

# P-Glycoprotein Activity in Steroid-Responsive vs. Steroid-Resistant Nephrotic Syndrome

Hassan S. Badr<sup>1</sup> · Mahmoud A. El-Hawy<sup>1</sup> · Mohammed A. Helwa<sup>2</sup>

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## Abstract

**Objectives** To explore the expression of P-glycoprotein (P-gp) in the peripheral blood nucleated cells (PBNCs) of children with nephrotic syndrome in relation to their clinical response to glucocorticoid treatment.

**Methods** Thirty-six children with nephrotic syndrome (20 cases of steroid-responsive and 16 cases of steroid-resistant) were examined. All the participants were subjected to complete history taking, thorough clinical examination, laboratory investigations (24-h urinary protein, serum albumin, complete blood count with differential white blood cell count, serum cholesterol, serum urea, serum creatinine) and functional assay of P-gp using FACS Calibur flowcytometry. P-gp assay was done in both groups during remission.

**Results** P-gp activity was significantly higher in steroid-resistant than steroid-sensitive cases.

**Conclusions** P-gp can be used as a predictor of outcome, as a part of laboratory evaluation of the cases before starting steroid therapy, so as to determine whether to use alternative line of therapy or use one of the P-gp inhibitors with steroid therapy.

**Keywords** P-gp · Steroid-sensitive · Steroid-resistant · Nephrotic syndrome

✉ Mahmoud A. El-Hawy  
mahmodelhawwy18@yahoo.com;  
mahmoud.elhawi@med.menofia.edu.eg

<sup>1</sup> Department of Pediatrics, Faculty of Medicine, Menoufia University, Shebin el Kom, 32511, Menoufia, Egypt

<sup>2</sup> Department of Clinical Pathology, Faculty of Medicine, Menoufia University, Menoufia, Egypt

## Introduction

Nephrotic syndrome (NS) is the most common kidney disease in childhood. In about 90 % of cases it involves primary glomerulopathy while the remaining 10 % encompass secondary glomerulopathies due to systemic, metabolic or infectious diseases [1].

Immune mechanisms, rather than primary structural defects of the filtration barrier, play a more prominent role in this syndrome. Idiopathic NS is currently considered an immune-mediated disease related to T-cell disorders. The treatment of primary NS is based on administration of synthetic glucocorticoids [1, 2].

However, not all patients respond positively to therapy which divides them into steroid-sensitive (SS) and steroid-resistant (SR) individuals. Many potential factors associated with steroid resistance have been identified so far. It seems that genetic factors associated with glucocorticoid receptor  $\alpha$  (GR $\alpha$ ), the structure of heterocomplex of GR, mutation or disruption of genes coding for several podocyte proteins as well as glycoprotein P or cytochrome P450 may play a role in the induction of glucocorticoid resistance [3, 4].

One of the most extensively studied gene is the so-called multidrug resistance-1 (*MDR-1*) gene, which is characterized by the over function of one 170 kDa P-glycoprotein (P-gp) [5]. P-gp belongs to the ATP binding cassette super family of transport proteins [6]. It is richly expressed in many tissues, and, in the hemopoietic system, peripheral blood mononuclear cells, macrophages, natural killer, dendritic cells and T and B lymphocytes all express P-glycoprotein at varying levels [7].

Studies found surface expression of P-gp on peripheral lymphocytes in patients with systemic lupus erythematosus (SLE) and a significant correlation between its expression level and disease activity. The use of both P-gp antagonists (*e.g.*, cyclosporine) successfully reduces the efflux of

corticosteroids (which are substrates of P-gp) from lymphocytes *in vitro*, suggesting that P-gp antagonists and P-gp synthesis inhibitors could be used to overcome drug-resistance *in vivo* and improve outcome [8].

Rhodamine 123 (Rho123) accumulations in peripheral blood cells were used as a surrogate indicator to evaluate the modulating effect of P-glycoprotein (P-gp) inhibitors in the multidrug resistance (MDR) [9]. The aim of this study was to explore the activity of P-gp in the peripheral blood mononuclear cells (PBMNCs) of children with nephrotic syndrome in relation to their clinical response to glucocorticoid treatment.

## Material and Methods

After approval of the local Institutional Ethical Committee of Menoufia University Hospital and an informed written consent obtained from the parents of the children, the cases were recruited from the Pediatric nephrology clinic, Menoufia University Hospitals from March 2014 through March 2015.

Cases diagnosed with NS were included if they fulfilled the following criteria; heavy proteinuria, hypoalbuminemia (serum albumin <2.5 g/dl) and hyperlipidemia (serum cholesterol >200 mg/dl) [10]. Those with nephropathy secondary to a well identified cause were excluded. Remission of NS was characterized by the disappearance of albuminuria for at least 3 consecutive days. Frequent relapsers were defined according to the International Study of Kidney Disease in Children (ISKDC) criteria (more than two relapses in the initial 6 mo after presentation or more than four per year during follow-up) [11]. Steroid dependency was defined according to the guidelines of the Arbeitsgemeinschaft für Pädiatrische Nephrologie (at least two relapses during alternate day treatment with prednisone or within 2 wk after cessation) [12].

All included cases were treated with the standard initial therapy, consisting of daily administration of prednisone 60 mg/m<sup>2</sup> body for 4 wk, followed by 40 mg/m<sup>2</sup> given on alternate days, followed by various taperings on alternate days. Relapses were treated with prednisone 60 mg/m<sup>2</sup> administered daily until remission was achieved, followed by 40 mg/m<sup>2</sup> on alternate days.

In steroid-dependent patients, maintenance alternate day dosage of prednisone was instituted. The alternate dose was gradually tapered to determine each patient's individual threshold at which relapse occurred.

In children with frequent relapses, steroid dependency or serious side effect of steroids, alternative treatment was used; Cyclophosphamide therapy was administered in an 8-wk course.

Patients were classified according to their initial response to steroids as steroid-sensitive (SS) or steroid-resistant (SR). steroid-sensitive “responders” NS patients were diagnosed if

they had; disappearance of proteinuria (negative to trace in a urine for 3 consecutive days, or a urine protein/creatinine level of <0.2) within the first 4 wk of full dose prednisone therapy. While steroid non-responders “resistant” NS patients had nephrotic range proteinuria >1000 mg/m<sup>2</sup> per day, serum albumin level < 2.5 g/dl and were resistant to steroid therapy [11]. A diagnosis of primary steroid resistance was made if the proteinuria persisted after 4 wk of daily prednisone therapy in a dose of 60 mg/m<sup>2</sup>/d in the absence of any evidence of underlying infection while secondary steroid resistance was defined as no response to 4 wk of daily prednisone therapy at a dose of 60 mg/m<sup>2</sup>/d in a child previously known to have a steroid-sensitive course [13].

The cases were divided into two groups according to their response to steroid therapy: Group I: Twenty cases of steroid-sensitive nephrotic syndrome and Group II: Sixteen cases of steroid-resistant nephrotic syndrome (steroid non-responders).

All of the patients were subjected to complete history taking, thorough clinical examination, routine investigations (24-h urinary protein, serum albumin, complete blood count with differential white blood cell count, serum cholesterol, serum urea, and serum creatinine).

Functional assay of P-gp was done using FACS Calibur flowcytometry (BD, USA). In both groups during disease remission, the activity of p-gp was assayed by measuring Rh-123 fluorescence at time zero (without further incubation of cells with the dye), after 10 min incubation with the dye, after 10 min incubation of the dye in the presence of cyclosporine as P-gp inhibitor at conc. of 5 mg/ml and after 10 min incubation of the dye in the presence of cyclosporine at conc. of 10 mg/ml.

Reagents used were Rhodamine 123 (ICN Biomedicals Inc. USA): First dissolved in DW for preparation of stock at 5 µg/ml, Before use, Rh-123 was diluted at 1/250 (20 µl from the stock solution in 5 ml DW); Cyclosporine (Novartis, Neoral): 50 mg/ml in DW was prepared as stock solution, 2 µl of stock solution diluted with 8 µl (1:5) and 18 µl (1:10) DW were used as a working solution and Ficoll-Hypaque (Sigma Diagnostics – USA) for separation of pbmncs.

PBMNCs were isolated from the fresh blood using a Ficoll-Hypaque density gradient centrifugation. After one wash with phosphate buffer saline (PBS), fresh mononuclear cells were adjusted at  $2 \times 10^6$  cells /ml in PBS. Three hundred µl of cell suspension were distributed in 5 test tubes: Tube 1- to evaluate cell autofluorescence, Tube 2- to evaluate Rh-123 uptake at time zero, Tube 3- to evaluate Rh-123 uptake after 10 min incubation, Tube 4- to evaluate Rh-123 uptake after 10 min incubation after preincubating cells with cyclosporine at conc. of 5 mg/ml for 5 min, Tube 5- to evaluate Rh-123 uptake after 10 min incubation after preincubating cells with cyclosporine at conc. of 5 mg/ml for 5 min, Ten µl of PBS were added in tube 1. In tube 4 and 5, cyclosporine was added at conc. of 5 and 10 mg/dl respectively. Then 25 µl of Rh-123 working

solution were added in tube 2,3,4 and 5, Rh-123 uptake was measured immediately in tube 2, after 10 min in tubes 3, 4 and 5 on green fluorescence detector (FL1). Data acquisition and data analysis were performed with cell Quest software (BD, USA). Mean fluorescence of Rh-123 are evaluated for each cell preparation, after acquisition of 10,000 viable cells.

Data were expressed as mean  $\pm$  SD. The results were computed statistically (SPSS for Windows, version 14.0; SPSS Inc., Chicago, Illinois, USA). The paired student t test was used to compare steroid-sensitive and steroid-resistant cases. *P* value  $<0.05$  and  $<0.001$  were considered statistically significant and highly significant respectively.

## Results

Among the 20 cases of steroid-sensitive nephrotic syndrome, 12 were girls and 8 were boys, with a mean age of  $7.02 \pm 3.38$  y. Among the 16 cases of steroid-resistant nephrotic syndrome, 9 were girls and 7 were boys with a mean age of  $6.98 \pm 3.70$  y (steroid non-responders); 10 of them showed primary resistance and 6 had secondary resistance.

Table 1 shows the baseline lab investigations (at initial diagnosis) between the studied groups.

At time zero incubation of cells with Rh-123, there was no statistically significant difference among the studied groups with regard to P-gp activity “Rh-123 uptake”.

After 10 min incubation with Rh-123, the Rh-123 fluorescence (and as a result of P-gp efflux) in steroid-resistant cases was lower than its level before incubation (time zero). On the other hand, Rh-123 after 10 min incubation was statistically higher in steroid-responders than at time zero.

Also after 10 min incubation, Rh-123 fluorescence was lower in steroid-resistant cases than in steroid-responders.

On pre-incubation with cyclosporine 5 mg/ml, the Rh-123 uptake after 10 min incubation with cells increased in steroid-resistant cases significantly in comparison to its uptake in absence of cyclosporine. Increasing the concentration of cyclosporine resulted in increasing Rh-123 uptake in steroid-resistant cases (by blocking P-gp activity).

This effect of cyclosporine was not noted in steroid-sensitive cases *i.e.*, pre-incubation of cells with cyclosporine did not increase Rh-123 uptake in steroid-responder cases.

At cyclosporine concentration of 10 mg/ml, there were no differences in Rh-123 uptake between steroid-responders and steroid-resistant cases (Table 2).

## Discussion

Glucocorticoids are still the mainstay of therapy for nephrotic syndrome (NS) in children. Poor response to glucocorticoids may relate, in part, to the overexpression of P-glycoprotein (P-gp) [14]. Glycoprotein P is a transmembrane protein coded by the *MDR1* gene located on chromosome 7q21; P-gp acts as a transporter responsible for cellular efflux of drugs and toxins with a molecular weight between 300 and 2000 Da, among which are included xenobiotics or drugs such as vinca alkaloids, verapamil or corticosteroids, among others [15].

P-gp is extensively expressed in various tissues including the blood-brain barrier, liver, kidney, intestines, and lymphocytes in peripheral blood [16].

In the present study, P-gp activity was found to be significantly higher in steroid-resistant cases than steroid-sensitive cases. This was shown by measuring Rh-123 (a substrate of P-gp) intensity in PMNCs. This effect can be abolished by pre-incubating cells with cyclosporine as a P-gp inhibitor.

This is in agreement with Wasilewska et al. [17] who studied the expression of peripheral lymphocytes (CD3) P-glycoprotein (P-gp) in children with steroid-dependent nephrotic syndrome (SDNS) during cyclosporine A (CyA) and ACE-inhibitor (ACE-I) treatment and revealed that, CyA + ACE-I in SDNS inhibits the expression of P-gp. CyA is an alternative therapy that may lead to the optimization of glucocorticoid (GC) doses, thus, reducing the risk that is associated with the treatment [18]. In another study, Wasilewska et al. studied the expression of P-glycoprotein (P-gp) in CD3 lymphocytes of children with nephrotic syndrome (NS) in relation to their clinical response to glucocorticoid (GC) treatment and concluded that “Worse” response to GC or dependency may be due to overexpression of P-gp [14].

**Table 1** Comparison of baseline lab investigations between the studied groups

Parameter	Group I (Steroid-sensitive) Mean $\pm$ SD	Group II (Steroid-resistant) Mean $\pm$ SD	<i>P</i> value
Serum creatinine (mg/dl)	0.62 $\pm$ 0.12	0.53 $\pm$ 0.15	> 0.05
BUN (mg/dl)	15.57 $\pm$ 4.58	13.59 $\pm$ 3.5	> 0.05
Serum albumin (g/dl)	2.40 $\pm$ 0.39	2.29 $\pm$ 0.27	> 0.05
24-h proteinuria (mg/m <sup>2</sup> /h)	76.47 $\pm$ 23.39	68.01 $\pm$ 23.51	> 0.05
P/C ratio	3.38 $\pm$ 0.93	3.07 $\pm$ 0.81	> 0.05

*P/C* Protein/creatinine ratio; *BUN* Blood urea nitrogen

**Table 2** Comparison of P-gp activity “Rh-123 uptake” between the studied groups

Group	Rh-123 uptake at time zero (A) Mean ± SD	Rh-123 uptake after 10 min incubation (B) Mean ± SD	Rh-123 uptake after cyclosporine 5 mg/ml (C) Mean ± SD	Rh-123 uptake after cyclosporine 10 mg/ml (D) Mean ± SD
Group I (Steroid-sensitive)	302.6 ± 23.4	549.3 ± 10.9	555.8 ± 20.5	563.1 ± 24
Group II (Steroid-resistant)	310.9 ± 29.2	162.6 ± 9.4	474.5 ± 22.3	559.2 ± 18.5
<i>P</i> value	> 0.05	< 0.001	< 0.001	< 0.001

Likewise, Prasad et al. [19] found overexpression of P-gp on lymphocytes during NS relapses. This overexpression of P-gp on lymphocytes might lead to the exclusion of corticosteroids from lymphocytes, resulting in steroid resistance in patients with highly active SLE as reported by Tsujimura et al. [20]. Reduction of P-gp expression achieved by intensive immunosuppressive treatment overcame the steroid resistance [20].

Türkmen et al. [16] investigated the relationship between renal P-glycoprotein (rP-gp) expression and response to corticosteroid therapy in childhood NS. They suggested that glomerular P-gp expression is increased in frequently relapsing, steroid-dependent or steroid-resistant childhood NS.

Inter-individual and intra-individual variability in response to exogenous GC may be partially due to differences in the activity of factors responsible for its systemic bioavailability, tissue distribution and elimination. It is becoming increasingly evident that genetic polymorphisms in the genes for membrane transporters (e.g., P-gp) and metabolizing enzymes may determine the outcome of drug therapies [21].

Funaki et al. [18] studied 14 patients with SSNS and *MDR1* mRNA gene expression of peripheral blood nucleated cells (PBNC), before and after CR using real-time PCR. They found that *MDR1* gene expression was high in the first attack or in relapse compared with those in remission. This may explain why patients in the first attack or in relapse require higher doses of corticosteroids than in remission to obtain the same drug action.

Similarly, Youssef et al. [22] found a significant increase *MDR1* gene in the patients with steroid-resistant than steroid-sensitive ones. Several *MDR1* gene polymorphisms were investigated. Jafar et al. [23] studied *MDR1* gene polymorphisms in 137 patients with SSNS, and 79 with SRNS and they found that patients with NS carrying homozygous mutants of single nucleotide polymorphism (SNP) G2677T/A are prone to develop SRNS.

In a Slovak study, Cizmarikova et al. [24] suggested that prednisone therapeutic response may be influenced by histology, age at the onset of INS, and *MDR1* 3435T > C polymorphism. Likewise, in Egypt, Youssef et al. [25] suggested that *MDR1* C3435T or G2677T/A gene polymorphisms are risk factors for increased susceptibility, earlier onset of NS, and steroid resistance.

## Conclusions

P-gp can be used as a predictor of outcome, as a part of case evaluation before starting therapy, so as to determine whether to use alternative line of therapy or use P-gp inhibitors with steroid.

**Contributions** HSB: Study design, data collection and will act as guarantor for the paper; MAEI-H: Statistics and paper writing; MAH: Lab work.

## Compliance with Ethical Standards

**Conflict of Interest** None.

**Source of Funding** None.

## References

- Banaszak B, Banaszak P, Adamczyk P, Ziara K. First detection of pediatric nephrotic syndrome: clinical characteristics of a steroid-sensitive and steroid-resistant patient. (In Polish) *Przegl Pediatr*. 2011;41:147–51.
- Jaroniec M, Ostalska-Nowicka D, Ąsmiech M, et al. Evaluation of single nucleotide polymorphism in direct vicinity of recognized mutations in *NPHS2* gene in children with nephrotic syndrome. (In Polish) *Nefrol Dial Pol*. 2010;14:111–5.
- Swierczewska M, Ostalska-Nowicka D, Kempisty B, et al. Molecular basis of mechanisms of steroid resistance in children with nephrotic syndrome. *Acta Biochim Pol*. 2013;60:339–44.
- Che R, Zhang A. Mechanisms of glucocorticoid resistance in idiopathic nephrotic syndrome. *Kidney Blood Press Res*. 2013;37:360–78.
- Ling V. Multidrug resistance: molecular mechanisms and clinical relevance. *Cancer Chemother Pharmacol*. 1997;40:S3–8.
- Hinoshita E, Uchiumi T, Taguchi K, et al. Increased expression of an ATP-binding cassette superfamily transporter, multidrug resistance protein 2, in human colorectal carcinomas. *Clin Cancer Res*. 2000;6:2401–7.
- Klimecki WT, Futscher BW, Grogan TM, et al. P-glycoprotein expression and function in circulating blood cells from normal volunteers. *Blood*. 1994;83:2451–8.
- Drigo I, Piscianz E, Valencic E, et al. Selective resistance to different glucocorticoids in severe autoimmune disorders. *Clin Immunol*. 2010;134:313–9.
- Li H, Yan Z, Ning W, et al. Using rhodamine 123 accumulation in CD8+ cells as a surrogate indicator to study the P-glycoprotein

- modulating effect of cepharanthine hydrochloride in vivo. *J Biomed Biotechnol.* 2011;2011:281651.
10. A Report of the International Study of Kidney Disease in Children. The primary nephrotic syndrome in children: identification of patients with minimal change nephrotic syndrome from initial response to prednisolone. *J Pediatr.* 1981;98:561–4.
  11. A report of the ISKDC early identification of frequent relapsers among children with minimal change nephrotic syndrome. *J Pediatr.* 1982;101:514–8.
  12. Report of Arbeitsgemeinschaft für pädiatrische Nephrologie. Cyclophosphamide treatment of steroid dependent nephrotic syndrome: comparison of eight week with 12 week course. *Arch Dis Child.* 1987;62:1102–6.
  13. Habashy D, Hodson EM, Craig JC. Interventions for steroid-resistant nephrotic syndrome: a systematic review. *Pediatr Nephrol.* 2003;18:906–12.
  14. Wasilewska A, Zoch-Zwierz W, Pietruczuk M, et al. Expression of P-glycoprotein in lymphocytes from children with nephrotic syndrome, depending on their steroid response. *Pediatr Nephrol.* 2006;21:1274–80.
  15. Meijer OC, de Lange EC, Breimer DD, et al. Penetration of dexamethasone into brain glucocorticoid targets is enhanced in *mdr1A* P-glycoprotein knockout mice. *Endocrinology.* 1998;139:1789–93.
  16. Türkmen M, Torun-Bayram M, Soylu A, et al. The relationship between renal P-glycoprotein expression and response to steroid therapy in childhood nephrotic syndrome. *Turk J Pediatr.* 2013;55:260–5.
  17. Wasilewska A, Zoch-Zwierz W, Pietruczuk M. Expression of multidrug resistance P-glycoprotein on lymphocytes from nephrotic children treated with cyclosporine A and ACE-inhibitor. *Eur J Pediatr.* 2007;166:447–52.
  18. Funaki S, Takahashi S, Wada N, et al. Multiple drug-resistant gene 1 in children with steroid-sensitive nephrotic syndrome. *Pediatr Int.* 2008;50:159–61.
  19. Prasad N, Jaiswal AK, Agarwal V, et al. Differential alteration in peripheral T-regulatory and T-effector cells with change in P-glycoprotein expression in childhood nephrotic syndrome: a longitudinal study. *Cytokine.* 2015;72:190–6.
  20. Tsujimura S, Saito K, Nakayama S, et al. Clinical relevance of the expression of P-glycoprotein on peripheral blood lymphocytes to steroid resistance in patients with systemic lupus erythematosus. *Arthritis Rheum.* 2005;52:1676–83.
  21. Sakaeda T, Nakamura T, Okumura K. Pharmacogenetics of drug transporters and its impact on the pharmacotherapy. *Curr Top Med Chem.* 2004;4:1385–98.
  22. Youssef DM, Elbehidy RM, Abdelhalim HS, et al. Soluble interleukine-2 receptor and MDR1 gene expression levels as inflammatory biomarkers for prediction of steroid response in children with nephrotic syndrome. *Iran J Kidney Dis.* 2011;5:154–61.
  23. Jafar T, Prasad N, Agarwal V, et al. MDR-1 gene polymorphisms in steroid-responsive versus steroid-resistant nephrotic syndrome in children. *Nephrol Dial Transplant.* 2011;26:3968–74.
  24. Cizmarikova M, Podracka L, Klimcakova L, et al. MDR1 polymorphisms and idiopathic nephrotic syndrome in Slovak children: preliminary results. *Med Sci Monit.* 2015;21:59–68.
  25. Youssef DM, Attia TA, El-Shal AS, et al. Multi-drug resistance-1 gene polymorphisms in nephrotic syndrome: impact on susceptibility and response to steroids. *Gene.* 2013;530:201–7.