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Expression of multidrug resistance protein 1 and multidrug resistance-associated protein 1 in peripheral blood lymphocytes amongst children and young adults

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Abstract

Multidrug resistance protein 1 (MDR1, ABCB1 or P-glycoprotein) and multidrug resistance-associated protein 1 (MRP1, ABCC1 or LTC4 transporter) are active membrane-associated efflux transporters with broad substrate specificity. The aim of this study was to assess the physiological expression of these proteins in the peripheral blood lymphocytes (PBLs) and selected polymorphisms in genes for these proteins in healthy children and young adults. The PBLs MDR1 expression in 41 subjects (aged from 2 months to 22.5 years of age) and PBLs MRP1 in 40 subjects (aged from 2 months to 22 years of age) were determined by using a flow cytometry analysis with the indirect immunofluorescence staining technique. Genotyping of rs1045642 (C3435T) and rs3213619 (T-129C) single nucleotide polymorphisms (SNPs) in ABCB1 gene and rs45511401 (G671V) SNP in ABCC1 gene was performed with TaqMan probes and by direct sequencing. We found a statistically significant dependence of PBLs MDR1 (but not PBLs MRP1) expression on age (Spearman R = –0.40014, p = 0.0095). The increased PBLs MDR1 expression was found in children up to 1 year of age and from 4 to 6 years of calendar age. In addition, this increased expression may reduce the concentration of MDR1-transported immunomodulatory and antiviral drugs in lymphocytes and thus lead to the attenuated efficacy of these drugs in younger children. The impact of selected gene polymorphisms on the expression was not found.

Key words: *MDR1, ABCB1, P-glycoprotein, MRP1, ABCC1, peripheral blood lymphocyte, age, C3435T, T-129C, drug resistance.*

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Introduction

Multidrug resistance protein 1 (MDR1) and multi-drug resistance-associated protein 1 (MRP1) are membrane-associated primary active transporters of a large family of ATP-binding cassette (ABC) transport proteins, utilizing the energy of ATP binding/hydrolysis. These proteins transport compounds from the intracellular to the extracellular space or between cell compartments [1].

Their designation is due to their ability to participate in the cell resistance to structurally different cytostatic drugs (multi-drug resistance) [2, 3]. Both proteins have a broad substrate specificity and may affect therapy with other drugs apart from cytostatic drugs through alteration of their intracellular concentration and plasma level, thus participating in drug-drug interactions. These proteins transport not only exogenous compounds but also various endogenous substances. In addition, a number of these endogenous substances are involved in the regulation of the immune response (Table 1).

MDR1 and MRP1 are predominantly expressed in the membranes of the cells located at the edge of tissues to protect the cells and subsequently the organism [4]. How-

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Table 1. MDR1 and MRP1-mediated transport of endogenous substances with immunological impact

MDR1	References	MRP1	References
sphingosine-1-phosphate (S1P)	[72]	sphingosine-1-phosphate (S1P)	[78]
platelet-activating factor (PAF)	[73]	conjugate of prostaglandin A1 (PGA1-GS)	[79]
IL-2	[74]	conjugate of prostaglandin A2 (PGA2-GS)	[79]
IL-4	[74]	conjugate of LTC4	[80]
IL-12	[75]	sulfatide	[81]
IFN-γ	[74]	monosialoganglioside	[81]
TNF-α	[75]	estrone	[82]
cortisol	[76]		
estrone	[77]		
estriol	[77]		

ever, MDR1 and MRP1 are also integrated into the membranes of the cells of immunologically privileged tissues, for example, in the endothelial cells of the blood-brain, blood-cerebrospinal fluid and blood-testis barriers [5-8].

Moreover, MDR1 and MRP1 are expressed in certain single immune cells, lymphocytes included. In addition, MDR1 protein mediates the transport of certain cytokines and thus participates in the control of lymphocyte activity, differentiation, proliferation and programmed cell death [9, 10]. The role of MRP1, amongst other things, is in the regulation of inflammatory activity of the cells through the transport of glutathione conjugate of leukotrienes and prostaglandins. It follows that MDR1 and MRP1 also act as immunomodulators.

MDR1 protein

MDR1 protein, also known as ATP-binding cassette sub-family B member 1 (ABCB1) or P-glycoprotein 1 (P-gp 1, PGY1 or GP170), is the product of the human ATP-binding cassette, sub-family B (MDR/TAP), member 1 (*ABCB1*) gene. *ABCB1* is located on chromosome 7q21.12, its cDNA spans about 4.5 kb and encompasses 29 exons. The first exon (exon 1a and 1b) and part of the second one are not translated. MDR1 has a molecular weight of 170 kDa and contains 1280 amino acids [11].

MDR1-transported drugs are, for example, a number of the anticancer agents, second-generation antihistamines, antibiotics, antivirals and immunomodulatory drugs – glucocorticoid drugs, chloroquine and colchicine (Table 2) [12-15].

The membrane expression of MDR1 differs in subpopulations of peripheral blood lymphocytes (PBLs). The highest expression is in CD3-CD16+CD56+ lymphocytes while the expression in CD3+CD8+, CD3+CD4+ and CD19+ is lower [16, 17].

In addition, a link between age and expression and activity of MDR1 in PBLs was also demonstrated [18-23]. The results of these studies are conflicting, however.

The expression and transport activity of MDR1 is regulated at several levels and is influenced by the cytokine micro surroundings, intracellular concentration and affinity of transportable substrates and by the intracellular presence of inducers or inhibitors of MDR1.

Additionally, the function of the MDR1 could be, to a lesser or greater degree, affected by the single nucleotide polymorphisms (SNPs) or other variants in the *ABCB1* gene. Although sporadic, the correlation between the expression of MDR1 and SNPs in the gene has also been demonstrated.

The relation of MDR1 expression to genotypes has been described, for example, in C3435T SNP polymorphism located in exon 26. The intestinal expression of MDR1 was approximately two times higher in persons with the CC genotype in comparison with individuals with the TT genotype [24]. Although non-significant, a similar relation between MDR1 expression and genotypes was also seen in human placentas [25]. In addition, *ABCB1* mRNA in peripheral blood mononuclear cells (PBMCs) revealed a similar trend based on the genotypes of C3435T SNP [26]. A significantly higher level of MDR1 in human placentas was also found in patients with the TT genotype in T-129C, SNP located in exon 1b [25].

MRP1 protein

MRP1 protein is also known as ATP-binding cassette sub-family C member 1 (ABCC1) or leukotriene C(4) transporter (LTC_4 transporter). This protein is composed of 1531 amino acids and is encoded by the ATP-binding cassette, sub-family C (CFTR/MRP), member 1 (*ABCC1*) gene which is located on chromosome 16p13.1 and encompasses 31 exons [27].

In addition to certain anticancer agents, drugs transported by MRP1 include antibiotics, antivirals as well as

Table 2. Examples of drugs which are MDR1-transported substrates

Anticancer agents	
daunorubicin, doxorubicin, etoposide, vincristine, vinblastine	
Antivirals	[65]
abacavir, lamivudine, stavudine, tenofovir	
amprenavir, atazanavir, darunavir, indinavir, nelfinavir, ritonavir, saquinavir, tipranavir	
Immunomodulatory drugs	[14, 15, 54]
chloroquine, colchicine, glucocorticoids	

immunomodulatory drugs such as colchicine, methotrexate and cyclophosphamide (such as cyclophosphamide glutathione conjugate) [28].

The expression of MRP1 differs in the lymphocyte subpopulations as well. The highest expression is in memory (CD45RO+) CD3+CD4+ and CD3+CD8+ lymphocytes while a lower expression is in CD3-CD16+CD56+ and CD19+ [29, 30].

The potential association between age and the physiological expression of MRP1 in PBLs has not been explored as yet.

Similar in function to MDR1, MRP1 transport activity and its expression are regulated at several levels [31, 32]. Although not as extensively as in the case of *ABCB1*, *ABCC1* variants as well as the correlation with the MDR1 expression have also been investigated. The mean *ABCC1* mRNA expression in CD3+CD4+ PBLs, for example, did not correlate with T825C, T1684C and G4002A polymorphisms in *ABCC1* in a healthy Caucasian [33].

A number of *ABCC1* polymorphisms may contribute, however, to the change in the transport activity of MRP1, for example, tryptophan in position 1246 (W1246C) eliminates the transport of the compounds (but not the transport of LTC_4) [34]. In a study of 4 different populations, R433S and G671V were indicated to have a potentially adverse effect on the MRP1 function [35].

Objectives

Lymphocytes have a central coordinating and effector role in the regulation of inflammatory activity. Adequate lymphocytic concentration of immunomodulatory and antiviral drugs transported by MDR1 or MRP1 is needed to achieve the expected therapeutic effect.

If the relationship between physiological MDR1 or MRP1 expression (and thus the activity as well [22]) in peripheral blood lymphocytes and age or selected polymorphisms is demonstrated, it should lead to the modification of therapy with MDR1 or MRP1-transported immunomodulatory drugs and antivirals, based on the identified dependencies.

The aim of this study was therefore to assess the relationship between the physiological expression of MDR1 and MRP1 in the cytoplasmic membrane of PBLs in the Central European Caucasian population at the age from 2 months to 23 years and selected polymorphisms of genes for these proteins amongst these children.

In the case of *ABCB1*, we chose the most frequently published SNP rs1045642 (C3435T) and SNP with a possible influence on the splicing of gene rs3213619 (T-129C). Polymorphisms in *ABCC1* were also studied, but there is no repeatedly associated SNP across several studies. We decided to choose one previously reported SNP rs45511401 (G671V) in exon 16 or the rarer variants, rs45544333 and rs8057331 in exons 28 and 29 of *ABCC1*, respectively.

Material and methods

The exclusion criteria for the subjects in the study were the presence of chronic inflammatory disease in the history, the use of drugs which can lead to interaction with MDR1 or MRP1, and the age of less than 2 months and more than 24 years of age. The selection procedure was performed amongst individuals who were hospitalized or were in ambulatory monitoring at the Teaching Hospital Olomouc, Czech Republic, in 2006-2011. Only subjects meeting the selection criteria and with signed informed consent (or their parents signed informed consent) were offered participation in the study. The study was approved by the Ethics Committee of the Teaching Hospital Olomouc and the Faculty of Medicine of Palacky University in Olomouc. The study was also in compliance with the Helsinki Declaration of 1975, as revised in 2000.

The criteria prior to collection of peripheral blood samples were modified to exclude the possible influence of inflammatory activity and the influence of flavonoids in the diet on the expression of MDR1 and MRP1 [36-38].

The collection of peripheral blood samples was carried out in subjects enrolled in the study at the time of previous 4 weeks without acute inflammatory diseases. Concurrently they did not drink or eat grapefruit, citrus, orange, garlic, onion, broccoli, celery, parsley, tea or St. John's wort over this 4 weeks' period (these contain flavonoids which are inducers or inhibitors of MDR1 or MRP1 [39-45]) and no drugs were used over this period apart from vitamin K and vitamin D_3, based on a valid

recommendation (vitamin K from birth to 6 months of age in a single dosage of 1 mg per month and vitamin D_3 from the 14th day after birth to 1-1.5 years of age in a dosage form of oral drops, daily in a dosage 600-800 IU).

The expression of MDR1 protein in PBLs and *ABCB1* gene polymorphisms rs1045642 (C3435T) and rs3213619 (T–129C) was determined in a total of 41 subjects. This MDR1 group ($n = 41$) consisted of 14 females and 27 males (aged from 2 months to 22.2 years, median of age 4.73 years).

The expression of MRP1 protein in PBLs and *ABCC1* gene polymorphisms rs45511401 (G671V), rs45544333 and rs8057331 was determined in a total of 40 subjects. This MRP1 group ($n = 40$) consisted of 18 females and 22 males (aged from 2 months to 22 years, median of age 2.44 years).

Flow cytometry analysis of the MDR1 and MRP1 expression

Peripheral blood lymphocytes (PBLs) were separated with density centrifugation on Ficoll Hypaque (density 1.077 g/ml; Pharmacia, SE) and washed twice in 1× phosphate-buffered solution (PBS). After the second wash, the cell pellet was fixed in ice cold methanol and stored in a freezer at –20°C. The expression of MDR1 and MRP1 on fixed and permeabilized cells was analyzed by a flow cytometry indirect immunofluorescence staining technique (modified according to Den Boer *et al*. [46]).

To measure the expression of MDR1, fixed cells were washed with 1× PBS, permeabilized with a permeabilizing solution based on the manufacturer's instructions (FACS Permeabilizing solution 2; Becton Dickinson, USA), washed in PBS with 0.5% BSA + 0.1% NaN_3 + 0.1% NP-40 and blocked in a solution containing a Fc receptor saturation reagent (Immunotech, Beckman Coulter, USA).

Another washing step was followed by incubation with mouse monoclonal antibodies against MDR1 (clone F4; Sigma Aldrich, USA) and isotypic control of mouse IgG1 (Sigma Aldrich, USA). The antibodies were used in a concentration of 0.5 µg/10^6 cells/100 µl of staining solution. The secondary anti-mouse fluorescein labeled F(ab')2 antibody fragment (Sigma Aldrich, USA) was consequently applied.

To determine the expression of MRP1, the fixed cells were permeabilized with a permeabilizing solution (FACS Permeabilizing solution 2; Becton Dickinson, USA), incubated and washed in PBS with 0.5% BSA + 0.1% NaN_3 + 0.1% NP-40. This was followed by incubation with specific IgG_{2a} mouse monoclonal antibodies against MRP1 (clone MRPm5, at a dilution of 1 : 20; Alexis, USA) and with mouse IgG_{2a} isotypic control (at a dilution of 1 : 200; Sigma Aldrich, USA) and after another washing step, the incubation with the secondary anti-mouse IgG (whole molecule)-FITC antibody (at a dilution of 1 : 250; Sigma Aldrich, USA) followed.

Immunostained cells were subsequently analyzed using a FACS Calibur flow cytometer and Cell Quest software (Becton Dickinson, USA). Fluorescent intensity values were acquired from 10,000 cells when the lymphocyte region was selected based on the size and granularity of the cells. PBLs expression values for individual subjects were defined as the median MDR1 expression in relative fluorescence units (RFU), as well as the median MRP1 expression in RFU.

Genotyping of *ABCB1* and *ABCC1* single nucleotide polymorphisms

DNA samples were isolated from peripheral blood by the standard salting out method. Genotypes were identified by using the allele discrimination/SNP's real time polymerase chain reaction (PCR). The probes for discrimination/SNP's real time PCR were obtained from the Applied Biosystem database of TaqMan® SNP genotyping assays (Applied Biosystem, USA). The PCR reaction was carried out in an overall volume of 11 µl with the following components: 6.3 µl of H_2O, 0.02 µl of reference dye Rox 50× (Finnzymes, FIN), 4.0 µl of DyNamo™ Probe qPCR Kit F-450S/L (Finnzymes, FIN), 0.2 µl of SNP genotyping assay (Applied Biosystems, USA) and 0.5 µl of DNA (100 ng/µl). Allele discrimination PCR was run on a Stratagene MxPro3005 thermal cycler (Stratagene, USA) under the following conditions: a starting denaturation at 95°C for 15 min and consequently 55 cycles including denaturation at 95°C for 15 s followed by an annealing/denaturising step at 60°C for 1 min. The dRn values (baseline subtracted fluorescent reading normalized to the reference dye) were used to determine fluorescence c_T values. The difference in c_T values and the shape of the curves from fluorescence readings (dRn data) were used to determine the genotypes.

The observed genotypes were confirmed by direct sequencing of the SNPs of interest in 15 patients. The sequencing reaction was carried out in a volume of 10 µl with reagents obtained from Applied Biosystems, the reaction mixture contained: 2 µl of primer, 2 µl of PCR water (Top-Bio, CZ), 2 µl of purified PCR amplicons and 4 µl of ABI PRISM® Big Dye® Terminator v3.1 (Applied Biosystems, USA), 4-times diluted by a 5× sequencing buffer. The sequence reaction ran under the following thermal conditions: denaturation at 94°C for 2 min and 34 cycles of 96°C for 10 s, with gradual 1°C per 1 s temperature decreasing to 50°C, 50°C for 5 s and 60°C for 4 min. After sequencing reaction products purification (ethanol precipitation), a capillary electrophoresis was performed on an ABI PRISM™ 310 Genetic Analyzer (Applied Biosystems, USA).

Statistical analysis

All the statistical analyses were performed with Statistica 8 (StatSoft Inc., USA) and R (R Foundation for

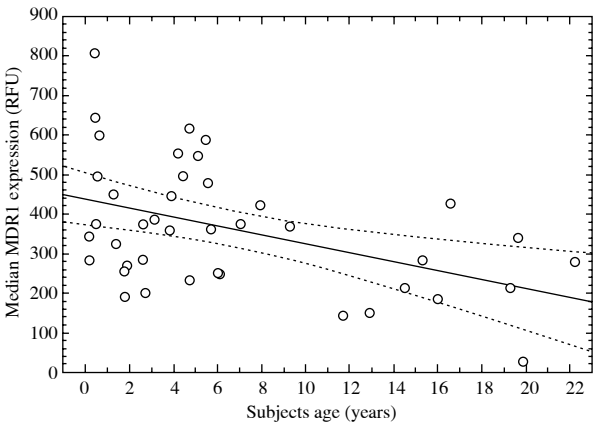

Fig. 1. PBLs MDR1 expression and correlation with age: median MDR1 expression (RFU). Spearman correlation coefficient R = –0.4001 (p = 0.0095). PBLs – peripheral blood lymphocytes, RFU – relative fluorescence units

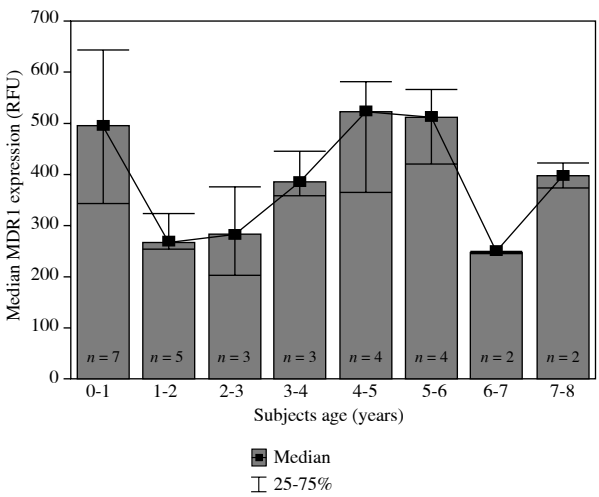

Fig. 2. Histogram of the PBLs MDR1 expression in age groups divided according to age in a one year interval up to 8 years of age: median MDR1 expression. PBLs – peripheral blood lymphocytes, RFU – relative fluorescence units, *n* – number of subjects

Statistical Computing, AT) software [47]. The statistically significant level was set at p = 0.05. The dependence of PBLs MDR1 and PBLs MRP1 expressions on the age of the subjects was clarified by the Spearman correlation coefficient. A histogram was used for visualization of PBLs MDR1 expression in the age groups divided according to age in a one-year interval up to 8 years of age. We made use of generalized linear models with an age adjustment to eliminate the age effect on the MDR1 and MRP1 expression to analyze the dependence of PBLs MDR1 and PBLs MRP1 expression on polymorphisms.

Results

The distribution of subjects into groups, age characteristics and PBLs MDR1 and PBLs MRPs protein expression values characteristics are shown in Table 3.

Analysis of MDR1 protein expression and *ABCB1* gene polymorphisms

The statistical analysis revealed the dependence of PBLs MDR1 protein expression on age. Higher expression values of MDR1 were observed amongst younger children. These differences in expression were statistically significant as demonstrated by a comparison of the median MDR1 expression (Spearman R = –0.4001, p = = 0.0095) (Fig. 1). The PBLs MDR1 expression has two peaks in younger children. The highest expression is in children younger than 1 year and consequently in the interval from 4 to 6 years of age (Fig. 2). In the MDR1 group there were 26 children younger than 6 years of age, the distribution of these children into age groups is shown in Fig. 2.

We attempted to associate the examined SNPs with the level of MDR1, but neither the rs1045642 (median MDR1 expression $p < 0.63$) nor rs3213619 SNPs (median MDR1 expression $p < 0.79$) genotypes correlated with the PBLs MDR1 expression (Fig. 3).

Analysis of MRP1 protein expression and *ABCC1* gene polymorphism

In contrast to previous results, the statistical analysis did not reveal the dependence of the PBLs MRP1 protein

Table 3. Distribution of subjects into groups, age characteristics and PBLs MDR1 and PBLs MRP1 protein expression values characteristics

Group	Total	Characteristic of age (years)					Median expression (RFU)			
	n	median	min.	max.	Q1	Q3	median	min.	Q1	Q3
MDR1 group	41	4.73	0.18	22.23	1.79	9.28	358.66	27.63	250.29	449.10
MRP1 group	40	2.44	0.15	22.00	0.65	6.2	181.06	155.38	171.54	195.44

PBLs – peripheral blood lymphocytes, n – number of subjects, RFU – relative fluorescence units, Q1 – first quartile, Q3 – third quartile

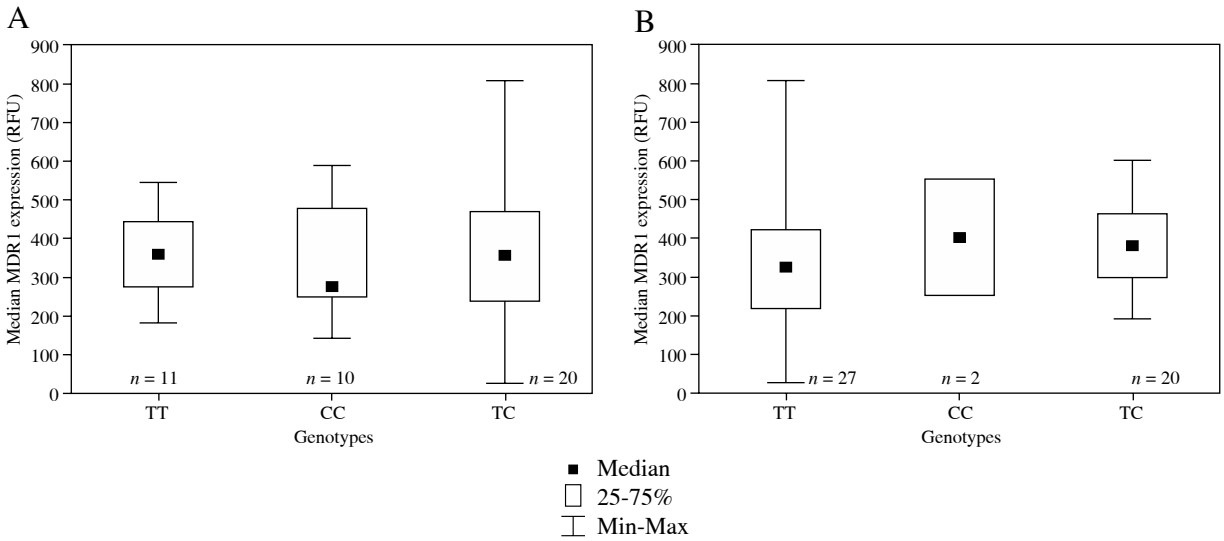

Fig. 3. PBLs MDR1 expression and *ABCB1* gene polymorphism: A) median MDR1 expression and genotypes of rs1045642 (C3435T) SNP ($p < 0.63$); B) median MDR1 expression and genotypes of rs3213619 (T-129C) SNP ($p < 0.79$). PBLs – peripheral blood lymphocytes, RFU – relative fluorescence units, *n* – number of subjects, SNP – single nucleotide polymorphism

expression based on age in the MRP1 group. The median MRP1 expression (Spearman R = 0.0633, $p = 0.6980$) was compared as well (Fig. 4).

Apart from this, the genotypes analysis of the examined SNPs in *ABCC1* did not find a linkage to the PBLs MRP1 level. Minor alleles of variants in exons 28 and 29 (rs45544333 and rs8057331) were relatively rare (frequency 0.009 and 0.007, respectively) and were consequently not used for the final analysis. There was no difference in the distribution of minor alleles among the examined groups. The genotype of exon 16 SNP (rs45511401) did not correlate with the PBLs MRP1 level (median MRP1 expression $p < 0.36$) (Fig. 5).

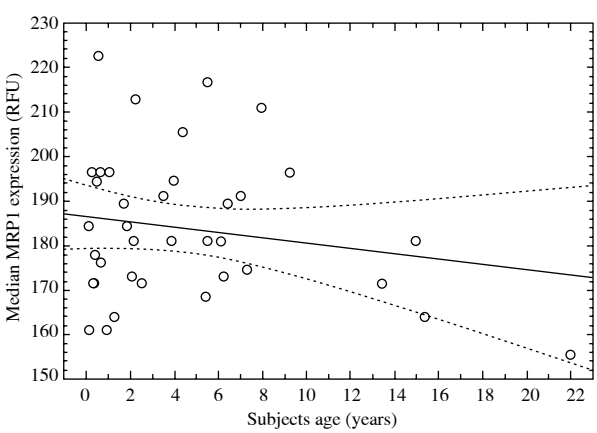

Fig. 4. PBLs MRP1 expression and age: median MRP1 expression. Spearman correlation coefficient $R = 0.0633$ ($p = 0.6980$). PBLs – peripheral blood lymphocytes, RFU – relative fluorescence units

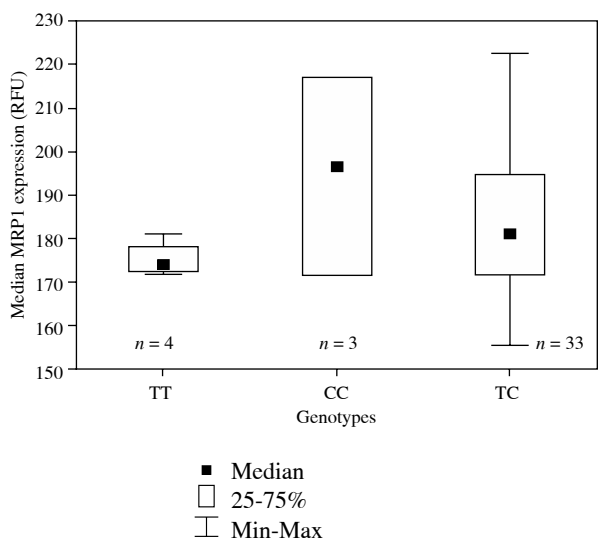

Fig. 5. PBLs MRP1 expression and ABCC1 gene polymorphism: median MRP1 expression and genotypes of exon 16 SNP rs45511401 (G671V) ($p < 0.36$). PBLs – peripheral blood lymphocytes, RFU – relative fluorescence units, *n* – number of subjects, SNP – single nucleotide polymorphism

Table 4. Expression or activity of MDR1 in peripheral blood lymphocytes or in peripheral blood mononuclear cells (age groups have been modified)

Study	Subjects			Tested parameter	Methods	Materials	Association with age	Comment	References
	number	age interval (years)	health status						
Pilarski *et al.* (1995)	*n* = 21	0-18, 5 NB	healthy	activity	efflux assay (rhodamine 123)	PBLs CD3+CD4+, CD3+CD8+	yes	1*	[18]
	n = 25	20-81	healthy			PBLs CD20+	yes	2*	
Machado *et al.* (2003)	*n* = 50	0-18, 10 NB	healthy	activity	efflux assay (rhodamine 123)	PBLs CD3+CD4+, CD3+CD8+	yes	3*	[20]
	n = 40	20-86	healthy			PBLs CD19+, CD16+CD56+	no		
Giraud *et al.* (2009)	*n* = 61	0-18, 28 NB	healthy	gene expression	PCR	PBMCs	no		[23]
	n = 33	0-18	HIV-infected	activity	efflux assay (DiOC6)	PBLs CD3+, CD56+	yes	4*	
	n = 10	> 18	healthy			PBLs C19+	no		
	n = 15	> 18	HIV-infected						
Vilas-Boas *et al.* (2011)	*n* = 34	18-30	healthy	expression	monoclonal antibody (UIC2)	PBMCs	yes	5*	[22]
	n = 31	60-78	healthy	activity	efflux assay (rhodamine 123)	PBMCs	no		
				activity	shift assay (UIC2)	PBMCs	no		
Cortada *et al.* (2009)	*n* = 61	19-90	healthy	activity	efflux assay (rhodamine 123)	PBLs	no		[49]
Aggarwal *et al.* (1997)	*n* = 10	22-26	healthy	expression	monoclonal antibody (MRK16)	PBLs CD3+CD4+, CD3+CD8+	yes	6*	[19]
	n = 10	70-90	healthy	gene expression	quantitative PCR	PBMCs	yes	6*	
				activity	efflux assay (rhodamine 123)	PBLs CD3+CD4+, CD3+CD8+	yes	6*	
Brenner *et al.* (2004)	*n* = 21	"young adults"	healthy	activity	efflux assay (rhodamine 123)	PBLs CD16+CD56+	no		[48]
	n = 18	"elderly"	healthy						
	n = 20	"elderly"	not healthy						
Storch *et al.* (2007)	*n* = 48	40-80	not only healthy	activity	efflux assay (rhodamine 123)	PBMCs	yes	7*	[21]

PBLs – peripheral blood lymphocytes, PBMCs – peripheral blood mononuclear cells, NB – newborns, PCR – polymerase chain reaction, DiOC6 – 3,3´-diethyloxacarbocyanin iodide

Text to comment: 1 lower in childhood, increasing with age, decreasing after 60 years; 2* increasing in early childhood, the highest in adulthood, then decreasing with age; 3* the highest at birth, markedly decreasing with age, lowest after 70 years; 4* the highest at birth, higher to 6 months, from 6 months on similar to that in adults; 5* increasing with age; 6* higher in ageing subjects; 7* decreasing with advancing age.*

Discussion

Lymphocyte expression levels of MDR1 and MRP1 proteins versus age

Although several studies had already highlighted the connection between age and the expression or activity of MDR1 in PBLs, their results are often conflicting (Table 4). Moreover, only one study examined the MDR1 expression and only three studies examined MDR1 activity in a comparison of age groups which included children.

The connection between age and the activity of MDR1 in PBLs was shown for the first time in a study by Pilarsky *et al*. (1995). They found the highest activity of MDR1 in CD3+ PBLs amongst newborns (samples from cord blood). It consequently decreased rapidly with a higher activity being observed once again in adults older than 20 years of age and decreasing once again after 61 years of age [18]. In a study by Aggarwal *et al*. (1997), there was a relation between age and both the expression and activity of MDR1 in PBLs. In subjects older than 70 years of age, there was a significantly higher expression and activity in CD3+ PBLs compared with younger adults, although childhood subjects were not included in the study [19]. A number of studies did not fully correspond with these results, however. For instance, Machado *et al*. (2003) demonstrated the highest activity in CD3+ PBLs (cord blood), which markedly decreased with age, and reached the lowest levels after 70 years of age [20]. Storch *et al*. (2007) demonstrated decreasing MDR1 activity with advancing age in PBMCs in adults as well, although, there were no tested subjects in childhood [21]. In contrast to the previous one, Brenner *et al*. (2004) in young and elderly adults as well as, Cortada *et al*. (2009) in adults, did not detect the relation between activity and age in PBMCs [48, 49]. In addition, Vilas-Boas *et al*. (2011) demonstrated a significant age-dependent increase in the MDR1 expression in adults after 60 years of age, although, this increase did not correspond to MDR1 activity [22].

Apart from the above-mentioned studies focused on childhood, Giraud *et al*. (2009) demonstrated the highest activity in newborns in CD3+ and in CD3-CD16+CD56+ PBLs. This activity gradually decreased to 6 months and was stabilized before the age of 2 years at a level similar to that in adults [23]. Nevertheless, no association was observed between age and *ABCB1* mRNA expression.

In the present study, we demonstrated the dependence of the PBLs MDR1 expression on age, as well. There were significantly higher MDR1 expression values in younger children amongst the Central European Caucasian population sample at an age from 2 months to 23 years of age as was clarified by the Spearman correlation coefficient. In younger children the highest expression was observed at the age under 1 year and consequently from 4 to 6 years of age, as the histogram used to identify these age groups revealed.

The increase in expression of MDR1 in the first year of life could be due to a higher proportion of CD3+ cells co-expressing CD45RA (naive T cells) which revealed a significantly higher activity than CD3+ cells co-expressing CD45RO (memory T cells) in peripheral blood, as well as the impact of the maternal hormonal level [50, 51]. However, this could not explain another peak of MDR1 expression at the age from 4 to 6 years.

We can consequently only speculate that increasing the MDR1 expression could be a consequence of higher lymphocyte activity. During the first year of life, escalated antigen stimulation, including vaccination, occurs. There is also a higher probability of infectious diseases in children from 3 years of age, usually attending collective preschools.

Our results and the results of previous studies focused on childhood are difficult to compare due to the different design. The study by Vilas-Boas *et al*. (2011) revealed a significant correlation between the lymphocyte MDR1 expression and MDR1 activity using sensitive methods amongst young adults up to 30 years of age [22]. Our evidence of increased expression in younger children, generally concurs with the similar results of those studies which assessed the highest activity amongst the youngest children by Machado *et al*. and Giraud *et al*. [20, 23]. We are not in agreement, however, with the results of the study by Pilarski *et al*., which apart from the higher activity in newborns, revealed a higher MDR1 activity in adults than in children [18]. In addition, we demonstrated the unexpected peak of the MDR1 expression between 4 to 6 years of age. We can presume, based on these previous studies, that CD3+ lymphocytes and presumably CD3–CD16+CD56+ lymphocytes participated in the increased expression of PBLs [20, 23].

As far as we are aware, the dependence of the MRP1 expression or activity in PBLs on age has not been published as yet. Although there is a noticeable trend toward a higher expression amongst younger children with a gradual decline in adulthood in our study (Fig. 4), these expression changes are not statistically significant. We can therefore assume that the expression of MRP1 in PBLs is not dependent on age in the subjects under 24 years of age. In addition, we demonstrated the distinct inter-individual variability of the physiological MRP1 expression in PBLs as had been determined previously [33].

The absence of determination of MDR1 and MRP1 expression levels in lymphocyte subpopulations, as well as the lack of transport activity evaluation of these proteins with sensitive methods, was a weak point in the present study.

Nevertheless, the higher MDR1 expression in PBLs in younger children could influence the effect of immunomodulatory glucocorticoid therapy. An increased MDR1 expression may reduce the intracellular concentration of glucocorticoid drugs in cells including lymphocytes and can in this manner modulate their availability to cytoplasmic glucocorticoid receptors and consequently influence the immunomodulatory effect of glucocorticoids [52, 53]. Furthermore, only slight variations in glucocorticoid drugs, such as the number of hydroxyl groups or the position of these groups, have an impact on their affinity and the transport activity of MDR1 [14].

MDR1-mediated transport of glucocorticoid drugs was compared using L-MDR1 and Caco-2 cell lines *in vitro* transport studies by Yates *et al.* (2003) and Crowe *et al.* (2012) [14, 54]. In these studies, methylprednisolone had distinctly higher efflux parameters of MDR1-mediated transport than prednisolone (an efficient form of administered prednisone precursor), hydrocortisone and dexamethasone.

Based on these *in vitro* transport studies and based on our results, methylprednisolone in immunomodulatory glucocorticoid therapy should therefore be considered for the supposed increased risk of attenuated efficacy, or drug resistance, in children under 6 years of age.

Moreover, an increased expression or activity of MDR1 in PBLs has been reported as one of the causes of glucocorticoid resistance, for example, in the treatment of rheumatoid arthritis [55-57], systemic lupus erythematosus [58-61], myasthenia gravis or in childhood as the cause of attenuated efficacy in the treatment of nephrotic syndrome [62, 63].

In a similar fashion, in the treatment of HIV-1 infection, with the well-known tropism for CD3+CD4+ T lymphocytes, an overexpression of MDR1 could lead to a decrease in lymphocytic accumulation of certain antiviral drugs (for example, ritonavir and saquinavir) thereby affecting the treatment outcome or contributing to drug resistance [64]. In addition, many of the antiviral drugs used in treating HIV infection are MDR1-transported substrates [65] (Table 2).

MDR1 and MRP1 lymphocyte expression levels versus gene polymorphisms

The study of the effect of *ABCB1* polymorphisms on the MDR1 level or function is an exciting field of MDR1 research. The majority of the attention has been paid to the study of C3435T SNP. Firstly, Hoffmeyer *et al.* (2000) discovered that the CC genotype of the SNP had been linked to an approximately 2-times higher MDR1 intestinal expression [24]. Similar trends have been further observed in additional studies by Tanabe *et al.* (2001), Hitzl *et al.* (2001) and Fellay *et al.* (2002) in different tissues including PBMCs [25, 26, 66].

These data were in concordance, for example, with the higher maximal plasma level of digoxin, the transported substrate of *ABCB1*, in subjects with the TT genotype [24]. In comparison with these results, Albermann *et al.* (2005) did not demonstrate a significant association between C3435T or G2677T polymorphisms and the MDR1 mRNA expression and MDR1 activity in PBMCs. Likewise, the relationship between these polymorphisms and the expression in selected tested tissues in Europeans was not demonstrated in the above-mentioned study [67]. In the same manner, Sakaeda *et al.* (2001) reported a higher digoxin level in persons with a CC genotype consistent with *ABCB1* mRNA expression for the genotype in the Japanese population [68, 69].

These conflicting results could reflect the important and well-known fact that the C3435T SNP is a synonymous polymorphism. The synonymous polymorphism has no effect on the amino acids sequence in protein and is not able to affect the splicing of *ABCB1* mRNA. There is actually no reason to believe that the SNP has any effect on MDR1 expression, no matter the significant associations. We also were unable to associate this SNP with the expression level of MDR1. The actual reason for the altered expression of MDR1 must be in the co-occurrence of additional SNP with the C3435T SNP, in other words the C3435T SNP must be in linkage with certain additional powerful SNP or SNPs in the same gene. This theory is supported by the observation of the linkage of two other *ABCB1* SNPs with the occurrence of the C3435T, in this case of G2677T [70]. It is consequently reasonable to focus on the other SNPs. Polymorphism T-129C is an intronic/promoter region SNP and could have an effect on gene expression (splicing). Significantly higher levels of MDR1 in human placentas were observed in subjects with the TT genotype [25]. Unfortunately, we did not find an association of this SNP with the MDR1 level.

Although the T-129C seemed to be in linkage with the G2677T [70], this was also not associated with *ABCB1* mRNA expression levels in duodenal enterocytes in the study by Moriya *et al.* (2002) [71]. We therefore hypothesize that T-129C SNP would have no effect on the MDRA1 expression.

Summary and recommendations

This study demonstrated a higher MDR1 expression in PBLs in younger children significantly decreasing with age. Moreover, we discovered two MDR1 expression peaks dependent on age. The first peak was observed in the first year of life, as could be expected on the basis of certain earlier studies assessing the activity of MDR1.

Furthermore, our study was the first one to reveal the second unexpected MDR1 expression peak in 4 to 6 year old children. In addition, this increased MDR1 expression may affect therapy with MDR1-transported immunomodulatory and antiviral drugs. A reduction in the concentration of these drugs in target cells (lymphocytes) may lead to attenuated efficacy, or drug resistance.

The efficacy of MDR1-transported drugs in specific lymphocyte subpopulations in children should be consequently verified in further studies.

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