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## Elevated plasma ADMA contributes to development of endothelial dysfunction in children with acute lymphoblastic leukemia\*

Podwyższone stężenie ADMA w osoczu przyczynia się do rozwoju dysfunkcji śródbłonna u dzieci z ostrą białaczką limfoblastyczną

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### Summary

#### Background:

Childhood acute lymphoblastic leukemia (ALL) survivors are at higher cardiovascular risk than the general population, which may result from anthracycline-related endothelial dysfunction (ED). However, a few studies indirectly show that ED may appear in ALL children before treatment begins. Hence, in this study we intended to verify the hypothesis that ED is part of the ALL phenotype.

#### Patients/Methods:

Twenty-eight ALL children and 14 healthy age-matched control children were recruited. The study group was examined at baseline, then at the 33<sup>rd</sup> and 78<sup>th</sup> day of treatment. At each step of the protocol endothelial vasodilative function was assessed by a laser Doppler flowmeter, which was followed by blood collecting for subsequent analyses.

#### Results:

Compared to controls, the study group at baseline was characterized by significantly lower endothelial vasodilative responsiveness, accompanied by elevated asymmetric dimethylarginine (ADMA) and symmetric dimethylarginine (SDMA) concentrations, which were correlated with lactate dehydrogenase (LDH) and aspartate transaminase (AST). Initial ALL treatment restored endothelial function, which followed changes in ADMA and LDH concentrations.

#### Discussion:

This is the first demonstration that functionally assessed ED is present in ALL children at the diagnosis and results from elevated ADMA and parallel inflammatory ED.

#### Keywords:

acute lymphoblastic leukemia • endothelial dysfunction • laser Doppler • ADMA

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## BACKGROUND

Asymmetric dimethylarginine (ADMA) is a competitive inhibitor of endothelial nitric oxide synthase (NOS-3), and numerous studies have demonstrated its significant contribution to endothelial dysfunction [2,3]. Furthermore, several studies show that elevated ADMA level is associated with increased cardiovascular risk as well as poorer prognosis in both acute and chronic diseases [4,7,12,14].

The main source of ADMA are L-arginine-rich proteins, previously methylated during post-translational modifications (i.e. histones) [6]. Proteolysis of methylated proteins leads to formation of ADMA and symmetric dimethylarginine (SDMA) without inhibitory action on NOS3.

Clinical disorders which are characterized by increased cellular turnover could be related to ADMA overproduction, leading to development of endothelial dysfunction due to decreased nitric oxide (NO) bioavailability. Persistent endothelial dysfunction (ED) in this population may be linked to an imbalance between concentrations of molecules involved in nitric oxide synthesis such as L-arginine and ADMA [19]. Several studies have demonstrated that oxidative stress, mostly by increased lipid peroxidation, may limit nitric oxide bioavailability by inhibiting dimethylarginine dimethylaminohydrolase (DDAH), an enzyme which degrades ADMA [10,17].

In this study we intended to verify the hypothesis that increased cellular turnover in ALL children results in ADMA overproduction, contributing to development of endothelial dysfunction. The conceptual hypothesis is presented in Figure 1.

## MATERIAL AND METHODS

All experiments were conducted and approved in accordance with the guidelines of the Bioethics Committee at Wrocław Medical University and adhered to the principles of the Declaration of Helsinki and Title 45, U.S. Code of Federal Regulations, Part 46, Protection of Human Subjects (revised November 13, 2001, effective December 13, 2001). All participants provided

their informed consent which was followed by its written approval by a legal representative, as appropriate. The study and the written consent form were approved by the Bioethics Committee at Wrocław Medical University.

We enrolled in our study 28 children with acute lymphoblastic leukemia and 14 healthy demographically matched children (Table 1).

## MATERIAL AND STUDY PROTOCOL

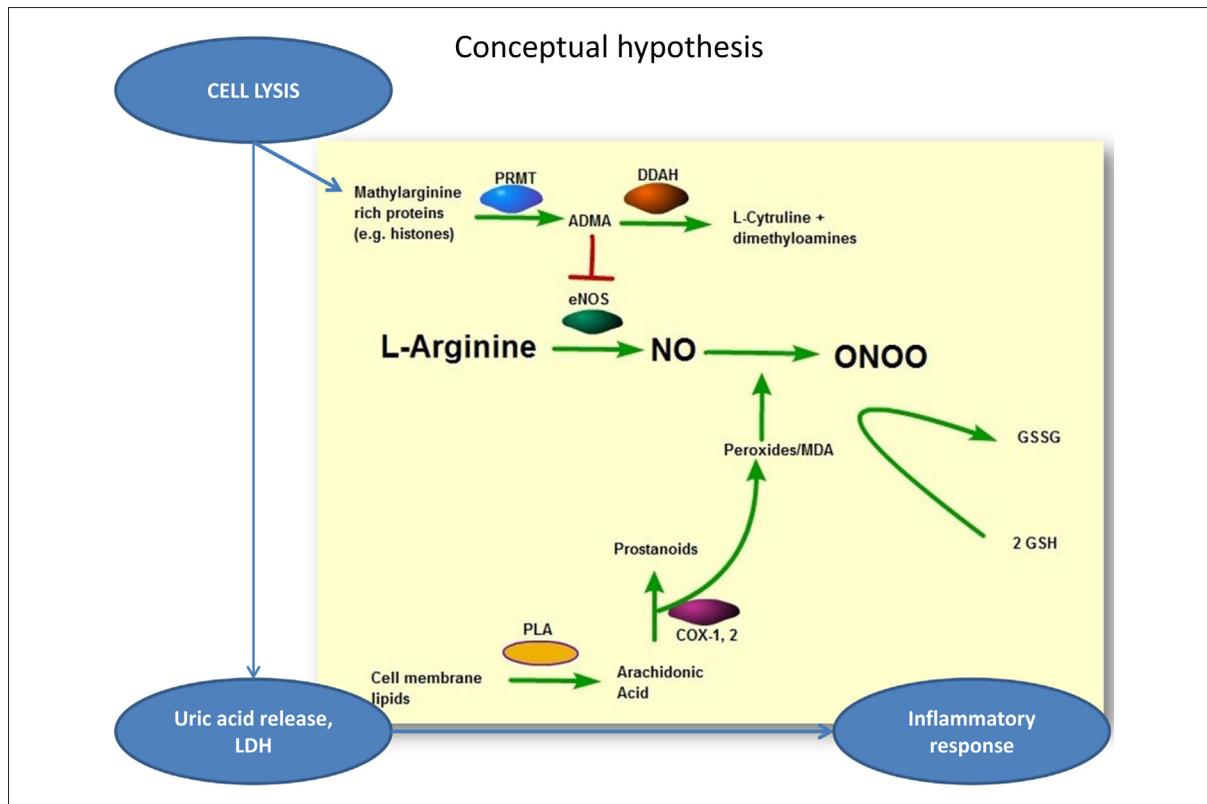
The study group comprised children with an established diagnosis of acute lymphoblastic leukemia treated strictly according to the guidelines published in the ALL-IC BMF 2002 protocol [18] (Fig. 2B) at the Department of Pediatric Bone Marrow Transplantation, Oncology and Hematology, Wrocław Medical University (Wrocław, Poland). Children were examined at the baseline (day of diagnosis) and on the 33<sup>rd</sup> and 78<sup>th</sup> day of therapy. In all three points blood samples were obtained and endothelial function was assessed (Fig. 2A).

ALL children were categorized into three risk groups: standard (n=8), intermediate (n=14) and high risk (n=6) [18]. Independently of this fact, during the first 78 days all of them were treated the same way – the only difference was a doubled dose of daunorubicin in intermediate and high risk groups (Fig. 2B). Remission was observed in all patients at the 33<sup>rd</sup> day of treatment.

The control group was formed by healthy children observed at a general pediatric ward (Wrocław Medical University, Wrocław, Poland) due to disorders that do not affect endothelial function. Controls were examined only once, since no changes in the profile of endothelial function were suspected.

## LASER DOPPLER FLOWMETRY

We used a laser Doppler device (PeriFlux System 5000, Perimed, Järfälla, Sweden) to assess the forearm skin blood flow. The PeriFlux System 5000 flowmetry device uses a laser light Doppler shift to measure blood flow in skin capillaries. Obtained information is visible in real time due to dedicated software. The whole proce-



**Fig. 1.** Conceptual hypothesis of the study. Briefly, increased cellular lysis in the course of ALL results in overproduction of methylated arginine-rich proteins, which are precursors for Asymmetric dimethylarginine /ADMA/. Increased ADMA synthesis (by protein arginine methyltransferase /PRMT/) is responsible for development of nitric oxide /NO/ deficiency and endothelial dysfunction. ADMA can be degraded by dimethylarginine dimethylaminohydrolase /DDAH/ to L-Cytrulline and metyloamines. Lower part of the figure presents that decreased bioavailability of NO may be also associated with oxidative stress resulting from inflammatory response to tissue damage; PLA - indicates Phospholipase A2; COX-1,2, Cyclooxygenase-1,2; MDA - malonyldialdehyde; GSH - reduced glutathione and GSSG, oxidized glutathione

ture was performed strictly according to the manufacturer's instructions, with a constant skin temperature (33°C) maintained by a PeriFlux heating unit. Briefly, the forearm was immobilized by a vacuum pillow to prevent limb movements. The skin was cleaned with an isopropyl alcohol soaked swab before placing the probe and electrodes on the anterior part of the forearm, free from visible superficial veins. When the signal was stable, the investigators began recording baseline flow values. Afterwards, pilocarpine (800 µl of 2% stock solution, Polfa Warszawa, Warszawa, Poland) was delivered to the skin by iontophoresis (Perimed Perilont Micro Pharmacology System). Seven pulses of pilocarpine were administered during each examination. Subsequently, the flow increase was recorded until a flow plateau was observed (mean time 10 min 16 s). Baseline flow values were compared with those observed after pharmacological stimulation. In order to avoid any influence of baseline flow variability, the data were presented as a percentage of change from baseline values. The protocol was adapted from a study conducted by another group [15]. We calculated the 5-minute response index (Fig. 2C).

## BIOCHEMICAL TESTS

Blood was collected using the Sarstedt S-Monovette system (Sarstedt AG & Co., Nümbrecht, Germany). EDTA plasma (9.0 mL; 1.6 mg-EDTA/ml of blood) was separated, immediately centrifuged (1000 x g for 15 minutes at 4°C) and frozen at -20°C for evaluation of ADMA, SDMA, L-arginine levels and markers of endothelial activation and oxidative stress.

## ASSESSMENT OF ADMA, SDMA AND L-ARGININE LEVELS

Plasma concentrations of L-arginine, ADMA, and SDMA were measured by high-performance liquid chromatography (HPLC) and precolumn derivatization with ophthaldialdehyde (OPA) by a modification of a previously published method [2]. L-Homoarginine (10 µM) was added to 0.5 ml of plasma as an internal standard. Plasma samples and standards were extracted on solid-phase extraction (SPE) cartridges (Bond Elute SCX, Varian Inc, Palo Alto, Calif). Recovery rates were 82.9±3.8%. Eluates were dried over nitrogen and resuspended in double-distilled water for HPLC analysis. HPLC was per-

**Table 1.** Baseline characteristics of acute lymphoblastic leukemia children

Parameter	Study group		
	(mean ± SEM)		
n	28		
Men (n)	12		
Age (years)	8.1	±	1
WBC (k/ $\mu$ L)	30,4	±	10,6
Hemoglobin (g/dL)	8,1	±	0,4
Neutrophiles	37%	±	12%
Platelets (k/ $\mu$ L)	90,1	±	22,0
Serum creatinine (mg/dL)	0.6	±	0.0
Plasma glucose (mg/dL)	100	±	5.9
ALT (U/L)	51.6	±	32.3
AST (U/L)	49.2	±	13
hsCRP (mg/L)	23.2	±	5.73
Total bilirubin (mg/dL)	0.57	±	0.11
LDH ( $\mu$ kat/L)	10.4	±	1.6
Total protein (g/dL)	6.8	±	0.1
Uric acid (mg/dL)	5.9	±	0.5
Sodium (mmol/L)	140	±	0.4
Potassium (mmol/L)	4.2	±	0.1

Abbreviations: hsCRP – high sensitive C-reactive protein. LDH – lactate dehydrogenase

formed on a computer-controlled Varian Star chromatography system consisting of a ternary gradient HPLC pump (Varian Pro Star 240), an automatic injector with automated sample-reagent mixing capabilities (Varian Pro Star 410), and a fluorescence detector (Varian Pro Star 363). Samples and standards were incubated for exactly 1 minute with OPA reagent (5.4 mg/ml OPA in borate buffer, pH 8.4, containing 0.4% 2-mercaptoethanol) before automatic injection into the HPLC. The OPA derivatives of L-arginine, ADMA, and SDMA were separated on a 150x4.6-mm-ID 5- $\mu$ m column (Symmetry C18 HPLC column [Waters Co., Milford, USA] with the fluorescence detector set at ex=340 nm and em=450 nm. Samples were eluted from the column with 0.96% citric acid/methanol (70:30), pH 6.8, at a flow rate of 1 ml/min. Variability of the method was < 7%, and the detection limit of the assay was 0.05  $\mu$ mol/l.

#### MARKERS OF OXIDATIVE STRESS

Malondialdehyde (MDA) level was assessed with a lipo-peroxidation marker using a colorimetric assay (LPO-586, BIOXYTECH, OxisResearch, Portland, Oregon, USA). In this method, the reaction of N-methyl-O-2-phenyl-indole with MDA and hydroxyalkenal (HAE) is used and results in the synthesis of a chromogenic product

( $\lambda_{max}$  =586 nm). Addition of HCl inhibits cross-reactivity for HAE. Thus, the results reflect only the level of MDA [8]. The intra-assay and inter-assay % coefficients of variation (CVs) for MDA were 4.5% and 6.0%.

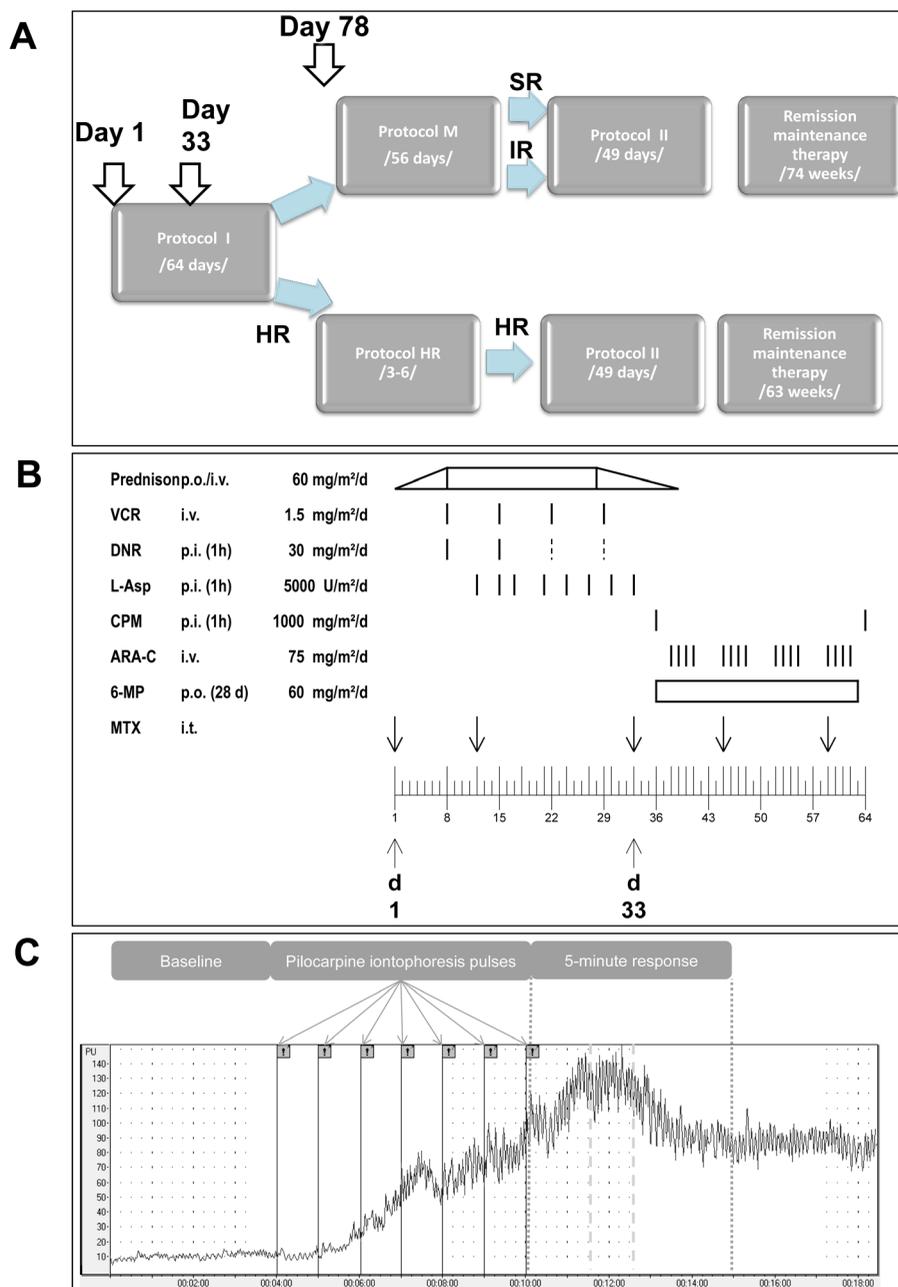
#### OTHER BIOCHEMICAL ANALYSES

Plasma concentrations of prostanoids (6-keto prostaglandin F1-alpha [6-keto-PGF<sub>1 $\alpha$</sub> ] as a marker of prostacyclin synthesis and thromboxane B2 [TxB<sub>2</sub>] reflecting thromboxane formation) were measured using commercial ELISA kits (Assay Designs – enzyme immunoassay kit 6-keto-PGF<sub>1 $\alpha$</sub>  and TxB<sub>2</sub> enzyme immunoassay kit).

Concentrations of serum creatinine, urea, fasting plasma glucose, aspartate transaminase (AST), alanine transaminase (ALT), lactate dehydrogenase (LDH), high-sensitivity C-reactive protein (hsCRP), potassium and uric acid were measured using standard commercial laboratory assays.

#### STATISTICAL ANALYSIS

Data is expressed as the mean ± SEM. The differences between two continuous parameters were assessed using the Mann-Whitney U-test or Student's t-test, fol-

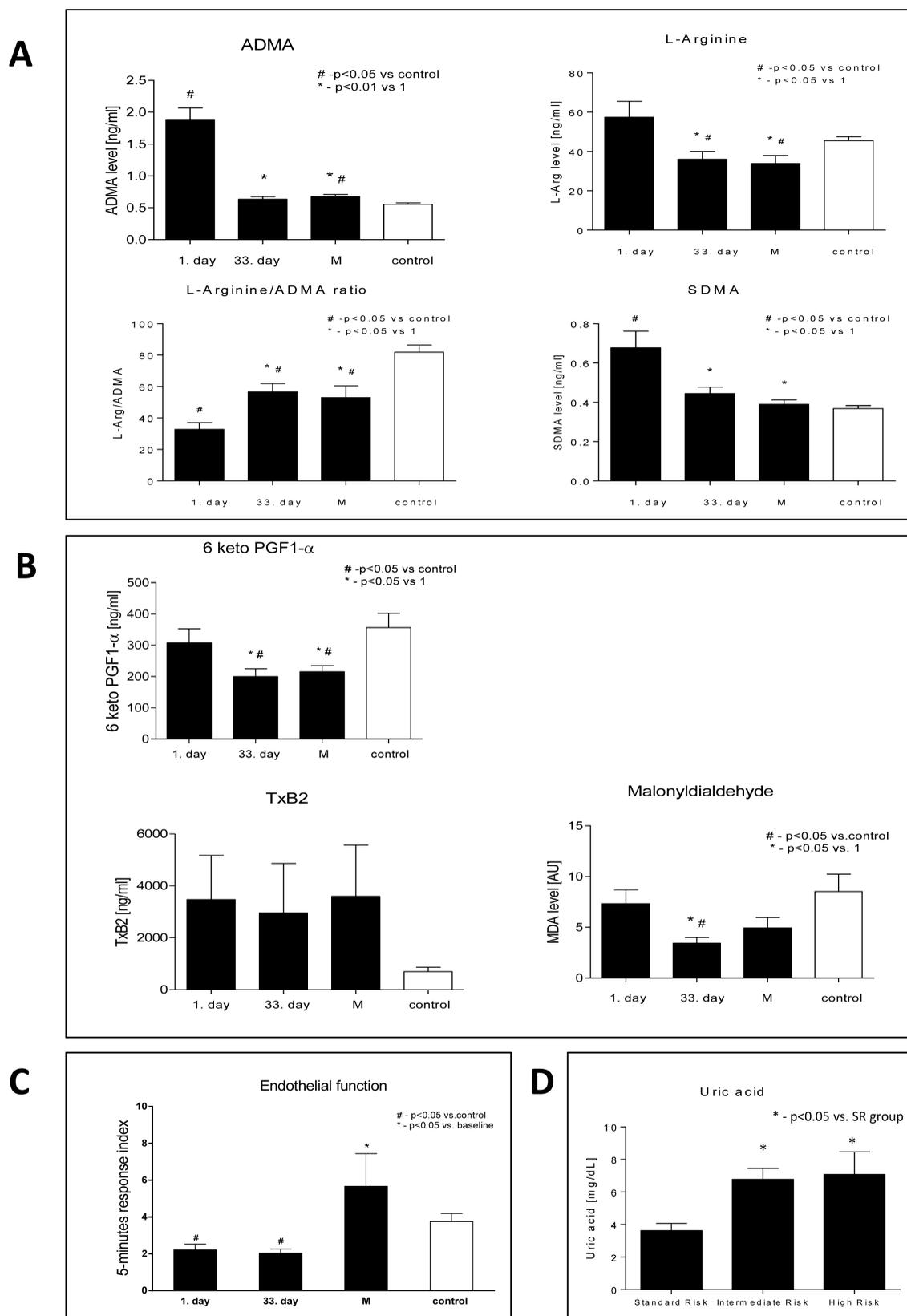


**Fig. 2.** A - Study design, B - Treatment protocol Protocol I in details. Study covered the period of treatment common to all risk groups; VCR - indicates vincristine; DNR - daunorubicin; L-Asp - L-Asparaginase; CPM - cyclophosphamide; ARA-C, cytarabine; 6-MP - mercaptopurine/mercaptopurine; MTX - methotrexate/methotrexate; p.o. - per os; p.i. - per infusionem; i.t. - intrathecal; d 1 - day 1; d 33 - day 33; SR - standard risk; IR - intermediate risk and HR, high risk; C - Laser Doppler examination protocol

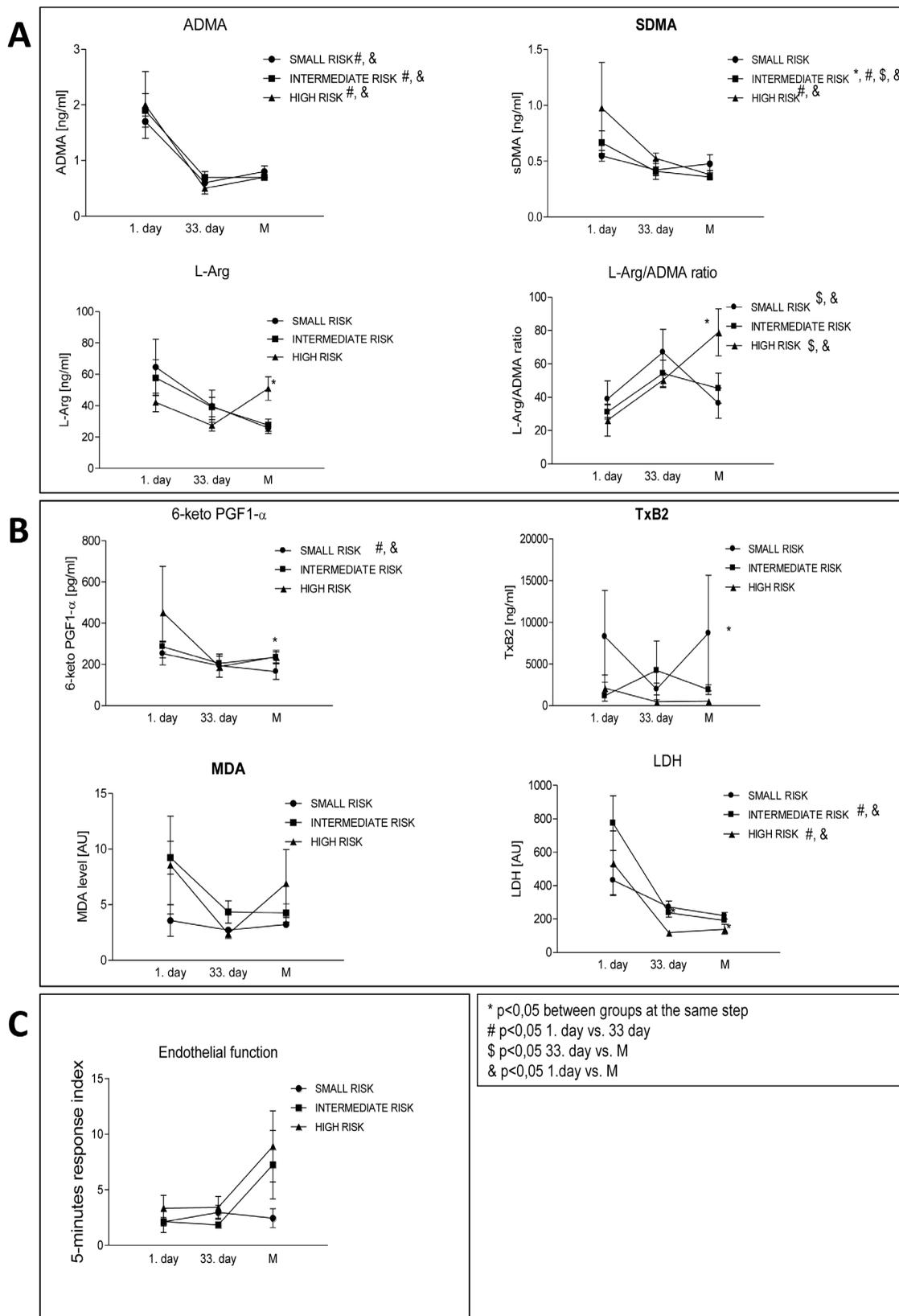
lowed by the Shapiro-Wilk test and Levene’s test, as appropriate. For comparison of more than two groups, an ANOVA followed by Tukey’s test, or a Friedman ANOVA test (for non-parametric statistics) was performed. Correlations between continuous variables were calculated using the Spearman test. All calculations were performed using Statistica 10.0 StatSoft, and the graphical representation of the data was performed using GraphPad 5 Prism.

## RESULTS

Baseline characteristics of the group of children with ALL is presented in Table 1. The control group was demographically matched and constituted children without any chronic diseases. Significant differences between these two groups were found for white blood cell count, hemoglobin level, platelet count, plasma glucose level and AST activity.



**Fig. 3.** A - Metabolites of the nitric oxide synthesis pathway at particular steps of the study protocol in children with ALL and in healthy control group. B - Endothelial markers of oxidative stress and inflammatory response at particular steps of the study protocol in children with ALL and in healthy control group. C - Endothelial function assessed by a Laser Doppler flowmetry at particular steps of the study protocol in children with ALL and in healthy control group. D - Analysis of the uric acid concentrations at particular steps of the study protocol in children with ALL and in healthy control group



**Fig. 4.** A - Analysis of metabolites of the nitric oxide synthesis pathway at particular steps of the study protocol in children with ALL assigned to the subgroups separated according to the risk stratification. B - Analysis of endothelial markers of oxidative stress and inflammatory response and cellular lysis in children with ALL assigned to the subgroups separated according to the risk stratification. C - Analysis of endothelial function assessed by a Laser Doppler flowmetry in children with ALL assigned to the subgroups separated according to the risk stratification

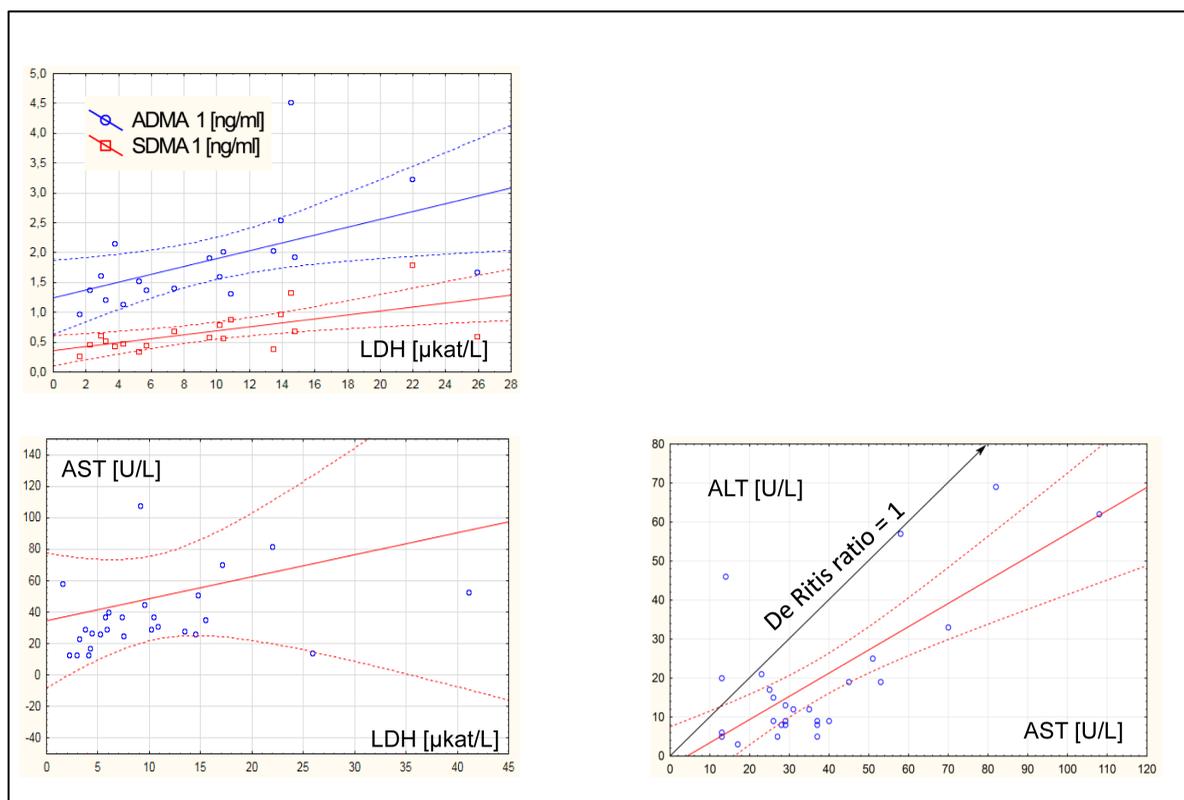


Fig. 5. Correlations between metabolites of the nitric oxide synthesis pathway and tissue/organ damage markers in children with ALL

### METABOLITES OF THE NITRIC OXIDE SYNTHESIS PATHWAY, ARACHIDONIC ACID CASCADE AND MARKERS OF OXIDATIVE STRESS

Baseline ADMA and SDMA levels in ALL children were significantly higher than in the control group and decreased during the treatment (Figure 3A). However, the ADMA level still remained significantly higher than in controls at the 78<sup>th</sup> day. A similar decreasing trend of ADMA levels was observed in each risk group (Figure 4A). However, the decrease in SDMA levels was significant only in the intermediate and high risk groups. The bioavailability of a substrate for NO synthesis (the L-arginine/ADMA ratio) was lower than in healthy controls at all steps of this study. Moreover, the lowest values of the ratio were observed at the first day of observation. Furthermore, the ratio was markedly rising in the high risk group (Figure 4A). We also verified whether changes in the NO biosynthesis metabolite levels are correlated with concentrations of tissue damage indicators (Figure 5). Statistically significant positive correlations between ADMA and LDH ( $R=0.66$ ,  $p=0.002$ ) as well as between AST and LDH ( $R=0.46$ ,  $p=0.013$ ) were demonstrated (Fig. 5, Table 2).

In the course of treatment (the 33<sup>rd</sup> day and the beginning of the M protocol) the 6-keto-PGF<sub>1α</sub> levels were significantly lower as compared to both the control group and the beginning of observation (Figure 3B). The decreasing trend of 6-keto-PGF<sub>1α</sub> levels was particularly

present in the intermediate risk group (Figure 4B). Furthermore, the low risk group was characterized by the lowest level of 6-keto-PGF<sub>1α</sub> and the highest level of TxB<sub>2</sub> at the beginning of the M protocol, when compared to both groups with greater risk.

The highest concentration of lipid peroxidation products (MDA) in the study group was observed at baseline. It was significantly higher than at the 33<sup>rd</sup> and 78<sup>th</sup> day of therapy. Moreover, the 33<sup>rd</sup> day value was also significantly lower than the value in the control group (Fig. 3B). Analysis of the subgroups separated according to the risk did not provide any additional information (Figure 4B). Uric acid level in both groups with greater risk was significantly higher when compared to the low risk group (Figure 3D).

### ENDOTHELIAL VASODILATIVE FUNCTION

From the beginning of the study until the 33<sup>rd</sup> day of treatment the ALL children had significantly poorer endothelial response to pilocarpine assessed by laser Doppler as compared to the healthy group (Figure 3C). At the 78<sup>th</sup> day of therapy a significant improvement of endothelial function in the study group was observed (Figure 3C). No significant differences in endothelial function were observed between risk subgroups at particular steps of the study protocol (Figure 4C).

**Table 2.** Correlation between parameters assessed in study group at baseline

	Uric acid		ADMA		SDMA		L-Arg		L-Arg/ADMA	
	r	p	r	p	r	p	r	p	r	p
ALT	<b>0.42</b>	<b>0.03</b>	0.26	0.28	0.29	0.22	-0.15	0.54	-0.27	0.27
AST	0.01	0.95	0.28	0.25	0.25	0.29	-0.36	0.13	-0.42	0.07
LDH	0.19	0.32	<b>0.66</b>	<b>0.00</b>	<b>0.64</b>	<b>0.00</b>	0.12	0.63	-0.26	0.27
Uric acid	x		0.11	0.67	0.30	0.22	<b>-0.46</b>	<b>0.05</b>	<b>-0.51</b>	<b>0.03</b>
Creatinine	<b>0.49</b>	<b>0.01</b>	-0.15	0.53	0.03	0.90	<b>-0.50</b>	<b>0.03</b>	-0.42	0.07
Urea	<b>0.71</b>	<b>0.00</b>	0.29	0.22	0.39	0.10	-0.25	0.30	-0.41	0.08

Abbreviations: ADMA - asymmetric dimethylarginine, ALT - Alanine transaminase, AST - Aspartate transaminase, L-Arg/ADMA – L-Arginine to asymmetric dimethylarginine ratio, LDH – lactate dehydrogenase, r - Spearman’s rank correlation coefficient, SDMA - symmetric dimethylarginine.

## DISCUSSION

To our knowledge this is the first verification of a hypothesis regarding the presence of endothelial dysfunction in ALL children at the onset of the disease by use of functional testing. Our data supports the theory that ED is part of acute lymphoblastic leukemia and the theory that ALL should be regarded as a multi-organ disease.

We postulate that there is no single reason for decreased endothelial reactivity to physiological stimuli in ALL children. In our study we demonstrated that synthesis of nitric oxide in children with ALL is substantially impaired. The bioavailability of a substrate for its synthesis (assessed as the L-arginine to ADMA ratio [1]) is decreased due to the high concentration of ADMA, which is a competitive inhibitor of the NO synthase [20]. ADMA in ALL may be a product of neoplastic cell degradation, as it is positively correlated with LDH concentration. Our observation for the first time points to the neoplastic cells as the source of ADMA. Taking into account all the above considerations, we postulate an additional novel mechanism of endothelial dysfunction in ALL – resulting from increased ADMA production. However, we have no direct evidence of ADMA release from neoplastic cells, and oxidative stress induced DDAH inhibition may also contribute to ADMA elevation. Also, since there was no correlation between ADMA and transaminases (AST, ALT), it seems not to be associated with hepatic failure as described by other investigators [16]. Furthermore, the study group differs from controls only in AST, but not ALT level, and in most cases AST>ALT, which suggests their non-hepatic origin [5].

SDMA does not exert a significant inhibitory effect on NOS-3 and is considered to be a marker of early kidney dysfunction [9]. Its levels have been shown to be elevated prior to a marked increase in creatinine level and decrease in estimated glomerular filtration rate (which is also based on the creatinine level) [13]. In our study SDMA was assessed in order to exclude a possible effect of early kidney dysfunction on the profile of endothelial function. The mean creatinine and urea levels in the

ALL children were maintained within physiological ranges at the onset of therapy. Since no significant correlation between SDMA and either urea or creatinine levels were observed, we may presume that the elevated SDMA level did not reflect the primarily impaired kidney function in this group but was rather associated with increased SDMA production (due to increased cell lysis), as it is strongly positively correlated with ADMA production as well as with LDH levels. The analysis of other markers of early kidney damage, such as cystatin-C [11], would be an interesting point to confirm this thesis.

Of note, a strong positive correlation between uric acid and creatinine and urea levels was observed. This observation confirms that increased cell lysis and nuclear breakdown in the course of ALL, by generating large quantities of nucleic acids converted to uric acid, are apt to precipitate as monosodium urate crystals, leading finally to the development of acute uric acid nephropathy (AUAN) in a concentration-dependent manner. Assessment of the urine uric acid/creatinine ratio in a random urine sample and the analysis of its correlation with markers of early kidney damage (such as cystatin-C or SDMA) would be of interest in verification of whether early kidney damage is also induced by uric acid similarly to that observed in AUAN.

We found that ADMA and markers of endothelial injury and oxidative stress normalize after the first month of treatment according to the ALLIC protocol. At the 33<sup>rd</sup> day of therapy we observed that concentrations of ADMA and MDA significantly decreased from the baseline values. It was followed – with some latency – by endothelial function recovery observed at the 78<sup>th</sup> day of the protocol.

## CONCLUSIONS

Our data reveals pathophysiological abnormalities involved in the pathogenesis of endothelial dysfunction in ALL children. ED is present in children with ALL prior to the treatment and may result from elevated ADMA levels, oxidative stress and systemic inflammation.

These new aspects of ALL pathophysiology should be taken into consideration in future therapeutic strategies. Further studies are needed in order to identify patients at high risk for late cardiovascular events and to determine whether they require a different ALL treatment protocol or prophylactic use of agents improving endothelial function to prevent overt cardiovascular disease development.

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