

Biochemical markers of endothelial dysfunction in pediatric nephrotic syndrome

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Abstract

Introduction: Endothelial dysfunction occurs in many diseases particularly in renal disease. The participation of the different endothelial mediators has been implicated in the pathogenesis of nephrotic syndrome and its complications. The aim of this study was to assess the value of endothelial mediators including nitric oxide (NO) metabolites, cyclic guanosine monophosphate (cGMP), thromboxane A2 (TxA2), and prostacyclin (PGI2) in pediatric patients with nephrotic syndrome at different stages of the disease.

Material and methods: Urinary nitrite, serum nitrites and nitrates, as well as serum cGMP, TxA2, and PGI2 were compared between 32 pediatric patients with nephrotic syndrome and 15 controls.

Results: Urinary nitrite level was significantly higher among all patient groups compared to controls. However, there were highly significant differences in plasma nitrite and nitrate levels between patient groups and control; plasma nitrite in contrast to plasma nitrate, was significantly higher in children with minimal change glomerulonephritis (MCNS) and non minimal lesion groups than remission group. There was a significant difference between all groups of patients and control as regard cGMP, PGI2 and TxA2.

Conclusions: Endothelial dysfunction is related to the activity of the disease and it is reversible. Modulation of endothelial function in NS may offer a novel strategy to reduce progressive renal insult.

Key words: nephrotic syndrome, nitrites, nitrates, cGMP, thromboxane A2, prostacyclin.

Introduction

Endothelial dysfunction occurs from the earliest stages of renal disease, particularly in patients with nephrotic range proteinuria. Studies in chronic renal failure as in patients with diabetes, hypertension, and hyperlipidemia demonstrate that abnormalities in endothelial function are potentially reversible [1]. Endothelial dysfunction has emerged as potential final common pathway which may promote systemic atherosclerotic as well as renal disease progression [2]. Nephrotic syndrome in pediatric patients is associated with an increased risk of cardiovascular disease that may be a consequence of dyslipoproteinemia, hypoalbuminemia, hyperoxidative stress, hypercoagulable state and/or inflammation. Endothelial dysfunction is an early phase of atherogenesis associated with impaired nitric oxide (NO) bioavailability [3].

Nitric oxide, a typical free radical gas, elicits a wide range of physiological and pathological effects [4]. It is synthesized in the body from L-arginine by enzyme nitric oxide synthase (NOS). It easily decomposes into nitrite and nitrate in biological fluids. The concentration of these can be measured and used as markers of nitric oxide production [5]. Nitric oxide diffuses to the vascular smooth muscle cells and activates guanylate cyclase which leads to an increased production of cyclic guanosine 3', 5'- monophosphate [6].

Other important endothelium-derived contractile factors include cyclooxygenase product thromboxane A2 and prostaglandin intermediates. Although altered glomerular metabolism of arachidonic acid (AA) has already been demonstrated in experimental nephrotoxic nephritis, Remuzzi *et al.* concluded that there is an abnormality of glomerular AA metabolism in experimental nephrotic syndrome, which leads to increased TxA2 production [7].

Prostacyclin which is produced from arachidonic acid under basal condition and upon stimulation with agonist such as histamine and 5HT. Prostacyclin vasodilates through a rise of cyclic AMP with effects being less than, although additive to, those of NO [1].

The study aimed to assess endothelial mediators, including urinary nitrite as well as plasma nitrite and nitrate levels, cGMP, TxA2, and PGI2, in pediatric patients with nephrotic syndrome during different stages of the disease (activity and remission).

Material and methods

Thirty two children with primary nephrotic syndrome were studied in Cairo University Specialized Children Hospital from 2004 to 2006. Informed consents were taken from the parents of our children according to guidelines of the ethical committee of National Research Centre (NRC), Dokki, Egypt. They were 21 males and 11 females, ranging in age from 12 months to 13 years. Fifteen healthy subjects served as control. They are chosen from children attending the pediatric surgery ward for elective surgery as hernia. Exclusion criteria included hypertension and infection to avoid the effect of disease or its treatment as ACE inhibitors, non-steroidal anti-inflammatory drugs or aspirin on biochemical markers. None of these patients was taking drugs that affect the mediator levels such as immunosuppressive drugs, or nitrate-contained drugs, converting enzyme inhibitors, statin, NSAIDs, aspirin, et. The patients were divided into three groups according to the activity of nephrotic syndrome and response to steroid. Group 1 – 13 patients with minimal lesion during activity before starting steroid therapy. The diagnosis of minimal change nephrotic syndrome (MCNS) was made in

children with steroid responsive proteinuria. Their age ranged from 18 months to 10 years with mean 3.99 ± 2.3 years. Group 2 – 9 patients with non minimal lesion diagnosed by renal biopsy in patients who were steroid resistant or frequent relapser more than 3 times. These patients were studied while they were on steroid and not yet on immunosuppressive drugs. The relapse was defined as proteinuria $\geq 2+$ for 3 consecutive days. The renal biopsy of these patients revealed focal segmental glomerulosclerosis in 5 patients, membranoproliferative glomerulonephritis in 4 patients with crescent formation in only one patient. Their age ranged from 12 months to 13 years with mean 7.97 ± 4.78 years. Two girls with mesangial proliferative glomerulonephritis (MPGN) and focal segmental glomerulosclerosis (FSGS) who had creatinine concentration of 4.4, and 1.6 mg/dl, blood urea nitrogen 66 and 28 mg/dl, and GFR 19 and 83 ml/min/1.73 m² respectively. These two patients were excluded from the study because of renal impairment. During the period of the study another two patients with non minimal lesion progressed to chronic renal failure. Group 3 – 10 patients during remission and started steroid withdrawal. Remission in response to treatment was achieved when the urine was free of protein for 3 consecutive days. Routine laboratory assessment including CBC, urea, creatinine, albumin, cholesterol, GFR, and 24 h urinary protein excretion was done. Endothelial mediators were assessed in all groups of patients and control.

Biochemical evaluation

Citrated plasma was used for assay of nitric oxide metabolites. Indomethacin was added at a concentration of 10 µg/ml to all serum samples used for assay of cGMP, TxA2, and PGI2. Isopropanol was added to all urine samples at a concentration of 6.5% (v/v). All serum and urine samples were subjected to ultrafiltration through a 10,000 molecular weight cut off filter prior to storage at -80°C. Plasma nitrites and nitrates as an index for NO were assayed according to the method described by David *et al.*, 1991 [8]. In brief nitrites and nitrates (after reduction to nitrites by nitrate reductase enzyme) form chromophore with Griess reagent (one part 1% naphthylethylene diamine dihydrochloride in distilled water plus one part 1% sulfanilamide in 5% concentrated phosphoric acid). Plasma was incubated with Griess reagent at 37°C for 10 min and absorbance was read at 550 nm. Sodium nitrite was used as standard. Serum cGMP, TxA2, and PGI2 were assayed by ELISA kits obtained Quantikine, R/D system, Minneapolis, MN, USA, according to manufacturer recommendations.

Statistical analysis

Statistical analysis was done using SPSS software version 10. Comparison between each

Table I. Comparison between different groups of nephrotic syndrome and control

Demographic data and chemical markers	Group 1 MCNS (n = 13)	Group 2 Non minimal lesion (n = 7)*	Group 3 Remission (n = 10)	Control (n = 15)
Age [years]	3.99 ±2.35	6.81 ±5.04	2.94 ±0.75	4.2 ±2.36
Sex (M/F)	11/2	4/3	6/4	7/8
Urinary nitrite [mg/g creatinine]	16.8 ±10.33 ^A	15.64 ±2.73 ^A	14.68 ±4.12 ^A	7.66 ±2.36 ^B
Plasma nitrite [μmol/l]	45.87 ±20.85 ^A	68.85 ±17.51 ^A	25.62 ±9.84 ^B	12.33 ±7 ^C
Plasma nitrate [μmol/l]	45.58 ±19.7 ^A	39.24±29.53 ^A	38.02 ±21.24 ^A	14.54 ±3.04 ^B
Serum cGMP [pmol/ml]	0.55 ±0.078 ^A	0.45 ±0.08 ^B	0.59 ±0.14 ^{AB}	0.26 ±0.08 ^C
Serum thromboxane A2 [ng/l]	360.5 ±243.7 ^A	475.1 ±259.6 ^A	447.8 ±349.6 ^A	36.67 ±6.7 ^B
Serum prostacyclin [ng/l]	0.62 ±0.38 ^A	0.34 ±0.17 ^A	0.5 ±0.33 ^A	2.26 ±0.44 ^B
Serum albumin [g/dl]	1.82 ±0.55 ^A	1.31 ±0.2 ^A	2.98 ±0.14 ^B	
Serum cholesterol [mg/dl]	419.1 ±170.2 ^A	351 ±100.3 ^A	182 ±21.5 ^B	
Serum creatinine [mg/dl]	0.42 ±0.2 ^A	0.51 ±0.31 ^A	0.34 ±0.2 ^A	
Urinary protein [g/24 h urine]	3.13 ±1.5 ^A	3.13 ±1.11 ^A	0.56 ±0.11 ^B	

Means do not share a superscript letter are significantly different at $p < 0.05$, e.g., A is different from B and A is not different from AB
 *2 cases were excluded because of decreased GFR

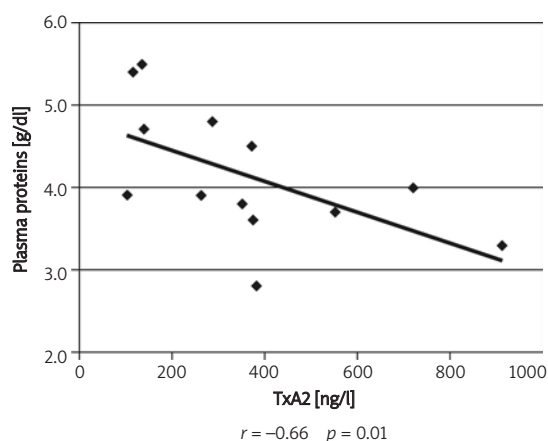
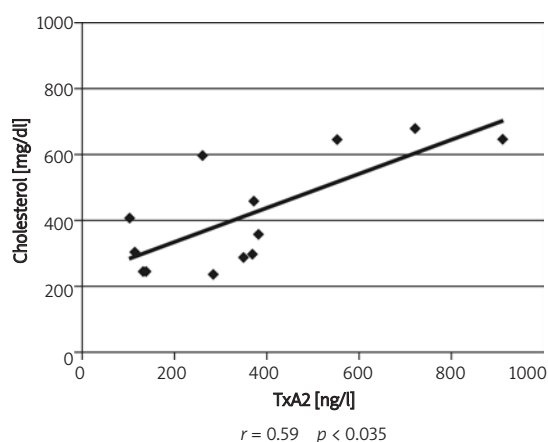
2 independent continuous variables was performed using Mann-Whitney U test. Spearman's rho " r " correlation coefficient was used to measure the linear relationship between continuous variables. Data were expressed as mean \pm SD, a p -value less than 0.05 was considered statistically significant.

Results

The serum creatinine concentration, blood urea nitrogen, and glomerular filtration rate (GFR) were within the normal range in all subjects. As expected, the NS group during activity had statistically higher urinary proteins, and serum cholesterol, and a significantly lower serum albumin than remission group (all $p < 0.001$).

Urinary nitrite level was significantly higher among all groups of patients compared to controls ($p < 0.001$, 0.001, 0.01 respectively), but the comparison between groups revealed no significant difference. Plasma nitrite and nitrate levels showed highly significant differences between all groups of patients and control. However, plasma nitrite in contrast to plasma nitrate, was significantly higher in children with MCNS and non minimal lesion groups than remission group (Table I).

Furthermore, there was a high significant difference between all groups of patients and control as regard cGMP, and PGI₂. The comparison between the groups of patients revealed significant increase in cGMP in MCNS than non minimal lesion (p value = 0.02). However, there was a high significant difference between all groups of patients and control as regard TxA₂, there was no significant difference between the groups of patients (Table I). Urinary nitrite concentrations may be related to


Figure 1. Scatter diagram and regression line of plasma proteins vs. TxA₂ in MCNS

Figure 2. Scatter diagram and regression line of cholesterol vs. TxA₂ in MCNS

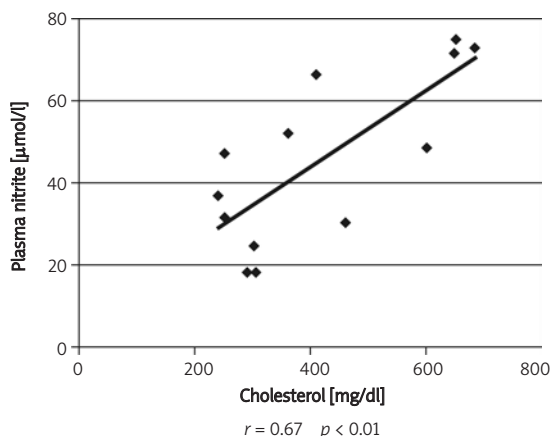


Figure 3. Scatter diagram and regression line of cholesterol vs. plasma nitrite in MCNS

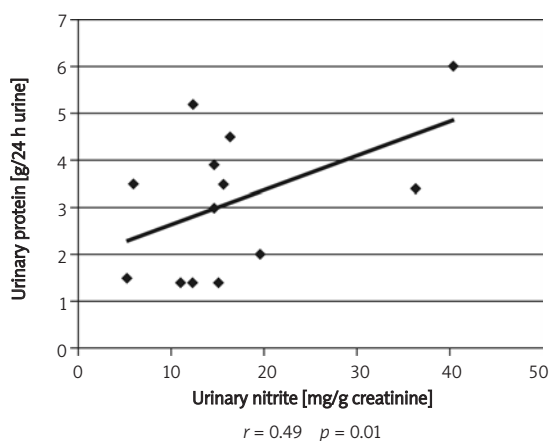


Figure 4. Scatter diagram and regression line of urinary protein vs. urinary nitrite in MCNS

proteinuria because they were correlated in MCNS. Cholesterol also correlates positively significant with plasma protein, plasma nitrite, and TxA2 in MCNS. TxA2 correlates negatively with plasma proteins in pediatric patients with MCNS and non minimal lesion (Figures 1-4). The correlation between serum albumin level and all endothelial mediators was assessed with no significant correlation.

Discussion

This study is a cross sectional examination from a single time point and employs only simple bivariate comparisons in the analysis. The findings in this study indicate that urinary nitrite as well as plasma nitrite and nitrate are significantly elevated in all groups of patients compared to control group.

Similar to our study, Trachtman *et al.* study, showed that urinary nitrite excretion was elevated in children with MCNS regardless of the activity of the disease or whether the patients were on steroid therapy or not, but there was no difference between controls

and patients with FSGS [5]. Balat *et al.* and Dubey *et al.* measured plasma and urinary NO in children with MCNS and the study revealed increased urinary nitrite excretion compared to controls and also plasma nitrite levels were high in relapse [9, 10]. In contrast to this study, Duan *et al.* showed that urinary levels of NO among nephrotic syndrome patients were not significantly different with controls but the urinary levels in patients with NS was lower than in patients with nephritic syndrome. It is assumed that the mechanism is the decreased expression of endothelial NOS (eNOS) caused by ischemia of kidney tissues in a progressive period of NS [11].

In this study, the higher level of nitrites and nitrates (NOx) in MCNS and non minimal lesion than remission group can be explained by progressive renal insult and more renal ischemia. Kawashima *et al.* study, revealed that NO in serum of NS patients was significantly elevated irrespective of rate of relapses [12]. Kettlar and Narita studies suggested that NO contributes to glomerular injury in experimental models of nephritis where inhibition of NO in acute mesangioproliferative glomerulonephritis in rats induced by an anti-thymocyte antibody resulted in reduced proteinuria and diminished mesangial matrix expansion [13, 14]. High serum NO may also be related to an increased apoptotic rate of circulating lymphocytes [12]. Measurement of urinary nitrite may be a useful test to predict the response to steroid therapy in patients with MCNS. In this study, the persistently elevated urinary nitrite excretion during activity and in remission; in addition to significant correlation between urinary nitrite excretion and urinary protein excretion suggests that the glomerular permeability might be still affected in remission. Trachtman *et al.* study, reported that the persistently elevated nitrite excretion in relapse and remission suggested that renal NO synthesis does not modulate proteinuria in MCNS [5]. In contrast to our study, Trachtman *et al.* study, concluded that increased urinary nitrite excretion is highly diagnostic of MCNS in a child with new-onset nephrotic syndrome but the presence of excess urinary nitrite has a low sensitivity to detect MCNS, and therefore therapeutic trials of corticosteroids is recommended even in children with normal or low urinary nitrite excretion. In this study, there was significant positive correlation of urinary nitrites and 24 h urinary proteins. Ozen *et al.* study suggested that increased NO production might be responsible for proteinuria [15]. The suggested mechanisms from other studies included that NO may be damaging glomerular basement membrane through complexing with structural proteins. The damaged membrane would thus be expected to spill proteins. Overproduction of NO may also be contributing to

proteinuria by enhancing glomerular damage through interaction with superoxide anion [16, 17]. Also it may reflect enhanced NO production by glomerular mesangial or tubular epithelial cells that contain calmodulin and calcium-independent-inducible NOS [5]. The plasma nitrites in this study were significantly higher in MCNS than remission group and this can be explained by the ability of dexamethazone to inhibit NO synthesis in glomeruli suggested induction of the inducible nitric oxide synthase (iNOS) enzyme [18, 19].

Nitric oxide had taken center stage as a pro-inflammatory mediator in kidney diseases where glomerular mesangial cells, and endothelial cells can express iNOS which is responsible for overproduction of cytotoxic NO. Enzymes producing proinflammatory mediators, such as cyclooxygenase 2 and secretory phospholipase A2 are transcriptionally affected by NO [20]. Furuu *et al.* reported that NO derived from eNOS and iNOS may be involved in the progression of renal diseases [21]. Walker *et al.* reported that NO formation during puromycin aminonucleoside (PAN)-induced NS is increased but does not participate in the development of glomerular injury because of the results of an increased NOx concentrations and lack of iNOS protein expression in either the glomeruli or the cortex [22]. The iNOS was also hardly detected in control kidneys and pediatric NS [12]. cGMP in this study was significantly increased in all groups of patients in relation to control, and did not show significant correlation with urinary nitrite. Our findings therefore support the concept that plasma cGMP concentrations are not an indicative of NO synthesis status [23, 24]. Nitric oxide acting as a messenger molecule mediates vascular relaxation, inhibit platelet aggregation and adhesion to the endothelium and modulates leukocyte chemotaxis and adhesion. All these effects of NO are mediated by activation of soluble guanylate cyclase after NO binding to its heme iron, resulting in increased levels of cGMP [6, 16]. The endothelial prostaglandins may control the synthesis of NO. Prostacyclin (PGI₂) inhibits NO release from cultured bovine aortic endothelial cells and modulates lipopolysaccharides (LPS) stimulated iNOS in mouse macrophages [25]. It has also been shown that NO stimulates the inducible cyclooxygenase enzyme (COX2) *in vitro* [26, 27]. The obvious involvement of COX2 in tubulovascular signaling has raised a number of questions regarding the co-ordinated action of prostaglandin and NO on glomerular vasculature [28]. The pediatric NS patients in this study had statistically significant higher plasma TxA₂ than control and prostacyclin was significantly lower than control. Remuzzi *et al.* 1985 study concluded that: there is an increased TxA₂ production in experimental nephrosis which correlates with protein excretion and might be responsible for altered the glomerular basement

membrane permeability to protein [7]. In our study, the TxA₂ levels were negatively correlated with plasma proteins. Tsygin *et al.* 1991 showed that the pathogenesis of the NS of paramount importance is imbalance of the output of renal prostanoids, manifesting in the predominance of vasopressor and proaggregate fraction TxA₂ and in the deficiency of its antagonist prostacyclin that exerts a protective action on glomerular filtration [29]. Watanabe *et al.* 1996 demonstrated enhanced platelet sensitivity to TxA₂ with an improvement of hypoalbuminemia and increased biological production of TxA₂ in patients with NS, suggesting that TxA₂ metabolism in the platelet is deeply involved in pathophysiology of NS [30]. Similar to our study, Dogra *et al.* study, 2001 showed that the NS patients had statistically higher plasma arachidonic acid levels which are significantly positively correlated with cholesterol [31]. Benigni *et al.* study 1990 suggested that renal TxA₂ could be regarded as one of the possible mediators of the altered glomerular permeability to proteins in MCNS [32]. These findings may explain the thrombotic tendency and endothelial dysfunction in patients with significant proteinuria. Joles *et al.* 1999 study showed that hypoalbuminemia itself or other aspects of the dyslipidemia characteristic of the NS impair endothelial function. Hypoalbuminemia may disturb endothelial function, either by directly affecting G-protein dependent signal transduction or indirectly by changing the configuration of the cell membrane. Such a change in cell membrane configuration will disturb binding of ligands to receptors and of endothelial nitric oxide synthase to caveolin [33].

Fuiano *et al.* 1996 study showed that increased cholesterol levels can induce endothelial cell dysfunction and thus reduction of cholesterol is associated with a significant increase in renal blood flow and this effect may contribute to increase the risk of ischemic acute renal failure in nephrotic patients and, along with changes induced in the mesangium by other mechanisms, to contribute to the progression of renal disease [34]. Nephrotic syndrome in children often behaves very differently than that of adults (which is also more heterogeneous) where nephrotic syndrome in children is due to minimal change disease in more than 90% of cases, while in adult is 10-15% of cases. In adults, the most common of glomerulopathy causing nephrotic syndrome is membranous glomerulonephritis followed by FSGS. Focal segmental glomerulosclerosis is increasing in incidence in adults [35]. Minimal change nephrotic syndrome is an immune mediated disorder of primary T-cell dysfunction, where the lymphocytes are altered with IL-2 expression during a relapse [36]. Increased urinary and serum nitric oxide metabolites in pediatric patients with nephrotic syndrome as NO is considered to have proinflammatory effect [20].

Furusu *et al.* study found that a decreased expression of endothelial nitric oxide synthase in adult patients with IgA nephropathy and lupus nephritis suggesting diminished effect of eNOS (decreased generation of NO) in damaged glomeruli [21]. Moreover, Trachtman *et al.* found that there was no difference between control subjects and patients with FSGS as regard urinary nitrite excretion [5]. Chemokine (C-C) ligand 13 (CCL13) and a novel galectin-related protein (HSPC159) gene expressions in MCNS patients were significantly higher than those in nephrotic membranous nephropathy (MN) patients and healthy controls. The mRNA expression patterns were variable among 10 MN patients [37]. This study concluded that endothelial dysfunction occurs from the earliest stages of NS and it is reversible. The exact etiology of endothelial dysfunction is still unclear and it is likely to be multifactorial. Modulation of endothelial function in NS may offer a novel strategy to reduce progressive renal insult, thus continued research on endothelial mediators (NO and its metabolites) will help to develop a new pathway for prevention and treatment of NS. However, it remains to be shown whether NO participates directly in glomerular injury as measured by proteinuria. Biochemical markers need further study to be applicable in treatment and diagnosis of nephrotic syndrome.

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