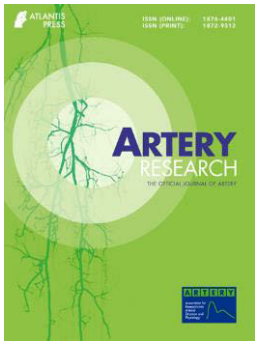

Artery Research

ISSN (Online): 1876-4401 **ISSN (Print): 1872-9312**
Journal Home Page: https://www.atlantis-press.com/journals/artres

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To cite this article: Venkatesh Arumugam, Abhijeet Saha, Manpreet Kaur, Kanika Kapoor, Nimisha Arora, Trayambak Basak, Shantanu Sengupta, Ajay Bhatt, Nitin Bharadwaj, Vineeta V. Batra, Ashish Datt Upadhyay (2017) Endothelin 1 and endothelial dysfunction in children with idiopathic nephrotic syndrome, Artery Research 18:C, 36–40, DOI: https://doi.org/10.1016/j.artres.2017.02.006

To link to this article: https://doi.org/10.1016/j.artres.2017.02.006

Published online: 3 December 2019

Artery Research (2017) 18, 36–40

Available online at www.sciencedirect.com

ScienceDirect

journal homepage: www.elsevier.com/locate/artres

Endothelin 1 and endothelial dysfunction in children with idiopathic nephrotic syndrome

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Received 11 September 2016; received in revised form 8 February 2017; accepted 21 February 2017
Available online 17 March 2017

KEYWORDS
Endothelin 1;
Nephrotic syndrome;
Endothelial dysfunction;
Children

Abstract *Background:* Endothelial dysfunction is the initial step for atherogenesis. Children with idiopathic nephrotic syndrome are at risk of endothelial dysfunction due to altered cholesterol metabolism which can lead to early atherosclerosis.
Methods: In this analytical study with longitudinal follow up 25 children with first episode of nephrotic syndrome (FENS) aged 1–16 years along with 25 age and gender matched healthy controls were enrolled. Endothelin 1 (ET 1) levels were measured by ELISA (Cloud Clone Corp. and assembled by USCN Inc, U.S.A). Other variables were measured using standard biochemical methods. Primary outcome measure was plasma ET 1 level in children with FENS and in controls. Secondary outcome measure was to observe the levels of ET 1 in children with FENS at 12 weeks in remission.
Results: The level of ET 1 was significantly higher ($p < 0.001$) in children with FENS as compared to controls. The level of ET 1 was significantly higher ($p = 0.006$) in SSNS at the onset of nephrotic syndrome and was significantly lower ($p = 0.04$) after 12 weeks of drug induced remission as compared to controls. SRNS children had higher levels of ET 1 than the steroid sensitive patients at onset but in was not statistically significant ($p = 0.062$). ET 1 showed significant positive correlation with total

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http://dx.doi.org/10.1016/j.artres.2017.02.006

cholesterol ($r = 0.462$; $p = 0.001$) and negative correlation with serum albumin ($r = -0.565$; $p = 0.001$).
Conclusion: Plasma ET 1 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 ET 1 needs to be studied.

Introduction

Endothelial dysfunction is characterised by a shift of the actions of the endothelium towards reduced vasodilation, a proinflammatory state and prothrombotic properties. In idiopathic nephrotic syndrome (INS), hyperlipidemia and raised apo-lipoproteins lead to oxidative stress in the endothelial cells.[1] Mechanisms that participate in the reduced vasodilatory responses in endothelial dysfunction include reduced nitric oxide generation and excess oxidative stress. INS is also a proinflammatory state associated with elevated levels of tumour necrosis factor α.[2] These factors may contribute to endothelial dysfunction in idiopathic nephrotic syndrome (INS) and subsequently lead to accelerated atherosclerosis.[3] There are several reports of atherosclerosis in children with idiopathic nephrotic syndrome.[4,5]

Studies in adult population suggest a stronger relationship between the elevated plasma levels of novel biochemical markers of endothelial dysfunction and atherosclerosis.[6] Many studies showed increased excretion of markers of endothelial dysfunction in patients with nephrotic syndrome. Adults who suffered from nephrotic syndrome in childhood have increased risk of atherosclerosis.[7] Long term studies in adult population indicate the association of cardiovascular diseases in nephrotic patients.[8] Studies in paediatric population have revealed that the risk factors of atherosclerosis occur in the patients having INS in various stages of the disease, which lead to the assumption that children with INS are predisposed to accelerated atherosclerosis.[7]

Endothelin 1 (ET 1) is produced by endothelial, vascular smooth muscle cells, and macrophages. It acts through G-protein-coupled ET (A) and ET receptors. There is evidence that ET 1 has an important role in the initiation and progression of cellular pathways leading to atherogenesis. In adults with proteinuria ET 1 levels are increased in urine.[9] Moreover ET 1 has been shown to promote microvascular platelet thrombus formation and therefore may contribute to acute coronary syndromes in this manner.[10] Oxidized low-density lipoprotein (LDL), one of the major participants in the atherogenic process, is a strong stimulus for ET production and secretion. The aim of this study was to evaluate the status of ET 1, in children with first episode idiopathic nephrotic syndrome (FENS).

Patients & methods

Study design and patient groups

This study was an analytical study with longitudinal follow-up conducted in a tertiary care hospital in New Delhi from October 2012 to March 2014. Study was approved by the institutional review board and written informed consent was taken from all the participants. For ET 1, considering mean of 54 pg/ml and standard deviation of 25 pg/ml in cases and mean of 29 pg/ml and standard deviation of 10 pg/ml in controls, using alpha error of 5% (two tail test) and power of 90% estimated sample of 13 cases and 13 controls were required. We decided to enrol 25 cases and equal number of age and gender matched controls attending the pediatric nephrology clinic. Controls were selected from the outpatient department coming for 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 functions, pre-existing hypertension, diabetes mellitus, recent history of blood transfusion, who refused to give an informed consent were excluded from the study. Samples were collected at the time of initial diagnosis and before starting second line drugs. The patients were enrolled at the onset of the disease and were followed up till the end of the study period of one year. Guidelines by Indian Society of Pediatric Nephrology were used for the diagnosis and treatment of FENS, steroid dependent nephrotic syndrome (SDNS) and steroid resistant nephrotic syndrome (SRNS).[11] The primary outcome was to compare the levels of ET 1 in children with FENS compared to controls. Secondary outcome measure was levels of ET 1 in children with FENS at 12 weeks in remission.

Measurement of level of ET 1

ET 1 was measured at onset of disease and at 12 weeks of drug induced remission and sampling was done longitudinally. The blood sample (3 ml) was collected by venepuncture into a vacutainer tube (EDTA vial). Plasma was obtained by centrifuging the blood at 2800×g for 10 min. Plasma was stored at −80 °C until analysed. ELISA based measurement of ET 1 was done at Institute of Genomics and Integrative Biology. ET 1 kits were designed by Cloud Clone Corp. and assembled by USCN Inc., U.S.A. The kits contained pre-coated, standard 2 standard diluent 1 × 20 mL, detection reagent A 1 × 120 μL Assay, detection reagent B 1 × 120 μL Assay, TMB substrate and wash buffer (30 × concentrate). The measurement was done on Tecan Eliza machine present at the Institute of Genomics and Integrative Biology. This assay employed the competitive inhibition enzyme immunoassay technique. The intra-assay and inter-assay coefficients of variation were <10% and <12% respectively. The protein binding of ET 1 is 98%; hence due to this high protein binding, the measured values were corrected to plasma albumin.[12]

Statistical analysis

Statistical analysis was done using SPSS version 20. Continuous variables were presented as the mean ± standard deviation (SD) or median (interquartile range) while categorical variables were presented as the frequency and percentage. Comparison between independent continuous variables was performed using Mann–Whitney U test and paired variables using Wilcoxon rank sum test. Correlations were assessed by Spearmann rank tests. Spearman's rho "r" correlation coefficient was used to measure the linear relationship between continuous variables. p value of less than 0.05 was considered statistically significant.

Results

In the present study, a total of 50 children (25 cases and 25 controls) aged between 1 and 15 years were enrolled. In the study group 17 were steroid sensitive nephrotic syndrome (SSNS) and 8 patients turned into initial SRNS. Renal biopsy was done in all patients with SRNS. The demographic profile was comparable between the two groups (Table 1). Children with INS had high serum cholesterol, serum LDL, serum VLDL, serum triglycerides and nephrotic range proteinuria as expected ($p < 0.001$). The level of endothelin 1 was significantly higher ($p < 0.001$) in children with first episode of nephrotic syndrome (FENS) as compared to controls (Table 2). The level of ET 1 was significantly higher ($p = 0.006$) (Table 2) in SSNS at the onset of nephrotic syndrome and was significantly lower ($p = 0.049$) after 12 weeks of drug induced remission as compared to controls. SRNS children had higher levels of ET 1 than the steroid sensitive patients at onset but in was not statistically significant ($p = 0.062$). ET 1 showed significant positive correlation with total cholesterol ($r = 0.462$; $p = 0.001$) and negative correlation with serum albumin ($r = -0.565$; $p = 0.001$). ET 1 did not show any significant correlation with systolic blood pressure, BMI and proteinuria.

Discussion

In our pilot study we found that the ET 1 levels were raised in plasma of children with FENS. ET 1 is highly bound to plasma proteins and plasma proteins act as a protective factor for endothelial dysfunction by preventing the binding of ET 1 to the endothelin receptors. In a study conducted by Wu-Wong JR et al., the addition of serum albumin decreased the binding of ET 1 to the receptors by 84%.[14] In children with FENS, the presence of hypoalbuminemia and associated increase in the level of free endothelin 1 may lead to increased risk of endothelial dysfunction by acting as a strong chemoattractant for circulating monocytes, and activating macrophages, constriction of blood vessels and inducing smooth vascular smooth muscle proliferation.[15] Vlachojannis JG et al. studied the renal excretion of ET 1 in patients with nephrotic syndrome and found it increased, there by suggesting a possible relationship between proteinuria and renal ET-1 production.[9] Our results are in line with that of Vlachojannis J et al.; however we selected a homogenous study group, whereas the previous one consisted with proteinuric non-homogenous population with systemic and renal inflammation like AGN, HUS and AKI.[13] It is found that increased renal production and excretion of ET 1 causes renal injury by stimulating hypertrophy, proliferation, and extracellular matrix accumulation in the kidneys, primarily by autocrine/paracrine manner via ET A receptor stimulation and blockade of endothelin receptors, and attenuates the renal injury by an anti-inflammatory mechanism.[16–18] Yang F et al., also studied plasma ET-1

Table 1 Baseline characteristics of patients with INS and controls.

Parameter	INS (mean ± SD) (n = 25)	Control (mean ± SD) (n = 25)	p value
Age (years)	4.52 ± 3.429	4.80 ± 3.674	0.782
Gender (male/female)	16/9	16/9	1.000
Height (cms)	100.40 ± 20.394	104.20 ± 23.775	0.547
Weight (kgs)	16.908 ± 7.872	17.016 ± 10.506	0.967
BMI (kg/m^2)	14.104 ± 2.104	14.332 ± 3.058	0.760
Systolic BP (mmHg)	101.60 ± 9.309	95.92 ± 11.143	0.056
Diastolic BP (mmHg)	67.28 ± 8.773	63.28 ± 6.997	0.081
Total protein (g/dl)	4.056 ± 0.588	6.820 ± 0.590	<0.001
S.Albumin (g/dl)	1.788 ± 0.292	4.188 ± 0.355	<0.001
Urine protein creatinine ratio	7.391 ± 4.844	0.212 ± 0.114	<0.001
Total Cholesterol (mg/dl)	432.84 ± 138.021	141.40 ± 28.277	<0.001
Low density lipoprotein (LDL) (mg/dl)	321.16 ± 132.568	69.88 ± 27.736	<0.001
Very low density lipoprotein (VLDL) (mg/dl)	66.80 ± 31.832	24.88 ± 10.940	<0.001
High density lipoprotein (HDL) (mg/dl)	44.88 ± 15.004	46.64 ± 8.534	0.613
Triglycerides (TGL) (mg/dl)	305.44 ± 206.763	121.80 ± 34.064	<0.001
INR	0.993 ± 0.234	1.072 ± 0.141	0.157

*Test name: unpaired T test.
#Abbreviations: INS – Idiopathic nephrotic syndrome, mg/dl – milligram per decilitre, mEq/L – milliequivalent per litre, g/dl – gram per decilitre, ml/min/1.732m^2 – millilitre per minute per 1.732 metre squared, eGFR – estimated glomerular filtration rate, Up/UC ratio – urine protein to creatinine ratio, INR – international normalised ratio, SD – standard deviation.

Table 2 Endothelin 1 levels in cases and controls.

	FENS (n = 25)	SSNS (at onset) (n = 17)	SSNS (at 12 weeks remission) (n = 17)	SRNS (at onset) (n = 8)	Controls (n = 25)
Endothelin 1 Median (IQR) pg/ml	18.72 (12.07–25.87)	17.17 (10.57–23.41)	2.28 (0.92–12.97)	25.05 (17.64–38.53)	11.16 (6.47–13.92)
p value	0.001*	0.006*	0.002**	0.062***	

¥Abbreviations: SSNS – steroid sensitive nephrotic syndrome; SRNS – steroid resistant nephrotic syndrome.
* Statistical difference as compared to controls and Wilcoxon's rank sum test applied.
** Statistical difference as compared to FENS and Wilcoxon's rank sum test applied.
*** Statistical difference as compared to SSNS at onset and Wilcoxon's sign rank test applied.

concentrations in children with INS and found significantly higher levels as compared to healthy children. The ET-1 gene SNP (rs10478694) correlated with the risk of NS and also with plasma ET-1 concentrations when comparing healthy children to children with NS. Moreover, the ET-1 gene SNPs (rs5370 and rs10478694) was related to the plasma cholesterol level in primary NS in children. Thus plasma cholesterol was found to be associated with genetic variations within the human ET-1 gene.[19]

The level of ET 1 was higher in the SRNS patients at the onset of the disease as compared to the infrequent relapsing nephrotic syndrome patients explaining the higher risk of endothelial dysfunction in these patients. This might be because of higher, persistently elevated cholesterol levels in patients with SRNS. INS is known to have a relapsing course in up to 70% of children. It is likely that repeated episodes of raised ET 1 can lead to a more pronounced endothelial damage.

We have shown risk of endothelial dysfunction due to elevated ET 1 in short term, in children with FENS. Modulation of endothelial dysfunction in children with FENS may be considered a therapeutic strategy, to decrease the risk of future adverse cardiovascular events. Long term prospective studies are needed in children with INS. 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 ET 1 levels and are at risk of endothelial dysfunction. Larger studies with long term follow up are needed in children with INS.

Ethical approval

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Funding

None.

Contributions

AS and SS conceived the idea, VA collected the data; TB, NB, AB and SP did the biochemical analysis, SS supervised the analysis, VB did the histological evaluation, AU did the statistical analysis; AS, VA, MK, KK and NA did the literature search; AS, MK, KK and NA drafted the manuscript; AS, MK and KK critically reviewed the manuscript.

Informed consent

Informed consent was obtained from all individual participants included in the study.

Conflict of interest

The authors declare that they have no conflict of interest.

Acknowledgement

This paper was presented in the 12th Asian Congress of Pediatric Nephrology, 4-6th December 2014, New Delhi, India.

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