DOI: 10.7860/JCDR/2024/70589.19617

Original Article

Biochemistry Section

Evaluating Serum Apelin and Nitric Oxide in Primary Hypertensive Patients with or without Microalbuminuria: A Cross-sectional Study

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ABSTRACT

Introduction: Hypertension (HTN) is the third leading risk factor contributing to the disease burden in Southeast Asia. Understanding its underlying mechanism, such as endothelial dysfunction, is very important. Apelin is a recently discovered endogenous peptide hormone that, along with Nitric Oxide (NO), is implicated in endothelial dysfunction and the severity of HTN. This can significantly reduce renal function and lead to microalbuminuria/proteinuria in 5-15% of patients.

Aim: To measure serum apelin and NO in patients with newly diagnosed primary HTN with or without microalbuminuria and to investigate the correlation of serum apelin with HTN in the same study population.

Materials and Methods: This cross-sectional study was conducted from February 2019 to January 2021 in the Department of Biochemistry In collaboration with the Department of Medicine at SCB Medical College and Hospital, Cuttack, Odisha, India. A total of 180 participants were included in the present study. Among them, 90 were newly diagnosed hypertensive patients, and 90 were non hypertensive. The study comprised of 90 hypertensive patients, with 46 having microalbuminuria (Group-A), 44 without microalbuminuria (Group-B) and 90 normotensive controls (Group-C). All participants underwent evaluation of their biochemical profile which included Fasting Plasma Sugar (FBS), serum urea, creatinine, Total Cholesterol (TC), Triglyceride (TG), High-density Lipoprotein cholesterol (HDL-c), Low-density Lipoprotein cholesterol (LDL-c), Very Low-Density Lipoprotein (VLDL), serum apelin and NO levels, and urine albumin. The comparison between the three groups was done by using Analysis of Variance (ANOVA) test with post-hoc analysis. The Chi-square test was used to compare categorical variables.

Results: The mean age of the participants was 50.48±9.73 years. study comprised of 90 hypertensive patients, with 46 having microalbuminuria, 44 without microalbuminuria, and 90 normotensive controls. The mean serum apelin levels were 394.45±84.2, 386.9±86.12, and 62.73±22.87 ng/L, respectively, among patients with microalbuminuria, without microalbuminuria, and normotensive patients. Similarly, the mean serum NO levels were 6.31±2.94, 8.32±2.63, and 20.15±4.37 µmol/L, respectively, among the three groups. The comparison of mean values indicated a significant ($p<0.001$) positive correlation between the level of serum apelin and HTN and its complications, such as microalbuminuria.

Conclusion: The positive correlation between serum apelin levels and blood pressure underscores the potential role of apelin in hypertensive pathophysiology.

Keywords: Endothelial dysfunction, Non hypertensive, Normotensive, Peptide hormone

INTRODUCTION

Hypertension (HTN) is defined when an individual has a Systolic Blood Pressure (SBP) >140 mmHg and/or Diastolic Blood Pressure (DBP) >90 mmHg [1]. The majority, approximately 82%, out of more than 1.3 billion people with HTN worldwide belong to low and middle economic status countries. India alone is home to an average of 220 million adults with HTN [2].

Apelin is a recently discovered endogenous peptide hormone. It was discovered in 1998 from bovine stomach extract by Tatemoto. The human gene of apelin produces a 77-amino acid prepropeptide, which is cleaved into several active forms: 36, 17, 13, and 12 amino acids in length. Of these, the 36-amino acid isoform is the most widely expressed in different organs, including adipocytes and the endothelia of small arteries [3]. An in-vitro study has shown that apelin can antagonise the angiotensin II signalling cascade by negatively regulating the angiotensin II type 1 receptor [4]. Moreover, apelin acts as a second catalytic substrate for angiotensin-converting enzyme 2, a negative regulator of the Renin-angiotensin-aldosterone System (RAAS) [5]. It is widely recognised that activation of the renin-angiotensin-aldosterone cascade is an etiological hallmark in the development of HTN [6].

Despite the abundance of data indicating a link between endothelial dysfunction and HTN, the exact nature of their relationship remains intricate and unclear. It has been stated that NO can reduce RAAS activation and, because of its vasodilation activity, can buffer angiotensin-II-induced peripheral vasoconstriction [7]. It has further been shown that reduced NO bioavailability can exacerbate the prohypertensive effects of the Sympathetic Nervous System (SNS), augment sympathetic outflow, and increase oxidative stress [7].

Essential HTN can significantly reduce renal function and produce microalbuminuria/proteinuria in 5-15% of patients [8]. Microalbuminuria is diagnosed when albumin excretion is between 30-300 mg/24 hours in urine [8]. It has been suggested that microalbuminuria is an independent predictor of cardiovascular morbidity and mortality in patients with essential HTN [8]. The risk of developing renal failure, ischaemic and haemorrhagic stroke, and peripheral arterial disease is doubled in the presence of microalbuminuria. This may be due to increased renal endothelial permeability and diffuse endothelial dysfunction [8].

The NO and apelin are suggested to have a role in endothelial dysfunction and the severity of HTN [7,9]. A study conducted by Wang T et al., revealed that there was no association between

apelin polymorphism and the prevalence of HTN [10]. In the study conducted by Hemmati M et al., it was found that apelin has a definite protective effect in preventing HTN [11]. According to WHO report 2002, it has been suggested that microalbuminuria is an independent predictor of cardiovascular morbidity and mortality in patients with essential HTN [12]. Hence, considering the controversial role of apelin in HTN and the severity of HTN, the present study aimed to evaluate the serum apelin and NO in hypertensive patients with or without microalbuminuria, which was not studied in the previous studies, and also to determine the correlation of serum apelin with HTN in the same study group.

MATERIALS AND METHODS

It was a cross-sectional study conducted at the Department of Biochemistry, in collaboration with the Department of Medicine, SCB Medical College and Hospital, Cuttack Odisha, India, for a period of two years, from February 2019 to January 2021. The study was approved by the Institutional Ethical Committee of SCB Medical College, Cuttack (IEC/IRB No. 800/15.01.19). Informed consent was taken from each participant, and only those who gave consent for the study were included.

Inclusion criteria: The selection of hypertensive patients involved clinically newly diagnosed cases of primary HTN according to World Health Organisation (WHO) and Joint National Committee (JNC)- VII [13]. Patients aged 25 to 60 years from the Department of Medicine were included in the study. For each case selected, an age and sex-matched control who was normotensive was also included.

Exclusion criteria: Patients with HTN and other complications like Cardiovascular Diseases (CVD), peripheral vascular disorders, HTN due to renal causes, any congenital anomaly of the cardiovascular system, a history of diabetes mellitus or any other endocrine disorders, and a history of alcohol intake and smoking were excluded from the study. The same exclusion criteria were applied for the selection of normotensive patients to ensure compatibility.

Sample size calculation: The sample size was determined using Open Epi web-based software [14], considering the mean difference in serum apelin levels between hypertensive and normotensive patients as previously reported. With a confidence interval of 95% and a study power of 95%, and considering the mean±SD of serum apelin as 0.21±0.11 and 0.27±0.1 ng/L among hypertensive and normotensive patients, respectively [15], the minimum required sample size was calculated to be 160 participants. Consequently, a total of 180 participants were included in the final study. Out of these, 44 patients with microalbuminuria were grouped as hypertensive with microalbuminuria (Group-A), while the remaining 46 patients were grouped as HTN without microalbuminuria (Group-B). The remaining 90 normotensive patients were grouped as the control group (Group-C).

A consecutive sampling method was used for the selection of the study participants for the present study. The participants were selected in a sequential order as they became available. Those who met the inclusion and exclusion criteria were included in the study. Once a hypertensive patient was included, the consecutive non hypertensive patients were selected by matching their age, sex, and locality with the previously selected hypertensive patient.

Study Procedure

All study participants underwent physical and biochemical investigations. Blood pressure was measured as per the recommended guidelines for each participant [16]. The biochemical profile included Fasting Plasma Glucose (FBG), serum urea, serum creatinine, TC, TG, HDL-C, LDL-C, and VLDL, which were estimated using standard commercial kits adapted to an auto-analyser TBA 120 FR. Serum levels of apelin and NO were measured using the Enzyme-linked Immunosorbent Assay (ELISA) technique at the Postgraduate Laboratory of the Department of Biochemistry. The reference range for the parameters was taken from [Table/Fig-1] [17-26].

Parameters	Method of estimation	Normal reference range
Fasting Plasma Glucose (FPG) [17]	GOD-PAP	70-110 mg/dL
Serum urea [18]	Urease method	15-40 mg/dL
Serum creatinine [19]	Enzymatic method	0.6-1.2 mg/dL in men; 0.5-1.1 mg/dL in women
Serum Total Cholesterol (TC) [20]	CHOD-PAP	<200 mg/dL
Serum Triglyceride (TG) [21]	GPO-TOPS	<150 mg/dL
Serum HDL [22]	Selective inhibition method	35-80 mg/dL
Serum LDL [23]	Calculated by Friedwald's formula	<100 mg/dL
Serum level of apelin [24]	ELISA	37-121 ng/L
Serum nitric oxide [25]	ELISA	10.3-66.8 µmol/L
Urine albumin [26]	Pyrogallol red method	<30 mg/d

[Table/Fig-1]: Normal range of different parameters [17-26].
GOD-PAP: Glucose oxidase-4-Amino-antipyrine, CHOD-PAP: Cholesterol oxidase-Peroxidase, GPO-TOPS: glycerol phosphate oxidase (GPO)-Topological descriptions of protein structures

Estimation of serum apelin [27]: Serum apelin was done by enzyme immunoassay, which uses two highly specific monoclonal antibodies in a "sandwich ELISA" format-a biotinylated human Apelin (APLN)/AP antibody and an antibody-human apelin-HRP conjugated antibody.

Estimation of Serum Nitric Oxide (Sr. NO) [28]: Serum NO was estimated using a "sandwich ELISA" method where a sandwich of NO antibody-human and NO-HRP conjugated antibody was formed. During the estimation of serum apelin and serum NO by ELISA, a standard curve was generated by plotting the absorbance versus the respective human apelin and NO concentrations of each standard on a point-to-point curve. The concentrations of human apelin and NO in the patient samples and controls were determined directly from the standard curve generated.

Measurement of urine albumin [29]: Albumin excreted in urine was quantitatively measured by the Pyrogallol Red method in a spectrophotometer.

STATISTICAL ANALYSIS

The collected information was tabulated in Microsoft Excel (Version 16) and analysed in R-statistics (Version 3.4). All quantitative variables were expressed as mean and standard deviation, while categorical variables were presented as percentages and proportions. The comparison among the three groups was done by using the ANOVA test with post-hoc analysis. The Chi-square test was employed for comparing categorical variables. A p-value less than 0.05, with a 95% confidence interval, was considered statistically significant.

RESULTS

The study included 90 hypertensive patients, with 46 in Group-A (HTN with microalbuminuria) and 44 in Group-B (HTN without microalbuminuria). Group-C consisted of 90 age and sex-matched normotensive participants. The mean age of the participants was 50.48±9.73 years, with no significant difference across the groups. Each group exhibited nearly equal proportions of males and females. Regarding the biochemical profile, both Group-A and Group-B showed significantly higher levels of Fasting Blood Sugar (FBS) (p=0.002 and 0.022), serum TC (p<0.001 and 0.044), and LDL levels (p<0.001 and 0.023) compared to Group-C. However, no significant difference was observed in urea, creatinine, TG, and VLDL levels across the three groups [Table/Fig-2].

In the present study, both SBP and DBP showed a substantial elevation in Group-A and B compared to Group-C (p<0.001).

Characteristics	Hypertension with microalbuminuria (Group-A) n=46	Hypertension without microalbuminuria (Group-B) n=44	Normotensive control group (Group-C) n=90	*p-value between A and C	*p-value between B and C	p-value between A a nd B
Socio-demographic profile						
Age (Y) Mean±SD	49.89±9.2	51.56±10.4	50±9.6	0.56	0.58	0.42
Sex						
Male	24 (52.17%	22 (50%)	48 (53.33%)	0.89**	0.13**	0.83**
Female	22 (47.83%)	22 (50%)	42 (46.67%)			
Total	46 (100%)	44 (100%)	90 (100%)			
Biochemical profile						
FBS (mg/dL)	100.26±6.48	99.36±6.79	96.53±6.28	0.002	0.022	0.521
Urea (mg/dL)	28.06±6.12	28.54±9.12	28.61±6.38	0.628	0.965	0.769
Creatinine (mg/dL)	0.98±0.19	0.93±0.2	0.96±0.17	0.531	0.495	0.227
Total Cholesterol (TC) (mg/dL)	214.78±40.87	199.15±43.95	182.23±47.23	<0.001	0.044	0.083
TG (mg/dL)	159.88±73.7	146.97±38.13	141.01±37.24	0.115	0.386	0.302
HDL (mg/dL)	44.68±8.73	46.67±9.1	47.96±8.33	0.0410	0.442	0.292
LDL (mg/dL)	136.58±35.07	122.97±34.55	106.30±47.88	<0.001	0.023	0.067
VLDL (mg/dL)	30.09±10.48	28.97±7.89	27.95±7.41	0.23	0.467	0.569

[Table/Fig-2]: Comparison of baseline profile between three groups.
*p-value was calculated by using ANOVA with post-hoc test for mean±SD; **p-value was calculated by using Chi-square test

Regarding the specific biochemical profile, serum apelin levels and urine albumin levels were markedly higher in Group-A and Group-B compared to Group-C (p<0.001). Similarly, Sr. NO levels were significantly higher in Group-C compared to both Group-A and B (p<0.001) [Table/Fig-3].

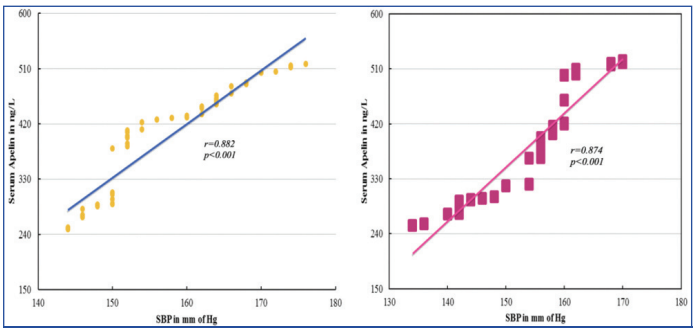
A significant positive correlation (p<0.001) was observed between serum apelin with SBP and DBP among the hypertensive patients with or without microalbuminuria (Group-A and B). The correlation coefficient between serum apelin and SBP and DBP in Group-A were (r=0.88, p<0.001) and (r=0.87, p<0.001), respectively, indicating

Characteristics	Hypertension with microalbuminuria (Group-A) n=46	Hypertension without microalbuminuria (Group-B) n=44	Normotensive control group (Group-C) n=90	p-value between A and C	p-value between B and C	p-value between A and B
Clinical assessment						
SBP (mmHg)	157.13±8.98	154.22±9.1	122.55±8.5	<0.001	<0.001	0.81
DBP (mmHg)	103.04±5.29	101.36±5.02	81.71±4.1	<0.001	<0.001	0.78
Specific biochemical profile						
Sr. apelin (ng/L)	394.45±84.2	386.9±86.12	62.73±22.87	<0.001	<0.001	0.95
Sr. NO (µmol/L)	6.31±2.94	8.32±2.63	20.15±4.37	<0.001	<0.001	0.61
Urine Albumin (mg/dL)	86.17±51.83	24.27±3.23	18.52±6.23	<0.001	<0.001	0.24

[Table/Fig-3]: Comparison of hypertensive profile and specific biochemical profile between three groups.
p-value was calculated by using ANOVA with post-hoc test for mean±SD

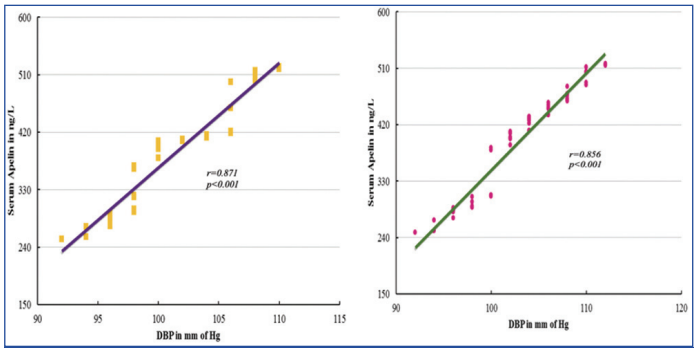
Characteristics	Correlation coefficient*	p-value
Group-A (Hypertension with Microalbuminuria)		
Sr. apelin Vs SBP	0.882	<0.001
Sr. apelin Vs DBP	0.871	<0.001
Group-B (Hypertension without Microalbuminuria)		
Sr. apelin Vs SBP	0.874	<0.001
Sr. apelin Vs DBP	0.856	<0.001

[Table/Fig-4]: Showing the correlation between apelin and blood pressure.
*Pearson correlation coefficient



[Table/Fig-5]: Correlation of serum apelin (in ng/L) with serum SBP (mmHg) in hypertensive patients with microalbuminuria (a) and without microalbuminuria (b).

a highly significant positive correlation. Similarly, the correlation coefficient between apelin with SBP and DBP in Group-B were (r=0.87, p<0.001) and (r=0.85, p<0.001), also indicating a highly significant positive correlation [Table/Fig-4-6].



[Table/Fig-6]: Correlation of serum apelin (in ng/L) with serum DBP (mmHg) in hypertensive patients with microalbuminuria (a) and without microalbuminuria (b).

DISCUSSION

In the present study, a total of 180 subjects were selected, out of which 90 were newly diagnosed primary hypertensive cases, and 90 were age and sex-matched healthy volunteers. The study aimed to evaluate the levels of serum apelin and Sr. NO in hypertensive

individuals, with or without microalbuminuria. During the study, it was found that both SBP and DBP exhibited substantial elevation in Group-A and B compared to Group-C ($p<0.001$ for all comparisons). Additionally, the level of serum apelin was significantly higher in the hypertensive group with microalbuminuria than in the hypertensive group without microalbuminuria and the control group, showing a significant positive correlation of serum apelin with HTN with or without microalbuminuria.

A study conducted by Zhu P et al., found a negative correlation between plasma apelin levels and BP in a study comprising 1,031 subjects in the coastal areas of China [30]. However, the present study's finding of high serum apelin levels in HTN compared to normotensive controls is similar to the study conducted by Kanmuru BM et al., who suggested a significant increase in the level of apelin in hypertensive patients compared to normotensive and prehypertensive individuals [31].

El Wakeel MA et al., suggested that the level of apelin was significantly higher in obese children versus controls, and the research supports the hypothesis that apelin may play a significant role in the development of obesity-related health issues in children, such as insulin resistance, HTN, and an increased risk of metabolic syndrome [32]. Apelin is a recent marker of endothelial dysfunction. It contributes to the pathophysiology of HTN by increasing the inotropic activity of cardiac muscle and significantly increasing shortening in cardiac myocytes. This effect is caused by enhancing the Na^+/H^+ exchanger activity of cardiac muscles, making the intracellular environment alkaline, which renders microfilaments more sensitive to Ca^{2+} [33]. Increased Na^+/K^+ exchanger activity increases intracellular Ca^{2+}, which, in turn, increases the contractility of cardiac muscle fibers. HTN is highly related to endothelial dysfunction and arterial stiffness.

Davern PJ et al., observed that sympathetic overactivation could contribute to HTN. Activation of the renal sympathetic nerve also exerts an effect on the development of HTN by stimulating renal renin release to increase angiotensin-II activity, decrease renal blood flow, and enhance tubular sodium retention [34]. The central apelin involved in the regulation of HTN is by over-activating the SNS. Under pathological conditions, a high concentration of central apelin could further increase BP.

The level of NO in the present study is lower in the hypertensive group compared to the control group. The finding of hypercholesterolemia in the present study is associated with endothelial dysfunction and HTN, which aligns with the study conducted by Daugherty A et al., who demonstrated that hypercholesterolemia exerts stimulatory effects on angiotensinogen, contributing to a robust activation of the RAAS that leads to an increase in vascular tone [35]. Another mechanism has been proposed by Ferons O et al., which explains the inhibitory effects of hypercholesterolemia on the synthesis of NO in vascular endothelial cells [36]. They described that elevated levels of LDL cholesterol stimulate the production of the structural protein, caveolin, and promote its inhibitory interaction with endothelial Nitric Oxide Synthase (eNOS), resulting in a reduction in the generation of NO, which is the endothelial-derived relaxing factor. Hence, there is an attenuation of the vasodilatory effects of NO in hypertensive cases.

In the present study, microalbuminuria was associated with higher blood pressure in Group-A compared to less elevated blood pressure in Group-B and the control group (Group-C). Hence, the increase in the severity of HTN leads to increased excretion of albumin in urine, consistent with the findings of Kumar AH et al., who found that the percentage of patients with microalbuminuria is more in patients with essential HTN with a longer duration of HTN and an unfavourable lipid profile [8]. Patel S and Savlani P found that, compared to normoalbuminuric patients, microalbuminuric patients had significantly higher blood pressure values ($p<0.05$) and renal vascular resistance ($p<0.05$) [37]. They proposed that renal haemodynamic changes result from the direct transmission of increased systematic pressure to the glomeruli, leading to selective permeability changes of the glomerular basement membrane and insufficient tubular reabsorption of albumin.

Limitation(s)

Cases of secondary HTN were not included in the present study.

CONCLUSION(S)

In the present study, serum apelin levels were higher in hypertensive patients with or without microalbuminuria compared to controls, showing a positive correlation with SBP and DBP. Sr. NO levels were significantly lower in hypertensive patients compared to controls. Based on the findings, it can be concluded that serum apelin has a direct relationship with the pathogenesis of HTN, both with and without microalbuminuria. However, more multicentric research may elucidate the diagnostic role of serum apelin in HTN. Hence, routine follow-up with serum apelin can be advised for the early detection of microalbuminuria among newly diagnosed hypertensive patients.

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PLAGIARISM CHECKING METHODS: [Jain H et al.]
- Plagiarism X-checker: Mar 09, 2024
- Manual Googling: Apr 16, 2024
- iThenticate Software: May 22, 2024 (17%)

ETYMOLOGY: Author Origin

EMENDATIONS: 8

AUTHOR DECLARATION:
- Financial or Other Competing Interests: None
- Was Ethics Committee Approval obtained for this study? Yes
- Was informed consent obtained from the subjects involved in the study? Yes
- For any images presented appropriate consent has been obtained from the subjects. NA

Date of Submission: **Mar 08, 2024**
Date of Peer Review: **Apr 13, 2024**
Date of Acceptance: **May 23, 2024**
Date of Publishing: **Jul 01, 2024**