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**Original Research Article**

# Association of Soluble α-Klotho and Klotho Polymorphisms (KL-VS and G-395A) with Essential Hypertension in Indian Population

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## Abstract

Klotho, a novel anti-aging gene, is known to ameliorate aging phenotypes and extend lifespan. Recent studies have highlighted arterial aging as a risk factor for hypertension. Thus, Klotho appears to be a link between aging and cardiovascular diseases (CVD) including hypertension. KL-VS and G-395A polymorphisms of Klotho and its soluble levels have been associated with CVD incidence. We analyzed the prevalence of α-Klotho polymorphisms – KL-VS and G-395A and their influence on soluble α-Klotho levels in essential hypertension (EH) patients in Indian population. One-hundred-twelve EH patients and age, BMI-matched 103 normotensive controls were recruited. Soluble α-Klotho levels were measured by Enzyme-Linked-Immunosorbent-Assay. Genotyping of single nucleotide polymorphisms (SNP) was performed using TaqMan assays. Soluble α-Klotho levels in patients were significantly lower (26.9%,p=0.001) than controls. ROC curve analysis for soluble α-Klotho showed a cut-off of 580pg/ml with AUC 0.798(SE=0.031, 95%CI=0.737-0.859). Genotype distributions for both polymorphisms did not differ between the groups. Distribution of soluble α-Klotho levels in both groups did not vary across KL-VS genotypes. In patient group, GA and AA genotypes of G-395A polymorphism were associated with significantly lower (15.4%,p=0.014) soluble α-Klotho levels compared to GG genotype. Soluble α-Klotho levels were significantly lowers in EH patients as compared to healthy normotensive individuals.

**Keywords:** Gene polymorphism; α-Klotho; anti-aging protein; cardiovascular disease.

## 1. Introduction

Essential hypertension is probably the most studied but least understood of the complex human disorders [1]. It is currently recognized as a multifactorial disease arising from the combined action of several genetic and environmental factors. Numerous physiological mechanisms are involved in the maintenance of normal blood pressure (BP), and an aberration in any one of these may play a part in the development of essential hypertension [2]. It is a major risk factor for coronary heart disease, ischemic as well as hemorrhagic stroke and contributes to ~13% of global deaths [3]. The high and increasing worldwide burden of hypertension is a major global health challenge because it increases morbidity and mortality from cardiovascular and kidney diseases as well as financial costs to society [4].

Recent studies have highlighted arterial aging as a risk factor for the development of hypertension [5,6]. Klotho, an anti-aging gene identified by Kuro-o *et al*. [7], was found to ameliorate aging phenotypes and extend lifespan. They developed a transgenic mouse model with several age related disorders caused by insertional mutation in the Klotho gene. The homozygous transgenic mice exhibited phenotypes resembling human aging including atherosclerosis, kyphosis, ectopic calcification in arterial walls and various soft tissues, pulmonary emphysema,

osteoporosis, skin, thymus and genital atrophy, cardiovascular diseases (CVD) and accelerated death [7]. Subsequent studies in animal models showed that Klotho deficiency resulted in salt-sensitive hypertension [8]. Thus, Klotho appears to be a link between aging and CVD including hypertension.

The human Klotho gene (KL), located on chromosome 13q12, spans over 50kb in length and comprises of five exons and four introns [9]. More than 10 mutations or single nucleotide polymorphisms (SNPs) in the human Klotho gene have been distinguished [10]. Several studies have reported a genetic contribution of KL-VS (rs9536314) and G-395A (rs1207568) variants to the incidence of CVD [11]. The KL-VS haplotype is composed of six SNPs that occur in an 800bp region spanning exon 2 and flanking sequence [12]. Of the three SNPs that lie in the exon region, one is silent while two code for amino acid changes. The SNP variations at (T1054G; TTT⇒GTT) and (G1117C; TGC ⇒ TCC) result in amino acid substitutions F352V and C370S respectively. The KL-VS refers to the V and S alleles of these SNPs. Since all six SNPs occur in perfect linkage disequilibrium, a single variant, F352V, can be used to tag the haplotype and generally defines the KL-VS variant [12,13]. The G-395A polymorphism lies in the promoter region of the gene [10].

The Klotho gene encodes a single pass transmembrane protein, Klotho, or more precisely α-Klotho with an extracellular amino-terminal domain and a small intracellular carboxy-terminal domain [7]. It is expressed primarily in the kidney [14], the parathyroid gland [15], and the choroid plexus in brain [16]. The extracellular domain of Klotho has KL1 and KL2 internal repeats separated by a proteolytic cleavage site [9]. The membrane form acts as co-receptor for bone-derived fibroblast growth factor-23 (FGF-23), thereby regulating phosphate and vitamin D metabolism [17]. Apart from the membrane form, a secreted form of α-Klotho, attributed with humoral activity, is found in circulation. This soluble form is produced by alternative mRNA splicing of the Klotho gene as well as cleavage of extracellular domain of the transmembrane protein by proteases [7,9]. Soluble α-Klotho is present in blood, urine and cerebrospinal fluid [18] and works independently of FGF-23 signaling as a humoral factor exerting diverse biological effects [19]. Circulating α-Klotho plays an important role in endothelial maintenance via regulation of nitric oxide availability [20,21], calcium and phosphorous homeostasis in the kidney [22,23], and inhibition of intracellular insulin and insulin-like growth factor-1 signaling [24].

The importance of Klotho in hypertension has been demonstrated in several animal models. Zhou *et al*[8] showed that, in mice, Klotho is essential for the maintenance of normal BP, and its deficiency increased salt sensitivity and elicited salt-sensitive hypertension resulting in significant and persistent elevation of systolic blood pressure. Saito *et al*[20]observed that nitric oxide synthesis and vasodilation are impaired in Klotho deficient Otsuka Long-Evans Tokushima Fatty (OLETF) rat, which can be rectified by parabiosis with wild-type mice. Further, adenovirus-mediated Klotho gene delivery to these rats ameliorated vascular endothelial dysfunction, increased nitric oxide production, reduced elevated BP, and prevented medial hypertrophy and perivascular fibrosis [20]. In a similar study by Wang & Sun [25], Klotho gene delivery via an adeno-associated virus in spontaneous hypertensive rats stopped further increase in BP, though it did not decrease the BP levels to that of normotensive controls. Given the implications of these discoveries, there is increasing interest in α-Klotho as a potential longevity-modulating therapeutic agent for human health. The development of a sensitive and specific assay for the measurement of soluble α-Klotho in humans has facilitated investigations in the potential utility of measuring soluble α-Klotho in clinical disorders [14].

The present study aimed at analyzing the prevalence of KL-VS and G-395A polymorphisms of α-Klotho gene and their influence on soluble α-Klotho levels in essential hypertension patients in Indian population.

## 2. Material and Methods

### 2.1 Study subjects

One hundred and twelve patients diagnosed with essential hypertension [systolic blood pressure (SBP)/diastolic blood pressure (DBP) ≥140/90 mmHg] and 103 age and body mass index (BMI) matched healthy normotensive (SBP/DBP≤120/80mmHg) controls were recruited for the study. Recruitment of hypertensive individuals was in accordance with the Seventh Joint National Committee (JNC7) [26] report guidelines. Only individuals with persistent high BP recommended by the consultants as essential hypertensive were recruited. Detailed information regarding demographic status, clinical history, family history and medication was obtained from all the subjects recruited in the study. All study subjects were not on any medication and were included in the study only after obtaining written consent. The study protocol was approved by the Institutional Ethics Committee, which follows the ethical standards laid down by the Indian Council of Medical Research's (ICMR) Ethical Guidelines for Biomedical Research on Human Participants.

### 2.2 Biochemical and Experimental Analysis

Fasting venous blood samples were collected in plain and $K_2$-EDTA vacutainers (at the first encounter, prior to starting hypertensive medication). Serum was used to perform biochemical analyses and detect soluble α-Klotho levels. Serum aliquots were stored at -80˚C until further use. Routine biochemical investigations (lipid profile, renal profile and liver profile tests) were performed for all study

subjects wherein those healthy individuals with levels beyond normal range were excluded from the control group. Soluble α-Klotho levels in serum samples were detected using Enzyme-Linked Immunosorbent Assay (ELISA) method (Immuno-Biological Laboratories Co., Ltd., Hamburg, Germany).

### 2.3 DNA Extraction and Genotyping

Genomic DNA was extracted from $K_2$-EDTA blood using Purelink Genomic DNA extraction kit (Invitrogen). SNP genotyping was performed using Real-Time Polymerase Chain Reaction (RT-PCR; Step One Plus, Applied Biosystems) with TaqMan genotyping assays. TaqMan assaysC___2983037_20 for KL-VS (rs9536314) and C___7604792_10 for G-395A (rs1207568) were employed.

### 2.4 Statistical Analysis

Results are expressed as frequency and percentage and mean ± standard deviation (SD) for parametric variables; and median with inter quartile ($25^{th}/75^{th}$) ranges for non-parametric variables. Student's unpaired t test and Mann–Whitney U test were used to determine the significance of differences between the two study groups for parametric and non-parametric variables respectively. Correlations were evaluated by Spearman's rank correlation test. Genotype frequencies were estimated by gene-counting method. Genotypes were tested for deviations from Hardy-Weinberg equilibrium. Chi-square ($\chi^2$) statistics with Yates correction was used to determine whether allele or genotype frequencies were significantly different between hypertension and control groups. Receiver-operating characteristic (ROC) curve analysis was performed to identify soluble α-Klotho cut-offs for the study population. For all tests, p value <0.05 was considered statistically significant. Analyses were performed using statistical software SPSS (version 21.0, Chicago, IL).

## 3. Results

The baseline demographic characteristics of patients and controls are depicted in Table 1. The comparison of lipid profile of patients and controls is given in Table 2. There was significant difference in the total cholesterol (p=0.004), triglycerides (p=0.001), VLDL cholesterol (p=0.001) and LDL cholesterol (p=0.035) levels between patient and control groups.

**Table 1: Comparison of demographic parameters between patient and control groups**

| Parameters | Controls (N=103) | Patients (N=112) | Increase (%) |
|---|---|---|---|
| Age (years) | 47.0 ± 7.3 | 47.8 ± 6.8 $^{NS}$ | 1.7 |
| Male/Female | 79/24 | 101/11 | - |
| BMI (kg/m$^2$) | 26.1 ± 3.8 | 26.5 ± 3.3 $^{NS}$ | 1.5 |
| SBP (mmHg) | 124.5 ± 11.6 | 165.6 ± 16.5$^{***}$ | 33.0 |
| DBP (mmHg) | 78.8 ± 7.5 | 102.3 ± 8.6$^{***}$ | 29.8 |

NS: Non-Significant, $^{***}$p<0.001

Values are expressed as mean ± SD. BMI, Body Mass Index; SBP, Systolic Blood Pressure, DBP, Diastolic Blood Pressure

**Table 2: Comparison of lipid profile between patient and control groups**

| Parameters | Controls (N=103) | Patients (N=112) | Increase (%) |
|---|---|---|---|
| TC (mmol/L) | 4.44 ± 0.79 | 4.85 ± 0.99$^{**}$ | 9.2 |
| HDL-C (mmol/L) | 1.16 ± 0.36 | 1.23 ± 0.48 $^{NS}$ | 6.0 |
| TC/HDL-C | 4.1 ± 1.3 | 4.3 ± 1.3 $^{NS}$ | 4.9 |
| Triglycerides (mmol/L) | 1.19 ± 0.47 | 1.58 ± 0.88$^{***}$ | 32.9 |
| VLDL-C (mmol/L) | 0.24 ± 0.09 | 0.32 ± 0.18$^{***}$ | 33.3 |
| LDL-C (mmol/L) | 3.04 ± 0.77 | 3.30 ± 0.92$^{*}$ | 8.6 |
| LDL-C/ HDL-C | 2.89 ± 1.19 | 3.01 ± 1.19 $^{NS}$ | 4.2 |

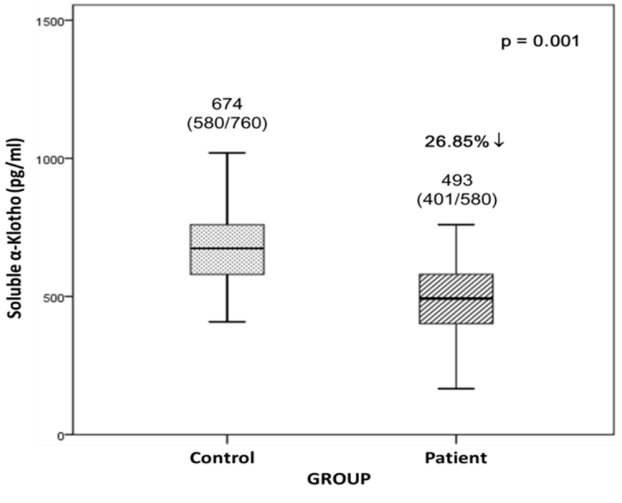
NS: Non-Significant,*p<0.05, **p<0.01, ***p<0.001

Values are expressed as mean ± SD. TC, Total cholesterol; HDL-C, High Density Lipoprotein Cholesterol; VLDL-C, Very Low Density Lipoprotein Cholesterol; LDL-C, Low Density Lipoprotein Cholesterol

### 3.1 Soluble α-Klotho levels

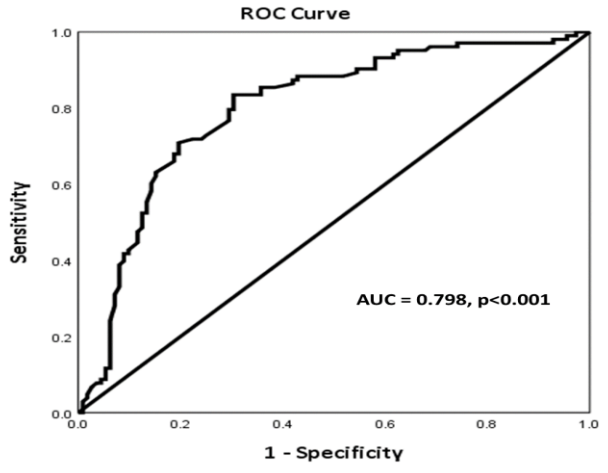
The levels of soluble α-Klotho in serum of hypertensive patients was significantly lower (26.9%, p=0.001) as compared to controls (Figure 1). Soluble α-Klotho levels showed positive correlation with HDL cholesterol (rs = 0.285, p=0.004) and inverse correlation with serum triglycerides (rs = -0.269, p=0.006) in control group. In the patient group, soluble α-Klotho levels were negatively correlated with BMI (rs = -0.318, p=0.001). ROC curves are used to show the optimal balance between clinical sensitivity and specificity for every possible cut-off for a test. The area under the ROC curve (AUC) represents the probability that a randomly selected patient will have a lower or higher test result than a randomly selected control. ROC curve analysis for soluble α-Klotho showed a cut-off of 580 pg/ml with AUC of 0.798 (SE=0.031, 95% CI= 0.737-0.859) (Figure 2). This suggested that on an average an essential hypertension patient would have lower soluble α-Klotho levels than approximately80% of healthy individuals.

**Figure 1: Soluble α-Klotho levels in patient and control groups**




Values are expressed as median with inter quartile ($25^{th}/75^{th}$) ranges

**Figure 2: ROC curve for soluble α-Klotho in the study population**




### 3.2 Klotho polymorphisms

The genotype and allele frequencies of KL-VS and G-395A polymorphisms for patients and controls in this study are displayed in Tables 3 and 4 respectively. The genotype distributions for both polymorphisms satisfied the Hardy-Weinberg equilibrium ($\chi 2$ test, $p>0.05$), and did not differ between the patient and control groups. However, in our study population, there were four hypertensive patients with homozygous variant VV genotype against one in control group for KL-VS polymorphism and six hypertensive patients with homozygous variant AA genotype as compared to none in the control group for G-395A polymorphism.

**Table 3: Genotype and allele frequencies of KL-VS polymorphism of Klotho gene**

| KL-VS | Genotype frequencies, N (%) | | | Allele frequencies | |
|---|---|---|---|---|---|
| | FF | FV | VV | F | V |
| Controls (N=103) | 82 (79.6) | 20 (19.4) | 1 (1.0) | 0.89 (N=184) | 0.11 (N=22) |
| Patients (N=112) | 82 (73.2) | 26 (23.2) | 4 (3.6) | 0.85 (N=190) | 0.15 (N=34) |
| | $\chi^2 = 0.66$, p=0.42 | | | $\chi^2 = 1.92$, p=0.17 | |

**Table 4: Genotype and allele frequencies of G-395A polymorphism of Klotho gene**

| G-395A | Genotype frequencies, N (%) | | | Allele frequencies | |
|---|---|---|---|---|---|
| | GG | GA | AA | G | A |
| Control (N=103) | 71 (68.9) | 32 (31.1) | 0 | 0.84 (N=174) | 0.16 (N=32) |
| Patient (N=112) | 71 (63.4) | 35 (31.3) | 6 (5.3) | 0.79 (N=177) | 0.21 (N=47) |
| | $\chi^2 = 0.33$, p=0.57 | | | $\chi^2 = 2.12$, p=0.15 | |

### 3.3 Influence of Klotho polymorphisms on soluble α-Klotho levels

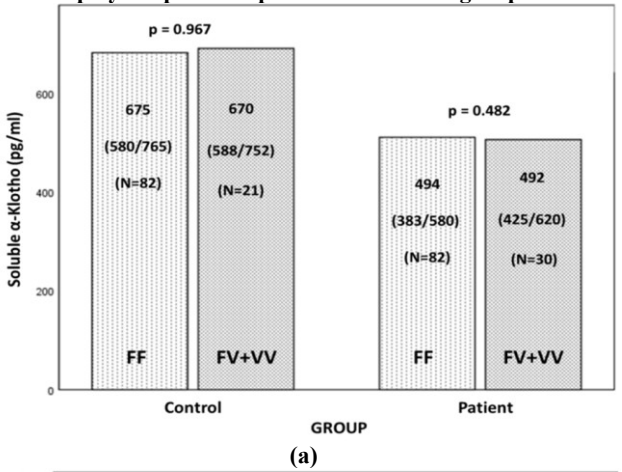
The distribution of soluble α-Klotho levels according to genotypes in KL-VS and G-395A polymorphisms in patient and control groups is shown in Table 5. Distribution of soluble α-Klotho levels in the two study groups did not vary across KL-VS genotype when compared between wild-type genotype against combination of heterozygous and variant genotypes (Figure 3a) or between heterozygous genotype against wild-type and variant genotypes combined (Figure 3b). Since the variant genotypes were found in few cases, analysis of soluble α-Klotho levels according to genotypes was done by combining heterozygous and variant genotypes, which were then compared against wild-type homozygous genotype for G-395A polymorphism. In the patient group, the presence of GA and AA genotypes was associated with significantly lower (15.4%, p=0.014) soluble α-Klotho levels as compared to wild-type GG genotype (Figure 4).

**Table 5: Distribution of soluble α-Klotho levels according genotypes for KL-VS and G-394A polymorphisms in patient and control groups**

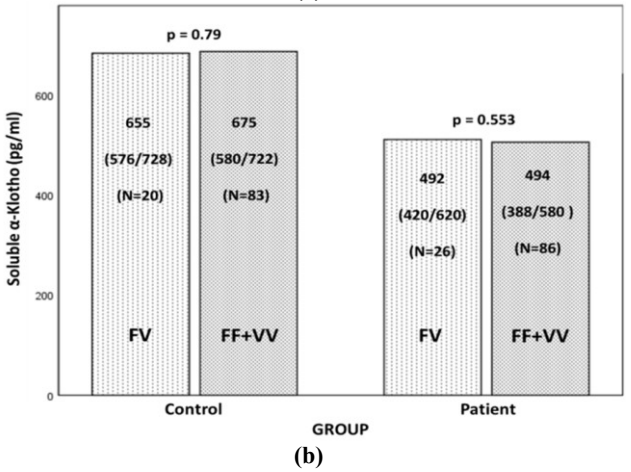
| Soluble α-Klotho Levels (pg/ml) | | | | | |
|---|---|---|---|---|---|
| **KL-VS** | **Controls** | **Patients** | **G-395A** | **Controls** | **Patients** |
| FF | 675 (580/765) (N=82) | 494 (383/580) (N=82) | **GG** | 674 (580/760) (N=71) | 520 (428/600) (N=71) |
| FV | 655 (576/728) (N=20) | 492 (420/620) (N=26) | **GA** | 675 (566/800) (N=32) | 440 (343/520) (N=35) |
| VV | 772 (N=1) | 505 (462/565) (N=4) | **AA** | - | 477 (369/516) (N=6) |

Values are expressed as median with inter quartile ($25^{th}/75^{th}$) ranges

**Figure 3: Distribution of soluble Klotho levels according to (a) FF and FV+VV, (b) FF+VV and FV genotypes of KL-VS polymorphism in patient and control groups**



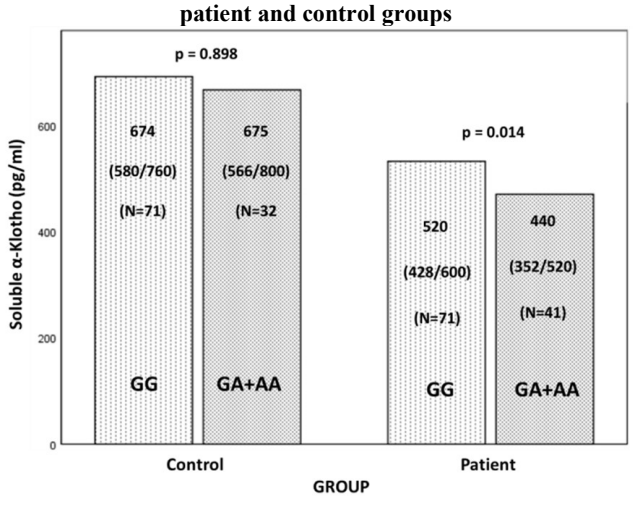

**(a)**




**(b)**

Values are expressed as median with inter quartile ($25^{th}/75^{th}$) ranges

**Figure 4: Distribution of soluble Klotho levels according to GG and GA+AA genotypes of G-395A polymorphism in patient and control groups**




Values are expressed as median with inter quartile ($25^{th}$/$75^{th}$) ranges

## 4. Discussion

To our knowledge, this is the first study investigating circulating levels of soluble α-Klotho and variants KL-VS and G-395A of Klotho gene in Indian hypertensive population. Since advanced age and obesity are established factors for essential hypertension, we have attempted to eliminate their effect on our analysis by recruiting age and BMI matched healthy controls.

In the present study, soluble α-Klotho levels were significantly lower in patient group as compared to the control group. Soluble α-Klotho levels showed positive correlation with HDL cholesterol and inverse correlation with triglycerides and BMI. These findings support its role in CVD where these factors have an influential role. In Chinese hypertensive patients, Su & Yang [27] have demonstrated that systolic or elderly hypertension may be partially attributed to reduction in serum α-Klotho levels. Semba *et al* [21] have shown independent association of plasma α-Klotho with CVD in Italian cohort, where they found lower risk of CVD in adults who had higher plasma α-Klotho concentrations. The association of reduced circulating levels of α-Klotho with the presence and severity of coronary artery disease (CAD) [28]and CVD [21], and as an independent marker of arterial stiffness in patients with chronic kidney disease [29] have been reported in Spanish, Italian and Japanese cohorts respectively. Our results also suggest that α-Klotho levels are depleted in essential hypertension patients.

With respect to the KL-VS polymorphism, frequency distribution of F (0.87) and V (0.13) alleles in our study population was in compliance with the reported frequencies of F (0.861, 0.89) and V (0.139, 0.11) in previous studies by Majumdar *et al*[30] and Sivaraman *et al*[31]respectively in Southern and Western Indian population. The F/V allele distribution of the present study was also similar to UK Caucasian (0.85/0.15) [13], Spanish (0.86/0.14) [32], Italian (0.85/0.15) [33] and French (0.84/0.16) [34] populations. However, the frequency of 352V allele in our study was lower than Chinese population (0.335) [10], while in Korean population it has been reported to be absent [35,36].

A large discrepancy prevails over the risk related to KL-VS polymorphism. Donate-Correa *et al*[11]reported a significantly higher incidence of CVD, and a similar trend in incidence of CAD, in Spanish individuals with 352V allele. Arking *et al*[37] studied apparently healthy siblings of individuals with premature incident CAD, and demonstrated that the 352V allele was an independent risk factor for occult CAD. In a subsequent study in Ashkenazi Jews, they also found higher SBP associated with VV genotype as compared to the heterozygous FV genotype [38]. However, in a previous study, Arking *et al*[12] have proposed that there exists a heterozygous advantage and a marked homozygous disadvantage of KL-VS polymorphism, for survival and longevity as observed in ethnically distinct Bohemian Czech, Baltimore Caucasian and Baltimore African-American populations. Majumdar *et al* [30] have reported similar finding in South Indian ischemic stroke patients where they found an over representation of FF and VV homozygotes in patients as compared to controls. In the present study, however, we did not find any significant association of KL-VS polymorphism with essential hypertension. Freathy *et al*[13] have found KL-VS not to be a risk factor for type 2 diabetes in UK Caucasian population. In fact, Nzietchueng *et al* [34] have found that VV genotype was significantly associated with lower SBP and pulse pressure, as well as with lower cardiovascular risk in French population. Studies in Korean population by Kim *et al*[34] and Rhee *et al* [36] have reported the lack of the variant 352V allele in Korean population, contending that it does not have any effect on aging or life-span.

Though the distribution of genotypes in our study satisfied the Hardy-Weinberg equilibrium, interestingly, we did not find the homozygous variant AA of G-395A polymorphism in the control group. The G/A allele distribution in our study population (0.82/0.18) was similar to previous studies in Indian (0.83/0.17) [31], Korean (0.852/0.148) [36], (0.854/0.146) [39], (0.845/0.155)[40], Chinese (0.809/0.191) [41], and Japanese (0.88/0.12) [42] populations. However, it differed from allele distribution in the Spanish population (0.65/0.35)[11].

The variant allele A of G-395A polymorphism has been reported to be associated with hypertension, CAD, vascular access, and cardioembolism in Korean [39,40, 43,44], Japanese [42] and Chinese[10] populations. A meta-analysis performed by Zhang *et al* [10] revealed that G-395A was a susceptibility factor for CAD in East Asia population, wherein individuals carrying the A allele

(GA+AA) were found to be at a higher risk for CVD as compared to those with GG genotype. In contrary reports, the G-395A variant was associated with protective role in Chinese population for hypertension [41,45,46]. This difference can be attributed to the variation in genetic constitution of the diverse ethnic populations. In the present study however, the difference in genotype distribution of G-395A polymorphism between essential hypertension patients and normotensive controls was not found to be significant.

The present study did not find any variation in levels of soluble α-Klotho in patients and controls with respect to the KL-VS genotypes. Donate-Correa *et al*[11] also have not observed any influence of KL-VS variant on Klotho expression in human vasculature. The homozygous AA and heterozygous GA genotypes of G-395A polymorphism showed significant association with lower soluble α-Klotho levels in the patient group of our study. Donate-Correa *et al* [11] have also reported similar results where they observed reduced Klotho gene expression levels in vascular tissue of subjects with GA and AA genotype as compared to GG genotype. Thus, in essential hypertension patients, G-395A polymorphism may have an influence on soluble α-Klotho levels. However further studies with larger cohort are warranted.

In conclusion, we observed significantly lower soluble α-Klotho levels in essential hypertension patients as compared to healthy normotensive individuals and its association with variant genotypes of G-395A polymorphism. Genotype distributions for both polymorphisms did not differ between the two study groups. Previous studies have suggested the use of soluble α-Klotho as a biomarker for severe disorders such as acromegaly [47], renal cell carcinoma and renal fibrosis [19,48], CVD, related cardiac pathologies and abdominal aortic calcification [49,50]. The fact that similar results are observed in essential hypertension as well, opens new perspectives for a more effective disease monitoring, prevention of disease escalation and future therapeutic interventions at much earlier presentation of risk factor. The limitation of our study is that our case and control groups had a few more than hundred participants each. However, extending this study in a larger cohort may reveal soluble α-Klotho as a novel, relevant biomarker.

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## Conflicts of interest

The authors report no conflicts of interest.

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