Original Article

# Plasma Free Homocysteine Levels in Children with Idiopathic Nephrotic Syndrome


## Abstract

Altered metabolism of homocysteine in children with idiopathic nephrotic syndrome leads to raised plasma-free homocysteine levels. Elevated free homocysteine causes endothelial cell dysfunction and promotes early atherosclerosis and glomerulosclerosis. In this analytical study with a longitudinal follow-up, 29 children with first episode of nephrotic syndrome (FENS) aged 1–16 years along with 30 age andgender-matched healthy controls were enrolled. Plasma-free homocysteine was measured using high-performance liquid chromatography (HPLC). Other variables were measured using standard biochemical methods. The primary outcome measure was plasma-free homocysteine level in children with FENS and in controls. The secondary outcome measure was to observe the levels of plasma-free homocysteine in children with FENS at 12 weeks in remission and in steroid resistant states. Plasma-free homocysteine levels were significantly elevated in children with FENS at disease onset [Median (IQR) 2.170 (1.54–2.71); $N = 29$; $P < 0.001$], at 12 weeks of steroid-induced remission [Median (IQR) 1.946 (1.53–2.71); $N = 22$; $P < 0.001$], and in steroid-resistant states [Median (IQR) 2.262 (1.53–2.74); $N = 7$; $P < 0.001$] compared to controls. The levels did not decrease significantly at 12 weeks of steroid-induced remission compared to onset of nephrotic syndrome. Plasma-free homocysteine levels correlated positively with serum total cholesterol ($P = 0.005$; $r = 0.362$) and negatively with serum albumin ($P = 0.032$; $r = 0.281$). Plasma-free homocysteine levels are raised in children with FENS posing a risk of endothelial dysfunction which persists at least in short term. Long-term effects of raised plasma-free homocysteine needs to be studied.


**Keywords:** *Children, endothelial dysfunction, free homocysteine, nephrotic syndrome*

## Introduction

Adults who suffered from idiopathic nephrotic syndrome in childhood have an increased risk of atherosclerosis.[1,2] Studies in the pediatric population have revealed that the risk factors of atherosclerosis occur in patients having nephrotic syndrome in various disease stages, which lead to the assumption that such children are predisposed to accelerated atherosclerosis.[1] Recently, several novel risk factors of atherosclerosis have been described. These risk factors include raised homocysteine, asymmetric dimethylarginine, endothelin-1, proteins of chronic inflammatory process, pro-thrombotic factors, and adhesion molecules.[3] In an analytical study, we recently demonstrated that the levels of soluble thrombomodulin, tissue plasminogen activator, plasminogen activator inhibitor 1, and von-Willebrand factor are raised in children with first episode of nephrotic syndrome (FENS), pointing towards endothelial dysfunction.[4]

Homocysteine is a sulfhydryl containing amino acid, a homologue of cysteine with one additional methylene group in its side chain.[5] Hyperhomocystenemia is an independent risk factor for coronary heart disease, andnormotensive patients with hyperhomocysteinemia display endothelial dysfunction.[6,7] In plasma, 70–80% of homocysteine occurs in a protein-bound form, and the remaining 20–30% occurs in free form as oxidized cysteine or homocysteine; free reduced homocysteine contributes to 1% of the total levels.[8] Elevated homocysteine levels are found in almost one-third of all patients with atherosclerosis, and levels more than 12% above the upper limit are associated with a three-fold increase in the risk of myocardial infarction.[9,10] Folic acid supplementation can reduce homocysteine

**How to cite this article:** Arumugam V, Saha A, Kaur M, Deepthi B, Basak T, Sengupta S, *et al.* Plasma free homocysteine levels in children with idiopathic nephrotic syndrome. Indian J Nephrol 2019;29:186-90.

This is an open access journal, and articles are distributed under the terms of the Creative Commons Attribution-NonCommercial-ShareAlike 4.0 License, which allows others to remix, tweak, and build upon the work non-commercially, as long as appropriate credit is given and the new creations are licensed under the identical terms.

**For reprints contact:** reprints@medknow.com

**Venkatesh Arumugam[1], Abhijeet Saha[2], Manpreet Kaur[1], Bobbity Deepthi[2], Trayambak Basak[3,4], Shantanu Sengupta[3,4], Ajay Bhatt[3], Vineeta V. Batra[5], Ashish D. Upadhyay[6]**

*[1]Division of Pediatric Nephrology, Department of Pediatrics, Postgraduate Institute of Medical Education and Research and Associated, Dr. Ram Manohar Lohia Hospital, Baba Kharak Singh Marg, [2]Division of Pediatric Nephrology, Department of Pediatrics, Lady Hardinge Medical College and associated Kalawati Saran Children Hospital, [3]Genomics and Molecular Medicine CSIR-Institute of Genomics and Integrative Biology, [4]Academy of Scientific and Innovative Research, [5]Department of Pathology, GB Pant Institute of Postgraduate Medical Education and Research, [6]Department of Biostatistics, All India Institute of Medical Sciences, New Delhi, India*

***Address for correspondence:***
*Dr. Abhijeet Saha, Division of Pediatric Nephrology, Room No 102, Lady Hardinge Medical College and Kalawati Saran Children Hospital, New Delhi - 110 001, India.*
*E-mail: drabhijeetsaha@yahoo.com*

**Access this article online**

**Website:** www.indianjnephrol.org

**DOI:** 10.4103/ijn.IJN_293_17

**Quick Response Code:**



© 2019 Indian Journal of Nephrology | Published by Wolters Kluwer - Medknow

levels and improve endothelial dysfunction in children with chronic renal failure.[11]

We have found that total homocysteine levels are markedly elevated in urine of the children with idiopathic nephrotic syndrome along with vitamin $B_{12}$ and folic acid.[12] Chambers *et al*. reported that free plasma homocysteine concentrations may be a more accurate index of the biological activity of homocysteine *in vivo*.[13,14] Evidence to support the assertion of plasma homocysteine levels playing a significant role in predicting endothelial dysfunction eventually predisposing to atherosclerotic events also comes from *in-vitro* data. These studies show that free homocysteine species inactivate NO, promote the generation of copper-catalyzed, oxygen-derived free radicals, induce tissue factor release, and cause endothelial injury.[15-18] Martijn*et al*. conducted a prospective study in a cohort of 379 patients hospitalized for acute coronary syndrome showing that plasma-free homocysteine level is a strong and independent predictor of recurrent acute myocardial infarction, stroke, or death due to cardiovascular causes.[19] Our study was designed with a primary objective to measure plasma free homocysteine levels in children with FENS compared to controls. The secondary objective was to measure levels in drug-induced remission and in steroid resistant states.

## Materials and Methods

### Study design and patient groups

This was an analytical study with a longitudinal follow-up conducted in a tertiary care hospital in New Delhi from November 2012 to July 2014. The study was approved by the institutional review board and a written informed consent was taken from all the participants. The study group included children with FENS aged 1–16 years and 30 age and gender-matched healthy controls coming to the outpatient department for a health certificate needed for joining swimming classes or dance classes. Children who had secondary nephrotic syndrome, signs of thromboembolic complications, bleeding diathesis, on drugs known to affect endothelial function, pre-existing hypertension, diabetes mellitus, recent history of blood transfusion, who had taken vitamin $B_{12}$/folate within the last 2 weeks and who refused to give an informed consent were excluded from the study. The patients were enrolled at the onset of the disease and were followed for 1 year. Samples were collected at the time of initial presentation, in remission, and before starting second-line drugs. Standard laboratory methods were used to analyze serum albumin, cholesterol, and other laboratory parameters. Guidelines by the Indian Society of Pediatric Nephrology were used for the diagnosis and treatment of steroid sensitive nephrotic syndrome (SSNS), and steroid-resistant nephrotic syndrome (SRNS).[20,21] Initial episode of nephrotic syndrome was treated with prednisolone at a dose of 2 mg/kg per day (maximum 60 mg in single or divided doses) for 6 weeks, followed by 1.5 mg/kg (maximum 40 mg) as a single morning dose on alternate days for the next 6 weeks; therapy was subsequently discontinued. Patients who failed to achieve remission after 4 weeks of prednisolone at a dose of 2 mg/kg/day were diagnosed as SRNS.Renal biopsy was performed in all steroid-resistant children. The primary outcome was to compare the levels of free homocysteine in children with FENS and controls. Secondary outcome measure was to observe the levels of plasma-free homocysteine in children with FENS at 12 weeks in remission.

### Measurement of free homocysteine

Blood samples were collected in lithium heparin vials and centrifuged immediately at 2800 g for 10 min. Two hundred microlitres of plasma was taken in three alliquots each and then 100 µl of perchloric acid was added and centrifuged at 10000 g for 30 min. The supernatant fluid thus obtained was stored at −80°C until analyzed. Free homocysteine was measured using high-performance liquid chromatography (HPLC) machine present at the IGIB. In this method, the samples were treated with sodium borohydrate to reduce disulfide bonds. Amyl alcohol controls foaming and borohydrate reduction of disulfide bonds releases homocysteine and cleaves low molecular weight oxidized forms of homocysteine such as homocysteine, cysteinylhomocysteine, and glutathionylhomocysteine. The thiol group of free homocysteine was then derivatized with the fluorescent adduct monobromobimane to form a fluorescent homocysteine-bimane conjugate. Subsequently, perchloric acid was added and centrifuged. The supernatant fluid was then neutralized with Tris and injected into HPLC. Homocysteine bimane was resolved from other thiol bimane conjugates on a reverse-phase column and detected fluorometrically. Results were quantified by taking the area of the homocysteine-bimane peak and calculating its concentration using a regression equation from a standard curve.

### Statistical analysis

Data was analyzedusing Strata 11.1(Stata Corp, 4905 Lakeway Drive, College Station, Texas USA) and presented as frequency (%), mean (SD), and median (IQR), as applicable. Categorical variable (sex) was compared in the two groups using Fischer's exact test. Continuous variables with a normal distribution were compared among the groups using one-way analysis of variance (ANOVA) followed by *post hoc* comparison using Bonferroni test continuous, and non-normal variables were evaluated by Wilcoxon's rank sum test/Kruskal–Wallis test followed by multiple comparison using Wilcoxan's rank sum test with Bonferroni correction. Changes in continuous variables were assessed by Wilcoxon's sign rank test. Corelation of homocysteine with other variables was assessed by spearman correlation coefficient "(rho)." $P$ value of $<0.05$ was considered significant.

## Results

Twentynine children with FENS were included in the study. Baseline characteristics in children with SSNS ($N$ = 22), SRNS ($N$ = 7) and controls groups has been reported in Table 1. Children in SSNS group had significantly lesser height for age than controls ($P$ = 0.012), significantly higher weight for their age than controls ($P$ = 0.005) and SRNS patients ($P$ = 0.019). Systolic blood pressure was significantly higher in SSNS patients ($P$ = 0.006) and SRNS patients ($P$ = 0.036) compared to controls [Table 1]. All patients with SRNS had minimal change disease on histopathological examination.

Free homocysteine levels were significantly elevated in children with FENS at disease onset ($P$ < 0.001), at 12 weeks of steroid-induced remission ($P$ < 0.001), and in steroid-resistant states ($P$ < 0.001) compared to controls [Table 2]. Free homocysteine levels decreased after 12 weeks of drug-induced remission compared to the levels at onset but was not statistically significant ($P$ = 0.06) [Table 2]. Plasma total homocysteine levels were elevated in SSNS (median = 4.053; $P$ < 0.001), SRNS at disease onset (median = 9.254; $P$ = 0.004) compared to controls (median = 3.433). The proportion of free homocysteine compared to the total homocysteine was significantly higher in first episode nephrotic syndrome compared to controls (median 24.47 vs 6.22; $P$ < 0.001). Free homocysteine levels correlated positively with serum total cholesterol ($P$ = 0.005; $r$ = 0.362) and negatively with serum albumin ($P$ = 0.032; 0.281). There was no correlation of free homocysteine with age ($P$ = 0.078; $r$ = 0.333), systolic blood pressure ($P$ = 0.975; $r$ = −0.006), diastolic blood pressure ($P$ = 0.924; $r$ = −0.018), BMI ($P$ = 0.949; $r$ = 0.012), and urinary protein ($P$ = 0.892; $r$ = 0.026).

## Discussion

In this study, we found that the level of free homocysteine in plasma was significantly elevated in children with FENS compared to controls. Similar findings have also been reported by Tkaczyk *et al*.;[22] however, because the patient population was not homogenous, we selected only FENS in our study. We recently reported that children with FENS have plasma total homocysteine levels comparable to controls; however, this was associated with a marked increase in urinary homocysteine excretion.[12] This is plausible as most of the homocysteine in plasma is bound

**Table 1: Demographic profile of patients and controls**

| | SSNS (mean±SD) ($n$=22) | SRNS (mean±SD) ($n$=7) | Control (mean±SD) ($n$=30) | $P$ |
|---|---|---|---|---|
| Age (years) | 5.55±3.54 | 8.28±4.40 | 5.59±3.81 | 0.354 |
| #Gender (male/female) | 13/9 | 3/4 | 16/14 | 0.759 |
| Height (cms) | 104.27±20.07 | 112.43±22.62 | 105.33±21.264 | 0.584 |
| Height z score | -1.38±0.99 | -2.37±1.18 | -1.02±1.39 | 0.039 |
| Weight (kgs) | 18.68±8.56 | 19.85±7.80 | 17.07±8.950 | 0.421 |
| Weight z score | -0.87±1.02 | -2.31±2.2 | -1.99±1.37) | 0.008 |
| BMI (kg/m$^2$) | 18.68±8.56 | 19.86±7.80 | 14.400±2.98 | 0.834 |
| BMI z score | -0.09±1.57 | -1.6±1.93 | -2.5±3.66 | 0.021 |
| Systolic BP z score | 0.87±1.08 | 1.08±0.87 | 0.25±0.63 | 0.020 |
| Diastolic BP z score | (1.32±1.10) | (1.26±0.68) | (0.95±0.48) | 0.270 |
| Total protein (g/dl) | 3.86±0.51 | 3.9±0.40 | 6.807±0.726 | <0.001 |
| S.Albumin (g/dl) | 1.75±0.28 | 1.7±0.33 | 4.190±0.377 | <0.001 |
| Urine protein creatinine ratio | 10.68±±15.09 | 10.81±5.17 | 0.218±0.122 | <0.001 |
| Total Cholesterol (mg/dl) | 433.95±128.91 | 367.71±138.88 | 133.37±27.987 | <0.001 |
| Creatinine (µmole/L) | 39.78±15.9 | 74.27±60.11 | 42.69±9.28 | 0.219 |
| eGFR (ml/min/1.732m$^2$) | 113.66±49.73 | 81.94±46.68 | 94.967±25.189 | 0.296 |

All the values are expressed as Mean±SD *Test name: one way annova - for normally distributed data; Kruskal wallis test - for skewed data ; #categorical variable – Fischer exact test. FENS: First episode nephrotic syndrome, SSNS: Steroid sensitive nephrotic syndrome, SRNS: Steroid resistant nephotic syndrome, cms: Centimetres, kgs: Kilograms, mmHg: Millimetres of mercury, Kg/m$^2$: Kilograms per metre squared, SBP/DBP: Systolic/Diastolic blood pressure, BMI: Body mass index, eGFR: Estimated glomerular filtration rate

**Table 2: Free homocysteine levels in cases and controls**

| | SSNS (at onset) ($n$=22) | SSNS (at 12 weeks remission) ($n$=22) | SRNS (at onset) ($n$=7) | SRNS (at 4 weeks) ($n$=7) | Controls ($n$=30) |
|---|---|---|---|---|---|
| Free homocysteine (micromol/L) median (IQ range) | 1.946 (1.53-2.71) | 1.606 (0.96-2.14) | 2.262 (1.53-2.74) | 2.443 (2.24-2.74) | 0.784 (0.57-1.16) |
| $P$ | <0.001* | <0.001* | 0.760** | 0.499*** | |

*Statistical difference as compared to controls and Wilcoxon's ranksum test applied. **Statistical difference as compared to SSNS group at onset and Wilcoxon's ranksum test applied. ***Statistical difference as compared to SRNS at onset and Wilcoxon's sign rank test apllied SSNS: Steroid sensitive nephrotic syndrome, SRNS: Steroid resistant nephotic syndrome

to albumin. There was concurrent increased urinary excretion of vitamin $B_{12}$ and folate which normalized during remission. Elevation of urinary homocysteine was because of the presence of albumin-bound homocysteine in urine. Children with FENS had low plasma levels of vitamin $B_{12}$ and folate, a finding previously reported by Podda *et al*.[23] It is well known that vitamin $B_{12}$ and folate deficiency causes hyperhomocysteinemia by interfering with the methylation pathway.[24] Low plasma vitamin $B_{12}$and folate is seen in children with idiopathic NS because of increased urinary loss.[12]

Free homocysteine species inactivate NO, promote the generation of oxygen-derived free radicals, induce tissue factor release, and cause endothelial cell injury.[22] Endothelial dysfunction in the presence of elevated plasma total homocysteine levels may be mediated by oxidative stress by impairing intracellular glutathione peroxidase-1 activity and expression.[25] This effect reduces the ability of the endothelial cell to detoxify itself of hydroxyl radicals and permits further oxidative damage. Previous studies investigating homocysteine and vascular disease have relied on total plasma homocysteine as the sole index of homocysteine status. Chambers *et al*. reported that reduced free homocysteine was closely associated with endothelial dysfunction during oral methionine and oral homocysteine loading. They found an inverse relationship between free homocysteine peak concentration and flow-mediated dilatation which is a marker of endothelial function.[14] Free homocysteine rather than total homocysteine was found to be an accurate marker for the prognosis of patients with acute coronary symptoms.[19] Hence, elevated free homocysteine levels in children with FENS poses a risk of endothelial dysfunction.

The level of free homocysteine in the cases decreased after 12 weeks of drug-induced remission but it was insignificant and remained higher than the controls, pointing that children are at a risk of endothelial dysfunction even after drug-induced remission. The level of free homocysteine was persistently elevated in steroid-resistant patients and was higher indicating a higher risk of endothelial dysfunction. Long-term adverse effects of raised free homocysteine levels in children with idiopathic nephrotic syndrome also needs to be evaluated in well-designed studies. Thus, free homocysteine might play a significant role in causing endothelial dysfunction in children with idiopathic nephrotic syndrome. Modulation of endothelial dysfunction in children with FENS may be considered a therapeutic strategy to decrease the risk of future adverse cardiovascular events.

To conclude, we have shown risk of endothelial dysfunction due to elevated free homocysteine levels in short term in children with FENS. Long-term prospective studies are needed in children with idiopathic nephrotic syndrome. The limitations of our study are small sample size, short term follow up, and single-centred analysis; nevertheless, our study showed that children with FENS have higher plasma free homocysteine levels and are at a risk of endothelial dysfunction.

### Financial support and sponsorship

Nil.

### Conflicts of interest

There are no conflicts of interest.

## References

1. Kniazewska MH, Obuchowicz AK, Wielkoszynski T, Zmudzinska-Kitczak J, Urban K, Marek M, *et al*. Atherosclerosis risk factors in young patients formerly treated for idiopathic nephrotic syndrome. Pediatr Nephrol 2009;24:549-54
2. Lechner BL, Bockenhauer D, Iragorri S, Kennedy TL, Siegel NJ. The risk of cardiovascular disease in adults who have had childhood nephrotic syndrome. Pediatr Nephrol 2004;9:744-8
3. Cattaneo M. Hyperhomocysteinemia, atherosclerosis and thrombosis. Thromb Haemost 1999;81:165-76
4. Sharma B, Saha A, Dubey NK, Kapoor K, Anubhuti, Batra VV, *et al*. Endothelial dysfunction in children with idiopathic nephrotic syndrome. Atherosclerosis 2014;233:704-6
5. Welch GN, Loscalzo J. Homocysteine and atherothrombosis N Engl J Med 1998;338:1042-50
6. Virdis A, Ghiadoni L, Cardinal H, Favilla S, Duranti P, Birindelli R, *et al*.Mechanisms responsible for endothelial dysfunction induced by fasting hyperhomocystinemia in normotensive subjects and patients with essential hypertension. J Am Coll Cardiol2001;38:1106-15.
7. Refsum H, Smith AD, Ueland PM, Nexo E, Clarke R, McPartlin J, *et al*.Facts and recommendations about total homocysteine determinations: An expert opinion. Clin Chem 2004;50:3-32.
8. Ueland PM. Homocysteine species as components of plasma redox thiol status Clin Chem 1995;41:340-2
9. Graham IM, Daly LE, Refsum HM, Robinson K, Brattstrom LE, Ueland PM, *et al*.Plasma homocysteine as a risk factor for vascular disease. The European Concerted Action Project. JAMA 1997;277:1775-881.
10. Stampfer MJ, Malinow MR, Willett WC, Newcomer LM, Upson B, Ullmann D, *et al*.A prospective study of plasma homocyst(e)ine and risk of myocardial infarction in US physicians. JAMA 1992;268:877-81.
11. Bennett-Richards K, Kattenhorn M, Donald A, Oakley G, Varghese Z, Rees L, *et al*.Does oral folic acid lower total homocysteine levels and improve endothelial function in children with chronic renal failure? Circulation 2002;105:1810-5.
12. Kundal M, Saha A, Dubey NK, Kapoor K, Basak T, Bhardwaj G, *et al*. Homocysteine Metabolism in Children with Idiopathic Nephrotic Syndrome. Clin Transl Sci 2014;7:132-6
13. Chambers JC, Ueland PM, Obeid OA, Wrigley J, Refsum H, Kooner JS. Improved vascular endothelial function after oral B vitamins: An effect mediated through reduced concentrations of free plasma homocysteine. Circulation 2000;102:2479-83
14. Chambers JC, Ueland PM, Wright M, Dore CJ, Refsum H, Kooner JS. Investigation of relationship between reduced, oxidized, and protein-bound homocysteine and vascular endothelial function in healthy human subjects. Circ Res 2001;89:187-92

15. Stamler JS, Osborne JA, Jaraki O, Rabbani LE, Mullins M, Singel D, *et al*.Adverse vascular effects of homocysteine are modulated by endothelium-derived relaxing factor and related oxides of nitrogen. J Clin Invest 1993;91:308-18
16. Fryer RH, Wilson BD, Gubler DB, Fitzgerald LA, Rodgers GM. Homocysteine, a risk factor for premature vascular disease and thrombosis, induces tissue factor activity in endothelial cells. Arterioscler Thromb 1993;13:1327-33
17. Starkebaum G, Harlan JM: Endothelial cell injury due to copper-catalyzed hydrogen peroxide generation from homocysteine. J Clin Invest 1986:77:1370-6
18. Dudman NP, Temple SE, Guo XW, Fu W, Perry MA. Homocysteine enhances neutrophil-endothelial interactions in both cultured human cells and rats *in vivo*. Circ Res 1999;84:409-16
19. van Oijen MG, Claessen BE, Clappers N, van Schaik A, Laheij RJ, *et al*.Prognostic value of free plasma homocysteine levels in patients hospitalized with acute coronary syndrome. Am J Cardiol 2008;102:135-9.
20. Indian Pediatric Nephrology Group IAoP, Bagga A, Ali U, Banerjee S, Kanitkar M, Phadke KD, *et al*. Management of steroid sensitive nephrotic syndrome: Revised guidelines. Indian Pediatr 2008;45:203-14.
21. Indian Society of Pediatric N, Gulati A, Bagga A, Gulati S, Mehta KP, Vijayakumar M. Management of steroid resistant nephrotic syndrome. Indian Pediatr 2009;46:35-47.
22. Tkaczyk M, Czupryniak A, Nowicki M, Chwatko G, Bald E. Homocysteine and glutathione metabolism in steroid-treated relapse of idiopathic nephrotic syndrome. Pol Merkur Lekarski 2009;26:294-7
23. Podda GM, Lussana F, Moroni G, Faioni EM, Lombardi R, Fontana G, *et al*.Abnormalities of homocysteine and B vitamins in the nephrotic syndrome. Thromb Res 2007;120:647-52
24. Selhub J. Homocysteine metabolism. Annu Rev Nutr 1999;19:217-46
25. Upchurch GR, Jr., Welch GN, Fabian AJ, Freedman JE, Johnson JL, Keaney JF, Jr., *et al*. Homocystiene decreases bioavailable nitric oxide by a mechanism involving glutathione peroxidase. J Biol Chem 1997;1272:17012-7.