ASSESSMENT OF MICROVASCULAR REACTIVITY AND OXIDATIVE STRESS IN HYPERTENSIVE ADOLESCENTS AND HEMODIALYSIS PATIENTS

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LIST OF ABBREVIATIONS

ACE	Angiotensin-converting enzyme
ACH	Acetylcholine
AIx	Augmentation index
Ang II	Angiotensin II
ANOVA	Analysis of variance
BMI	Body mass index
BP	Blood pressure
CAT	Catalase
CKD	Chronic kidney disease
E-CAT	Erythrocyte catalase
E-MDA	Erythrocyte malondialdehyde
EPO	Erythropoietin
ESA	Erythropoiesis-stimulating agent
E-SOD	Erythrocyte superoxide dismutase
ESRD	End-stage renal disease
ET-1	Endothelin-1
FMD	Flow-mediated dilation
FOXO	Forkhead box class O
GPx	Glutathione peroxidase
GSH	Reduced glutathione
GSSG	Oxidized glutathione
GSSG/GSH	Ratio oxidized/reduced glutathione
Hb	Hemoglobin
HD	Hemodialysis; hemodialyzed
HDL	High-density lipoprotein
HPLC	High-performance liquid chromatography
JNK	cJun-*N*-terminal kinase
LDF	Laser Doppler flowmetry
LDI	Laser Doppler imaging
LDL	Low-density lipoprotein
MDA	Malondialdehyde
NO	Nitric oxide
NO_x	Plasma nitric oxide end-products
PON1	Paraoxonase-1
PORH	Postocclusive reactive hyperemia
PWV	Pulse wave velocity
RBC	Red blood cell
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SNP	Sodium nitroprusside
SOD	Superoxide dismutase

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1. INTRODUCTION

1.1. Reactive species and antioxidants: physiology and pathophysiology

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are products of the normal cellular metabolism and play important roles in various physiological processes [1, 2]. These include the defense against microorganisms (the oxidative burst in phagocytes); the redox regulation of the cell cycle; and the modulation of vascular tone, blood pressure (BP) and platelet aggregation, mediated by nitric oxide (NO) [1, 2].

Defense mechanisms against elevated concentrations of ROS and RNS involve enzymatic antioxidants such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and the peroxiredoxin–thioredoxin–thioredoxin reductase system [1-3]; and nonenzymatic scavenging antioxidants, represented by reduced glutathione (GSH), ascorbic acid (vitamin C), alfa-tocopherol (vitamin E), carotenoids, flavonoids, urate and other antioxidants (Figure 1). Under normal conditions, the cell maintains a balance between the prooxidants and antioxidants, a state referred to as “redox balance” or “redox homeostasis”. This equilibrium is essential for the survival of organisms and their health [1, 2]. Thus, antioxidants not only serve as defense mechanisms against an overproduction of reactive species, but their proper functioning also allows ROS and RNS to fulfil their physiological roles [1-3].

Oxidative stress is defined as a disturbance in the prooxidant-antioxidant balance in favor of the former, leading to potential damage to important biomolecules (nucleic acids, lipids and proteins), often mediated by transition metals (iron, copper) [1-4]. ROS can react with all components of the DNA molecule, both the purine and pyrimidine bases and the deoxyribose chain. Permanent modification of the genetic material by ROS is the first step involved in mutagenesis, carcinogenesis and ageing [1]. ROS also attack polyunsaturated fatty acid residues of phospholipids, resulting in a decreased membrane fluidity, increased leakiness of the membrane, and inactivation of receptors and ion channels, as well as the formation of lipid peroxides and their by-products malondialdehyde (MDA), 4-hydroxy-2-nonenal (both mutagenic and toxic) and F_2-isoprostanes (Figure 1) [1-3]. Additionally, ROS and RNS may

induce damage to proteins, either reversible (such as the formation of mixed disulfides between protein thiol groups and low-molecular-weight thiols, in particular GSH (*S*-glutathionylation)) or irreversible (carbonylation, nitrosylation and hydroxylation, resulting in generation of carbonylated proteins, nitrotyrosine, 3,4-dihydroxy-phenylalanine, ortho-tyrosine and meta-tyrosine) [1, 5, 6]. Such modifications can be detected in cardiovascular (e.g. hypertension, obesity and atherosclerosis), renal (chronic kidney disease (CKD) and end-stage renal disease (ESRD)) and pulmonary diseases (for example asthma and cystic fibrosis), and also in cancer and diabetes [1-7]. These modified molecules can serve as representative biomarkers of the oxidative/nitrosative damage involved in the pathophysiology of the diseases [4].

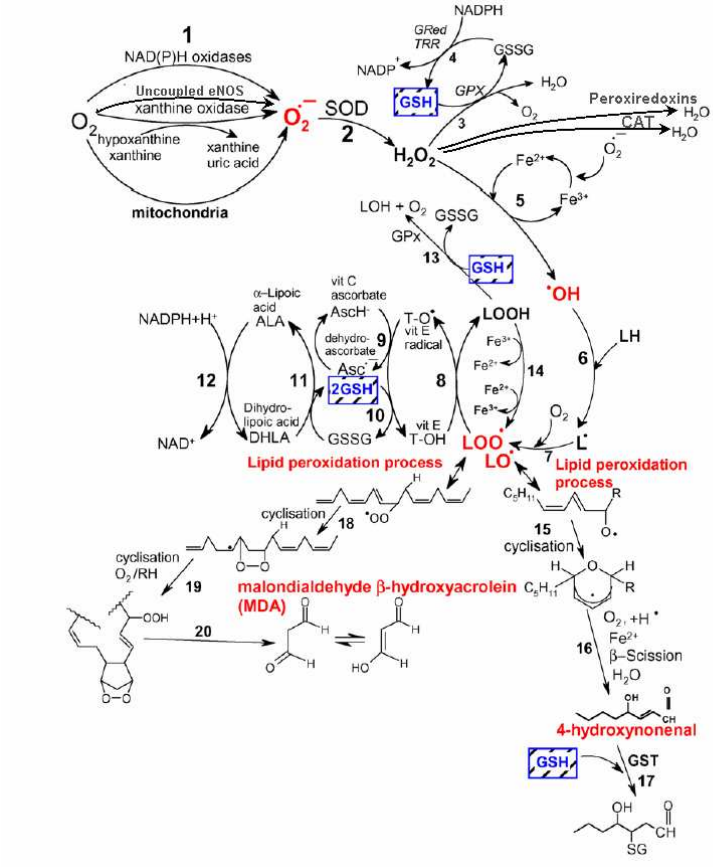

Figure 1: Pathways of ROS formation, the lipid peroxidation process and the role of enzymatic and nonenzymatic antioxidants in the management of oxidative stress. From Valko M. *et al.* Int J Biochem Cell Biol. 2007; 39: 44-84. [1]

1.2. Oxidative stress in pathological states

1.2.1. Primary hypertension and obesity in childhood: two interrelated diseases

The definition of juvenile hypertension is based on the normative distribution of BP in healthy children, as a systolic and/or diastolic BP $\geq 95^{th}$ percentile for age, gender and height (on at least three separate occasions) by The Fourth Report on the Diagnosis, Evaluation, and Treatment of High Blood Pressure in Children and Adolescents [8]. Even if hypertension in childhood is often of secondary origin [9-11] (in contrast with that in adulthood [12-14]), the prevalence of juvenile primary hypertension increases markedly with age, in particular from puberty [9, 10].

Primary (earlier term: essential) hypertension is a multifactorial and polygenic disorder in which the individual's genetic background, and various demographic and environmental factors play roles in the development of elevated BP [10, 12-15]. Factors that may contribute to the development and aggravation of hypertension include obesity, insulin resistance, renal microvascular alterations, oxidative stress and an endothelial dysfunction [13]. The major pathways leading to an elevation of BP via ROS formation are depicted in Figure 2 [16]. The inducing effects of various agents, such as salt, vasopressin, endothelin-1 (ET-1), renin, angiotensin II (Ang II) and aldosterone, lead to an increased abundance of ROS, which, in turn, can induce vasoconstriction, vascular and myocardial hypertrophy, a decreased kidney function, and increased sympathetic efferent activity from the central nervous system, contributing to the development of hypertension [16]. The closed reaction loops, leading to the uncoupling of nitric oxide synthase, the activation of xanthine oxidase, and the generation of ET-1 and Ang II, are possible mechanisms of persistence of the BP elevation (Figure 2) [16].

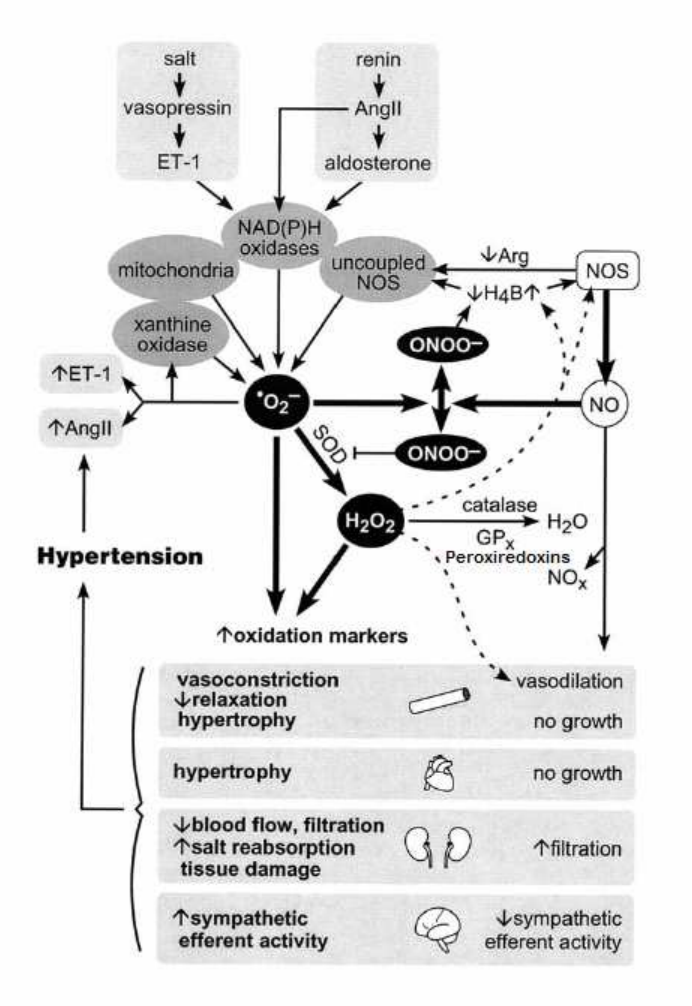

Figure 2: Major pathways leading to hypertension via reactive oxygen species formation. From Lassegue B, Griendling KK. Reactive oxygen species in hypertension. Am J Hypertens. 2004; 17: 852-860. [16]

An increasing number of children and adolescents exhibit excess weight, which comprises a major issue in public health due to the increased risk of development of chronic diseases [17]. The prevalence of overweight in adolescents varies from under 5% to more than 20%, being higher in the Western and Southern regions of Europe and in the United States [18, 19]. However, the exact number depends on geographical and ethnical factors, and the definition of obesity itself [18-20].

The body mass index (BMI) is the parameter most widely used to assess childhood overweight, even if it is only an indirect measure of adiposity and has limitations as compared with other techniques [17, 20]. Due to the fact that the BMI in children varies greatly with age and sex [20], percentiles (such as those specified by the Centers for Disease Control and

Prevention in 2000 [21]), or the age and sex-specific cut-off points set by the International Obesity Task Force [22] are used to define childhood overweight and obesity.

Obesity *per se* exerts unfavorable effects on several organs such as the lung, liver, kidney and heart [17], and is associated with a proinflammatory state [23] and activation of vascular endothelial cells and platelets [24], as well as higher prevalences of insulin resistance [25], type 2 diabetes [26] and dyslipidemia [27]. The above alterations and the increased cardiovascular risk of overweight subjects have been shown in obesity intervention programs to be partly reversible [28-31]. It is noteworthy that the level of oxidative stress is increased and the antioxidant defense is compromised in obesity [32], as shown by the increased formation of superoxide [33] and peroxyl radicals [34], higher levels of MDA [35] and F_2-isoprostanes [24], reduced concentrations of alfa-tocopherol and beta-carotene [36], and lower activities of SOD and GPx [37].

Excess weight is one of the most important factors predisposing to hypertension [38]. The interrelation between overweight and hypertension is complex, involving (among others) activation of the sympathetic and renin-angiotensin-aldosterone systems, and various metabolic and vascular changes (e.g. insulin resistance, decreased NO availability and renal sodium excretion, and increased levels of plasma lipids and ET-1), often manifested as the metabolic syndrome [38-40]. These effects have been suggested to be mediated, in part, by hyperleptinemia and leptin resistance in obesity [40].

In view of the facts that adult hypertension [41, 42] and obesity [37] are associated with an increased level of oxidative stress, we previously hypothesized that oxidative stress may be an important feature in juvenile hypertension too. In line with this, increased levels of oxidized glutathione (GSSG), elevated ratios of oxidized/reduced glutathione (GSSG/GSH), decreased GSH levels, impaired GSH regeneration and a more pronounced lipid peroxidation have been demonstrated in adolescent hypertensives [43]. However, no differences were observed in the paraoxonase-1 (PON1) activities, PON1 Q192R genotype or homocysteine levels in this patient group, irrespective of the degree of obesity [44]. Next, we set out to examine whether a short-term antihypertensive treatment could reveal differences in markers of oxidative stress and endothelial dysfunction between lean or obese hypertensive adolescents [45]. Before medication, elevated activities of the angiotensin-converting enzyme (ACE) were found in the lean hypertensives as compared with the controls. In contrast, the obese hypertensive group

demonstrated a higher heart rate and elevated levels of xanthine oxidase activity, ET-1 and leptin at baseline. In both groups, the levels of the plasma NO end-products (NO_x) and free thiols were decreased, and the ratios MDA/NO_x were increased before treatment. ACE inhibitor therapy normalized the levels of NO_x and the ratios MDA/NO_x only in the obese hypertensives. These results suggested that the pathomechanisms of juvenile hypertension associated with normal body weight or obesity may differ [45].

1.2.2. Hemodialysis

The incidences of CKD and ESRD show increasing trends, in part due to the ever more frequent prevalence of their underlying diseases, in particular diabetes [46]. Other causes include hypertension, glomerulonephritis, chronic pyelonephritis and cystic kidney disease [46]. The risk of cardiovascular events in ESRD patients is 3.5-50 times higher than that in the general population [7]. Besides classical cardiovascular risk factors (e.g. diabetes, hypertension and dyslipidemia), uremia-associated factors such as a chronic volume expansion, inflammation, oxidative stress and anemia also play roles [7, 47].

Uremia itself is regarded as a prooxidant state, and the oxidative stress becomes more severe together with the severity of CKD [7, 48]. Despite beneficial effects due to the removal of uremic toxins and excess fluid [49], hemodialysis (HD) sessions may themselves induce repetitive bouts of oxidative stress [7, 50, 51], as indicated by increased levels of MDA and organic hydroperoxides, decreased activities of SOD and GPx, and lower concentrations of selenium, zinc and copper [52]. The dialysis modalities, including the dialysis dose and frequency [49, 53], the type of dialysis membrane used [54-56], and the presence of glucose in the dialyzing fluid [57], may also affect oxidative stress.

Anemia is a common feature of CKD and ESRD, and is associated with elevated levels of morbidity and mortality in HD patients [58]. The current Revised European Best Practice Guidelines for the Management of Anemia in Patients with Chronic Renal Failure [59] state that the fundamental treatment choices in anemia correction are the administration of erythropoiesis-stimulating agents (ESAs), iron and adjuvant vitamins (for example, vitamin E, vitamin C and folate), and in HD patients, optimization of the dialysis process [59].

Despite a transient increase in the levels of oxidative markers after the initiation of ESA therapy [60] (preventable with simultaneous vitamin E administration [61]), long-term ESA treatment is thought to have antioxidant effects [62-66], though the mechanisms have not been well established [67]. These mechanisms are suggested to include elevated expressions of the enzymes SOD, CAT, GPx [62-65] and heme oxygenase-1 [66], and attenuated lipid peroxidation [64, 65]. Factors associated with the reduction of iron-dependent oxidative injury [68] and the correction of anemia [65, 69] may also play roles in the improvement of oxidative stress. Before our studies, however, it was not known whether a short-term withdrawal of ESA treatment has an influence on the indices of oxidative stress.

1.3. Assessment of the vascular system in pathological states

1.3.1. Studies on large vessels

Among the various techniques developed for the assessment of the macrovasculature, the measurement of arterial stiffness is being increasingly used in the clinical setting [70]. The pulse wave velocity (PWV) and the augmentation index (AIx) are reproducible parameters with which to characterize arterial stiffness and pulse wave reflection, respectively [70], as measured noninvasively by means of applanation tonometry, piezoelectricity or oscillometry (a recent technique developed by Illyés and colleagues) [71-73]. The carotid-femoral PWV (considered the gold-standard measurement of arterial stiffness [70]) is elevated in adult patients with obesity [74] and uncomplicated primary hypertension [75], and in those on HD [76]. The carotid-femoral PWV is of independent predictive value for all-cause mortality, cardiovascular morbidity, coronary events and stroke in hypertension [75] and HD [76]. In adult hypertensives with or without the metabolic syndrome, the peripheral (radial) PWV and AIx have likewise been reported to be increased [77]. As concerns juvenile primary hypertension, the brachial-ankle PWV has been reported to be greater in hypertensive adolescents, but the patients were not selected according to the degree of adiposity [78].

Another technique, forearm venous occlusion plethysmography, is based on the interruption of the venous outflow from the arm by the compression of a cuff at a pressure that leaves the arterial flow unaltered [79]. This causes the forearm blood volume to rise. The

rate and degree of swelling reflects the forearm vascular resistance, which, in turn, is related to the vascular endothelial function [79, 80]. Venous occlusion plethysmography is often used in combination with complete occlusion and subsequent release of the arm (postocclusive reactive hyperemia (PORH) test) or brachial artery catheterization [79, 80]. Even if the latter allows assessment of the responses to endothelium-dependent and -independent vasoactive agents, its applicability is limited due to its invasive nature [79]. As examples, the impaired endothelial function of the brachial artery was confirmed in both hypertensive adults [81] and HD patients [82] in earlier studies in which this technique was used.

The working principle of the third method, brachial artery flow-mediated dilation (FMD), is the change in the arterial diameter in response to increased shear stress [79]. Dilation of the artery after the PORH test or due to a sublingual dose of nitroglycerin (endothelium-independent vasodilation) is monitored by means of vascular ultrasonography [79]. Even if brachial artery FMD has some limitations (e.g. substantial intra- and inter-observer variance), this technique is most commonly performed and best validated for the noninvasive assessment of the conduit vessel function [83]. FMD has been reported to be impaired in obese children [23, 84] and in patients on HD [85]. A relationship between the brachial artery FMD, the carotid artery intima-media thickness and the levels of vascular inflammatory markers was recently demonstrated in obese hypertensive children [86]. Importantly, a decreased brachial artery FMD in adult hypertension [77, 87] has been suggested to have a prognostic role in identifying patients at higher risk of nonfatal or fatal cardiovascular events [88].

1.3.2. Assessment of the microvasculature

The definition of “microcirculation” is based on the vessel physiology rather than diameter or structure [89]. Accordingly, all vessels that respond to increasing pressure by a myogenic reduction in lumen diameter are considered part of the microcirculation. Such a definition includes the smallest arteries and arterioles in the microvasculature in addition to capillaries and venules [89]. The functions of the microcirculation include the delivery of nutrients and the removal of waste products from all cells of the body; the avoidance of large fluctuations in hydrostatic pressure at the level of the capillaries that would otherwise impair capillary

exchange; and a contribution to the overall peripheral resistance (due to a substantial drop in hydrostatic pressure at this level of the circulation) [89].

Laser Doppler flowmetry (LDF) and laser Doppler imaging (LDI) are noninvasive means of assessment of the microvascular function [90, 91]. This technique is based on the scattering and frequency-shift of monochromatic laser light due to its interaction with moving blood cells (Figure 3A) [90, 91]. Laser Doppler methods measure the microvascular blood perfusion (termed the flux), which is the product of the velocity and concentration of the moving blood cells within the measured volume [90]. LDF and LDI provide an estimate of perfusion up to a depth of 1 to 1.5 mm into the dermis and thus mainly measure the perfusion in the arterioles, venules and capillaries [91]. LDI makes use of a laser beam that scans across a predetermined area, resulting in a two-dimensional map of the blood perfusion. LDI has a better spatial resolution and smaller site-to-site variability as compared with LDF [91]. LDF, in turn, allows continuous monitoring of the flux at a specified point of the skin, offers higher sensitivity and has the ability to detect rapid perfusion changes [90].

Due to the relatively large spatial and temporal variabilities of the technique, the response of the microvessels to provocation tests is assessed, instead of the basal flux. These tests include thermal challenges (local or systemic heating or cooling), iontophoretic administration of vasoactive drugs, the PORH test, or a combination of them (Figure 3B) [90, 91]. For further details relating to the standardization of microvascular reactivity measurements with the LDF technique, see Section 2.1.1.

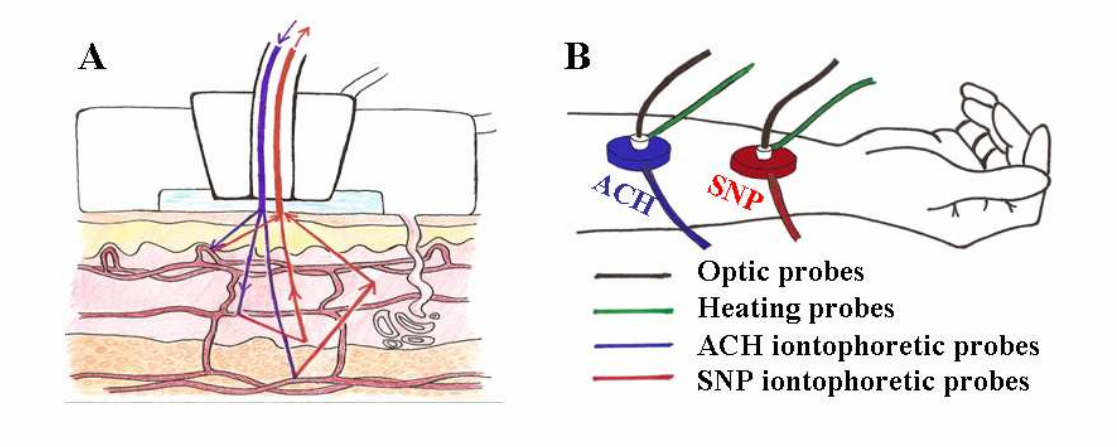

Figure 3: A: Theory of the laser Doppler flowmetry technique. B: Schematic plot for the probe settings during laser Doppler flowmetry measurement. ACH: acetylcholine; SNP: sodium nitroprusside.

The skin microvascular reactivity, as measured by means of the laser Doppler technique, has been reported to correlate with other methods applied to assess the vascular function in distinct vascular beds, including brachial artery FMD [92, 93] and transthoracic coronary echocardiography [94]. The functional impairment of the microvascular system in HD affects both the endothelium-dependent [95-97] and -independent vasodilations [96, 97]. In contrast, only the endothelium-dependent microvascular reactivity is affected in adult hypertension, as shown by attenuated LDF flux responses to occlusion of a limb and iontophoretic administration of vasodilators [98], or to local heating and iontophoresis [99], whereas the endothelium-independent function is preserved [98, 99].

Due to its simple, convenient and painless nature, LDF is highly appropriate for the examination of children and adolescents. Before our studies, however, no data were available regarding the functional properties of the microvasculature in juvenile primary hypertension.

1.4. Aims

We set out to answer the following questions:

1. Is there a detectable alteration in the microvascular reactivity in juvenile primary hypertension? Is the microvascular reactivity impaired or augmented? Is the endothelium-dependent or the -independent reactivity changed?
2. Is there any variance in the microvascular reactivity as a function of the BMI in adolescent hypertensives?
3. Does the level of oxidative stress correlate with the microvascular reactivity in juvenile hypertensives and HD patients?
4. Do the characteristics of the treatment with ESA (the type and the withdrawal of ESA) influence the oxidative stress in HD?

2. SUBJECTS AND METHODS

2.1. Microvascular reactivity assessment

2.1.1. Laser Doppler flowmetry

Microvascular reactivity studies were performed in a temperature-controlled room (24 °C) between 9 a.m. and noon, the subjects being in the fasted state. They were lying in a supine position with their feet at the heart level. After a 15-min equilibration, the volar surface of the right forearm (in HD patients, the forearm contralateral to the Cimino fistula) was gently cleaned with alcohol. The microvascular perfusion of the skin was continuously measured at two sites (distance of separation: 6-8 cm) by means of a DRT4 laser Doppler flowmeter (laser wavelength: 780 nm, Moor Instruments Ltd., Axminster, UK). The optic probes (DP12-V2) fit into the heater probes (SHP2), which, in turn, fit into the iontophoresis chambers (ION1, surface area: 0.71 cm^2; all Moor Instruments Ltd., Axminster, UK). This setting allows perfusion measurements at skin sites affected by iontophoretic and local thermal provocations (Figure 3B). The positions of the probes were chosen so as to avoid hair and injured skin. A MIC2 Iontophoresis Device (Moor) was utilized for the parallel delivery of freshly prepared 1% isotonic saline solutions of the endothelium-dependent vasodilator acetylcholine (ACH) and the endothelium-independent vasodilator sodium nitroprusside (SNP) (both Sigma-Aldrich, St. Louis, MO, USA), using separate chambers (at sites 1 and 2, respectively). The local skin temperature was standardized at 33 °C during the iontophoresis sequence, and subsequently increased to 44 °C by means of a SH02 Skin Heating Unit (Moor).

2.1.2. Validation of the laser Doppler flowmetry protocol

The LDF protocol used in the present work was adapted with modifications from Khan *et al.* [100]. It consisted of a 4-min measurement of the baseline perfusion, followed by 3 consecutive, increasing iontophoretic doses of ACH and SNP (current: 20 μA, duration: 20, 40 and 80 s, respectively; yielding a total charge of 2.8 mC and a total charge density of

3.94 mC/cm^2). Between iontophoretic doses, 4-min intervals with no current administration were allowed. Following an additional 10-min interval after completion of the iontophoresis sequence, both measurement sites were gradually heated at a rate of 0.1 °C/s [101] to reach and maintain a temperature of 44 °C for 25 min, corresponding to maximal vasodilation. A typical blood flow curve in response to the above LDF protocol is shown in Figure 4.

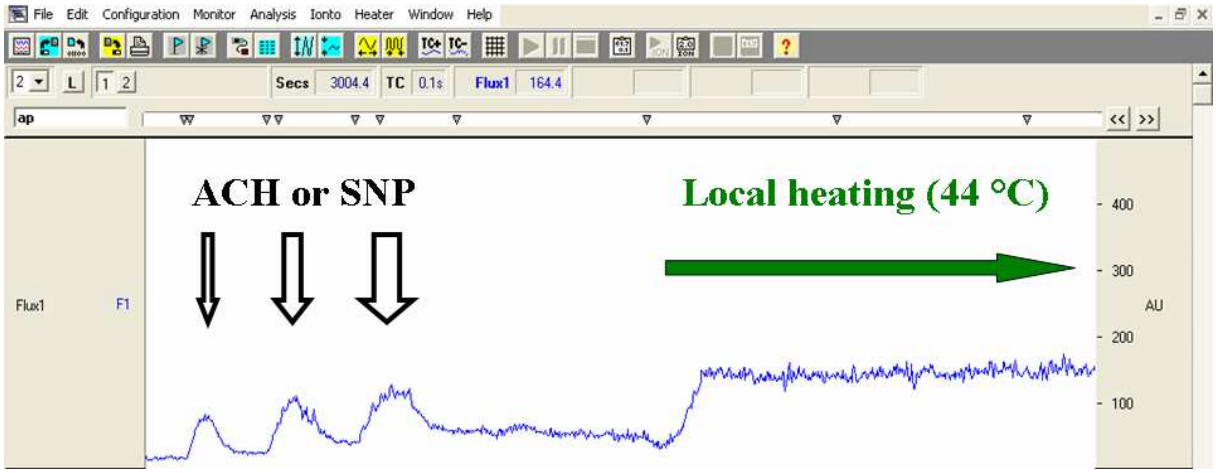

Figure 4: Typical blood flow curve. ACH: acetylcholine; SNP: sodium nitroprusside.

Flux values at baseline and in the plateau phase of the local heating response were evaluated by averaging 60-s intervals. Average values of 20-s intervals were used as peak perfusion responses after each iontophoretic dose. All counted perfusion values were expressed relative to the baseline values (baseline=100%).

The original LDF protocol [100] was modified in order to meet recent recommendations with which to avoid measurement artifacts such as current-induced hyperemia [102-104]. A lower current was applied (20 μA, instead of 100 μA) and isotonic saline was used instead of deionized water as the solvent for vasoactive drugs. In accord with this, we observed no changes in the flux when isotonic saline was iontophoresed by using the above protocol. The within-subject variabilities determined at fixed skin sites of healthy young subjects were 15-25% for both iontophoretic and local thermal provocations in our laboratory.

In order to validate the modified LDF protocol in the clinical setting, the microvascular reactivities of 12 HD patients were assessed as the presence of an impaired microvascular function had previously been demonstrated in this group [95-97]. In agreement with the literature, the endothelium-dependent and -independent vasodilation both proved to be markedly diminished as compared with those in healthy young subjects ($p<0.001$ *vs* the controls; data not published) (Table 1). The study protocol was well tolerated by both the patients and the controls, and no adverse local events (pain or red flare) or systemic effects (change in BP, heart rate or respiratory rate) were observed.

	Controls (*n*=19)	**HD patients (*n*=12)**
1st ACH iontophoresis	367.0 (100.0-499.0)	110.0 (100.0-473.0)
2nd ACH iontophoresis	860.0 (150.0-1854.0)	176.0 (100.0-752.0)***
3rd ACH iontophoresis	1339.0 (577.0-2187.0)	289.0 (151.0-914.0)***
Local heating to 44 °C (site 1)	2087.0 (909.0-3245.0)	513.0 (235.0-1263.0)***
1st SNP iontophoresis	367.0 (100.0-499.0)	110.0 (100.0-467.0)
2nd SNP iontophoresis	860.0 (150.0-1854.0)	333.0 (100.0-669.0)***
3rd SNP iontophoresis	1339.0 (577.0-2187.0)	412.0 (100.0-829.0)***
Local heating to 44 °C (site 2)	2087.0 (909.0-3245.0)	654.0 (221.0-1373.0)***

Table 1: Skin blood flux values in response to three doses of acetylcholine (site 1) and sodium nitroprusside (site 2) iontophoresis and local heating to 44 °C (LDF protocol validation). Data are expressed relative to the baseline values (medians (ranges), baseline=100%). LDF: laser Doppler flowmetry; HD: hemodialyzed. Kruskal-Wallis test and Dunn's multiple comparison test. *** $p<0.001$ *vs* controls.

2.2. Laboratory determinations

For determination of the biochemical, metabolic and hematological parameters, blood samples containing EDTA or heparin were used. In Study 1 (Microvascular reactivity in lean,

overweight and obese hypertensive adolescents), venous blood was obtained prior to the LDF measurements. In Study 2 (Erythropoiesis-stimulating agent withdrawal and oxidative stress in hemodialysis), blood samples were taken from the arterial line of the dialysis tubes. For the biochemical determinations, we used samples drawn at the end of the long interdialytic period so as to minimize the acute effects of the dialysis session or intravenous iron administration on the indices of oxidative stress [7, 105]. The possibly higher degree of fluid retention had no effect on the oxidative parameters, as either normalization to the hemoglobin (Hb) levels was performed (GSSG and GSH) or erythrocyte concentrations and activities were determined (MDA and antioxidant enzymes, respectively). The hematological parameters were measured from mid-week blood samples according to current guidelines [59].

2.2.1. Whole blood oxidized and reduced glutathione

Whole blood levels of GSSG and GSH were assayed by means of a spectrophotometric enzymatic recycling method [105]. In the presence of 5,5′-dithio-bis(2-nitrobenzoic acid), glutathione reductase and nicotinamide adenine dinucleotide phosphate (reduced form), the colored product 5-thionitrobenzoate is formed upon the progressive reduction of GSSG to GSH. The rate of color change at 412 nm, followed over 3 min, is proportional to the total glutathione (i.e. GSSG plus GSH) concentration. GSSG was determined via the blocking of GSH with the thiol-masking agent *N*-ethylmaleimide in a separate sample.

2.2.2. Plasma alfa-tocopherol

Plasma alfa-tocopherol was determined by means of high-performance liquid chromatography (HPLC), using a 4.0x250 mm Spherisorb ODS2 5 μm reverse-phase HPLC column (Waters Corp., Milford, Massachusetts, USA) and a variable-wavelength ultraviolet-visible detector (at 297 nm) [107]. The levels of plasma alfa-tocopherol are reported relative to the total cholesterol plus triglyceride levels.

2.2.3. Erythrocyte malondialdehyde

Erythrocyte MDA (E-MDA), after its reaction with thiobarbituric acid, was measured by means of HPLC, using a 3.9x300 mm μBondapak C18 10 μm reverse-phase HPLC column (Waters Corp., Milford, Massachusetts, USA) and a variable-wavelength ultraviolet-visible detector (at 532 nm) [108].

2.2.4. Erythrocyte superoxide dismutase and catalase activities

The activity of the erythrocyte SOD (E-SOD) was measured according to Misra and Fridovich [109], modified by Matkovics *et al.* [110]. The assay is based on the inhibition of the epinephrine-adrenochrome transformation by SOD, detected at 480 nm by means of spectrophotometry.

The activity of the erythrocyte CAT (E-CAT) was determined with the method of Beers and Sizer [111], measuring the breakdown of hydrogen peroxide by CAT, detected at 240 nm with spectrophotometry.

The enzyme activities are given relative to the protein concentration of the sample, determined at 675 nm with the spectrophotometric method of Lowry *et al.* [112].

2.2.5. Determination of metabolic and hematological parameters

Plasma levels of the total, high- and low-density lipoprotein (HDL and LDL) cholesterol and triglycerides, as well as Hb levels, proportions of reticulocytes, transferrin saturation values and ferritin levels, were determined with standard laboratory methods.

2.3. Study groups and interventions

2.3.1. Microvascular reactivity in lean, overweight and obese hypertensive adolescents (Study 1)

Primary hypertensive patients (aged 6-19) with a confirmed diagnosis were asked to participate in Study 1. Hypertension was defined as a 24-h systolic and/or diastolic mean BP

equal to or greater than the 95th percentile for age, height and sex [113], measured with an oscillometric ambulatory BP monitor (Meditech ABPM-04, Budapest, Hungary). Secondary causes of hypertension (renal parenchymal, renovascular, endocrinological, cardiological or neurological) were excluded. No proteinuria (defined as >10 mg/kg/day) or any impairment in renal function (creatinine clearance <80 ml/min/1.73 m^2) was observed. Of the 33 adolescents enrolled after establishment of the diagnosis of primary hypertension in the Department of Pediatrics, University of Szeged, 10 patients had a normal BMI (lean hypertensive (LH) group), while 13 adolescents were classified as overweight hypertensive (OWH) and 10 patients as obese hypertensive (OBH) according to the age and sex-specific cut-off points of child overweight and obesity defined by the International Obesity Task Force [22]. Most patients were enrolled within 3 months after establishment of their diagnosis (in 1, 4 and 2 subjects in the LH, OWH and OBH groups, respectively: within 6-10 months). Five LH, 6 OWH and 1 OBH patient had familiar predisposition to hypertension. Nineteen healthy adolescents with no evidence of cardiovascular or renal disorders served as controls, recruited from secondary schools in Szeged. For all groups, exclusion criteria included the presence of an acute illness, smoking, and taking medication (in the previous 5 days) or beverages (on the previous day) known to affect microvascular perfusion. Three OWH and 2 OBH patients had been on short-term metoprolol therapy antecedent to the present study. No signs of acute inflammation or systemic infection were revealed by means of physical examination and qualitative blood count in any of the patients or controls. Written informed consent was obtained from the patients, the controls and their parents prior to the study, which was approved by the Ethical Committee of the University of Szeged.

2.3.2. Erythropoiesis-stimulating agent withdrawal and oxidative stress in hemodialysis (Study 2)

Twenty-one patients on chronic HD were enrolled in Study 2, dialyzed in two distinct morning shifts (11 patients: age (mean)±SD 59.3±17.7 yrs, time on HD: 3.6±2.0 yrs; and 10 patients: age 56.6±13.6 yrs, time on HD: 4.3±2.1 yrs, respectively). All patients were on 4-h bicarbonate HD, performed on a Polyflux 21L dialyzer, 3 times a week. The single-pool Kt/V was greater than 1.4, in line with the current European Best Practice Guideline on Dialysis

Strategies [114]. Heparin was used as the anticoagulant during HD. The primary diagnoses were chronic glomerulonephritis, chronic pyelonephritis or hypoplastic kidney. No patients had hypertension or diabetes as etiological factors for chronic kidney disease in these shifts. Fifteen of the patients were undergoing antihypertensive treatment with amlodipine, enalapril, metoprolol or prazosin. Additional drugs regularly used by some patients included sodium polystyrene sulfonate, sevelamer, clopidogrel, acetylsalicylic acid and famotidine. All patients received 5 mg folate once weekly. Patients with diabetes were excluded. The regular examination of the patients did not reveal any sign of acute inflammation or systemic infection throughout the study.

The patients had been receiving epoetin beta twice weekly. As part of the treatment strategy, the latter 10 patients were to be switched to darbepoetin alfa, independently from the present study. Epoetin beta therapy was withdrawn for 14 days, after which ESA administration was resumed either with epoetin beta (11 patients, dose: 5000 IU twice a week), or with darbepoetin alfa (10 patients, dose: 50 µg once weekly after the first dialysis of the week), administered in subcutaneous bolus injections at the end of the dialysis. If the transferrin saturation of a patient fell below 30%, 62.5 mg iron gluconate was given intravenously during each of five consecutive dialysis sessions. Written informed consent was obtained from the patients prior to their entering the study, approved by the Ethical Committee of the University of Szeged.

2.4. Statistical analysis

For Study 1, the clinical data on the patients and the results of the microvascular reactivity measurements and biochemical determinations are reported as medians (ranges). For Study 2, the clinical data on the patients and the results of the biochemical analyses are reported as means±SD. Statistical analyses were performed with GraphPad Prism 4.00 software (GraphPad Software Inc., La Jolla, CA, USA). Statistical comparisons included the nonparametric Kruskal-Wallis test, followed by Dunn's multiple comparison test (Study 1), or repeated-measures two-way analysis of variance (ANOVA), followed by Bonferroni's *post hoc* test (Study 2). For both studies, a p value <0.05 was considered significant.

3. RESULTS

3.1. Microvascular reactivity in lean, overweight and obese hypertensive adolescents (Study 1)

3.1.1. Epidemiological and clinical data on the study groups

The plasma levels of HDL cholesterol were significantly elevated in the LH group, and significantly decreased in the OBH patients (both $p<0.05$ *vs* the controls). The plasma triglyceride levels were higher in the OWH and OBH patients as compared with the controls ($p<0.05$ and $p<0.01$, respectively). The plasma HDL cholesterol and triglyceride levels in the OBH group were also higher than those in the LH subjects ($p<0.01$ and $p<0.05$, respectively). The mean age, diastolic BP and plasma levels of total and LDL cholesterol were similar in the study groups (Table 2).

Variables	Controls (*n*=19)	Lean HT (*n*=10)	Overweight HT (*n*=13)	Obese HT (*n*=10)
Age (yrs)	15.4 (15.2-16.0)	16.5 (7.0-18.5)	15.3 (9.8-18.7)	14.2 (7.0-18,9)
Systolic BP (mmHg)	115 (105-120)	144 (125-160)**	138 (125-170)**	140 (125-150)*
Diastolic BP (mmHg)	80 (70-90)	84 (75-90)	90 (62-100)	83 (60-100)
BMI (kg/m^2)	22.4 (19.7-24.2)	22.0 (18.3-24.2)	25.3 (24.2-28.6)*,†	30.9 (23.0-38.2)***,†††
Total cholesterol (mmol/l)	3.9 (2.8-4.3)	4.1 (3.6-5.4)	4.8 (3.6-5.2)	4.6 (3.3-5.7)
HDL cholesterol (mmol/l)	1.5 (1.2-1.8)	1.9 (1.4-2.1)*	1.4 (0.9-1.7)†	1.1 (0.8-1.4)*,††
LDL cholesterol (mmol/l)	1.9 (1.1-2.7)	2.4 (1.9-3.2)	2.7 (1.8-3.1)	2.3 (1.9-3.7)
Triglycerides (mmol/l)	0.7 (0.4-1.0)	0.7 (0.4-1.1)	0.9 (0.6-3.1)*	1.2 (0.9-3.0)**,†

Table 2: Epidemiological and clinical parameters of the study groups (Study 1). Data are reported as medians (ranges). HT: hypertensive; BP: blood pressure; BMI: body mass index; HDL: high-density lipoprotein; LDL: low-density lipoprotein. Kruskal-Wallis test and Dunn's multiple comparison test. *** $p<0.001$, ** $p<0.01$, * $p<0.05$ *vs* controls. ††† $p<0.001$, †† $p<0.01$, † $p<0.05$ *vs* lean HT.

3.1.2. Biochemical parameters

Results of the biochemical analyses are summarized in Table 3. The whole blood ratios GSSG/GSH were significantly elevated in all the patient groups (all $p<0.001$ *vs* the controls). Also, the ratios GSSG/GSH in the OBH group were higher than those in the LH subjects ($p<0.05$). The activities of E-CAT were increased in the OWH group as compared with the controls ($p<0.05$). The levels of plasma alfa-tocopherol (relative to the total cholesterol plus triglyceride levels) and E-MDA, and the activities of E-SOD were similar in the study groups.

Variables	Controls (*n*=19)	Lean HT (*n*=10)	Overweight HT (*n*=13)	Obese HT (*n*=10)
GSSG/GSH (%)	0.144 (0.071-0.201)	0.322 (0.163-0.379)***	0.314 (0.203-0.401)***	0.367 (0.311-0.441)***,†
Plasma alfa-tocopherol/ /(total cholesterol plus triglycerides) *10^{-3}	3.1 (2.6-4.1)	3.0 (2.4-3.8)	2.8 (2.3-3.4)	2.8 (2.6-3.3)
E-MDA (nmol/g Hb)	5.1 (3.2-8.6)	6.2 (5.4-8.0)	4.9 (3.6-6.1)	5.5 (3.0-8.0)
E-SOD (U/mg prot)	3.7 (3.0-4.0)	3.7 (1.0-4.1)	3.4 (2.4-4.2)	3.2 (2.2-5.3)
E-CAT (BU/mg prot*10^{-3})	1.1 (1.0-1.6)	1.4 (1.0-1.9)	1.6 (1.1-2.1)*	1.4 (0.8-1.8)

Table 3: Oxidative stress markers in the study groups (Study 1). Data are reported as medians (ranges). HT: hypertensive; GSSG/GSH: whole blood ratio of oxidized/reduced glutathione; E-MDA: erythrocyte malondialdehyde; E-SOD: erythrocyte superoxide dismutase; E-CAT: erythrocyte catalase; Hb: hemoglobin. Kruskal-Wallis test and Dunn's multiple comparison test. *** $p<0.001$, * $p<0.05$ *vs* controls. † $p<0.05$ *vs* lean HT.

3.1.3. Laser Doppler flowmetry

The perfusion increments after iontophoresis of the endothelium-dependent vasodilator ACH did not differ significantly in the patient groups and the controls (Table 4, Figure 5A). In contrast, the second iontophoretic dose of the endothelium-independent vasodilator SNP increased the microvascular blood flux to a significantly smaller extent in the LH group than in the controls ($p<0.05$). After the third SNP dose, similar differences were revealed in the LH and the OBH groups as compared with the controls (both $p<0.05$) (Table 5, Figure 5B).

In response to local heating to 44 °C, the increase in the microvascular perfusion attained a plateau at similar relative flux values at site 1 in all groups (Table 4, Figure 5A). At site 2, significantly smaller flux increments were detected in the LH and the OBH groups than in the controls (Table 5, Figure 5B). The study protocol was well tolerated by all subjects and no adverse local or systemic effects were observed.

	Controls (*n*=19)	**Lean HT (*n*=10)**	**Overweight HT (*n*=13)**	**Obese HT (*n*=10)**
1st ACH iontophoresis	367.0 (100.0-499.0)	110.0 (100.0-1134.0)	123.5 (100.0-856.0)	267.0 (100.0-364.0)
2nd ACH iontophoresis	860.0 (150.0-1854.0)	420.5 (268.0-1465.0)	578.5 (264.0-1935.0)	526.0 (333.0-909.0)
3rd ACH iontophoresis	1339.0 (577.0-2187.0)	889.0 (730.0-2368.0)	1037.0 (380.0-2210.0)	994.5 (884.0-1492.0)
Local heating to 44 °C (site 1)	2087.0 (909.0-3245.0)	1687.0 (1068.0-3230.0)	1534.0 (836.0-2741.0)	1576.0 (1058.0-2137.0)

Table 4: Skin blood flux values in response to three doses of acetylcholine iontophoresis and local heating to 44 °C (site 1, Study 1). Data are reported as medians (ranges). HT: hypertensive; ACH: acetylcholine. Kruskal-Wallis test and Dunn's multiple comparison test.

	Controls (*n*=19)	**Lean HT (*n*=10)**	**Overweight HT (*n*=13)**	**Obese HT (*n*=10)**
1st SNP iontophoresis	341.0 (100.0-1131.0)	110.0 (100.0-949.0)	426.5 (100.0-897.0)	361.5 (164.0-774.0)
2nd SNP iontophoresis	847.0 (432.0-1708.0)	482.5 (157.0-842.0)*	776.5 (170.0-1494.0)	655.5 (240.0-1204.0)
3rd SNP iontophoresis	1195.0 (674.0-1774.0)	797.5 (270.0-1100.0)*	1078.0 (333.0-1618.0)	744.0 (390.0-1207.0)*
Local heating to 44 °C (site 2)	1870.0 (1029.0-2970.0)	1257.0 (585.0-1649.0)*	1682.0 (464.0-2367.0)	1188.0 (693.0-1815.0)*

Table 5: Skin blood flux values in response to three doses of sodium nitroprusside iontophoresis and local heating to 44 °C (site 2, Study 1). Data are reported as medians (ranges). HT: hypertensive; SNP: sodium nitroprusside. Kruskal-Wallis test and Dunn's multiple comparison test. * $p<0.05$ *vs* controls.

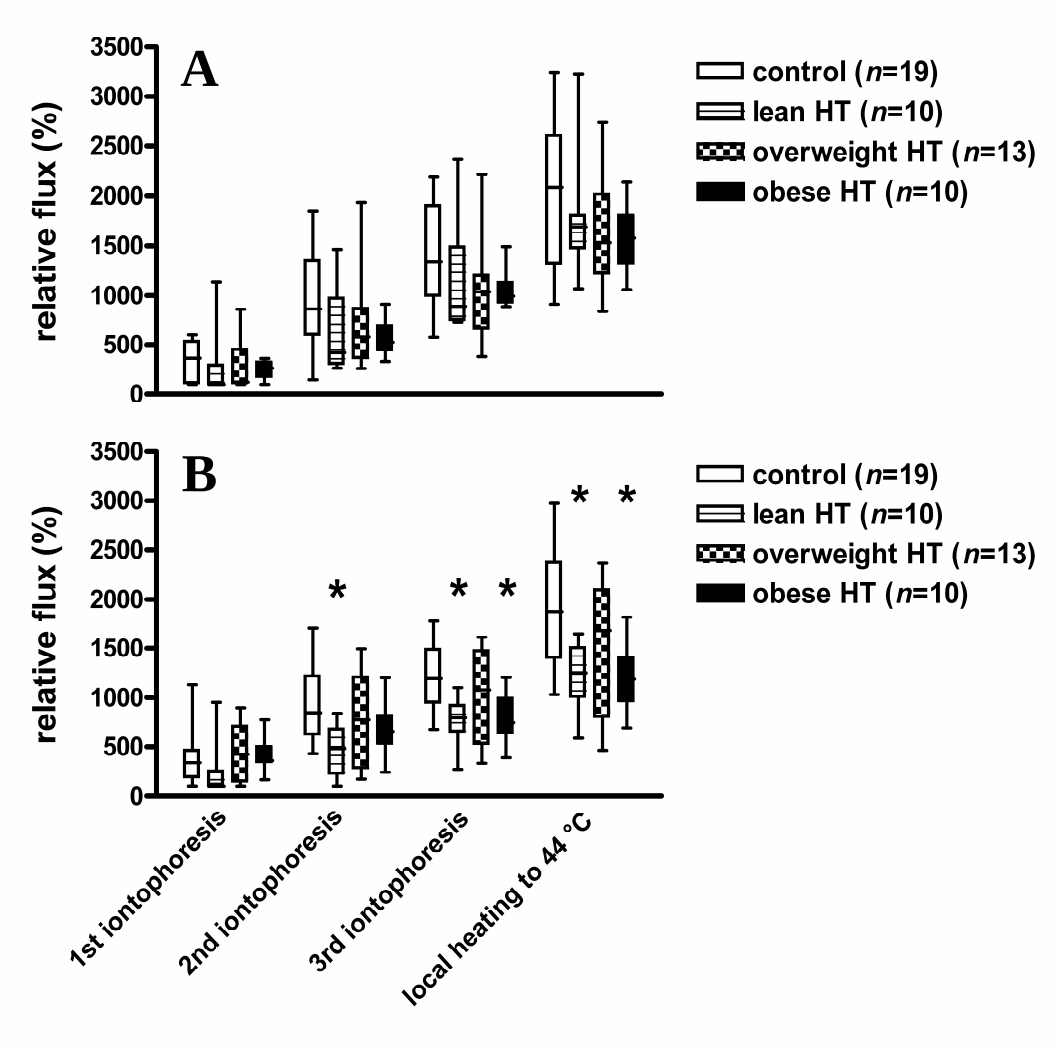

Figure 5: Changes in skin blood flux in response to three doses of acetylcholine [part A, site 1] and sodium nitroprusside [part B, site 2] iontophoresis and local heating to 44 °C (Study 1). Empty box: control group; striped box: lean hypertensives; squared box: overweight hypertensives; filled box: obese hypertensives. Data are expressed relative to the baseline values (medians (ranges), baseline=100%). HT: hypertensive. Kruskal-Wallis test and Dunn's multiple comparison test. * $p<0.05$ *vs* controls.

3.2. Erythropoiesis-stimulating agent withdrawal and oxidative stress in hemodialysis (Study 2)

3.2.1. Hematological indices

The Hb levels were decreased at week 6 in both groups ($p<0.05$ *vs* the baseline), and had returned to the initial values by week 14 (Table 6). The proportion of reticulocytes exhibited a decrease at week 2 and a subsequent elevation at weeks 6 and 10 (all $p<0.05$ *vs* the baseline) (Table 6). No differences were observed between the groups in either parameter. The transferrin saturation values and the levels of ferritin were not changed in either group (Table 6).

		Base-line	Week 2	Week 6	Week 10	Week 14	Repeated-measures two-way ANOVA		
							p value for interaction	*p* value for factor "drug"	*p* value for factor "time"
Hemoglobin (g/l)	**Darbepoetin alfa**	114.1± 8.6	111.9± 12.8	101.6± 13.0	103.4± 10.5	116.6± 11.0	n.s.	n.s.	<0.05
	Epoetin beta	120.9± 14.2	107.3± 20.4	103.0± 16.1	109.9± 17.3	114.3± 17.6			
Proportion of reticulocytes (‰)	**Darbepoetin alfa**	10.3± 4.2	7.6± 1.3	14.0± 7.7	14.4± 6.8	13.7± 3.1	n.s.	n.s.	<0.001
	Epoetin beta	11.5± 4.2	6.8± 2.2	13.9± 3.9	16.4± 4.3	12.3± 5.5			
Transferrin saturation (%)	**Darbepoetin alfa**	38.1± 25.5	37.9± 28.9	34.9± 9.9	34.3± 9.9	34.1± 14.4	n.s.	n.s.	n.s.
	Epoetin beta	39.3± 17.1	55.4± 23.8	42.6± 27.9	29.1± 8.3	49.0± 24.5			
Ferritin (ng/ml)	**Darbepoetin alfa**	-	793.5± 653.3	-	-	687.0± 468.8	n.s.	n.s.	n.s.
	Epoetin beta	-	808.6± 465.4	-	-	758.4± 529.0			

Table 6: Hematological parameters in the study groups (Study 2). The antecedent epoetin beta therapy was withdrawn between week 0 (baseline) and week 2. Data are presented as means±SD. n.s.: nonsignificant. Repeated-measures two-way ANOVA and Bonferroni's *post hoc* test.

3.2.2. Biochemical parameters

The ratios GSSG/GSH were significantly increased at weeks 2 and 6 in both groups (all $p<0.001$ *vs* the baseline), but had returned to the initial levels by week 14 (Figure 6). Similar tendencies were found in the levels of GSSG (Table 7). The GSH concentrations were increased at week 14 in the two groups ($p<0.001$ *vs* the baseline) (Table 7). The E-MDA levels exhibited an elevation at week 6 ($p<0.01$ *vs* the baseline) and a subsequent return to the initial levels by week 14 in both groups (Table 7). As compared with the baseline values, the activities of E-SOD were reduced significantly at week 6 in both study groups ($p<0.001$), and had returned to the baseline by week 14 (Table 7). The activities of E-CAT were significantly increased at week 14 in both groups ($p<0.001$ *vs* the baseline) (Table 7).

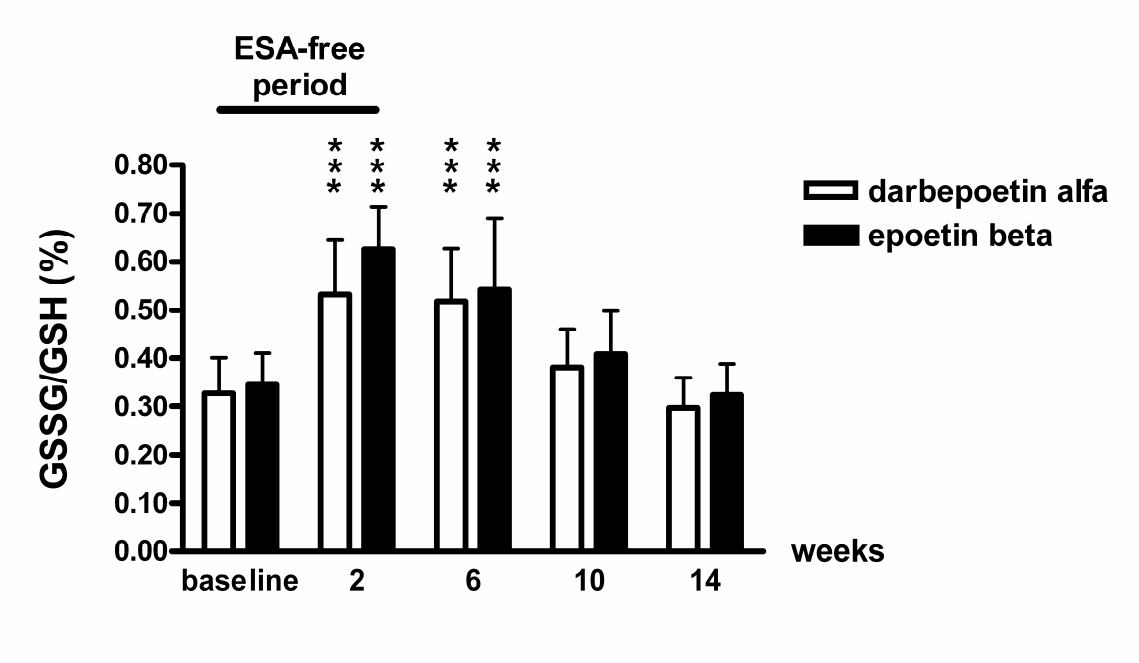

Figure 6: Whole blood oxidized/reduced glutathione ratios (GSSG/GSH) (Study 2). The antecedent epoetin beta therapy was withdrawn between week 0 (baseline) and week 2. Empty columns: darbepoetin alfa; filled columns: epoetin beta. Data are expressed as means±SD. ESA: Erythropoiesis-stimulating agent. Repeated-measures two-way ANOVA and Bonferroni's *post hoc* test: *** $p<0.001$ *vs* baseline (factor "time") and p=nonsignificant for both interaction and factor "drug".

		Base-line	Week 2	Week 6	Week 10	Week 14	Repeated-measures two-way ANOVA		
							p value for interaction	*p* value for factor "drug"	*p* value for factor "time"
GSSG (nmol/g Hb)	**Darbepoetin alfa**	25.9± 6.5	43.6± 10.7	44.6± 9.6	34.2± 9.4	28.8± 4.7	n.s.	n.s.	<0.001
	Epoetin beta	24.3± 4.4	44.2± 9.0	41.8± 9.8	32.6± 4.9	29.1± 4.9			
GSH (µmol/g Hb)	**Darbepoetin alfa**	7.9± 1.2	8.2± 1.5	8.7± 2.1	9.1± 2.0	10.0± 1.9	n.s.	n.s.	<0.001
	Epoetin beta	7.2± 1.6	7.1± 1.6	7.8± 1.1	8.1± 1.2	9.1± 1.5			
E-MDA (nmol/g Hb)	**Darbepoetin alfa**	12.3± 1.9	13.1± 1.2	14.0± 1.4	13.2± 1.1	12.0± 1.5	n.s.	n.s.	<0.01
	Epoetin beta	11.8± 1.1	12.2± 1.2	13.4± 1.0	12.5± 1.3	12.4± 1.1			
E-SOD activity (U/mg protein)	**Darbepoetin alfa**	3.3± 0.6	3.4± 0.8	2.6± 0.3	3.3± 0.4	3.0± 0.4	n.s.	<0.05	<0.001
	Epoetin beta	3.8± 0.6	3.2± 0.2	3.0± 0.7	3.5± 0.2	3.6± 0.2			
E-CAT activity (BU/mg protein $*10^{-3}$)	**Darbepoetin alfa**	1.1± 0.4	1.3± 0.4	0.9± 0.2	1.1± 0.1	1.6± 0.3	n.s.	n.s.	<0.001
	Epoetin beta	1.0± 0.4	1.1± 0.2	0.9± 0.2	1.1± 0.1	1.5± 0.3			

Table 7: Oxidative parameters in the study groups (Study 2). The antecedent epoetin beta therapy was withdrawn between week 0 (baseline) and week 2. Data are presented as means±SD. GSSG: whole blood oxidized glutathione; GSH: whole blood reduced glutathione; E-MDA: erythrocyte malondialdehyde; E-SOD: erythrocyte superoxide dismutase; E-CAT: erythrocyte catalase; Hb: hemoglobin; n.s.: nonsignificant. Repeated-measures two-way ANOVA and Bonferroni's *post hoc* test.

4. DISCUSSION

Cardiovascular risk factors such as hypertension, obesity and uremia are accompanied by the loss of endothelium-derived vasodilator and anti-thrombotic factors, and increased levels of inflammatory and oxidative stress markers in adults [7, 115]. Target-organ damage such as left ventricular hypertrophy or an increased carotid artery intima-media thickness can be present already in childhood [9, 10, 15], although there are at present no long-term data to link a level of childhood BP with cardiovascular events in later adulthood. The prevalence of hypertension in childhood and adolescence (about 3% in 2006 [116]) is showing an increasing trend, which is in part attributable to the high prevalence of obesity [9, 10, 15]. BP [117] and weight [118] show tracking from childhood into adulthood, which further emphasizes the importance of juvenile hypertension and overweight. Even if secondary causes of the BP elevation can be revealed in some hypertensive patients, late in the first decade of life and throughout the second, primary hypertension becomes the most common cause of sustained hypertension, particularly in those children with mild asymptomatic disease [9].

4.1. Microvascular reactivity in lean, overweight and obese hypertensive adolescents (Study 1)

To the best of our knowledge, this study was the first to assess the functional properties of the microvessels in juvenile primary hypertension. Moreover, a comparison of the microvascular reactivities of hypertensive adolescents grouped according to the degree of ponderosity (LH, OWH and OBH patients) was performed.

The microvascular responses to iontophoresis of the endothelium-dependent vasodilator ACH and local heating (site 1) were not significantly attenuated in these hypertensive groups (irrespective of the BMI) as compared with the controls (Table 4, Figure 5A). The endothelium-independent vasodilation in response to SNP iontophoresis was impaired to a similar degree in the LH and OBH patients, but not in the OWH adolescents (Table 5, Figure 5B). It was an interesting finding that local skin heating was associated with distinct responses when performed after either the ACH or SNP iontophoresis sequence (sites 1 and 2,

respectively). Local skin warming initiates a biphasic response: an initial rapid increase to a peak, mediated by neurotransmitter release from sensory afferents, followed by a prolonged plateau phase, predominantly mediated by NO generation [119]. This, in turn, raises the possibility of using the local thermal hyperemia test as a tool with which to assess the endothelium-dependent microvascular function [102]. In accordance, similar blood flux responses were observed to ACH iontophoresis and local heating at site 1 in our study. The different responses to local heating at site 2 were presumably influenced by the slower clearance of SNP as compared with ACH, as indicated by the reported time-response curves of the drugs [120] and the higher flux values at site 2 at the start of the heating test in the present study, as compared with those at site 1 (data not shown).

During the validation stage of Study 1, a markedly attenuated function of the microvascular system, both endothelium-dependent and -independent, was found in the HD patients. Moreover, a greatly increased oxidative stress was confirmed by elevated levels of E-MDA, GSSG and GSSG/GSH, and decreased concentrations of GSH (data not published). All changes were much more pronounced in the HD group than those in the hypertensives. Even if these findings are in agreement with previous observations [52, 56, 95-97], the mechanisms of these alterations and their possible relationship have not been fully elucidated. Further analysis of the HD group revealed that the most noteworthy variance among the patients was the difference in the ESA therapy. These observations led us to the assumption that characteristics of the ESA therapy and/or variances in the action of ESA may contribute to the alterations described above. Therefore, the aims of our second study were to examine the influence of the short-term withdrawal of ESA therapy on oxidative parameters in HD patients; and to follow the changes in oxidative stress after therapy resumption with two commonly used ESAs, darbepoetin alfa and epoetin beta.

4.2. Erythropoiesis-stimulating agent withdrawal and oxidative stress in hemodialysis (Study 2)

This study was the first to reveal alterations in oxidative stress, as shown by increased ratios GSSG/GSH, after the interruption of epoetin beta treatment in HD patients (Figure 6). Four weeks after either darbepoetin alfa or epoetin beta readministration (week 6), significantly elevated GSSG/GSH, GSSG and E-MDA levels, and decreased activities of E-SOD were found as compared with the respective baseline values (Table 4). These findings are in line with previous reports of a transiently increased oxidative stress after the initiation of epoetin alfa treatment (culminating at week 4 of the therapy) [60], an impairment of the antioxidant defense after ESA withdrawal [122], and elevations in the levels of circulating inflammatory markers after 4 weeks of ESA therapy [123]. By the end of the 14-week study, all parameters had returned to the respective initial values. The GSH and E-CAT activity levels at week 14 were higher than initially in both groups (Table 4), concordant with the reported adaptive response of the glutathione redox system to oxidative stress [1, 124], consisting of augmented GSH regeneration and synthesis.

4.3. Possible mechanisms and clinical significance of the findings

Controversies exist as to whether the development of a vascular dysfunction precedes or follows the elevation of the BP. The endothelial function in normotensive offsprings of hypertensive patients has been reported to be impaired [125, 126], although not consistently [127] (this issue has been previously reviewed [128]). This would suggest that an endothelial dysfunction might precede the development of hypertension. However, none of these studies were prospective, and adolescents were enrolled in only one study assessing brachial artery FMD [125]. On the other hand, a decreased vascular function may develop as a consequence of the elevated BP. Transient hypertension has been shown to give rise to a direct attenuation of the endothelium-dependent vasodilation of the human microvasculature *in vitro* [129]. In the Cardiovascular Risk in Young Finns Study, an elevated systolic BP in male adolescents was found to predict an impaired brachial artery FMD in adulthood [130]. However, there

have been no reports as concerns endothelium-dependent and -independent reactivities of the microvascular system in adolescents.

Our finding of a preserved endothelium-dependent microvascular reactivity in hypertensive adolescents (Figure 5A) may seem to contradict previous reports of a diminished availability of NO in this patient group [43, 131]. ACH iontophoresis releases NO, prostanoids and other vasoactive substances in the microvessels [132]. The compensatory activities of vasoactive mechanisms other than NO could prevent an impairment of the endothelium-dependent microvascular reactivity despite decreased NO levels. In accordance, prostanoids contribute to the vasodilator response to ACH to a greater extent in healthy young adults than in older subjects [133]. Taken together, these findings suggest that, in the pathogenesis of juvenile primary hypertension, an impairment of the endothelium-dependent microvascular reactivity is more likely to follow, rather than precede the elevation of the BP.

The responses to the endothelium-independent vasodilator SNP were found to be attenuated in the LH and OBH groups, but not in the OWH adolescents (Figure 5B). This result is in line with the hypothesis that the cardiovascular risk of OWH patients might be more favorable than that of their LH counterparts [134], as suggested by several [135, 136], albeit not all [137] studies. In contrast with the present findings, the endothelium-independent microvascular reactivity has been reported to be preserved in adult hypertensives [98, 99]. The different microvascular responses among the patient groups in Study 1 were not related to variances in the levels of oxidative markers, suggesting that factors other than oxidative stress play roles. SNP is an NO donor that reacts with tissue sulfhydryl groups under physiological conditions to produce NO directly and thereby stimulate smooth muscle relaxation [91]. The decomposition of SNP, the rate of NO liberation and consequently the effects of SNP iontophoresis on smooth muscle cells may be influenced by the local temperature, the abundance of tissue sulfhydryl groups, and the tissue oxygen tension and pH [138, 139]. Even if we standardized the local skin temperature during the iontophoresis sequence, the possible influences of the latter factors on the endothelium-independent microvascular responses cannot be excluded.

One may speculate that the microvascular responses reported here might have been influenced by individual differences in skin thickness, especially in obese subjects. Skin resistance is indeed an important factor that affects the delivery of vasoactive ions and

consequently the reactivity of the microvessels, as described by Ramsay *et al.* [140]. The MIC2 Iontophoresis Device used in the present study automatically modifies the applied voltage as a function of skin resistance, so that the current and the delivery of ions into the skin remain constant. Thus, individual differences in skin resistance are not likely to have had a substantial effect on the microvascular responses.

As to the microvascular function in the HD group, both the endothelium-dependent and -independent reactivities were markedly attenuated. Oxidative stress is a potential cause of the impaired vascular function in HD patients, characterized by increased levels of oxidative markers, a compromised antioxidant protection [7, 52], and periodical exogenous challenges that reportedly affect oxidative stress, such as the HD sessions [7, 50, 51] and the administrations of intravenous iron [50, 105] and ESA [67]. Although effects of the HD sessions and intravenous iron on oxidative markers have already been explored [7, 50, 51, 105], the issue of ESA therapy has not yet been fully elucidated. The episodic administration of ESA is in contrast with the smoother physiological changes in the endogenous erythropoietin (EPO) levels [121]. Elevations and descents may be observed in the plasma level of ESA between individual doses, despite unaltered Hb levels [121]. This, in turn, could have an influence on the reported antioxidant effect of the ESA treatment [67], so that the antioxidant protection would be transiently attenuated if the level of ESA declined. Such fluctuations in the antioxidant protection would add to the repeated effects of HD sessions on the oxidative stress and the microvasculature [50, 51, 141]. Possible clinical examples are the late phase of the widely extended administration intervals, and the temporary suspension of ESA therapy in consequence of the guideline Hb level being exceeded, conditions in which the action of ESA may be depressed [121]. To assess the hypothesis that variances in the action of ESA contribute to an alteration in the oxidative stress, we studied the effects of a 14-day suspension of ESA therapy on oxidative indices.

Directly after ESA withholding (Study 2), a marked increase in the ratio GSSG/GSH was revealed in HD patients (Figure 6). In terms of mechanisms that may contribute to this elevation, we first refer to the earlier study of Rice *et al.* [142], who reported that ESA therapy withdrawal induces hemolysis of the young red blood cells (RBCs) in renal failure [142]. Although we did not assess neocytolysis directly, a higher elimination rate of young erythrocytes could contribute to an increase in the ratio GSSG/GSH, due to low GSSG and

high GSH contents of young RBCs [143] and the release of free heme, promoting the oxidation of lipids and membrane-bound proteins [144]. However, the proposed theory of the mechanism of neocytolysis, which suggested the existence of an EPO-mediated endothelial cell-macrophage interaction [121], has been questioned by recent reports of an absence of EPO receptors on endothelial cells [145, 146].

Secondly, ESA withdrawal is associated with depressed erythropoiesis in HD, a disease characterized by diminished endogenous EPO production [147]. The consequently decreasing proportion of young RBCs, containing low levels of GSSG and high levels of GSH [143], may also contribute to the elevation of the whole blood GSSG/GSH levels.

Finally, EPO has been reported to activate cJun-*N*-terminal kinases (JNKs) in erythroid cells [122]. JNKs may, in turn, stimulate heme oxygenase-1 [148] and Forkhead box class O (FOXO) transcription factor FOXO3a in response to oxidative stress [149]. FOXO3a activation is reported to be parallelled by elevated SOD, CAT and GPx expressions and an increased survival [150, 151]. As mature RBCs do not have nuclei and cannot induce FOXO3a, they are dependent on antioxidant enzymes synthesized during early erythroid differentiation [151], even if post-translational modifications of the enzymes may have an influence on their activities [152153]. Therefore, a decrease of the JNK activity shortly after ESA withdrawal [122] could contribute to an attenuation of the FOXO3a-mediated stress tolerance in erythroid cells, and also an increased sensitivity of RBCs to free heme due to a lower activity of heme oxygenase-1.

Four weeks after ESA readministration, the level of oxidative stress was significantly elevated in both HD groups, with a subsequent return to the baseline level (Figure 6 and Table 4). Despite the distinct pharmacokinetic characteristics of epoetin beta and darbepoetin alfa [154, 155] and the marked changes in the oxidative parameters during the follow-up, no statistically significant difference between the groups, except for E-SOD, was found. Modest variances were present already at baseline and week 2 when identical interventions were performed in both groups. It was a noteworthy finding that the trend of the changes in the level of Hb was opposite to that shown by the oxidative parameters, in accordance with the suggested association between the degree of anemia and the level of oxidative stress [65, 69]. Moreover, the time courses and magnitudes of the oxidative alterations in the present study with darbepoetin alfa and epoetin beta, and those in a previous study with epoetin alfa [60],

were remarkably similar. These findings raise the possibility that the changes in oxidative stress during ESA therapy may be generated by factors associated with the correction of anemia, and not the direct effect of ESA. To confirm this hypothesis, assessments of the effects of different administration doses and frequencies of ESA, and studies comprising larger patient cohorts with a parallel-group or cross-over design, are suggested.

5. SUMMARY

The microvascular responses to endothelium-dependent and -independent vasodilators (ACH and SNP, respectively) and to local heating were studied in adolescents with primary hypertension grouped according to their BMI (LH, OWH and OBH). It emerged that the endothelium-dependent vasorelaxation was not significantly attenuated in any of the hypertensive groups. This finding does not support the (otherwise clinically attractive) hypothesis of predicting the development of juvenile hypertension via noninvasive assessment of the endothelial function with LDF. In contrast, the endothelium-independent vasodilation was significantly impaired in the LH and OBH patients as compared with the controls. The microvascular reactivities were not related to differences in the levels of oxidative markers, even if the presence of an increased oxidative stress was confirmed in all the hypertensive groups. Prospective studies on larger populations of adolescents, which also assess the mechanisms of the microvascular responses to different stimuli, could clarify these issues.

An impairment of the endothelium-dependent and -independent vasodilations and the presence of a markedly increased oxidative stress were confirmed in HD patients. Among possible factors influencing the oxidative stress, substantial characteristics of ESA treatment (type and withdrawal of ESA) were studied. A significant elevation of the ratio GSSG/GSH was revealed directly after the interruption of epoetin beta treatment. Four weeks after darbepoetin alfa or epoetin beta therapy resumption, the levels of GSSG/GSH, GSSG and E-MDA were significantly increased in both groups as compared with the baseline. In line with the reported antioxidant effect of the sustained ESA administration, these parameters had returned to the baseline values by the end of the 12-week follow-up, parallelled with increased GSH and E-CAT activity levels. The findings of an opposite trend in the levels of Hb and oxidative markers, and similarities in the time courses and magnitudes of the oxidative alterations during the treatment with different ESAs suggest that the observed changes may primarily be caused by factors associated with the correction of anemia, rather than the direct effect of ESA. This emerging hypothesis is yet to be confirmed.

6. ORIGINAL FINDINGS

1. The endothelium-dependent microvascular reactivity is not significantly attenuated in primary hypertensive adolescents being lean, overweight or obese.

2. The endothelium-independent vasodilation is significantly impaired in the LH and OBH groups, but not in the OWH patients, as compared with the controls.

3. The microvascular reactivities are not related to differences in the levels of oxidative markers, even if the presence of an increased oxidative stress is confirmed in all the hypertensive groups.

4. An increased oxidative stress is revealed by significantly elevated ratios GSSG/GSH directly after the withdrawal of epoetin beta treatment in HD patients.

5. The levels of GSSG/GSH, GSSG and E-MDA are significantly increased and the activities of E-SOD are significantly decreased four weeks after darbepoetin alfa or epoetin beta therapy resumption as compared with the baseline.

6. The levels of GSSG/GSH, GSSG and E-MDA return to the baseline values by the end of the 12-week follow-up after darbepoetin alfa or epoetin beta therapy resumption, parallelled with increased GSH and E-CAT activity levels.

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9. APPENDIX