of the height in meters) was used as a measure of ponderosity. According to their BMI both the hypertensive and the control groups were divided into normal (< 25 kg/m^2) and overweight (> 25 kg/m^2) subgroups. However, in order to acquire more information about the potential relation between the weight status and the oxidative stress parameters, the results of the biochemical examinations were analyzed and are also listed in the figures, as lean (BMI < 20 kg/m^2), normal (BMI = 20–25 kg/m^2), overweight (BMI = 25–30 kg/m^2) and obese (BMI > 30 kg/m^2) groups.

Demographic data, physical characteristics and some metabolic parameters of the biochemical study population are presented in **Table 2**. The concentrations of metabolic parameters (plasma glucose, cholesterol and triglyceride) revealed similar increase in overweight/obese subjects, both in the controls and in the patients with hypertension. Naturally, the BP values were significantly higher in hypertensive patients as compared with their appropriate BMI control groups (**Table 2**).

Table 2. Demographic data, physical characteristics and some metabolic parameters of the biochemical study population, related to their body mass index (BMI, kg/m^2)

	Controls (n = 48)		Hypertensive patients (n = 52)	
	BMI < 25 (n = 26)	BMI > 25 (n = 22)	BMI < 25 (n = 28)	BMI > 25 (n = 24)
Age (years)	14.6 ± 4.6	14.4 ± 5.1	14.4 ± 3.1	14.4 ± 2.5
Sex (male/female)	13/13	14/8	18/10	19/5
Body weight (kg)	51 ± 9	81 ± 12	54 ± 13	86 ± 11
Height (m)	1.60 ± 0.18	1.62 ± 0.12	1.65 ± 0.18	1.66 ± 0.15
Hearth rate (beats/min)	69 ± 12	75 ± 18	71 ± 16	81 ± 11
Office SBP (mmHg)	112.6 ± 5.1	118.4 ± 5.3	153.4 ± 9.7***	154.4 ± 9.6***
Office DBP (mmHg)	66.8 ± 6.1	69.1 ± 8.1	89.4 ± 9.4***	89.7 ± 8.4***
24-h SBP (mmHg)	–	–	143.6 ± 8.2	144.4 ± 9.9
24-h DBP (mmHg)	–	–	80.6 ± 6.1	79.4 ± 9.4
Blood glucose (mmol/l)	4.13 ± 0.33	4.51 ± 0.21	4.08 ± 0.22	4.68 ± 0.38*
Cholesterol (mmol/l)	4.16 ± 0.22	4.52 ± 0.26*	4.23 ± 0.37	4.55 ± 0.24*
Triglycerides (mmol/l)	1.17 ± 0.18	1.44 ± 0.41	1.21 ± 0.22	1.53 ± 0.33*
Creatinine (μmol/l)	67 ± 5	82 ± 8	76 ± 8	81 ± 7

Data presented as mean ± standard deviation. SBP, systolic blood pressure; DBP, diastolic blood pressure. $^{***}P < 0.001$ versus controls; $^{*}P < 0.05$ versus BMI < 25.

3.2. Clinical characteristics of subjects in the genetic study of renin-angiotensin system

Thirty-five adolescents with EH (mean age 14.4 ± 2.7 years, male/female 30/5), and 70 patients with ESRD (20 pediatric, mean age 14.9 ± 3.1 years, male/female 9/11 and 50 adult, mean age 48.7 ± 18.7 years, male/female 23/27) were genotyped for RAS polymorphisms in the genetic study. One hundred and thirty healthy randomly selected normotensive blood donors from the blood bank of our university (mean age 34.9 ± 8.1 years, male/female 66/64, BP 117.9 ± 8.7/78.7 ± 8.5 mmHg) and 20 healthy children with normal BP from a school screening program (mean age 13.2 ± 1.2 years, male/female 10/10, BP 109 ± 6.5/71 ± 5.9 mmHg) were also studied as controls to determine the frequency of gene polymorphisms within the population. No differences were observed regarding the genotype and allele distributions between the pediatric and adult control groups, therefore the values have been collected in one group as controls (n = 150). In the control group the genotype distribution is consistent with Hardy-Weinberg equilibrium.

The diagnosis of hypertension was set up according to the same definition and clinical evaluation protocol which we used in the biochemical study. All the hypertensive patients in the genetic study had a BMI below 30 kg/m^2, and normal serum cholesterol (< 5.2 mmol/l) and triglyceride (< 1.7 mmol/l) levels.

In patients with ESRD, the hereditary nephrological diseases, myeloma multiplex, and malignant disorders were excluded. The distribution of the original nephrological diagnoses in the adult ESRD group was as follows: chronic pyelonephritis 31, IgA nephropathy 6, focal segmental glomerulosclerosis (FSGS) 5, membranoproliferative glomerulonephritis 6, rapidly progressive glomerulonephritis 2. In the pediatric ESRD group the distribution was chronic pyelonephritis with reflux nephropathy 12, tubulointerstitial nephritis 2, membrano-proliferative glomerulonephritis 3, FSGS 2, and rapidly progressive glomerulonephritis 1. ESRD patients were on routine bicarbonate hemodialysis (all patients were treated three times per week, mean treatment time was 4.08 ± 0.59 h; Gambro dialysis machines were used). In ESRD patients the mean concentrations of total serum cholesterol (5.5 ± 1.2 mmol/l) and triglyceride (2.5 ± 0.7 mmol/l) were slightly above the laboratory references (normal cholesterol < 5.2 and triglyceride < 1.7 mmol/l).

Demographic data, physical characteristics and some related metabolic parameters of the hypertensive and ESRD patients in the genetic study are presented in **Table 3**.

Table 3. Demographic data, physical characteristics and some metabolic parameters of patient groups in the genetic study of renin-angiotensin system

	Hypertensive patients (n = 35)	ESRD patients (n = 70)	
		Pediatric (n = 20)	Adult (n = 51)
Age (years)	14.4 ± 2.7	14.9 ± 3.1	48.7 ± 18.7
Sex (male/female)	30/5	9/11	23/27
SBP (mmHg)	135.4 ± 7.4	149.1 ± 24	139 ± 14
DBP (mmHg)	72.4 ± 7.7	96.9 ± 12	91 ± 13
BMI (kg/m^2)	25.3 ± 2.7	–	
Cholesterol (mmol/l)	3.9 ± 0.5	5.5 ± 1.2*	
Triglyceride (mmol/l)	1.1 ± 0.4	2.5 ± 0.7*	
Creatinine (μmol/l)	80 ± 15.4	785 ± 129	

Data presented as mean ± standard deviation. ESRD, end stage renal disease; SBP, systolic blood pressure; DBP, diastolic blood pressure; BMI, body mass index. SBP and DBP values are expressed as 24-h mean in hypertensive patients and single measurement mean, before hemodialysis in ESRD patients. Serum cholesterol, triglyceride and creatinine were measured before hemodialysis. *mild increase compared to the laboratory reference values (normal cholesterol < 5.2 and triglyceride < 1.7 mmol/l).

The studies were previously approved by the Ethical Committee of the University of Szeged. Informed consent was obtained from the participants or from their parents.

4. METHODS

4.1. Biochemical methods

Nitric oxide end-products in plasma (nitrite + nitrate, NO_X)

NO reduction is considered the most important factor in the development of endothelial dysfunction. NO is oxidized in the blood and tissues to form nitrite and nitrate. These end-products are generally accepted markers of endogenous NO production. The plasma level of the sum of nitrite and nitrate (NO_X) is frequently used to assess NO bioavailability *in vivo*, which reflects the degree of endothelial dysfunction in humans [40, 41].

Nitrite and nitrate were determined simultaneously by an anion-exchange high-performance liquid chromatography (HPLC) method [64]. The Pharmacia LKB HPLC system was used with a Variable Wavelength Ultraviolet Detector (at 210 nm). The within-day (intra-assay) coefficient of variation (CV) for nitrate standards in pooled plasma ranged from 4.5% for 25 μmol/L to 2.5% for 50 μmol/L ($n = 10$). The day-to-day (inter-assay) variability was 5.2% for 25 μmol/L and 3.5% for 50 μmol/L ($n = 20$).

Plasma lipid peroxides as malondialdehyde equivalents

LPO is the oxidative degeneration of lipids caused by ROS. In the course of this process a number of by-products are generated, such as malondialdehyde (MDA), and they are commonly used as oxidative stress markers [23, 24].

Plasma lipid peroxides were quantified as MDA-thiobarbituric acid adducts by the HPLC method [65], using a 3.9 × 300mm μBondapak C18 (10 μm) (Waters-Millipore Corp., Milford, Massachusetts, USA) reverse-phase HPLC column and a Variable Wavelength Ultraviolet Detector (at 532 nm). The reproducibility of the results was evaluated by the analyses of commercial quality-control serum. Replicate daily analyses yielded a CV of 12.1% at 1.19 μmol/L ($n = 18$), while the intra-assay CVs were below 8% ($n = 10$).

Plasma free thiol groups

Thiols, such as glutathione, cysteine and homocysteine, are organosulfur compounds that contain a carbon-bonded thiol (sulfhydryl) group. Among all the antioxidants that are

available in the body, intracellular and extracellular thiols constitute the major portion of the total body antioxidants and they play a significant role in the defense against ROS. In the plasma both free and protein-bound thiols are presented. The plasma level of free thiol groups is a useful marker of oxidative stress; they correlate inversely with each other [66].

Plasma free thiol groups were assayed with 5,5-dithiobis (2-nitrobenzoic acid) at 412 nm; a molar extinction coefficient of 13 600 was used [67]. The values of both the inter- and the intra-assay precision were similar to those reported by other authors, with CVs of 8.9% and 6.7% in the presence of 300 μmol/L free thiol, as a GSH standard [67].

Biochemical analysis of the glutathione redox system

Glutathione tripeptide in its reduced state (GSH) is present in millimolar intracellular concentrations, and provides antioxidant protection in all the cells of the body. In the presence of oxidative stress GSH is converted to oxidized glutathione (glutathione disulfide, GSSG), which is reduced back to GSH by the enzyme glutathione reductase using NADPH as an electron donor. The GSH concentration in the plasma has previously been used as a marker of oxidative stress in hypertensive patients [68].

Red blood cells (RBC), contain millimolar concentrations of GSH, and circulate in the direct vicinity of the endothelial surface. Their membranes are permeable to oxidants and therefore can provide antioxidant protection to their surroundings [69]. Inter-related enzyme systems in the RBC function to achieve the efficient recycling of GSSG to GSH and to provide the reducing equivalent, NADPH. Hence, GSH concentration, and the redox ratio GSSG/GSH of the RBC can serve as a reliable parameter of the oxidative imbalance in patients with endothelial dysfunction (GSH concentration decreases, while GSSG/GSH ratio increases).

Highly sensitive and specific separate determinations of GSSG and GSH + GSSG concentrations were carried out by a previously published method [70]. This is a combination of standard methods [71] used after validation for accurate determination, especially of GSSG values in the presence of much higher concentrations of GSH (more than 50 times), in the presence of hemoglobin (Hb). The intra-assay and inter-assay variabilities of the assay, using GSSG standard at 50 nmol/l, resulted in CVs of 3.5% (n = 10) and 5.8% (n = 20), respectively [70].

The „GSH stability test" [72] was used to measure the recycling capacity of the RBC after an *in vitro* oxidative stress. Acetylphenylhydrazine (APH) (0.33 mmol/l) was added to the whole blood sample, together with sufficient glucose. Following incubation at 37 °C for 60 min with APH, RBC deficient in recycling, but not the normal RBC, suffer a marked fall in GSH level [72]. This method of calibrated oxidative challenge was established by Beutler in order to recognize patients with deficient glucose-6-phosphate-dehydrogenase [72] and was proved to be a sensitive biochemical marker of the oxidative susceptibility of the RBC both in population studies and during infection [73, 74].

The proportions of carbon monoxide hemoglobin and methemoglobin, and the total concentration of hemoglobin in the whole blood were measured with a Hemoximeter (Radiometer, Copenhagen, Denmark) within 15 min after venipuncture.

4.2. Determination of renin-angiotensin system gene polymorphisms

Genomic DNA was isolated from peripheral blood leukocytes by a standard phenol/chloroform method [75].

I/D polymorphism of the angiotensin-converting enzyme (ACE) gene

The I/D polymorphism in intron 16 of the ACE gene was determined according to the previously published method of Chiu and McCarthy [76] using polymerase chain reaction (PCR). A forward primer (5'-CTGGAGACCACTCCCATCCTTTCT-3'), a reverse primer (5'-TCGAGACCATCCCGGCTAAAAC-3'), and an insertion-specific primer (5'-GAT GTGGCCATCACATTCGTCAGAT-3') were used for the PCR. Amplification was carried out in a 25 µl reaction mixture containing 250 ng genomic DNA, 2 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 0.1% Triton X-100, 5% dimethylsulfoxide, 0.2 mM of each dNTP, 25 pmol of each primer and 0.25 U Taq DNA polymerase. The amplification involved the following steps: 5 min initial denaturation at 94 °C, followed by 30 cycles of 1 min denaturation at 94 °C, 1 min annealing at 64 °C, 1 min extension at 72 °C, and then 10 min final extension at 72 °C. The samples were visualized after electrophoresis on a 2% agarose gel with ethidium bromide staining. The DD genotypes were re-typed with the insertion specific primer and the consensus (forward) primer.

M235T polymorphism of the angiotensinogen gene (AGT)

The M235T polymorphism in exon 2 of the AGT gene was detected by a single-step Light Cycler technology published by Malin et al. [77] that uses a rapid PCR amplification followed by the analysis of the melting behaviour of fluorophore-labeled hybridization probes. As a reaction buffer in the PCR, the Light Cycler DNA Master Hybridization Probes 10× buffer (Roche Molecular Biochemicals) with a final Mg^{2+} concentration of 4 mmol/L was used. PCR was performed in a reaction volume of 20 µl with 0.3 µmol/L of each primer (5'-CTCTATCTGGGAGCCTTG-3' and 5'-GTTTGCCTTACCTTGGAA-3') and 0.2 µmol/ L of the anchor and detection probes. The detection probe was labelled at the 3' end with fluorescein (5'-CCCTGAGTGGAGCCAGTG-X); the anchor probe was labelled with LightCycler Red 640 at its 5' end and modified at the 3' end by phosphorylation to block extension (5'-LC Red 640-GACAGCACCCTGGCTTTCAACAC-P). The PCR was performed in a LightCycler instrument and included initial denaturation at 94 °C for 45 sec, followed by 50 cycles of denaturation (94 °C for 0 sec, with a temperature transition rate of 20 °C/sec), annealing (57 °C for 5 sec, 20 °C/sec), and single extension (72 °C for 20 sec, 3 °C/sec). After the amplification, the melting curve was recorded by cooling the reaction mixture to 50 °C for 3 min, and then by slowly raising the temperature to 85 °C at 0.2 °C/sec. The fluorescence signal (F) was continuously monitored during the temperature ramp and then plotted against the temperature (T) to obtain melting curves for the samples (F vs. T). The melting curves were subsequently converted to derivative melting curves [–(dF/dT) vs. T]. The melting peak of the samples homozygous for the M allele was at 63 °C, whereas in samples homozygous for the T allele, the melting peak was at 53 °C. The heterozygous samples contained both M and T alleles and produced both peaks.

A1166C polymorphism of the angiotensin II type 1 receptor (AT1R) gene

Determination of the A1166C polymorphism of the AT1 gene was also carried out with LightCycler technology according to the M235T polymorphism detection (described above) which was optimized for the analysis of A1166C polymorphism by us.

The same PCR reaction buffer with a final Mg^{2+} concentration of 3 mmol/L and 20 µl reaction volume with 0.4 µmol/L of each primer (5'-ATCCACCAAGAAGCCT-3' and 5'-AAAGTCGGTTCAGTCCA-3') and 0.2 µmol/L of hybridization probes were used for the

PCR. The detection probe was labelled at the 3' end with fluorescein (5'-AGGAGCAAGAGAACATTCCTCTGCA-X) and the anchor probe with LightCycler Red 640 at its 5' end and modified at the 3' end by phosphorylation (5'-LC Red 640-ACTTCACTACCAAAGTAGCCTTAGC-P). The amplification included an initial denaturation at 94 °C for 45 sec, followed by 50 cycles of denaturation at 94 °C for 5 sec, annealing at 57 °C for 15 sec and single extension at 72 °C for 25 sec. After the PCR the melting curves were recorded by cooling the reaction mixture to 40 °C for 2 min and then by slowly raising the temperature to 85 °C at 0.2 °C/sec. The melting peaks of the AA and CC genotypes were 61 °C and 67 °C.

4.3. Statistical analysis

Clinical data on the patients are reported as means ± standard deviations. The results of biochemical analyses are shown in the figures as means ± standard errors. Statistical analyses included both parametric (variance analysis, Tukey test and Student's *t* test) and non-parametric tests (Wilcoxon rank test, chi-square test) as appropriate. When the extent of variance between pairs of groups differed significantly ($P < 0.05$ in the F test), we used the Welch test (*d* probe) instead of the *t* test to compare the mean values. Correlations between parameters were characterized by calculation of the linear regression and correlation coefficients. The distribution of genotypes was expressed as percentage frequency and odds ratio (OR) values were also calculated. Deviation from Hardy-Weinberg equilibrium was assessed by chi-square test with Yates correction. P less than 0.05 was considered significant for all statistical tests.

5. RESULTS *(according to the main questions of the studies)*

Question 1. Is there any relationship between BP and BMI?

Result 1. In the hypertensive patients, the BMI displayed significant positive correlations with both the SBP and the DBP (r = 0.581 and r = 0.542, respectively; $n = 52$, $P < 0.001$).

Question 2. Does NO production, which is characterized by plasma concentration of its end-products (NO_x), decrease in juvenile EH? Does it depend on BMI?

Result 2. High decreases in plasma NO_x concentrations were only seen in hypertensive patients with a normal body mass (BMI < 25 kg/m^2) compared to the control subjects. In overweight groups (BMI > 25 kg/m^2) the NO_x levels were somewhat, but not significantly, lower, irrespectively of hypertension (**Figure 2**).

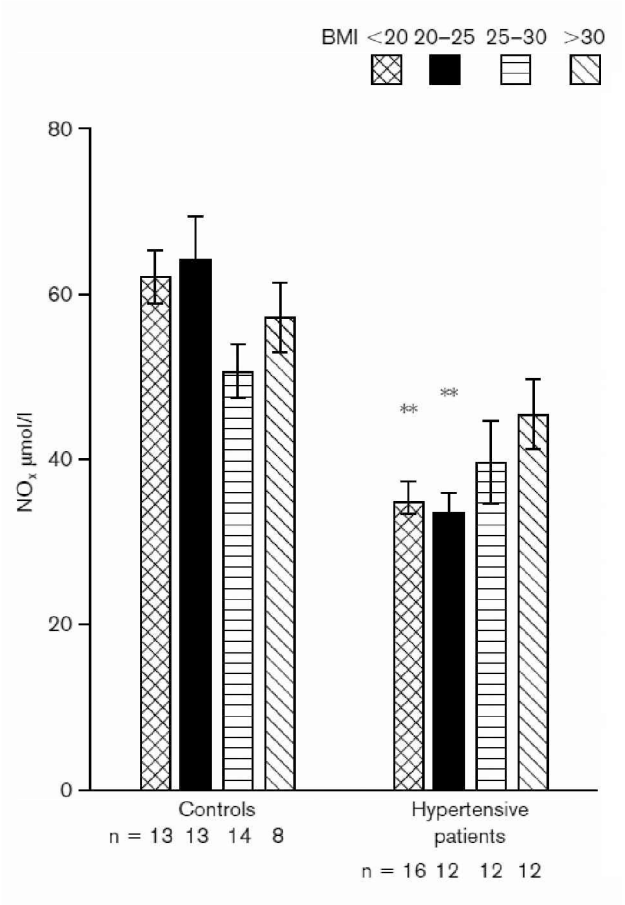

Figure 2. Plasma concentration of nitric oxide metabolites (nitrite + nitrate, NO_x) in controls and hypertensive patients, related to their body mass index (BMI), means ± standard errors. $^{**}P < 0.01$ versus BMI-matched controls.

The plasma NO_x concentration did not show significant correlations neither with BMI nor the investigated parameters of oxidative stress.

Question 3. Can we observe an increased oxidative stress state in juvenile EH? Is there any correlation between oxidative stress and BP? Does the oxidative stress correlate with BMI in normotensive and hypertensive adolescents?

Result 3. The MDA concentrations were significantly higher only in case of overweight hypertensive patients (BMI > 25 kg/m^2) as compared with the levels of the BMI-matched control subjects (**Figure 3**).

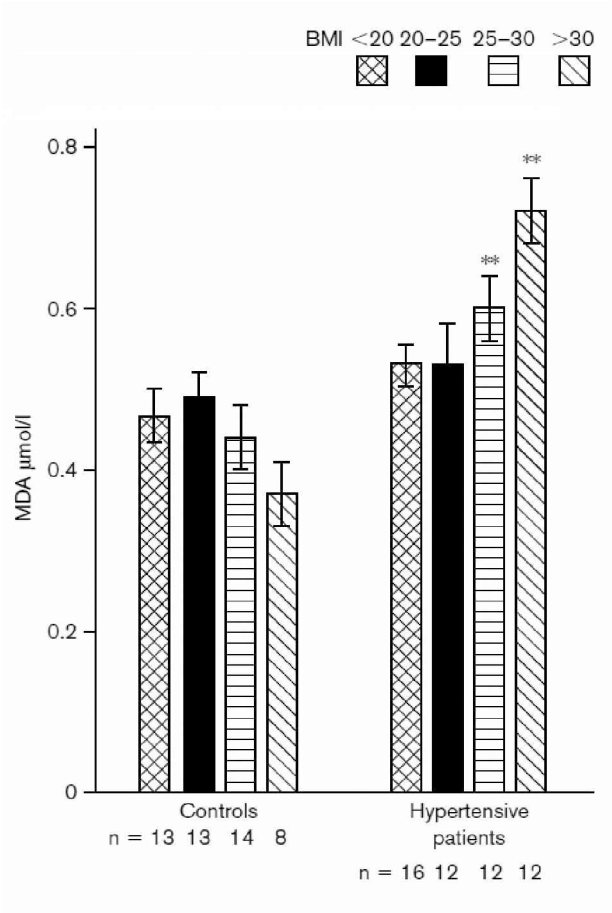

Figure 3. Plasma concentration of end-products of lipid peroxides (malondialdehyde, MDA) in controls and hypertensive patients, related to their body mass index (BMI), means ± standard errors. $^{**}P < 0.01$ versus BMI-matched controls.

The concentrations of free thiols displayed a marked decrease in overweight normotensive subjects, and a slight reduction in overweight hypertensive patients (**Table 4**), as a sensitive sign of a moderate pro-oxidant state related to overweight state even without hypertension.

Table 4. Concentration of some metabolic parameters in control subjects and hypertensive patients, related to their body mass index (BMI, kg/m^2)

	Controls (n = 48)		Hypertensive patients (n = 52)	
	BMI < 25 (n = 26)	BMI > 25 (n = 22)	BMI < 25 (n = 28)	BMI > 25 (n = 24)
Whole blood values				
Total hemoglobin (mmol/l)	9.2 ± 0.9	9.3 ± 0.7	9.9 ± 0.8*	9.8 ± 0.7*
Carboxyhemoglobin (µmol/l)	144 ± 43	165 ± 57	158 ± 63	179 ± 78
Methemoglobin (µmol/l)	82 ± 16	82 ± 13	86 ± 25	90 ± 23
Oxidized glutathione (GSSG, nmol/g Hb)	9.5 ± 2	11.4 ± 3†	12.5 ± 3*	14.1 ± 3†
Plasma values				
Free thiols (µmol/l)	215 ± 35	181 ± 24†††	209 ± 38	199 ± 30

Data presented as mean ± standard deviation. Hb, hemoglobin. $^*P < 0.05$ versus BMI-matched controls; $^\dagger P < 0.05$, $^{\dagger\dagger\dagger}P < 0.001$ versus BMI < 25.

The GSH levels were considerably decreased in hypertensive patients, irrespective of their BMI (**Figure 4a**). After *in vitro* oxidant insult, there was a gradual decrease in the proportion of residual GSH with increasing BMI, as a sign of the potential effects of certain metabolic factors on the GSH stability. Nevertheless, a further significant fall in the residual GSH was observed in the hypertensive non-obese patient groups (BMI < 30) as compared with the BMI-matched control groups (**Figure 4b**).

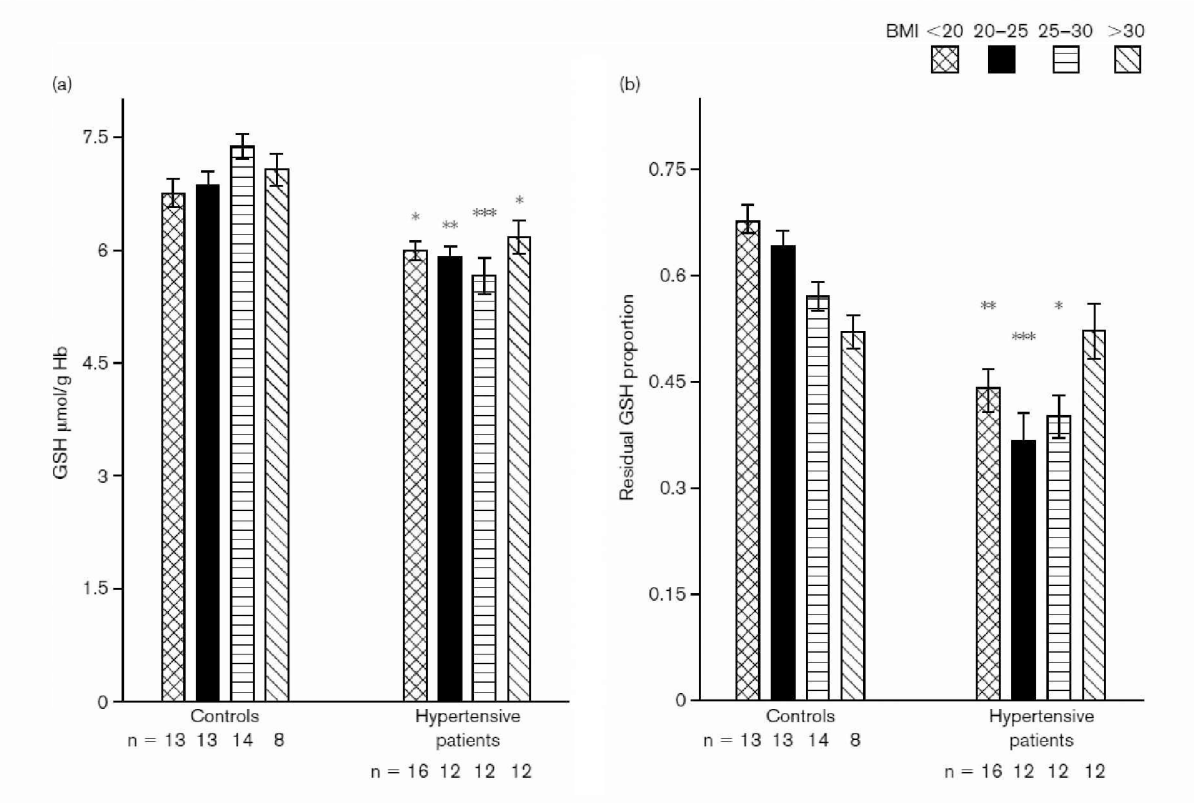

Figure 4. Concentrations of reduced glutathione (GSH) (a) and the proportion of GSH remaining after in vitro oxidative stress with acetylphenylhydrazine (b) in the red blood cells of controls and hypertensive patients, related to their body mass index (BMI), means ± standard errors. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus BMI-matched controls.

The significant depletion of erythrocyte GSH in hypertensive patients with somewhat increasing values of GSSG according to BMI (**Table 4**) resulted in a highly elevated redox ratio GSSG/GSH in patients with hypertension as compared with the BMI-matched controls (**Figure 5a**). However, there was not a significant direct relationship between the BP and GSSG/GSH.

When the concentrations of LPO end-products (MDA) were related to those of NO_x, a highly significant increase was seen in hypertensive patients, with or without overweight condition (**Figure 5b**). In addition, the ratios MDA/NO_x proved to correlate significantly with both the SBP and the DBP in the overall patient population ($r = 0.525$ and $r = 0.492$, respectively; $n = 100$, $P < 0.001$). Age was not a significant covariate in this correlation. None of the oxidative stress parameters correlated significantly with BMI.

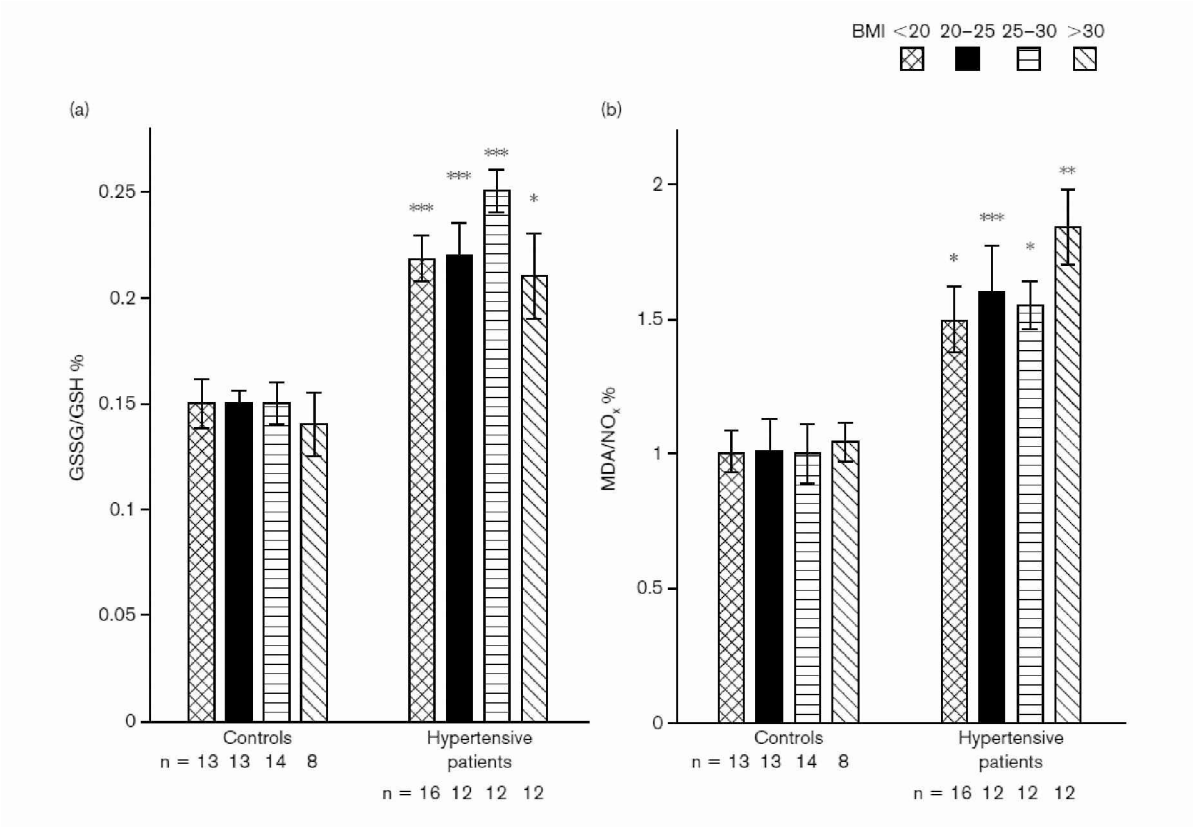

Figure 5. Ratios of oxidized/reduced glutathione (GSSG/GSH) (a) and lipid peroxides/nitric oxide metabolites (MDA/NO_x) (b) as parameters of oxidative stress in controls and hypertensive patients, related to their body mass index (BMI), means ± standard errors. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus BMI-matched controls.

Question 4. May the polymorphisms of the RAS genes have any influence on the development of juvenile EH?

Result 4. Genotype and allele frequencies for the polymorphisms of the RAS genes in hypertensive patients are listed in **Table 5** and **Table 6**. A significant increase in the frequency of the MT genotype of the AGT gene was observed in juvenile EH ($P < 0.02$), the OR was 2.9, respectively. The frequencies of the other AGT genotypes, and also frequencies of all ACE and AT1R genotypes did not differ significantly in the hypertensive and control group, only a weak non significant increase in the incidence of the II genotype of ACE gene was seen. Furthermore, the same allele frequencies of the M235T polymorphism of AGT gene, I/D polymorphism of the ACE gene, and A1166C polymorphism of the AT1R gene were found in the hypertensive as well as the control group.

Table 5. Genotype frequencies (%) for the M235T polymorphism of the angiotensinogen (AGT) gene, I/D polymorphism of the angiotensin converting enzyme (ACE) gene, and A1166C polymorphism of the angiotensin II type 1 receptor (AT1R) gene in hypertensive patients, and controls (OR, odds ratio)

Gene polymorphism	Genotype	Hypertensive patients ($n = 35$)		Controls ($n = 150$) n (%)
		n (%)	OR	
AGT M235T	MM	4 (12)	0.4	37 (25)
	MT	26 (74)**	2.9	75 (50)
	TT	5 (14)	0.5	38 (25)
ACE I/D	II	12 (34)	1.8	34 (23)
	ID	16 (46)	0.7	83 (55)
	DD	7 (20)	0.9	33 (22)
AT1R A1166C	AA	13 (37)	0.6	78 (51)
	AC	20 (57)	1.6	67 (45)
	CC	2 (6)	1.8	5 (4)

** $P < 0.02$

Table 6. Allele frequencies for the M235T polymorphism of the angiotensinogen (AGT) gene, I/D polymorphism of the angiotensin converting enzyme (ACE) gene, and A1166C polymorphism of the angiotensin II type 1 receptor (AT1R) gene in hypertensive and end stage renal disease (ESRD) patients, and controls

Gene polymorphism	Alleles	Hypertensive patients ($n = 35$)	ESRD		Controls ($n = 150$)
			Pediatric ($n = 20$)	Adult ($n = 50$)	
AGT M235T	M	0.49	0.50	0.55	0.50
	T	0.51	0.50	0.45	0.50
ACE I/D	I	0.56	0.35	0.47	0.50
	D	0.44	0.65*	0.53	0.50
AT1R A1166C	A	0.69	0.78	0.72	0.75
	C	0.31	0.22	0.28	0.25

*$P < 0.05$

Question 5. Is there any role of the RAS gene polymorphisms in the development of ESRD?

Result 5. There was a significant increase in the frequency of the MT genotype of the AGT gene in the group of pediatric ESRD patients ($P < 0.02$), the OR was 4.0, respectively. The DD genotype of the ACE gene was over-represented in pediatric ESRD patients compared with that of the control group (pediatric 45% versus controls 22%; $P < 0.05$). The OR was 2.9. In addition, a non-significant increase of the DD genotype frequency was found in the adult ESRD group. The similar genotype distributions of the AT1R gene polymorphism were observed in the ESRD and control groups. The slightly higher CC genotype frequency, which we have found in adult ESRD patients compared to that of the control group, was not significant (**Table 7**). Allele frequencies of the M235T polymorphism did not differ in pediatric and adult patients with ESRD compared with the controls. Similarly, there was no significant difference between the ESRD groups and the control group regarding the allele frequencies of AT1R gene polymorphism. The D allele of the ACE gene was significantly more frequent in the pediatric ESRD patients compared with the control subjects ($P < 0.05$) (**Table 6**).

Table 7. Genotype frequencies (%) for the M235T polymorphism of the angiotensinogen (AGT) gene, I/D polymorphism of the angiotensin converting enzyme (ACE) gene, and A1166C polymorphism of the angiotensin II type 1 receptor (AT1R) gene in end stage renal disease (ESRD) patients, and controls (OR, odds ratio)

Gene polymorphism	Genotype	ESRD				Controls (n = 150) n (%)
		Pediatric (n = 20)		Adult (n = 50)		
		n (%)	OR	n (%)	OR	
AGT M235T	MM	2 (10)	0.4	17 (34)	1.6	37 (25)
	MT	16 (80)**	4.0	21 (42)	0.7	75 (50)
	TT	2 (10)	0.3	12 (24)	0.9	38 (25)
ACE I/D	II	3 (15)	0.6	11 (22)	1.0	34 (23)
	ID	8 (40)	0.5	25 (50)	0.8	83 (55)
	DD	9 (45)*	2.9	14 (28)	1.4	33 (22)
AT1R A1166C	AA	11 (55)	1.1	26 (52)	1.0	78 (51)
	AC	9 (45)	1.0	20 (40)	0.8	67 (45)
	CC	–	–	4 (8)	2.5	5 (4)

*$P < 0.05$, **$P < 0.02$

6. DISCUSSION

6.1. Oxidative stress and endothelial dysfunction in juvenile essential hypertension and obesity

The BMI is a clinical indicator of overall body fat [78]. We used it as a definition of the mass status based on height and weight, because these can be obtained with reasonable precision. Comparisons of various weight-for-height indices for both adults and children have led to the selection of the BMI as the most desirable parameter [79]. The BMI cut-off points in the adolescent age group do not differ significantly from those of the adults; a value above 25 indicates overweight status, and one above 30 indicates obesity [79].

In our hypertensive patients, both the systolic and the diastolic BP correlated significantly positively with the BMI, indicating that the weight status is a confounding factor in the assessments of the potential relationship between oxidative stress and hypertension. The positive correlations between the BP values and weight, BMI and height in childhood were demonstrated by several studies in large populations of children, among others in Hungarian adolescents [80].

Impairment of NO bioavailability, which is a major component of endothelial dysfunction, plays an important role in the mechanism of hypertension [39]. Determination of stable end-products of NO, nitrite and nitrate (NO_X) in plasma is often used as an index of systemic NO formation [40, 41], which reflects not only NO production and NOS activity, but also NO quenching by ROS, therefore may allude to an oxidative stress state. Plasma NO_X level significantly decreased in hypertensive patients with normal BMI. In overweight children, irrespectively of hypertension, a non-significant decrease in plasma NO_X was observed. In a previous study from our group a marked decrease was seen in plasma NO_X in non-obese and also in obese hypertensive patients [44]. These data suggest that endothelial dysfunction, characterized by decreased plasma NO_X may be a more important mechanism in EH of lean patients than in OH, but the presence of endothelial dysfunction is detectable in obesity and OH. Moreover, decrease in plasma NO_X may be an indirect sign for the increased oxidative stress in hypertensive and obese subjects.

In the present study, the concerted action of the mass status and hypertension was measured in terms of the different biochemical parameters of oxidative stress. However, the results from hypertensive patients, compared with BMI-matched control subjects, permit identification of the differences resulting exclusively from the hypertensive state. As a sensitive sign of the compromised antioxidant status the plasma level of free thiols was reduced in overweight patients, even in case of those without hypertension. In contrast, a significant increase in the end-products of lipid peroxides, in MDA concentrations, was observed only in cases involving both hypertension and overweight state. However, the ratio MDA/NO_X, as a measure of the increase in LPO relative to NO, proved to be a useful marker of the link between BP and oxidative stress, presenting a highly significant increase in hypertensive patients irrespective of their BMI. The significant correlation between MDA/NO_X and the systolic and diastolic BP from a clinical point of view makes this parameter important.

In adult patients with uncontrolled EH, the levels of MDA were high, whereas those of NO_X were low [81]. When the ratio of lipid peroxides/NO was calculated, a far more significant rise in the concentration of lipid peroxides as compared to NO_X levels was noted [81]. Our results in young hypertensive patients with a different mass status are in accordance with the conclusion that the ratio MDA/NO_X is a BMI-independent sensitive parameter of the oxidative stress in childhood and adolescent hypertension. Additionally, the ratio was normalized following the control of hypertension in the study of Kumar and Das [81]. Thus, this ratio may be used as a relevant target for future clinical trials aimed at controlling the effects of antioxidant or antihypertensive treatment in patients with juvenile EH.

However, an oxidative stress status irrespective of BMI was also consistently manifested by a depleted GSH level and a pronounced GSH lability after APH loading in hypertensive adolescents. Another marker of oxidative stress, the redox ratio GSSG/GSH in RBC, also increased significantly in hypertensive patients, independent of their BMI. Although GSH depletion was the predominant cause, a higher GSSG in overweight patients had some influence as well. This could be the explanation for the absence of direct correlation between the BP values and GSSG/GSH.

These findings prove the presence of increased oxidative stress in childhood EH. In addition, a significantly positive correlation was observed between oxidative stress, characterized by MDA/NO_X ratio and BP. The extent of oxidative stress was not correlated

directly to BMI, but obese hypertensive patients exhibited a slightly more pronounced alterations in oxidative stress parameters than hypertensive patients with normal BMI. A compromised antioxidant status as the sign of increased oxidative stress was observed in obese control subjects. Obesity *per se* is a pro-oxidant state, and may aggravate the oxidative stress state if it is associated with hypertension.

The present study does not give an answer to the question of whether the oxidative stress is the cause or consequence of hypertension. GSH depletion resulted in a perturbation of the NO system and caused severe hypertension in normal animals [82]. The administration of antioxidant vitamins ameliorated hypertension and improved the urinary nitrate-nitrite excretion, supporting the notion that oxidative stress is involved in the pathogenesis of this experimental hypertension [82]. On the other hand, thiol supplementation with GSH, given by intravenous infusion, selectively improved a human endothelial dysfunction by enhancing NO activity [83]. Moreover, a GSH infusion caused a reduction of BP in adult hypertensive patients [84].

In conclusion, an imbalance between the available NO and LPO end-products, with a simultaneous alteration in the glutathione redox system of the RBC, was present in young hypertensive patients, irrespective of their weight status. The population studied was relatively small and rather heterogenous with regard to age. Thus, further studies are warranted to clarify the relationship between the hormonal, SNS activity and the redox status during the age of puberty.

6.2. Gene polymorphisms of the renin-angiotensin system in juvenile essential hypertension and uremia

Genetic association studies based on the comparison of genotype and allele distribution in cases and controls are considered a useful approach in studying the role of candidate genes in the development and progression of multifactorial diseases, such as hypertension, and other cardiovascular or chronic renal diseases. Among the candidate genes, AGT, ACE and AT1R genes of the RAS seem to be particularly biologically and clinically relevant to cardiovascular and renal diseases [85].

Evidence has been suggesting that RAS and Ang II have a central role in the pathogenesis of cardiovascular and chronic renal diseases, such as LVH and fibrosis, vascular media hypertrophy or neointima formation, and structural alterations of the heart and the kidney [60, 86]. Therefore, genetic variants of the RAS, which have influence on the production or action of Ang II, may have a pathogenetic role in these disorders.

Large numbers of association studies have been published about the possible role of polymorphisms of the RAS genes in cardiovascular and renal diseases. The results are often inconclusive and sometimes different studies may even be conflicting. The interpretation of data is dependent on several aspects, for example the sample size, clinical and genetic homogenity of the study population, definition and relevancy of the outcome, the role of acquired and environmental factors [87]. All of these factors may have a significant influence on the results and conclusions. The differences in the prevalence of a polymorphism between various ethnic groups may also be an influential factor.

The distribution of the ACE gene polymorphisms in the Caucasian population is similar in different European countries; with incidences of II, ID, and DD genotypes in Germany [88] of 26%, 50%, and 27%, respectively; in France [89] of 22%, 51%, and 27%; in Sweden [90] of 23%, 50%, and 27%. In the present study we accumulated data for the Hungarian population (23%, 55%, 22%) that were in accordance with these studies. In Japanese [61] and South Asian [91] control groups the incidence of II genotype was higher (41% and 39.8%, respectively) than in the European population. In the United States it was lower, but the incidence of DD genotype was higher than in the European countries [92].

In a large study of hypertensive siblings, a strong association was demonstrated between M235T polymorphism of the AGT gene and hypertension, which was later confirmed in patients with no family history of hypertension [93, 94]. In several other studies, the role of M235T polymorphism was demonstrated in cardiovascular disorders and hypertension. These reported that the TT genotype of AGT was associated with an increased risk of hypertension and coronary heart disease [95, 96]. Theoretically, this polymorphism accounts for higher angiotensinogen levels, which could translate into higher Ang II levels and may lead to pathological changes in the cardiovascular tissues. This concept was confirmed by a case-control study of Winkelmann et al., in which a higher plasma angiotensinogen levels were linked to the number of T235 alleles and to elevated DBP. Furthermore, they observed a weak

association between the M235T variant and coronary artery disease and myocardial infarction [48]. In contrast, Caulfield et al. failed to show an association between AGT gene polymorphism and hypertension [97].

The results of Gumprecht et al. [98] suggest that the AGT gene M235T polymorphism contributes to the increased risk for the development of CRF. The T235 allele was transmitted more frequently to patients with CRF caused by interstitial nephritis. In type 2 diabetes an association of AGT gene polymorphism with renal dysfunction and coronary heart disease was also found [99]. T allele and TT genotype showed a significant association with albuminuria in Chinese patients with type 2 diabetes and Japanese children with IgA nephropathy [100, 101]. Others were not able to confirm the role of this polymorphism in the development or progression of chronic renal diseases [102–104]. A fairly conclusive meta-analysis of the association of AGT gene M235T polymorphism with ESRD was published by Zhou et al. based on sixteen literatures. T allele and TT genotype were associated with ESRD susceptibility in Caucasians, which were not observed in overall populations, Asians and Africans [105].

We did not demonstrate an increased frequency of the TT genotype in juvenile EH and ESRD as expected from previous studies, but there was a significant increase in MT genotype in the groups of EH and pediatric ESRD. It is conceivable, that the small number of patients in these groups did not allow detection of an increased incidence of the TT genotype.

A study by Zee et al. [106] is the only report that demonstrates a positive association between the ACE gene and hypertension. A higher frequency of I allele and lower frequency of the DD genotype were found in adult hypertensive patients with a family history of hypertension compared with normotensive subjects. Similarly, a weak, non significant increase in the incidence of the II genotype, but normal frequencies of the ID and DD genotypes were found in our adolescent essential hypertensive patients. Schunkert et al. [107] and Iwai et al. [108] reported a positive association between DD genotype and LVH, but did not identify a relationship between hypertension and ACE gene polymorphism. The relevance of ACE I/D polymorphism in cardiovascular diseases was also showed by the ECTIM study [109]. Subjects with DD genotype had an increased risk of myocardial infarction. Data from Butler et al. suggest that DD genotype is associated with arterial dysfunction limited to NO pathway [110].

We found an increased prevalence of the D allele and DD genotype in the pediatric ESRD population, suggesting that certain pathogenetic mechanisms associated with the D allele result in a faster progression of ESRD in pediatric kidney disorders. These results are in accordance with those of the multicenter study of Hohenfellner et al. [111]. They found that the ACE DD genotype is a significant risk factor for children with congenital renal malformations associated with progressive CRF. Although hereditary renal diseases were excluded from our study, reflux nephropathies associated with pyelonephritis were included.

The role of D allele and DD genotype in the progression of CRF has been demonstrated in a large number of nephropathies of different origin. Ozen et al. found that the DD genotype had a significant impact on renal scar formation in reflux nephropathy [112]. This association was also confirmed in our study of patients with vesicoureteric reflux [113]. In a group of children with FSGS homozygotes for the I allele were less likely to have progressive renal disease than in case of patients with other genotypes (ID and DD), so the II genotype had a protective, while the D allele had a detrimental effect on the outcome in this study [114]. Studies in IgA nephropathy showed that ACE genotype is a risk factor for the worsening of nephropathy clinical course; patients with II genotype have more favorable prognosis than those with ID and DD [115, 116]. The role of the D allele in progression of non-diabetic CRF patients was confirmed by the study of Samuelson et al. [117]. The progression of CRF to ESRD was faster in patients with autosomal dominant polycystic kidney disease (ADPKD) who had DD genotype, compared with other genotypes [118]. The II genotype is protective against the development and progression of diabetic nephropathy and is associated with a slower progression of non-diabetic proteinuric kidney disease published in a review by Ruggenenti et al. [87]. Our adult ESRD group showed a slight increase in DD genotype frequency (OR = 1.4). The difference between pediatric and adult ESRD patients in DD genotype frequency may be an explanation for the faster progression in the decline of renal function. In contrast to our findings and studies cited above, others failed to demonstrate a relationship between ACE I/D polymorphism and the progression of various chronic renal diseases [102, 103, 119].

Most of the known actions of Ang II are mediated by the AT1R [53]. The functional significance of the A1166C non-coding polymorphism of AT1R gene is uncertain, and its influence on Ang II responsiveness is not confirmed. Nevertheless, studies have revealed an

association between this polymorphism and hypertension [120] and aortic stiffness in hypertensive patients [121]. A significant association of the C1166 allele to essential hypertension was confirmed by other studies [122, 123]. Moreover, a synergistic interaction between the AT1R and ACE gene polymorphism on the risk of myocardial infarction was demonstrated [124], but in the ECTIM study this genetic variant of AT1R did not affect the risk of myocardial infarction [125]. In addition, other studies in Caucasian populations or in Japanese persons were not able to demonstrate an obvious link between A1166C polymorphism and hypertension [53]. An increased response to Ang II was observed in isolated arteries from patients with CC genotype. This association suggests that the A1166C polymorphism may alter Ang II responsiveness indirectly (mostly in linkage with a functional mutation), which may explain the relation between this polymorphism and cardiovascular abnormalities [126].

About the role of A1166C polymorphism of AT1R gene in chronic renal diseases conflicting results were published, an association was found between this polymorphism and renal function in diabetic nephropathy [99], but was not in IgA nephropathy [103] and ADPKD [104].

We found a non-significant increase of the CC genotype in the groups of juvenile EH and adult ESRD patients (the ORs were 1.8 and 2.5, respectively, $P > 0.05$). The frequency of the AC genotype in hypertensive patients was higher (57%) compared with that of the control group (45%), but it was not significant either.

In summary, there is no difference in the ACE genotype distribution between Hungarian and other Caucasian populations. We conclude that the DD genotype of ACE was more frequent in pediatric ESRD. This genotype, which is associated with higher circulating and tissue ACE levels, could be a genetic risk factor for renal parenchymal destruction, renal scarring, and the development of ESRD in children, independent of the original renal disease. Furthermore, a significant increase was observed in the occurrence of AGT MT genotype in the groups of hypertensive and pediatric ESRD. The role of the AT1R gene polymorphism in juvenile EH and ESRD needs to be further investigated.

7. SUMMARY OF OUR FINDINGS AND CONCLUSIONS *(according to the main questions of the studies)*

Conclusion 1. Obesity is directly related to blood pressure (BP) and hypertension. In accordance to this statement, the body mass index (BMI) displayed significant positive correlations with both the systolic and the diastolic BP in our hypertensive patients.

Conclusion 2. A significant decrease in plasma concentration of nitric oxide end-products (NO_x) was observed in hypertensive patients with normal BMI. In overweight hypertensive patients and overweight control subjects the plasma concentration of NO_x was also lower, but not significantly. These findings suggest that endothelial dysfunction, characterized by decreased plasma NO_x, may be more important pathogenetic mechanism in the essential hypertension (EH) of lean patients than in obesity-induced hypertension (OH), but the presence of endothelial dysfunction is also detectable in obesity and OH.

Conclusion 3. Increased plasma concentration of lipid peroxides, as malondialdehyde (MDA) and a slight decrease in free thiol groups in overweight hypertrensive patients, elevated ratio of oxidized/reduced glutathione (GSSG/GSH) due to decreased GSH and increased GSSG concentrations in red blood cells and increased ratio of lipid peroxides/nitric oxide end-products (MDA/NO_x) in all hypertensive groups provide strong evidence of the presence of oxidative stress in juvenile EH. In addition, the oxidative stress, characterized by MDA/NO_x ratio showed a significant correlation with BP. Oxidative stress seemed to be more pronounced in overweight hypertensive patients than in hypertensive patients with normal BMI. The decrease of plasma free thiols and increase of red blood cell GSSG in normotensive overweight children allude to the presence of oxidative stress in obesity, irrespectively of hypertension. Our data suggest that oxidative stress may be a pathogenetic factor in juvenile EH, including OH. Obesity *per se* is a pro-oxidant state, and may aggravate oxidative stress, if associated with hypertension.

Conclusion 4. In adolescents with EH the frequency of MT genotype of angiotensinogen (AGT) gene M235T polymorphism was higher than in the normotensive subjects, but no increase in the frequencies of the TT genotype and T allele has been detected. Therefore, the presence of the T allele is not an obvious risk factor for EH. In addition, we failed to demonstrate any other association between the gene polymorphisms of the renin-angiotensin system (RAS) and juvenile EH. We conclude that gene variants of the RAS have no significant influence on the development of EH in adolescents. Clarification of the exact role of these gene polymorphisms in juvenile EH needs further investigations in wider patient population.

Conclusion 5. I/D polymorphism of the angiotensin-converting enzyme (ACE) gene and M235T polymorphism of the AGT gene showed significant associations with pediatric end stage renal disease (ESRD). The increased prevalence of the D allele and DD genotype was demonstrated in this group of patients. The presence of the DD genotype could be a genetic risk factor for renal parenchymal destruction, renal scarring, and the development of ESRD in children, independent of the original renal disease. The MT genotype of AGT gene, which was more frequent in pediatric ESRD, may contribute to the progression of CRF. These genetic associations of pediatric ESRD were not established in adult patients.

Major findings of the thesis

I. **The endothelial dysfunction may play a role in the pathogenesis of juvenile essential hypertension, mainly in patients with normal body weight, but its presence is also detectable in obesity and obesity-induced hypertension.**
II. **Increased oxidative stress state is observed in essential hypertension of adolescents, irrespectively of the body weight. Obesity *per se* (without hypertension) is associated with increased oxidative stress.**
III. **Gene polymorphisms of the renin-angiotensin system have no significant influence on the development of essential hypertension in adolescents.**
IV. **Some gene polymorphisms of the renin-angiotension system (I/D polymorphism of the ACE gene and M235T polymorphism of the angiotensinogen gene) may affect the development of end stage renal disease in pediatric chronic kidney diseases.**

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9. REFERENCES

1. Lawes CM, Vander Hoorn S, Rodgers A; International Society of Hypertension. Global burden of blood-pressure-related disease, 2001. *Lancet* 2008; 371:1513–1518.
2. Falkner B. Prevalence of hypertension in childhood. In: Flynn JT, Ingelfinger JR, Portman RJ (eds). Pediatric hypertension, 2nd ed. Humana Press, New York, 2011, pp. 137–138.
3. Din-Dzietham R, Liu Y, Bielo MV, Shamsa F. High blood pressure trends in children and adolescents in national surveys, 1963 to 2002. *Circulation* 2007; 116:1488–1496.
4. Muntner P, He J, Cutler JA, Wildman RP, Whelton PK. Trends in blood pressure among children and adolescents. *JAMA* 2004; 291:2107–2113.
5. Sorof JM, Lai D, Turner J, Poffenbarger T, Portman RJ. Overweight, ethnicity, and the prevalence of hypertension in school-aged children. *Pediatrics* 2004; 113:475–482.
6. McNiece KL, Poffenbarger TS, Turner JL, Franco KD, Sorof JM, Portman RJ. Prevalence of hypertension and pre-hypertension among adolescents. *J Pediatr* 2007; 150:640–644.
7. Katona É, Zrínyi M, Lengyel Sz, Komonyi É, Paragh Gy, Zatik J, Nagy G, Fülesdi B, Páll D. The prevalence of adolescent hypertension in Hungary-The Debrecen hypertension study. *Blood Pressure* 2011; 20:134–139.
8. Raj M. Essential hypertension in adolescents and children: Recent advances in causative mechanisms. *Indian J Endocr Metab* 2011; 15:367–73.
9. Samuels J. The increasing burden of pediatric hypertension. *Hypertension* 2012; 60:276–277.
10. Ogden CL, Carroll MD, Kit BK, Flegal KM. Prevalence of obesity and trends in body mass index among US children and adolescents, 1999-2010. *JAMA* 2012; 307:483–490.
11. Wijnhoven TMA, van Raaij JMA, Spinelli A, Rito AI, Hovengen R, Kunesova M, Starc G, Rutter H, Sjöberg A, Petrauskiene A, O'Dwyer U, Petrova S, Farrugia Sant'Angelo V, Wauters M, Yngve A, Rubana I-M, Breda J. WHO European Childhood Obesity Surveillance Initiative 2008: weight, height and body mass index in 6–9-year-old children. *Pediatric Obesity* 2013; 8:79–97.
12. Baráth Á, Boda K, Tichy M, Károly É, Túri S. International comparison of blood pressure and BMI values in schoolchildren aged 11–16 years. *Acta Paediatrica* 2010; 99:251–255.
13. Sorof J, Daniels S. Obesity hypertension in children. A problem of epidemic proportions. *Hypertension* 2002; 40:441–447.
14. Oparil S, Zaman MA, Calhoun DA. Pathogenesis of hypertension. *Ann Intern Med* 2003; 139:761–776.
15. Hall JE. Pathophysiology of obesity hypertension. *Curr Hypertens Rep* 2000; 2:139–147.
16. Hall JE, da Silva AA, Brandon E, Stec DE, Ying Z, Jones DW. Pathophysiology of obesity-induced hypertension and target organ damage. In: Lip GYP, Hall JE (eds). Comprehensive hypertension. Elsevier, New York, N.Y., 2007, pp. 447–468.
17. Hall JE, da Silva AA, do Carmo JM, Dubinion J, Hamza S, Munusamy S, Smith G, Stec DE. Obesity-induced hypertension: Role of sympathetic nervous system, leptin, and melanocortins. *J Biol Chem* 2010; 285:17271–17276.

18. Kotchen TA. Obesity-related hypertension: epidemiology, pathophysiology, and clinical management. *Am J Hypertens* 2010; 23:1170–1178.
19. Adly AAM. Oxidative stress and disease: An updated review. *Res J Immunol* 2010; 3: 129–145.
20. Vaziri ND. Causal link between oxidative stress, inflammation, and hypertension. *Iranian J Kidney Dis* 2008; 2:1–10.
21. Deshmukh LP, Rode SD, Wagh PV, Thakur P, Bandawane DD. Oxidative stress and antioxidants in focus: A review. *Inventi Impact: Molecular Pharmacology* 2013:127–134.
22. Shinde A, Ganu J, Naik P. Effect of free radicals & antioxidants on oxidative stress. *J Dent Allied Sci* 2012; 1:63–66.
23. Kohen R, Nyska A. Oxidation of biological systems: Oxidative stress phenomena, antioxidants, redox reactions, and methods for their quantification *Toxicol Pathol* 2002; 30:620–650.
24. Montuschi P, Barnes PJ, Roberts LJ. Isoprostanes: markers and mediators of oxidative stress. *FASEB J* 2004; 18:1791–1800.
25. Lassèque B, Griendling KK. Reactive oxigen species in hypertension. An update. *Am J Hypertens* 2004; 17:852–860.
26. Grossman E. Does increased oxidative stress cause hypertension? *Diabetes Care* 2008; 31:S185–S189.
27. Hirata Y, Satonaka H. Hypertension and oxidative stress. *JMAJ* 2001; 44:540–545.
28. Rodrigo R, Prat H, Passalacqua W, Araya J, Guichard C, Bächler JP. Relationship between oxidative stress and essential hypertension. *Hypertens Res* 2007; 30:1159–1167.
29. Keaney JF, Larson MG, Vasan RS, Wilson PWF, Lipinska I, Corey D, Massaro JM, Sutherland P, Vita JA, Benjamin EJ. Obesity and systemic oxidant stress: clinical correlates of oxidative stress in the Framingham Study. *Arteroscl Thromb Vasc Biol.* 2003; 23:434–439.
30. Furukawa S, Fujita T, Shimabukuro M, Iwaki M, Yamada Y, Nakajima Y, Nakayama O, Makishima M, Matsuda M, Shimomura I. Increased oxidative stress in obesity and its impact on metabolic syndrome. *J Clin Invest* 2004; 114:1752–1761.
31. Morrow JD. Is oxidant stress a connection between obesity and atherosclerosis? *Arterioscler Thromb Vasc Biol.* 2003; 23:368–370.
32. Visser M, Bouter LM, McQuillan GM, Wener MH, Harris TB. Low-grade systemic inflammation in overweight children. *Pediatrics* 2001; 107:1–6.
33. Ceriello A. Possible role of oxidative stress in the pathogenesis of hypertension. *Diabetes Care* 2008; 31(Suppl 2):S181–S184.
34. Böhm F, Pernow J. The importance of endothelin-1 for vascular dysfunction in cardiovascular disease. *Cardiovasc Res* 2007; 76:8–18.
35. Endemann DH, Schiffrin EL. Endothelial dysfunction. *J Am Soc Nephrol* 2004; 15:1983–1992.
36. Verma S, Anderson TJ. Fundamentals of endothelial function for the clinical cardiologist. *Circulation* 2002; 105:546–549.
37. Schiffrin EL. Oxidative stress, nitric oxide synthase, and superoxide dismutase. A matter of imbalance underlies endothelial dysfunction in the human coronary circulation. Hypertension 2008; 51:31–32.

38. Paravicini TM, Touyz RH. NADPH oxidases, reactive oxygen species, and hypertension. *Diabetes Care* 2008; 31(Suppl 2):S170–S180.
39. Hermann M, Flammer A, Lüscher TF. Nitric oxide in hypertension. *J Clin Hypertens* 2006; 8(Suppl 12):17–29.
40. Kleinbongard P, Dejam A, Lauer T, Jax T, Kerber S, Gharini P, Balzer J, Zotz RB, Scharf RE, Willers R, Schechter AN, Feelisch M, Kelm M. Plasma nitrite concentrations reflect the degree of endothelial dysfunction in humans. *Free Radic Biol Med* 2006; 40:295–302.
41. Lauer T, Preik M, Rassaf T, Strauer BE, Deussen A, Feelisch M, Kelm M. Plasma nitrite rather than nitrate reflects regional endothelial nitric oxide synthase activity but lacks intrinsic vasodilator action. *Proc Natl Acad Sci USA* 2001; 98:12814–12819.
42. Pollock DM. Endothelin, angiotensin, and oxidative stress in hypertension. *Hypertension* 2005; 45:477–480.
43. Bereczki Cs, Túri S, Németh I, Sallai É, Torday Cs, Nagy E, Haszon I, Papp F. The roles of platelet function, thromboxane, blood lipids and nitric oxide in hypertension of children and adolescents. *Prostaglandins Leukot Essent Fatty Acids* 2000; 62:293–297.
44. Haszon I, Papp F, Bors M, Bereczki Cs, Túri S. Platelet aggregation, blood viscosity and serum lipids in hypertensive and obese children. *Eur Journal Pediatr* 2003; 162:385–390.
45. Kato N. Genetic analysis in human hypertension. *Hypertens Res* 2002; 25:319–327.
46. Zhu M, Zhao S. Candidate gene identification approach: Progress and challenges. *Int J Biol Sci* 2007; 3:420–427.
47. Malik FS, Lavie CJ, Mehra MR, Milani RV, Re RN. Renin-angiotensin system: genes to bedside. *Am Heart J* 1997; 134:514–526.
48. Winkelmann BR, Russ AP, Nauck M, Klein B, Bohm BO, Maier V, Zotz R, Matheis G, Wolf A, Wieland H, Gross W, Galton DJ, Marz W. Angiotensinogen M235T polymorphism is associated with plasma angiotensinogen and cardiovascular disease. *Am Heart J* 1999; 137:698–705.
49. Bloem LJ, Manatunga AK, Tewksbury DA, Pratt JH. The serum angiotensinogen concentration and variants of the angiotensinogen gene in white and black children. *J Clin Invest* 1995; 95:948–953.
50. Rigat B, Hubert C, Alhene-Gelas F, Cambien F, Corvol P, Soubrier F. An insertion/deletion polymorphism in the angiotensin I-converting enzyme gene accounting for half the variance of serum enzyme levels. *J Clin Invest* 1990; 86:1343–1346.
51. Costerousse O, Allegrini J, Lopez M, Alhene-Gelas F. Angiotensin I-converting enzyme in human circulating mononuclear cells: genetic polymorphism of expression in T-lymphocytes. *Biochem J* 1993; 290:33–40.
52. Danser AH, Schalekamp MA, Bax WA, van den Brink AM, Saxena PR, Riegger GA, Schunkert H. Angiotensin-converting enzyme in the human heart. Effect of the deletion/insertion polymorphism. *Circulation* 1995; 92:1387–1388.
53. Duncan JA, Scholey JW, Miller JA. Angiotensin II type 1 receptor gene polymorphisms in humans: physiology and pathophysiology of the genotypes. *Curr Opin Nephrol Hypertens* 2001; 10:111–116.
54. Tanira MOM, Al Balushi KA. Genetic variations related to hypertension: a review. *J Hum Hypertens* 2005; 19:7–19.

55. Baráth Á, Endreffy E, Bereczki C, Gellén B, Szűcs B, Németh I, Túri S. Endothelin-1 gene and endothelial nitric oxide synthase gene polymorphisms in adolescents with juvenile and obesity-associated hypertension. *Acta Physiol Hung* 2007; 94:49–66.
56. Liu Y. Renal fibrosis: New insight into the pathogenesis and therapeutics. *Kidney Int* 2006; 69:213–217.
57. Eddy AA. Molecular basis of renal fibrosis. *Pediatr Nephrol* 2000; 15:290–301.
58. El Nahas AM, Muchaneta-Kubara EC, Essawy M, Soylemezoglu O. Renal fibrosis: Insight into pathogenesis and treatment. *Int J Biochem Cell Biol* 1997; 29:55–62.
59. Mezzano SA, Ruiz-Ortega M, Egido J. Angiotensin II and renal fibrosis. *Hypertension* 2001; 38:635–638.
60. Matsusaka T, Hymes J, Ichikawa I. Angiotensin in progressive renal diseases: theory and practice. *J Am Soc Nephr* 1996; 7:2027–2043.
61. Mizuiri S, Hemmi H, Kumanomidou H, Iwamoto M, Miyagi M, Sakai K, Aikawa A, Ohara T, Yamada K, Shimatake H, Hasegawa A. Angiotensin-converting enzyme (ACE) I/D genotype and renal ACE gene expression. *Kidney Int* 2001; 60:1124–1130.
62. National High Blood Pressure Education Working Group on Hypertension Control in Children and Adolescents. Update on the Task Force Report (1987) on High Blood Pressure in Children and Adolescents: a Working Group Report from the National High Blood Pressure Education Program. *Pediatrics* 1996; 98:649–658.
63. Soergel M, Kirschstein M, Busch C, Danne T, Gellermann J, Holl R, et al. Oscillometric twenty-four-hour ambulatory blood pressure values in healthy children and adolescents: a multicenter trial including 1141 subjects. *J Pediatr* 1997; 130:178–184.
64. Marzinzig M, Nussler AK, Stadler J, Marzinzig E, Barthlen W, Nussler NC, et al. Improved methods to measure end-products of nitric oxide in biological fluids: nitrite, nitrate, and S-nitrosothiols. *Nitric Oxide* 1997; 1:177–189.
65. Wong SHY, Knight JA, Hopfer SM, Zacharia O, Leach CN, Sunderman FW. Lipoperoxides in plasma as measured by liquid-chromatographic separation of malondialdehyde–thiobarbituric acid adduct. *Clin Chem* 1987; 33:214–220.
66. Prakash M, Shetty MS, Tilak P, Anwar N. Total thiols: Biomedical importance and their alteration in various disorders. *Online J Health Allied Scs* 2009; 8:2.
67. Koster JP, Biemond P, Swaak AJ. Intracellular and extracellular sulphydryl levels in rheumatoid arthritis. *Ann Rheum Dis* 1986; 45:44–46.
68. Kristal B, Shurtz-Swirski R, Chezar J, Manaster J, Levy R, Shapiro G, Weissman I, Shasha SM, Sela S. Participation of peripheral polymorphonuclear leukocytes in the oxidative stress and inflammation in patients with essential hypertension. *Am J Hypertens* 1998; 11:921–928.
69. Winterbourn CC, Stern A. Human red cells scavenge extracellular hydrogen peroxide and inhibit formation of hypochlorous radical. *J Clin Invest* 1987; 80:1486–1491.
70. Németh I, Boda D. Blood glutathione redox ratio as a parameter of oxidative stress in premature infants with IRDS. *Free Radic Biol Med* 1994; 16:347–353.
71. Tietze F. Enzymic method for quantitative determination of nanogram amounts of total and oxidized glutathione: application to mammalian blood and other tissues. *Anal Biochem* 1969; 27:502–522.
72. Beutler E. The glutathione instability of drug-sensitive red cells. A new method for the in vitro detection of drug sensitivity. *J Lab Clin Med* 1957; 49:84–95.

73. Beutler E. G6PD: population genetics and clinical manifestation. *Blood Rev* 1996; 10:45–52.
74. Baechner RL, Nathan DG, Castle WB. Oxidant injury of caucasian glucose-6-phosphate dehydrogenase deficient red blood cells by phagocytosing leukocytes during infection. *J Clin Invest* 1971; 50:2466–2473.
75. Woodhead JL, Fallon R, Figuerido H, Langdale J, Malcolm ABO. Alternative methods of gene diagnosis. In: Davies KE (ed). Human genetic diseases. A practical approach. IRL Press, 1987, pp. 56–57.
76. Chiu KC, McCarthy JE. The insertion allele at the angiotensin I converting gene locus is associated with insulin resistance. *Metabolism* 1997; 46:395–399.
77. Malin R, Wirta V, Hiltunen TP, Lehtimaki T. Rapid detection of angiotensinogen M/T235 polymorphism by fluorescence probe melting curves (letter). *Clin Chem* 2000; 46:880–881.
78. Troiano RP, Flegal KM. Overweight children and adolescents: description, epidemiology, and demographics. *Pediatrics* 1998; 101:497–504.
79. Cole TJ, Bellizi MC, Flegal KM, Dietz WH. Establishing a standard definition for child overweight and obese worldwide: an international survey. *BMJ* 2000; 320:1240–1243.
80. Túri S, Baráth Á, Boda K, Tichy M, Károly É. Blood pressure reference tables for Hungarian adolescents aged 11–16 years. *Kidney Blood Press Res* 213
81. Kumar CA, Das UN. Lipid peroxides, anti-oxidants and nitric oxide in patients with pre-eclampsia and essential hypertension. *Med Sci Monit* 2000; 6:901–907.
82. Vaziri ND, Wang XQ, Oveisi F, Rad B. Induction of oxidative stress by glutathione depletion causes severe hypertension in normal rats. *Hypertension* 2000; 36:142–146.
83. Prasad A, Andrews NP, Padder FA, Husain M, Quyyumi AA. Glutathione reverses endothelial dysfunction and improves nitric oxide bioavailability. *J Am Coll Cardiol* 1999; 34:507–514.
84. Ceriello A, Giugliano D, Quatraro A, Lefebvre PJ. Antioxidants show an antihypertensive effect in diabetic and hypertensive subjects. *Clin Sci* 1991; 81:739–742.
85. Buraczynska M, Ksiazek P, Drop A, Zaluska W, Spasiewicz D, Ksiazek A. Genetic polymorphisms of the renin-angiotensin system in end-stage renal disease. *Nephrol Dial Transplant* 2006; 21: 979–983.
86. Pratt RE (1999) Angiotensin and control of cardiovascular structure. *J Am Soc Nephrol* 10:S120–S128.
87. Ruggenenti P, Bettinaglio P, Pinares F, Remuzzi G. Angiotensin converting enzyme insertion/deletion plymorphism and renoprotection in diabetic and nondiabetic nephropathies. *Clin J Am Soc Nephrol* 2008; 3:1511–1525.
88. Hohenfellner K, Hunley TE, Brezinska R, Brodhag P, Shyr Y, Brenner W, Habermehl P, Kon V. ACE I/D gene polymorphism predicts renal damage in congenital uropathies. *Pediatr Nephrol* 1999; 13:514–518.
89. El-Essawy AB, Berthoux P, Cécillon S, Depréle C, Thibaudin D, De Fillippis J-P. Alamartine E, Berthoux F. Hypertension after renal transplantation and polymorphism of genes involved in essential hypertension: ACE, AGT, AT1R and ecNOS. *Clin Nephrol* 2002; 57:192–200.

90. Bengtsson K, Orho-Melander M, Lindblad U, Melander O, Bøg-Hansen E, Ranstam J, Rastam L, Groop L. Polymorphism in the angiotensin converting enzyme but not in the angiotensinogen gene is associated with hypertension and type 2 diabetes: the Skaraborg Hypertension and diabetes project. *J Hypertens* 1999; 17:1569–1575.
91. Sagnella GA, Rothwell MJ, Onipinla AK, Wicks PD, Cook DG, Capuccio FP. A population study of ethnic variations in the angiotensin-converting enzyme I/D polymorphism: relationships with gender, hypertension and impaired glucose metabolism. *J Hypertens* 1999; 17:657–664.
92. Freire MBS, van Dijk DJ, Erman A, Boner G, Warram JH, Krolewski AS. DNA polymorphisms in the ACE gene, serum ACE activity and the risk of nephropathy in insulin-dependent diabetes mellitus. *Nephrol Dial Transplant* 1998; 13:2553–2558.
93. Jeunemaitre X, Soubrier F, Kotelevtsev YV, Lifton RP, Williams CS, Charru A, Hunt SC, Hopkins PN, Williams RR, Lalouel JM. Molecular basis of human hypertension: role of angiotensinogen. *Cell* 1992; 71:169–180.
94. Jeunemaitre X, Charru A, Chatellier G, Dumont C, Sassano P, Soubrier F, Menard J, Corvol P. M235T variant of the human angiotensinogen gene in unselected hypertensive patients. *J Hypertens* 1993; 11:S80–S81.
95. Katsuya T, Koike G, Yee TW, Sharpe N, Jackson R, Norton R, Horiuchi M, Pratt RE, Dzau VJ, MacMahon S. Association of angiotensinogen gene T235 variant with increased risk of coronary heart disease. *Lancet* 1995; 345:1600–1603.
96. Ishigami T, Umemura S, Iwamoto T, Tamura K, Hibi K, Yamaguchi S, Nyuui N, Kimura K, Miyazaki N, Ishii M. Molecular variant of angiotensinogen gene is associated with coronary atherosclerosis. *Circulation* 1995; 91:951–954.
97. Caulfield M, Lavender P, Farrall M, Monroe P, Lawson M, Turner P, Clark AJL. Linkage of the angiotensinogen gene to essential hypertension. *N Engl J Med* 1994; 330:1629–1633.
98. Gumprecht J, Zychma MJ, Grzeszczak W, Zukowska-Szczechowska E, End-Stage Renal Disease Study Group. Angiotensin I-converting enzyme gene insertion/deletion and angiotensinogen M235 T polymorphisms: risk of chronic renal failure. *Kidney Int* 2000; 58:513–519.
99. Lin J, Hu FB, Qi L, Curhan GC. Genetic polymorphisms of angiotensin-2 type I receptor and angiotensinogen and risk of renal dysfunction and coronary heart disease in type 2 diabetes mellitus. *BMC Nephrology* 2009; 10:9.
100. Young RP, Chan JCN, Critchley JAJH, Poon E, Nicholls G, Cockram CS. Angiotensinogen T235 and ACE insertion/deletion polymorphisms associated with albuminuria in Chinese type 2 diabetic patients. *Diabetes Care* 1998; 21:431–437.
101. Maruyama K, Yoshida M, Nishio H, Shirakawa T, Kawamura T, Tanaka R, Nakamura H, Iijima K, Yoshikawa N. Polymorphisms of renin-angiotensin system genes in childhood IgA nephropathy. *Pediatr Nephrol* 2001; 16:350–355.
102. Wong TY, Chan JC, Poon E, Li PK. Lack of association of angiotensin-converting enzyme (DD/II) and angiotensinogen M235T gene polymorphism with renal function among Chinese patients with type II diabetes. *Am J Kidney Dis* 1999; 33:1064–1070.
103. Frimat L, Philippe C, Maghakian MN, Jonveaux P, Hurault de Ligny B, Guillemin F, Kessler M. Polymorphism of angiotensin converting enzyme, angiotensinogen, and angiotensin II type 1 receptor genes and end-stage renal failure in IgA nephropathy: IGARAS-a study of 274 Men. *J Am Soc Nephrol* 2000; 11:2062–2067.

104. Lee KB, Kim UK. Angiotensinogen and angiotensin II type 1 receptor gene polymorphism in patients with autosomal dominant polycystic kidney disease: effect on hypertension and ESRD. *Yonsei Med J* 2003; 44:641–647.
105. Zhou TB, Yin SS, Qin YH. Association of angiotensinogen M235T gene polymorphism with end-stage renal disease risk: a meta-analysis. *Mol Biol Rep* 2013; 40:765–772.
106. Zee RYL, Lou YK, Lyn R, Morris BJ. Association of a polymorphism of the angiotensin I-converting enzyme gene with essential hypertension. *Biochem Biophys Res Commun* 1992; 184:9–15.
107. Schunkert H, Hense HW, Holmer SR, Stender M, Perz S, Keil U, Lorell BH, Riegger GA. Association between a deletion polymorphism of the angiotensin converting enzyme and left ventricular hypertrophy. *N Eng J Med* 1994; 330:1634–1638.
108. Iwai N, Ohmichi N, Nakamura Y, Kinoshita M. DD genotype of the angiotensin-converting enzyme is a risk factor for left ventricular hypertrophy. Circulation 1994; 90:2622–2628.
109. Cambien F. The angiotensin-converting enzyme (ACE) genetic polymorphism: its relationship with plasma ACE level and myocardial infarction. *Clin Genet* 1994; 46:94–101.
110. Butler R, Morris AD, Burchell B, Struthers AD. DD angiotensin-converting enzyme gene polymorphism is associated with endothelial dysfunction in normal humans. *Hypertension* 1999; 33:1164–1168.
111. Hohenfellner K, Wingen AM, Nauroth O, Wühl E, Mehls O, Schaefer F. Impact of ACE I/D gene polymorphism on congenital renal malformations. *Pediatr Nephrol* 2001; 16:356–361.
112. Ozen S, Alikasifoglu M, Saatci U, Bakkaloglu A, Besbas N, Kara N, Kocak H, Erbas B, Unsal I, Tuncbilek E. Implications of certain genetic polymorphisms in scarring in vesicoureteric reflux: importance of ACE polymorphism. *Am J Kidney Dis* 1999; 34:140–145.
113. Haszon I, Friedman AL, Papp F, Bereczki C, Baji S, Bodrogi T, Károly E, Endreffy E, Túri S. ACE gene polymorphism and renal scarring in primary vesicoureteric reflux. *Pediatr Nephrol* 2002; 17:1027–1031.
114. Frishberg Y, Becker-Cohen R, Halle D, Feigin E, Eisenstein B, Halevy R, Lotan D, Juabeh I, Ish-Shalom N, Magen D, Shvil Y, Sinai-Treiman L, Drukker A. Genetic polymorphisms of the renin-angiotensin system and the outcome of focal segmental glomerulosclerosis in children. *Kidney Int* 1998; 54:1843–1849.
115. Syrjänen J, Huang XH, Mustonen J, Koivula T, Lehtimäki T, Pasternack A. Angiotensin-converting enzyme insertion/deletion polymorphism and prognosis of IgA nephropathy. *Nephron* 2000; 86:115–121.
116. Stratta P, Canavese C, Ciccone G, Barolo S, Dall'Omo AM, Fasano ME, Mazzola G, Berutti S, Fop F, Curtoni ES, Piccoli G. Angiotensin I-converting enzyme genotype significantly affects progression of IgA glomerulonephritis in an italian population. *Am J Kidney Dis* 1999; 33:1071–1079.
117. Samuelson O, Attman P-O, Larsson R, Mulec H, Rymo L, Weiss L, Ricksten A. Angiotensin I-converting enzyme gene polymorphism in non-diabetic renal disease. *Nephrol Dial Transpl* 2000; 15:481–486.
118. Pérez-Oller L, Torra R, Badenas C, Milà M, Darnell A. Influence of the ACE gene polymorphism in the progression of renal failure in autosomal dominant polycystic kidney disease. *Am J Kidney Dis* 1999; 34:273–278.

119. Saggar-Malik AK, Afzal AR, Swissman JS, Bland M, Sagnella GA, Eastwood JB, MacGregor GA, Jeffery S Lack of association of ACE/angiotensinogen genotype with renal function in autosomal dominant polycystic kidney disease. *Genet Test* 2000; 4:299–303.
120. Bonnardeaux A, Davies E, Jeunemaitre X, Fery I, Charru A, Clauser E, Tiret L, Cambien F, Corvol P, Soubrier F. Angiotensin II type 1 receptor gene polymorphisms in human essential hypertension. *Hypertension* 1994; 24:63–69.
121. Benetos A, Topouchian J, Ricard S, Gautier S, Bonnardeaux A, Asmar R, Poirier O, Soubrier F, Safar M, Cambien F. Influence of angiotensin II type 1 receptor polymorphism on aortic stiffness in never treated hypertensive patients. *Hypertension* 1995; 26:44–47.
122. Hingorani AD, Jia H, Stevens PA, Hopper R, Dickerson JE, Brown MJ. Renin-angiotensin system gene polymorphisms influence blood pressure and the response to angiotensin converting enzyme inhibition. *J Hypertens* 1995; 13:1602–1609.
123. Wang WY, Zee RY, Morris BJ. Association of angiotensin II type 1 receptor gene polymorphism with essential hypertension. *Clin Genet* 1997; 51:31–34.
124. Tiret L, Bonnardeaux A, Poirier O, Ricard S, Marques-Vidal P, Evans A, Arveiler D, Luc G, Kee F, Ducimetiere P, Soubrier F, Cambien F. Synergistic effects of angiotensin-converting enzyme and angiotensin-II type 1 receptor gene polymorphism on risk of myocardial infarction. *Lancet* 1994; 344: 910–913.
125. Poirier O, Georges JL, Ricard S, Arveiler D, Ruidavets JB, Luc G, Evans A, Cambien F, Tiret L. New polymorphisms of the angiotensin II type 1 receptor gene and their associations with myocardial infarction and blood pressure: the ECTIM study. *J Hypertens* 1998; 16:1443–1447.
126. van Geel PP, Pinto YM, Voors AA, Buikema H, Oosterga M, Crijns HJ, van Gilst WH. Angiotensin II type 1 receptor A1166C gene polymorphism is associated with an increased response to angiotensin II in human arteries. *Hypertension* 2000; 35:717–721.

Appendix

Copy of the original papers directly related to the thesis