

Blood concentration of aminothiols in children with relapse of nephrotic syndrome

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Background: The role of idiopathic nephrotic syndrome (INS) in the pathogenesis of atherosclerosis in childhood has not been clearly elucidated. However, antioxidative defense in INS is thought to be imbalanced. This study aimed to assess the changes of plasma concentration of selected aminothiols in the blood of children with INS at various stages of the disease.

Methods: This cross-sectional study was conducted in 125 children aged 2-18 years. The children were divided into 4 groups: group A, early relapse ($n=37$); group B, early remission for 4-6 weeks from the onset ($n=37$); group C, late steroid-free remission ($n=31$); and group D, long-term remission for 2-5 years ($n=20$). Control group (E) consisted of 30 age- and gender-matched healthy children. The study protocol comprised an analysis of plasma concentrations of glutathione, homocysteine, cysteine and cysteinylglycine by high-performance liquid chromatography. Fractions of protein-bound and free aminothiols were measured. Endothelial injury was assessed by thrombomodulin, PAI-1 concentration, and von Willebrand factor activity.

Results: The children with INS had unbalanced aminothiol metabolism only in relapse and early remission, that shifted towards increased oxidative processes. Administration of cyclosporine A caused a significant increase in homocysteine and cysteine concentration. Changes in aminothiol metabolism were significantly related to endothelial injury.

Conclusion: The findings of this study may be helpful in elucidating the pathogenesis of premature atherosclerosis in patients with INS refractory to the treatment or in the case of frequent relapse.

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Key words: aminothiols; children; cyclosporine A; homocysteine; nephrotic syndrome

Introduction

Idiopathic nephrotic syndrome (INS) is associated with the presence of several classical risk factors of cardiovascular disease such as obesity, hypercholesterolemia, increased thrombinogenesis, impaired fibrinolysis, enhanced platelet activation, oxidative stress, insulin resistance, immunological dysregulation and subclinical inflammation.^[1,2]

Among them, oxidative stress in humans is described as an excessive production of reactive oxygen species (ROS) or a diminished function of the antioxidative defense system. It can cause direct injury of human cells by interference with DNA structure and protein production, and function of multiple cellular organelles with subsequent cellular apoptosis and death.^[3] Therefore, it is essential to maintain the redox balance. However, antioxidative defense in INS was described imbalanced.^[4-6] Two aspects of this issue should be taken into account: general and local (renal tissue) antioxidant status. The general effect could be responsible for the aggravation of clinical complications (i.e. atherosclerosis), whereas the local one was hypothesized to influence the course of the disease and it correlates with the intensity of proteinuria.^[7] The redox imbalance dependent on changes of aminothiols' concentration (aminoacids with sulphuric residues) is regarded as intra- and extracellular antioxidant. In general, aminothiols can be divided into two different groups: proatherogenic [cysteine (CY) and homocysteine (HCY)] and anti-atherogenic [glutathione (GSH) and cysteinylglycine (CG)]. The function and mechanism of

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action were well recognized for HCY and GSH. The former was described to serve as a direct and indirect factor of endothelial injury, whereas the latter as a potent antioxidant.^[8,9]

The present study was designed to assess the changes in plasma concentration of selected aminothiols in the blood of nephrotic children after the relapse or remission of the disease.

Methods

A total of 125 children with INS aged 2-18 years (Table 1) were divided into 4 groups: group A, early relapse with proteinuria for 2-5 days ($n=37$); group B, early steroid-induced remission without proteinuria for 4-6 weeks from the onset ($n=37$); group C, late steroid-free remission for 5-10 months ($n=31$); and group D, long-term remission for 2-5 years without any treatment ($n=20$). Cyclosporine A was given to 7 patients from group A, 12 patients from group B and 15 patients from group C. The control group (group E) consisted of 30 age- and gender-matched healthy children aged 2-18 years.

The diagnosis of the children with INS met the criteria recommended by the International Study for Kidney Diseases in Children.^[10] In this series, 65 children with INS were confirmed by renal biopsy, and the remaining 60 by clinical data. In the children with biopsy-proven INS, 54 had minimal change in nephrotic syndrome, 9 presented with mesangial proliferation, and 2 showed focal segmental glomerulosclerosis. The clinical course of INS was defined upon the criteria suggested by Wyszynska et al.^[11] Steroids were given to group A at a dose of 2 mg/kg per day and to group B at a dose of 1.5 mg/kg every 48 hours. No steroid-resistant patients were observed. In group A, 16 children also received

acetylosalicylic acid (1 mg/kg every 48 hours). When cyclosporine A was administered, children received a median dose of 3.3 mg/kg per day with no significant differences between the study groups. Children from all groups were normotensive or their blood pressure was adequately controlled by ACEI (30%). Children with an acute or chronic kidney injury were excluded. Additional analysis of measured variables was performed for those treated or not treated with cyclosporine A at every chosen stage of the disease (groups A-C). Patients from group D received no treatment.

The study protocol comprised a cross-sectional analysis of plasma concentration of glutathione, homocysteine, cysteine and cysteinylglycine by high-performance liquid chromatography after immediate trapping of labile SH-groups.^[12] Fractions of protein-bound and free aminothiols were measured. To reveal clinical significance of the aminothiols, coagulation test and markers of endothelial injury [thrombomodulin-TBG-Asserachrom thrombomodulin (Diagnostica Stago, Switzerland) and coagulation cascade (F1+2-prothrombin fragments), PAI-1, thrombin-antithrombin complexes (TAT), with Enzygnost F1+2 Micro (Dade-Behring, Germany), Coaliza PAI-1 (Chromogenix, Italy), and Enzygnost TAT Micro (Dade-Behring, Germany), respectively] were measured as well as classical biochemical measures [Cobas Integra devices (Roche Diagnostics, Switzerland), Paragon (Dade-Behring, Germany), CellDyn 1700 (Abbot, US), and BCS device (Dade-Behring, Germany)]. Glomerular filtration was calculated according to the modified Schwartz formula.^[13] In the CsA-treated children, blood concentration of cyclosporine A (C_0) was assessed by the immunoenzymatic method (EMIT).

Data distribution was checked by the Kolmogorov-Smirnov test for normality. The Mann-Whitney U test or Kruskal-Wallis test was used for between-group statistical analysis. Spearman's rank-order correlation coefficient was used to assess relations between variables. Multistep regression model was used for the multiple regression analysis. *P* values of less than 0.05 were considered statistically significant. All non-normally distributed variables were presented as median or in a 25-75 interquartile range.

The parents of all the study subjects gave their written informed consent for the participation in the study. The study protocol was approved by the Local Ethics Committee.

Table 1. Clinical data in the study (A-D) and control (E) groups, median (25-75 interquartile range)

Variables	Group A	Group B	Group C	Group D	Group E
Age (y)	5.2 ^{*,†‡} (2.9-12.4)	7.8 (4.0-12.5)	8.0 (5.0-14.0)	15.0 ^{*,§} (12.5-17.5)	8.0 [†] (5.0-13.0)
Duration of INS (mon)	16 [†] (0.1-37.0)	29 [†] (3-86)	39 [†] (21-78)	104.5 (57-144)	-
Mean number of relapses	2 [†] (1-6)	4 (1-8)	5 (3-7)	3 (2-4.5)	-
Hypertension (%)	21.6	33	30	4.7	-
Overweight (%)	21.6	31.8	25.8	20	8%
Weight (kg)	22.0 ^{*,†‡} (17.0-43.0)	32.1 [†] (17.8-51.9)	34.22 (26.5-55.0)	51.9 ^{*,§} (39.5-60.5)	30.0 [†] (20.8-41.9)
Height (m)	1.0 ^{*,†‡} (0.9-1.3)	1.3 [†] (0.9-1.6)	1.3 [†] (1.1-1.6)	1.5 ^{*,§} (1.4-1.6)	1.2 [†] (1.1-1.5)
BMI (kg/m ²)	18.7 (15.5-20.6)	19.2 (16.0-21.7)	18.0 (15.7-22.6)	19.1 [†] (16.4-21.3)	17.2 [†] (14.8-20.1)

*: significantly different when compared to controls (E), $P<0.05$; †: significantly different when compared to group D, $P<0.05$; ‡: significantly different when compared to group C, $P<0.05$; §: significantly different when compared to group B, $P<0.05$. INS: idiopathic nephrotic syndrome; BMI: body mass index. "-": no data.

Results

The initial analysis comprised changes in the concentration of aminothiols by the stage of the disease (Fig. A). Total

GSH levels (Fig. 1A) showed no significant differences during the relapse of INS (groups A-C) and were comparable to those of the control group. However, long-term remission patients (group D) had higher GSH levels than the others. Both protein-bound GSH and free GSH levels were elevated in this group ($P<0.05$). Total HCY (Fig. 1B) in all groups of nephrotic children (groups A-D) was not significantly different from that in the control group (Fig. 1B). There was a significant increase between early relapse (group A) and early remission (group B) for total, free and protein bound GSH.

Total CY (Fig. 1C) in the groups A, B and D was comparable to that in the control group. Only patients with late remission (group C) had lower tCY than the others. Protein-bound fraction of CY was elevated in early remission (group B) when compared to the controls (group E), early relapse (group A) and late remission (group C). However, the patients with long-term remission (group D) had the highest values. Free CY was decreased in groups C and D when compared to the controls (group E).

Total CG (Fig. 1D) was decreased in patients with early relapse (group A) and late remission (group C) when compared to the controls (group E). The tCG of children with long-term remission was higher than that of others. Protein bound CG was significantly decreased in patients with early relapse (group A) but elevated in those with long-term remission (group D). Free fraction of CG was significantly decreased in patients with late

remission (group C) but elevated in those with long-term remission (group D) when compared with other groups.

We found that there were only small differences in the total HCY concentration. Patients with early relapse (group A) had lower tHCY than those with early remission, but tHCY was similar in other groups (Fig. 1).

Protein bound Hcy concentration was lower in patients with early relapse (group A) than in those with long-term remission (group D) (4.32 vs. 8.9 $\mu\text{mol/L}$ respectively), which was obviously associated with lower serum albumin concentration and higher urinary albumin losses in active phase of NS. Interestingly, the free fraction of Hcy in group A was almost twice as high as the concentration in group D (0.81 vs. 0.44 $\mu\text{mol/L}$, respectively).

The influence of cyclosporine A administration was analyzed separately at every stage of the relapse (Fig. 2). In CsA treated patients, total GSH was higher only at the beginning of the relapse. HCY and CY were elevated only in these patients at the beginning or after 2 weeks of treatment (groups A and B). No significant difference was observed in the CG and GSH concentrations in CsA treated or untreated children. When the whole study group in relapse was taken together (groups A, B, and C), a higher concentration of proatherogenic aminothiols (HCY, CY) was detected in children treated with CsA ($n=37$ vs. $n=58$, $P<0.02$) with no changes in GSH and CG levels (antiatherogenic aminothiols).

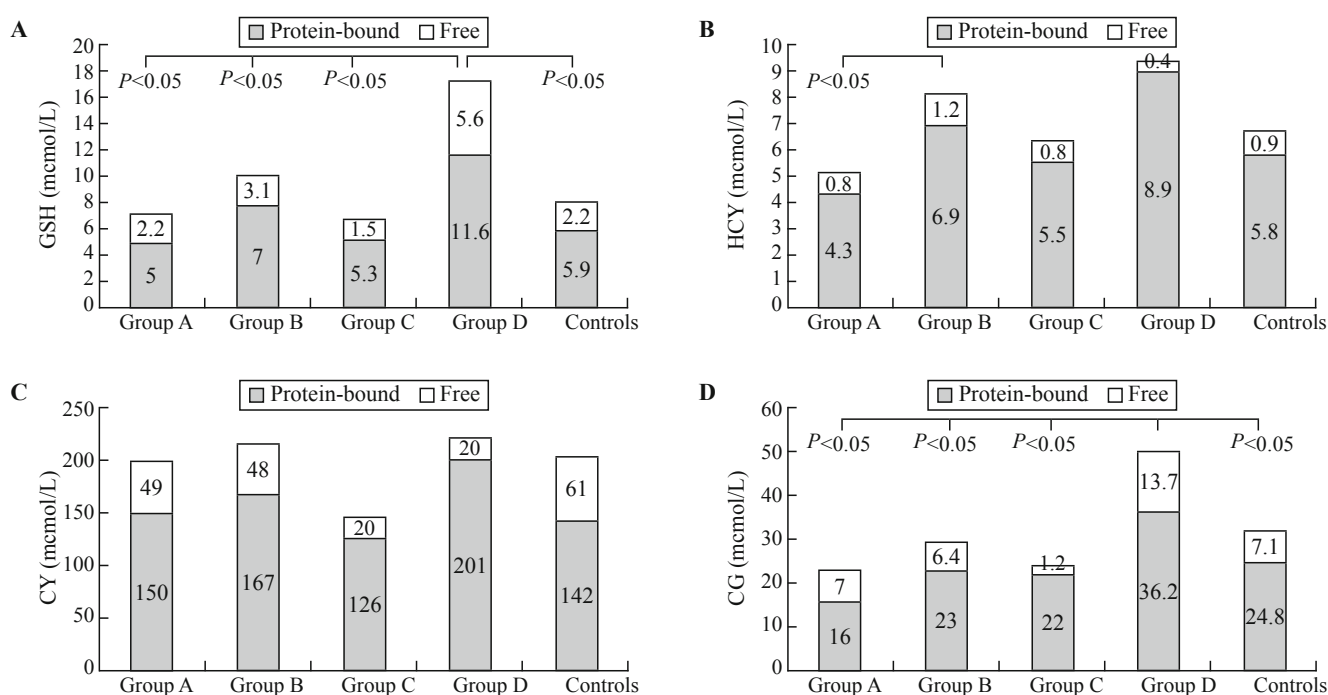


Fig. 1. Plasma concentration (protein-bound and free fraction; median value) of glutathione (A), homocysteine (B), cysteine (C) and cysteinylglycine (D) in the study (A-D) and control (E) groups. GSH: glutathione; HCY: homocysteine; CY: cysteine; CG: cysteinylglycine.

The results of the biochemical analysis are presented in Table 2. The concentrations of total cholesterol, total protein and fibrinogen were concordant with the description of INS relapse. No renal function impairment was found.

The highest thrombomodulin (TMB) concentration (Table 2) was observed in children at the start of INS relapse and subsequently decreased in groups B, C, and D, but remained elevated when compared to the control group. Similarly, the highest PAI-1 concentration was

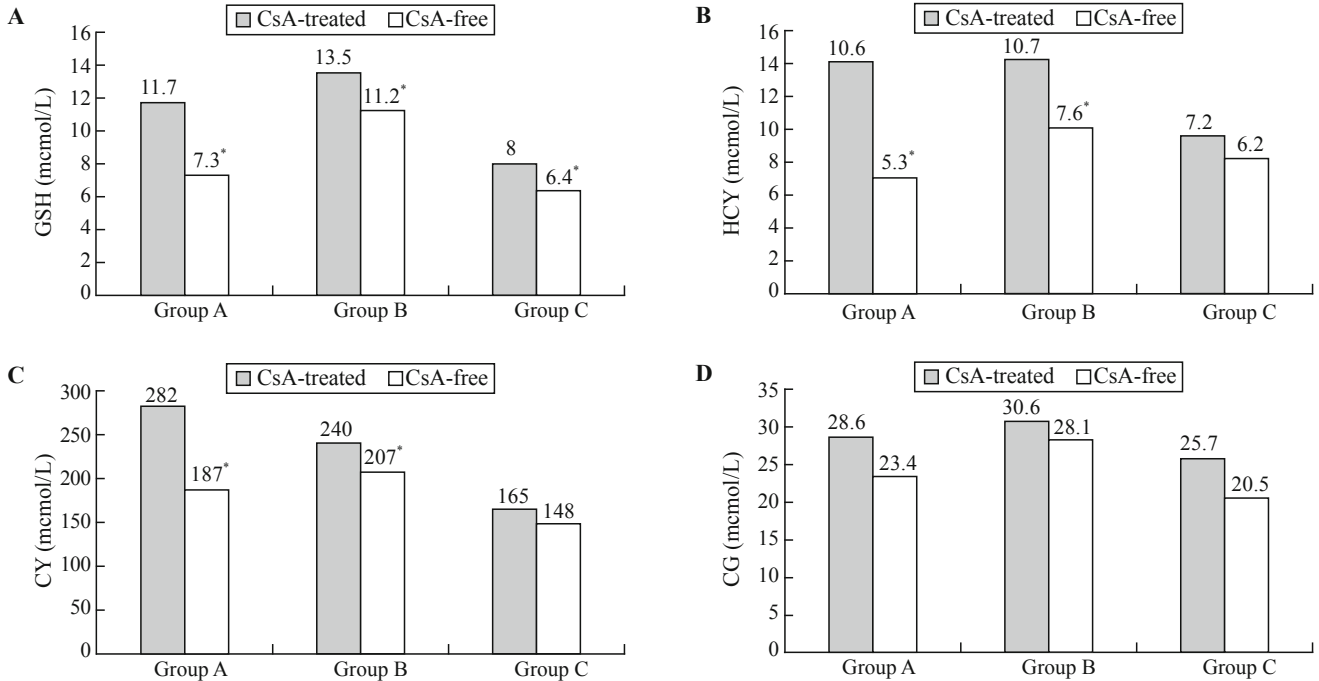


Fig. 2. The influence of cyclosporine A administration on plasma concentration of glutathione (A), homocysteine (B), cysteine (C) and cysteinylglycine (D) in the study groups. GSH: glutathione; HCY: homocysteine; CY: cysteine; CG: cystein-glycine; CsA: cyclosporine A.

Table 2. Biochemical data and markers of endothelial injury in the study (A-D) and control (E) groups, median (25-75 interquartile range)

Variables	Group A	Group B	Group C	Group D	Group E
GFR (mL/min/1.73 m ²)	149 ^{*,†,‡,§} (133-167)	130 (110-154)	131 (125-143)	133 (117-151)	135 (110-154)
Total cholesterol (mmol/L)	7.4 ^{*,†,‡,§} (5.3-11.3)	5.4 ^{*,†,‡} (4.2-6.7)	4.3 ^{†,‡} (4.0-5.2)	4.2 ^{†,‡} (3.7-4.5)	4.2 (3.6-4.6)
Hemoglobin concentration (g/L)	141 ^{*,†} (132-155)	136 ^{*,†} (129-142)	123 ^{†,‡} (119-130)	142 [†] (139-144)	127 [‡] (121-133)
Serum total protein (g/L)	44.5 ^{*,†,‡,§} (40.0-52.0)	61.0 ^{*,†,‡} (59.5-63.0)	67.0 [‡] (66.0-71.0)	70.5 (67.0-76.0)	71.0 [‡] (68.0-73.0)
Serum albumin (g/L)	21 ^{*,†,‡,§} (12-32)	41 ^{*,†,‡} (35-42)	44 ^{*,†,‡} (41-47)	46 ^{†,‡} (45-48)	45 ^{†,‡} (43-47)
Urinary albumin (g/L)	12.9 ^{*,†,‡,§} (0.2-14.7)	0.03 (0.02-0.06)	0.03 (0.02-0.07)	0.02 (0.02-0.13)	0.04 (0.02-0.10)
Urinary albumin/creatinine ratio (mg/mg)	2.4 ^{*,†,‡,§} (0.9-6.9)	0.005 (0.003-0.013)	0.008 (0.005-0.016)	0.004 (0.001-0.015)	0.005 (0.005-0.012)
TBM (ng/mL)	24.2 ^{*,†,‡,§} (14.2-64.3)	12.6 [†] (11.1-17.2)	12.7 ^{*,†} (11.0-16.0)	12.2 ^{*,†} (9.9-13.1)	7.60 ^{†,‡,§} (6.14-8.23)
PAI-1 (ng/L)	83.0 ^{*,†} (69.4-107.1)	66.9 ^{*,†} (48.0-93.1)	70.2 ^{*,†} (52.0-88.1)	36.4 ^{†,‡} (31.0-45.0)	53.2 ^{†,‡} (25.4-73.6)
vWF activity (%)	129 [†] (111-135)	128 [†] (124-131)	128 [†] (122-131)	132 [†] (126-133)	94 ^{†,‡,§} (74-130)
F1+2 (nmol/L)	4.11 ^{*,†,‡,§} (1.92-6.50)	2.21 (1.22-4.23)	2.47 (1.29-3.80)	1.89 (1.31-2.31)	1.70 (1.10-2.34)
TAT (ng/L)	13.4 ^{*,†,‡} (10.4-37.8)	12.7 ^{*,†,‡} (6.9-22.7)	8.8 ^{†,‡} (4.9-11.7)	7.1 [†] (4.3-8.9)	4.1 ^{†,‡,§} (3.0-6.2)
t-PA (ng/L)	7.6 ^{*,†,‡,§} (3.5-9.8)	4.5 [†] (1.9-6.6)	4.7 [†] (2.1-6.5)	1.8 ^{*,†,‡,§} (1.6-2.0)	5.8 [†] (2.1-6.7)

*: significantly different when compared to the controls (E), $P < 0.05$; †: significantly different when compared to group D, $P < 0.05$; ‡: significantly different when compared to group C, $P < 0.05$; §: significantly different when compared to group B, $P < 0.05$. GFR: glomerular filtration rate; TBM: thrombomodulin; PAI-1: plasminogen activator inhibitor-1; vWF: von Willebrand factor; TAT: thrombin-antithrombin complex; t-PA: tissue plasminogen activator; F1+2: fibrinogen fragments 1+2.

Table 3. Correlation coefficients by Spearman's rank-order test between plasma aminothiols and selected markers of endothelial injury and coagulation activation

Aminothiol	Markers of endothelial injury	R-Spearman	P value
Glutathione vs.	Thrombomodulin	-0.317151	0.000120
	F1+2 prothrombin fragments	-0.297100	0.000347
	PAI-1	-0.569144	0.000000
	tPA	-0.614133	0.000000
Homocysteine vs.	Thrombomodulin	0.331574	0.000056
	Trombin-antithrombin complexes	0.193919	0.021218
	PAI-1	0.211594	0.012085
	tPA	0.243151	0.003548
Cysteine vs.	Thrombomodulin	0.171836	0.040875
	Trombin-antithrombin complexes	0.311163	0.000183
	PAI-1	0.311163	0.000183
	Thrombomodulin	-0.304956	0.000224
Cysteinylglycine vs.	Trombin-antithrombin complexes	-0.270359	0.001185
	F1+2 prothrombin fragments	-0.307215	0.000211
	PAI-1	-0.508917	0.000000
	tPA	-0.489260	0.000000

PAI-1: plasminogen activator inhibitor-1; t-PA: tissue plasminogen activator; F1+2: fibrinogen fragments 1+2.

observed in group A, but there was a tendency to decrease in groups B and C. The concentration of this marker in patients with long-term remission (group D) was similar to that in the control group. vWF activity was higher in all groups of INS children than in healthy subjects with no apparent difference between the different stages of the disease. Enhanced thrombinogenesis was detected by highest F1+2 and TAT concentration in group A (early relapse). The latter parameter decreased in the course of INS but remained elevated in all nephrotic children as compared with the controls (Table 2).

Correlation analysis found that the concentrations of proatherogenic aminothiols (HCY, CY) were correlated positively with the markers of the endothelial injury and activation of coagulation cascade (thrombomodulin, thrombin-antithrombin complexes, PAI-1 and tPA). The concentrations of anti-atherogenic aminothiols (GSH, CG) were correlated reversibly with the parameters mentioned above (Table 3). In the multi-regression model (with preselected variables), the relation remained significant for thrombomodulin and PAI-1 for HCY, PA-1 I for CY, and PAI-1, tPA and thrombomodulin for GSH and CG. A weak correlation was found between total HCY, GSH, CY and the total protein concentration ($R=0.3-0.4$, $P<0.05$). No significant relation was found between plasma aminothiols concentration with dose or serum concentration of CsA or dose of glucocorticosteroids.

Discussion

In the present study, children with idiopathic nephrotic syndrome showed imbalanced concentration of sulfuric aminoacids due to the disease activity. Generally, it is aminothiols-atherogenic and anti-atherogenic and

limited to acute phase of the disease. The imbalanced concentration of sulfuric aminoacids could be briefly described as a diminished concentration of antiatherogenic aminothiols (CG) and shift to a high free fraction of HCY postulated previously to be more harmful. These findings suggest that proatherogenic disbalance persists preferably only in the active phase of the INS.^[14]

Oxidative stress is defined as a disturbance in the reactive oxygen species and antioxidant balance. Free radicals are reactive compounds that interact with lipids, proteins, and nucleic acids. Aminoacids that are donors of sulphhydryl residues play an important role in the antioxidant defense in humans (intra- and extra-cellularly).^[15] The oxidation processes may be specially significant in the renal tissue as described by Granqvist et al.^[7] Mishra et al^[16] reported the evidence of oxidative stress and impaired antioxidant defense during INS. The authors evaluated markers of ROS in children with active nephrotic syndrome (ANS) and age- and gender-matched healthy controls. Plasma (MDA) malonyldialdehyd and nitrite levels were significantly higher and selenium level was lower in ANS patients compared with controls but the selenium level increased and then normalized in a long-term remission. Furthermore, antioxidant status recovered completely only during a long-term remission.^[16] Admittedly, increased plasma levels of MDA and nitrite observed by Mishra et al^[16] in ANS indicate increased lipid and protein peroxidation but OS also leads to the damage of nucleic acids. Kaneko et al^[15] reported changes in 8-OH-DG in INS-highlighted the problem of interaction of ROS with cellular DNA. The authors demonstrated that in patients with INS, the urinary levels of 8-OHdG (ng/mL) corrected by creatinine (ng/mg Cr) were significantly higher in patients with relapse than in those in remission.

Markan et al^[17] evaluated the oxidative status in adult patients with primary glomerular diseases (PGD) in both: non-proliferative (NPGN) and proliferative glomerulonephritis (PGN). In the PGD patients, they found a significant increase in MDA, reactive nitrogen intermediates, tHCY, 8-IP levels, and decreased SOD. Total thiols and protein bound thiol levels were compared to controls. Simultaneously, they confirmed that oxidative stress in PGN was significantly higher than in NPGN independently of renal function.^[17] Significantly increased levels of serum lipid peroxide, HCY and decreased levels of serum total antioxidant capacity, copper, zinc and plasma vitamin C were observed by Dwivedi et al^[18] in NS patients as compared to controls.

Most HCY in the plasma is bound to albumin, and urinary losses and diminished plasma concentration

are the features of NS.^[19] Tenderenda et al^[20] evaluated the levels of serum and urinary homocysteine (stHCY and utHCY) in a small group of NS children. StHCY concentration were not different in controls from children with active proteinuria and those after regression of proteinuria; but utHCY concentration was significantly higher in both groups than that in the controls. In children with steroiddependent NS (SDNS), subclinical disturbances in hemostasis were independent of stHCY concentration, but urinary excretion of HCY significantly increased and correlated positively with serum AT III level.^[20] Aminzadeh et al^[19] reported that nephrotic rats had reduced plasma HCY (due to the reduction in albumin-bound HCY as opposed to the free HCY fraction), increased urinary albumin-bound HCY, and down-regulation of, but expression of methylenetetrahydrofolate reductase. Arnadottir et al^[21] demonstrated lower levels of total plasma HCY in adult nephrotic patients with idiopathic membranous nephropathy compared with non-nephrotic patients matched for renal function. These findings are similar to the results obtained by Tenderenda and Aminzadeh and to those obtained in the present study where stHCY was lower in group A (active) than in group D (remission) (4.34 vs. 8.9 $\mu\text{mol/L}$; $P < 0.05$).^[19,20] Dogra et al^[22] measured the total levels of plasma HCY (tpHCY) in primary INS patients and non-nephrotic controls (matched for age, BMI, and GFR) as well as brachial artery flow-mediated dilatation (FMD%), which reflects endothelial function and serves as an early cardiovascular risk marker. Unlike Tenderenda et al^[20] and Arnadottir et al,^[21] we found no difference in tpHCY between the groups. Within the INS group, HCY was significantly correlated with serum creatinine and GFR but not with urinary protein or serum albumin.^[20,21] We also found impaired brachial FMD in the NS group; however surprisingly, HCY did not correlate with FMD. Bafna et al^[23] observed relative defects of oxidant/antioxidant balance in NS which could predispose NS patients to increased oxidative stress and hyperhomocysteinemia. They concluded that in NS patients oxidative stress was enhanced, total antioxidant capacity was reduced due to dyslipidemia, hyperhomocysteinemia, hyperlipoproteinemia and hypoalbuminemia.^[23] By the review of recent studies, we may hypothesize that the long-term significance of aminothiol imbalance has not yet been elucidated. In our study, patients in a long-term remission of INS had elevated concentrations of pro- and antiatherogenic aminoacids. We hypothesize that due to the long stimulation of oxidative processes, the compensatory mechanisms were induced and antiatherogenic aminoacids were produced.

The second clinically important finding that arose

from the study was an unfavourable influence of cyclosporine A administration on plasma concentration of homocysteine and cysteine that was limited mostly to the acute phase of the nephrotic syndrome (relapse and early remission). Previous studies on this group of patients brought similar observations.^[24] Cyclosporine has been previously described to increase endothelial injury and raise the homocystein concentration.^[25]

In our study we related aminothiols balance to the biochemical markers of endothelial injury, that was also confirmed in early relapse of INS. As children with INS showed increased plasma concentration of markers of endothelial injury.^[26] The endothelial dysfunction was mostly pronounced in the early relapse of INS (A) with a tendency to decrease in the following several months (groups B, C and D). Endothelial function may be altered in proteinuric renal diseases.^[27,28] The injury was related to the degree of proteinuria and renal dysfunction. T endothelial function measured by NO-dependent relaxation of the brachial artery may be changed in INS relapse.^[29]

Our study does have some limitations as its results might be influenced by selection bias. The study center is of a tertiary reference level and admits more complicated patients, typically with advanced INS, often obese and hypertensive ones. Furthermore, the cross-sectional design was less appropriate to compare phases of the disease than longitudinal approach. But the uniformity of study groups was enhanced by strict criteria of qualification and the fact that children suffered from idiopathic nephritic syndrome.

In conclusion, the children with the idiopathic nephrotic syndrome showed imbalance in the aminothiol metabolism in the relapse and early remission, shifting it towards increased oxidative processes. The administration of cyclosporine A caused a significant increase in proatherogenic homocysteine and cysteine concentration. We postulate that the aminothiol imbalance role in disturbed red-ox mechanisms is limited to early phases of nephrotic syndrome relapse, thus it has a potential detrimental role only for severe steroid resistant or frequently relapsing children.

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Ethical approval: The study was approved by Independent Ethics Committee of Polish Mother's Memorial Hospital Research Institute.

Competing interest: The authors declare no competing interest
Contributors: TM proposed and performed the project, prepared the manuscript; MM contributed in preparation of project manuscript; LJ contributed in the project and laboratory analysis and preparation of manuscript; FW did the statistical analysis and contributed in preparation of manuscript.

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