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AHSG Gene Polymorphisms in renal stone disease: A Cross-sectional study

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Abstract

Alfa 2 HS glycoprotein (AHSG) also called as Fetuin-A,a plasma glycoprotein, is a strong calcification inhibitor whose polymorphism in kidney stone disease has received the least attention. The aim of the study was to compare patients with and without urinary oxalate stones in terms of the distribution of AHSG gene polymorphisms. A total of 100 participants, including 50 cases and 50 control subjects were enrolled in the study. Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR–RFLP) was used to analyse the AHSG c.742C>T and c.766C>G Single Nucleotide Polymorphisms (SNPs). Biochemical parameters were assayed. SPSS 23 was used for statistical analysis. The distribution of AHSG c.742C>T and AHSG c.766C>G mutant and wild alleles did not differ significantly. The polymorphisms in the AHSG gene were not significantly correlated with their expression. Serum AHSG was insignificantly lowered($p=0.43$), uric acid and creatinine were significantly higher ($p=0.012$ and $p=0.046$ respectively) in cases. eGFR was reduced significantly ($p<0.0001$) in cases suggesting a renoprotective role of AHSG. Significant positive correlation was observed between AHSG and calcium levels. According to the study, there is no conclusive link between renal stone disease and AHSG gene variations. Lowered AHSG levels in patients with renal stone disease suggest a protective role of AHSG. Small sample size is the limitation of the study. However, it may be considered as a pilot study and further investigations may be carried out to further explore the role of AHSG in kidney stone disease.

Keywords: AHSG, alleles, polymorphism, renal stone

Full length article *Corresponding Author, e-mail: ushachidu@yahoo.com

1. Introduction

Nephrolithiasis is a multi-step process with several contributing elements. The majority of urinary calculi, or around 80% of all renal stones, are calcium stones. Finding the chemicals and metabolic changes that influence this process may open the door to intervening in stone formation. AHSG is one such molecule that has received the least amount of research. AHSG is primarily released by the liver and is a 45-kDa plasma protein made up of two polypeptide chains resulting from post-transcriptional cleavage [1]. It exhibits adverse acute-phase reactivity. The development of the AHSG-mineral complex is partly responsible for the protein's activity as a powerful inhibitor of vascular calcification. It improves the solubility of calcium and phosphorus in the corresponding serums by forming reversible compounds with them [2]. The ability of serum to suppress calcium-phosphate product precipitation was noticeably reduced in dialysis patients whose serum AHSG levels were found to be low, and it was discovered that these individuals had coronary or other calcification foci. Additionally, the defective precipitation inhibition was restored to normal [3] by reconstituting the serum from these individuals with pure AHSG to attain physiological quantities. Price and Lim demonstrated that AHSG prevents the precipitation of hydroxyapatite from supersaturated calcium and phosphate solutions in vitro [4] by creating complexes with minerals. Additionally, it has been shown that urolithiasis patients have lower urine AHSG levels than healthy individuals. The same study found that urine AHSG levels were 100% specific and 97% sensitive for detecting urolithiasis [5].

AHSG levels were evaluated in the urine and serum of patients with stone problems by Arora et al., who concluded that these patients' levels were lower than those of individuals without renal stones [6]. However, contradictory results were seen by other researchers [7,8]. Research on the significance of a AHSG gene polymorphism in nephrolithiasis is limited, but Aksoy et al. explored the role of AHSG gene polymorphism in the pathophysiology of

calcium oxalate stone formation and concluded that this may increase the risk of calcium oxalate stone formation [9].

South Indian state of Karnataka's coastline region is home to the districts of Dakshina Kannada and Udupi. The influence of a certain diet distinctive to the coastal area with decreased calcium consumption and high intake of animal protein (whether meat, fish, or chicken) and oxalate could be attributed for the high incidence and prevalence of renal stone disease in coastal districts. Enteric hyperoxaluria and a higher incidence of calcium oxalate stone development are the results of this. Additionally, the likelihood of stone formation in coastal regions was raised by the reduced urine volumes brought on by the hot, humid, and dry climate [10]. Studies are required to confirm the part AHSG and its gene play in the development of calcium oxalate stones.

Study Objectives:

1. To compare urinary oxalate stone patients to healthy controls in terms of the distribution of AHSG gene polymorphisms.
2. To compare the biochemical parameters between kidney stones and those without them.
3. To determine whether serum AHSG levels in kidney stone patients correlate with other biochemical markers.

2. Materials and methods

2.1. Study design and setting

An observational cross-sectional study was carried out between June 2020 and March 2022. Patients who visited the Department of Urology at the Justice K. S. Hegde Charitable Hospital in Mangalore, Karnataka, India had kidney stone disease, as determined by ultrasonography were included. Additionally, blood samples were studied in the Molecular Genetics and Central Research Laboratory wings of the KS Hegde Medical Academy. Samples of kidney stones were taken either following surgery or extracorporeal shock wave lithotripsy for therapy. Chemical techniques were used to analyse calculi [11]. The study only included patients with calcium oxalate stones. The study was initiated after obtaining approval on 15/6/2020 from CEC Ref,NU/CEC/2020/0289 Nitte (Deemed to be University) and eligible patients were given information on the study procedure, goal and role in the study and written patient informed consent was obtained from all the participants in their respective native languages.

Fifty patients in the age group of 18-65yrs of either gender, with normal GFR with the calcium oxalate kidney stone confirmed by qualitative tests were included as cases.

Patients with uric acid / cysteine stones diagnosed by qualitative tests or those with primary hyperparathyroidism diagnosed by investigation were excluded.

2.2. Laboratory Investigations

2.2.1. Gene Analysis

Five ml of blood were drawn and placed in EDTA vacutainers to study gene polymorphisms. The salting out approach was used to isolate DNA[12]. The spectrophotometer (ratio of OD260/OD280) was used to verify the quantity and purity of the DNA. The following formula was used to determine the DNA concentration:

Concentration (µg/ml) of DNA in original solution= Absorbance x 100 x 50 µg/ml.

2.2.2. Amplification and Genotyping of the gene polymorphism

Genotyping of the genes was confirmed by PCR-RFLP

2.2.3. AHSG Genotyping

A forward and reverse oligonucleotide primers were used to amplify the AHSG 742C>T polymorphism 5'-CCTCCCACAAGCAGAAAC-3' and 5'-TGATGATTC-CGCATACCC-3'respectively using Primer 3Plus [9]. Amplification was performed in MiniAmp plus Thermal cycler (Thermofischer Scientific). PCR products was digested with 0.5 µl NlaIII(NEB) restriction endonucleases overnight at 37°C,and the digested products were separated by 3% agarose gel electrophoresis and visualized using ethidium bromide. For Analysis of AHSG 766C>G polymorphism was performed with the oligonucleotide primers forward 5'-GTCAC-CCCTCCTTGTAAC-3' and reverse 5'-CCCCAATGAGAC-CACA-3' [9]. Serum AHSG levels were assayed in the serum by ELISA Kit. Biochemical parameters like serum calcium, phosphorus and creatinine were estimated by semi-automated chemistry analyser. Estimated Glomerular Filtration Rate (eGFR) was calculated using Modification of Diet in Renal Disease (MDRD) formula.

2.3. Statistical analysis

The statistical analysis was carried out utilising SPSS version 23. In order to determine if observed and anticipated alleles are in equilibrium, the Hardy Weinberg equilibrium was calculated. The chi square test was used to examine the relationship between the genes and serum AHSG levels and stone formation. Unpaired t test or Mann Whitney U test were used to compare biochemical parameters for parametric and non-parametric parameters, respectively. Spearmann's correlation was used to conduct the correlations. A P value of 0.05 or less was regarded as statistically significant.

3. Results and Discussions

Age matching was evident from the cases' and controls' demographic data, with no age difference that was statistically significant (p=0.17) (table 1). In instances, a significantly reduced eGFR and greater creatinine (p=0.046 and p0.0001, respectively) were observed. The levels of serum phosphate and calcium differed insignificantly. (table 1). The Hardy Weinberg equilibrium study of the two AHSG SNPs revealed that there is no significant difference between the anticipated and observed alleles. The distribution of wild-type and mutant AHSG c.742C>T and AHSG c.766C>G alleles did not change significantly (tables 2 and 3). Figures 1 and 2 show RFLP allelic distribution patterns. In the present study, serum AHSG levels were insignificantly low in cases as compared to controls (table 1). Association between AHSG gene polymorphism and serum AHSG levels showed an insignificant difference(Chi-squared=1.75,p=0.18 with OR 2.29 (95% CI 0.66-7.95). There was no significant association between the wild and mutant alleles of c.742C>T of AHSG gene and its serum levels (chi square=1.203, p=0.27) (table 9). Similarly, the association of c.766C>G alleles with serum AHSG levels was also insignificant(chi square=0.90,p=0.34)(table 1).

Table 1. Biochemical Parameters in calcium oxalate stone disease

Parameters	Control group	Case group	P value
Age	33.7±10.47	46±13.87	<**0.01***
Gender(Male/Female)	34(68%)/16(32%)	18(36%)/32(64%)	-
Uric acid (µmol/L)	416.36(285.5-707.81)	273.61(136.8-523.42)	**0.012***
Creatinine(µmol/L)	71.62(58.36-86.65)	88.42(61.89-124.67)	**0.046***
Calcium(mmol/L)	2.32±0.46	2.11±0.95	0.146
Albumin(g/L)	27.6±12.11	19.2±11.59	**0.007****
Phosphorus(mmol/L)	1.74(1.43-2.03)	1.55(1.35-1.84)	0.181
eGFR	122.9(96.6-208.1)	78.2(53.8-105)	<**0.0001*****
AHSG(µg/mL)	0.04±0.0145	0.037±0.016	0.43

* significant
** highly significant
*** very highly significant

Table 2. Genotype distribution of AHSG c742C>T

AHSG (c.742 C>T) Genotype	Control(n=50) %	Case (n=50)%	OR(95% CI, p value)
CC CT TT	27(54%) 22(44%) 1(1%)	30(60%) 18(36%) 2(4%)	X2=0.891, df=2 ,p=0.640

Table 3. Genotype distribution of AHSG 766C > G

AHSG (c.766 C>G) Genotype	Control (%)	Case (%)	P value
CC CG GG	32(64%) 17(32%) 1(4%)	30(60%) 18(36%) 2(4%)	X^2=0.426,df=2,p=0.808

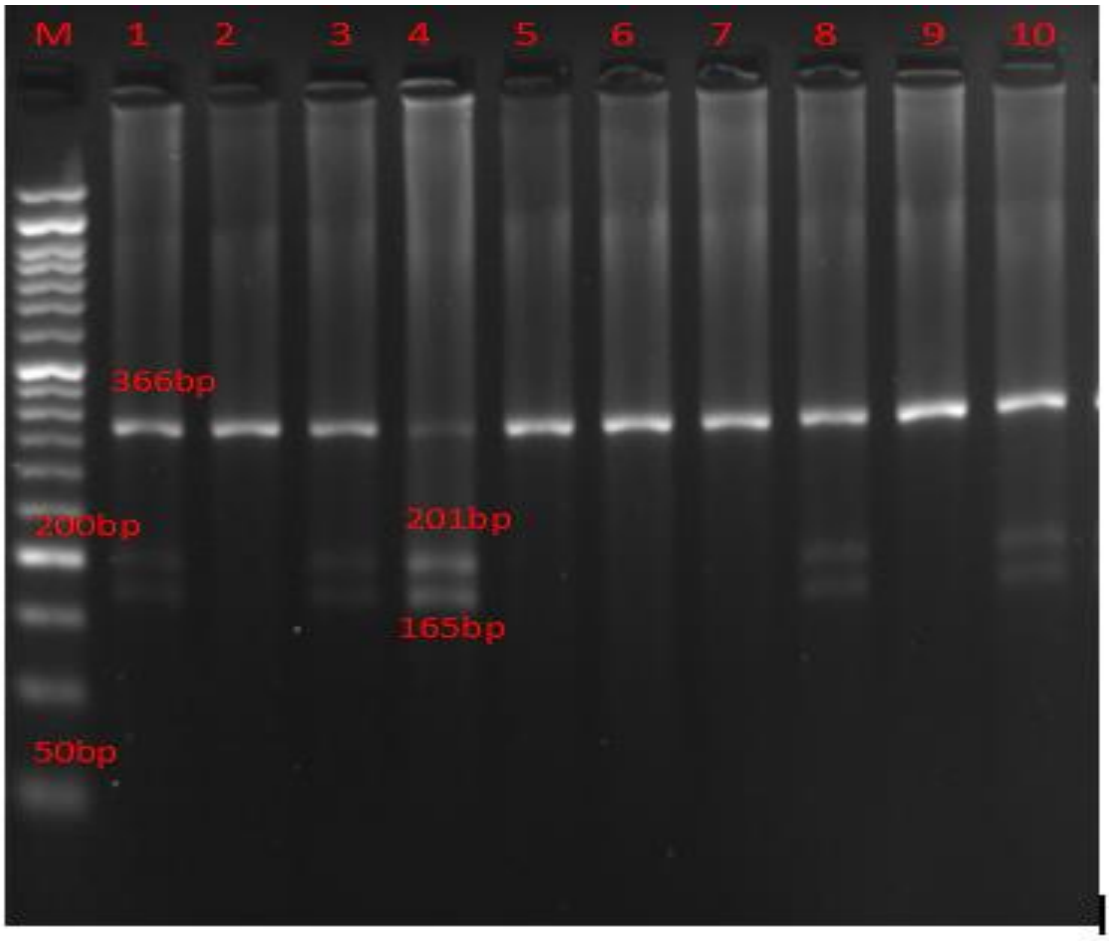

Fig. 1. PCR RFLP analysis c.742C>T polymorphism in the of AHSG.Lane M: 50 bp marker: Lanes 1,3,8,10: CT alleles ;Lanes 2,5,6,7,9: CC alleles ; Lanes 4:TT alleles

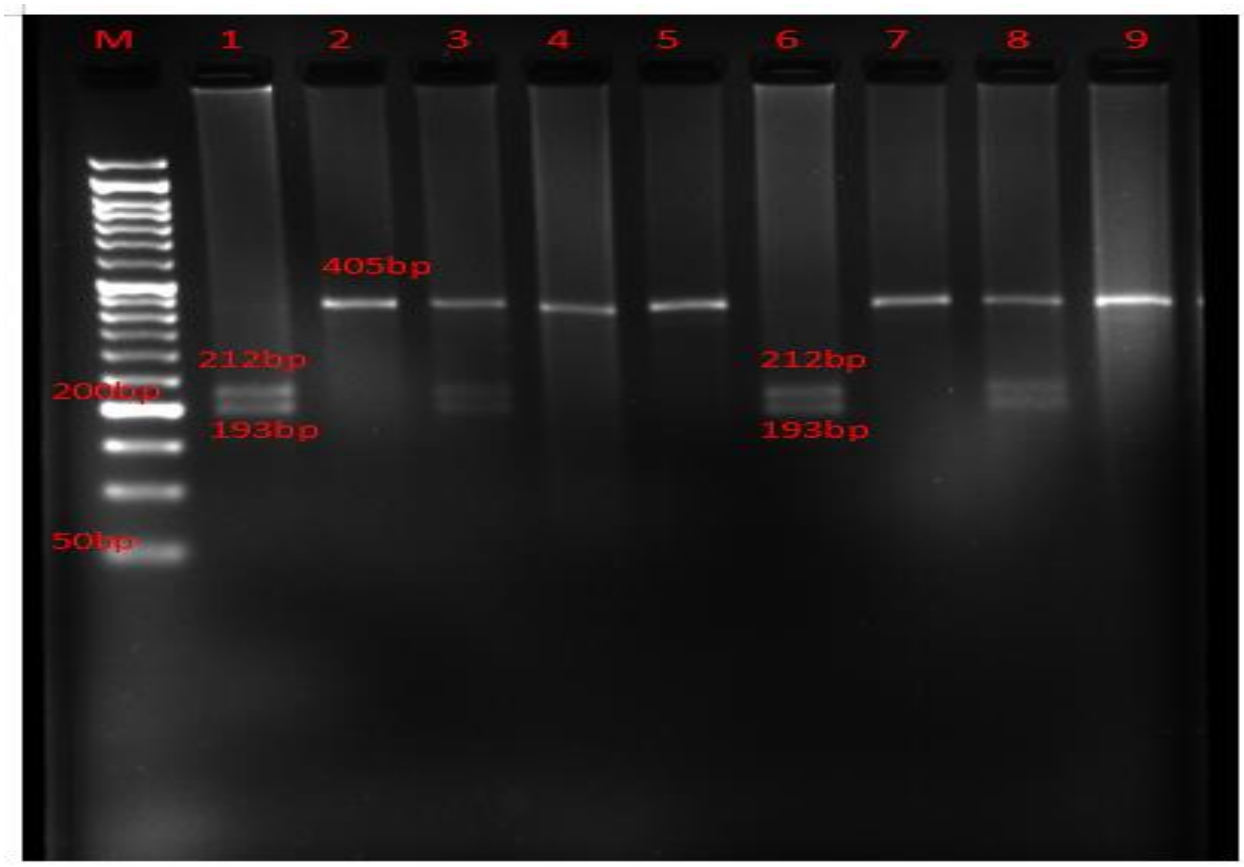

Fig. 2. PCR RFLP analysis c.766C>G polymorphism in the of AHSG.Lane M: 50 bp marker: Lanes 1,6: GG alleles ;Lanes 2,4,5,7,9: CC alleles ; Lanes 3,8 :CG alleles

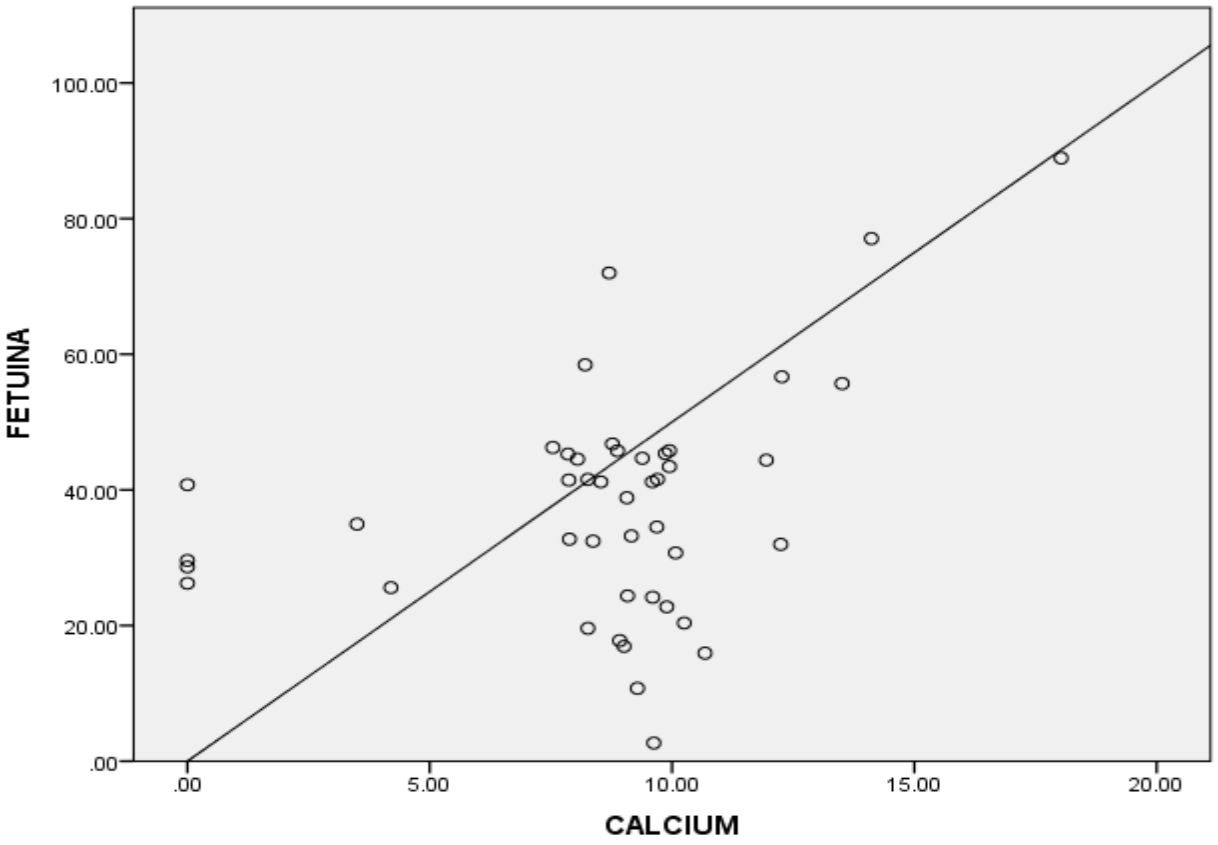

Fig. 3. Correlation between Calcium and AHSG (r =0.377 P=0.012)

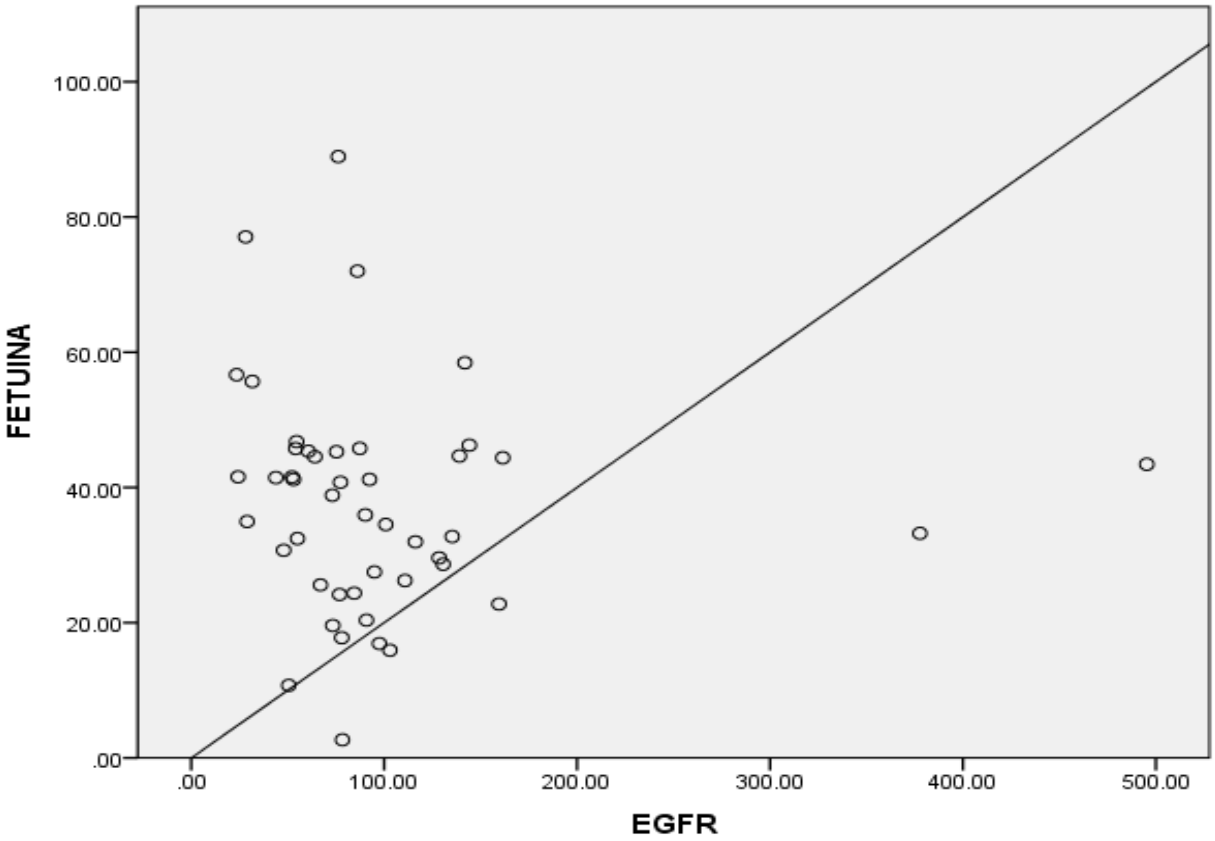

Fig. 4. Correlation between eGFR and AHSG (r = -0.070 P=0.661)

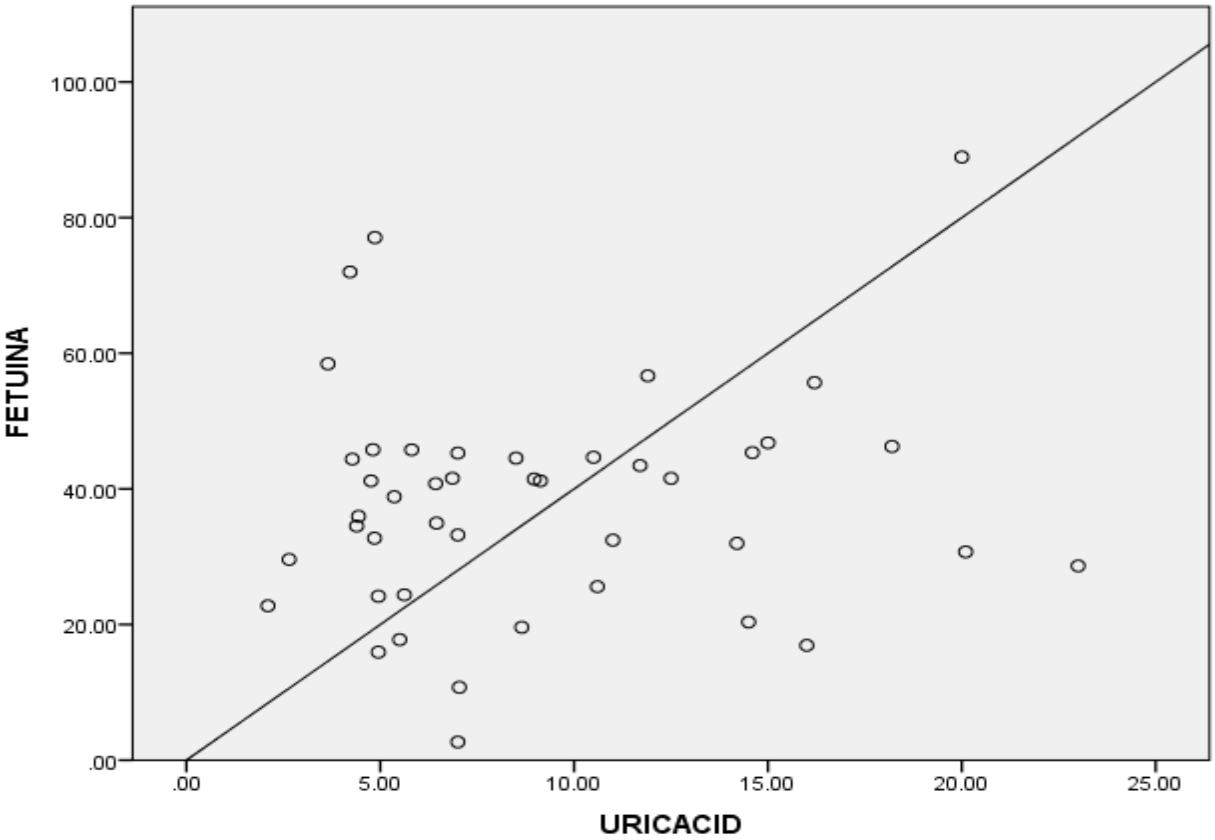

Fig. 5. Correlation between Uric acid and AHSG (r = 0.126, p =0.413)

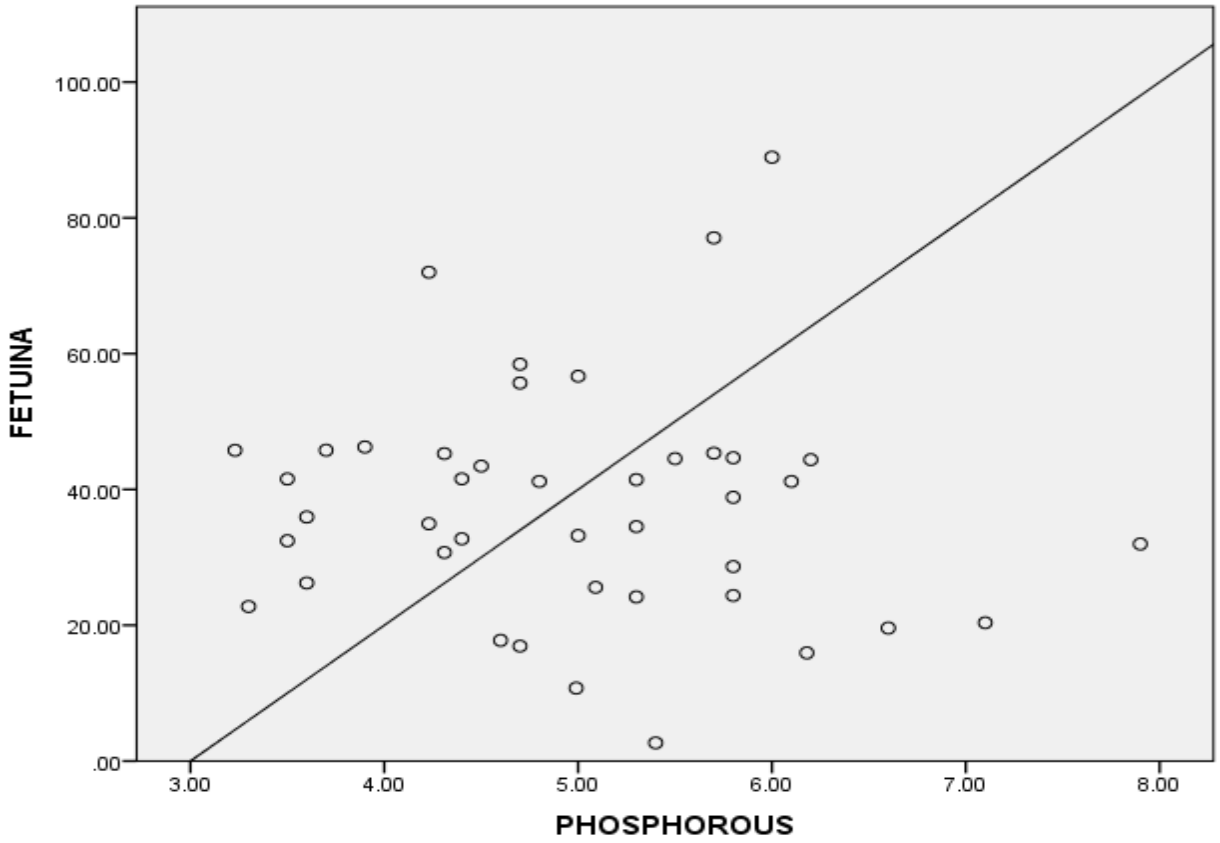

Fig. 6. Correlation between Phosphorous and AHSG (r = -0.070, p =0.661)

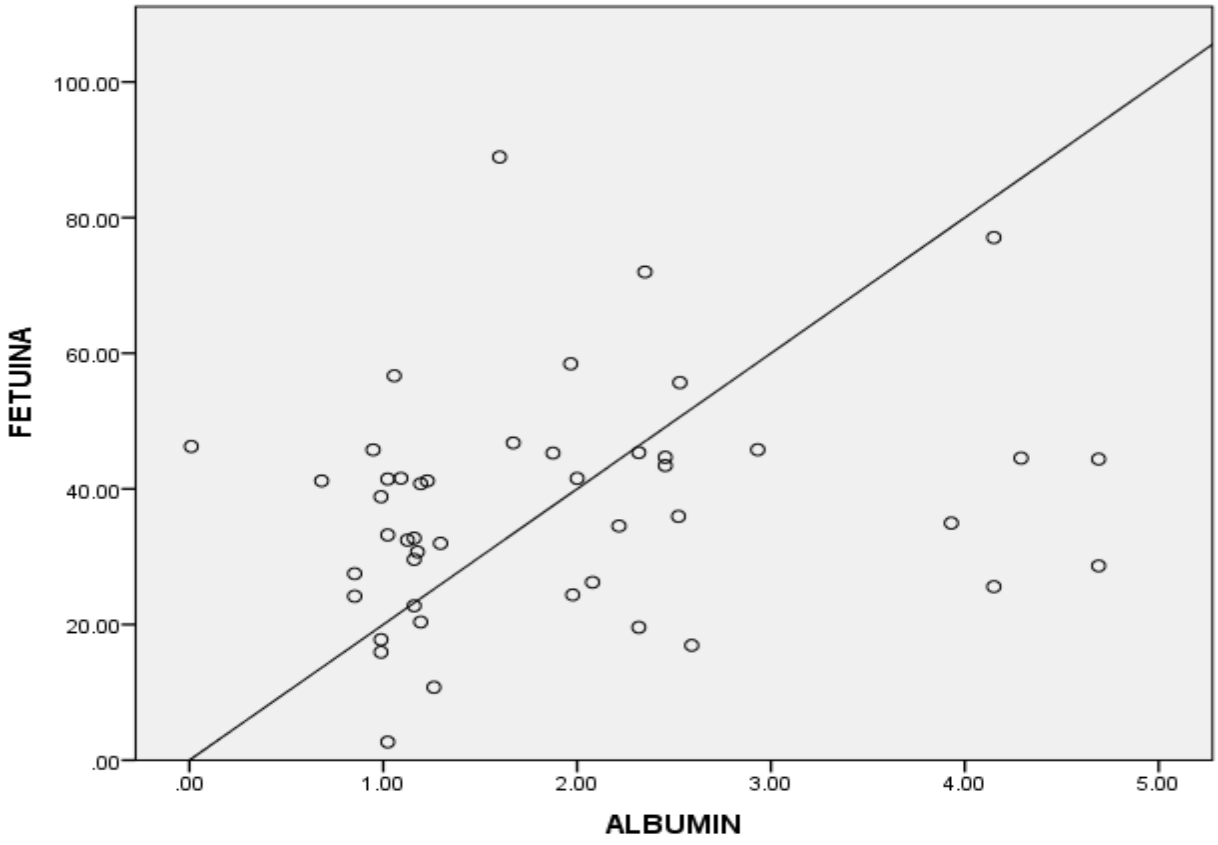

Fig. 7. Correlation between Albumin and AHSG (r =0.203,p =0.177)

SNPs of AHSG did not bear any significant association with eGFR in cases (Chi-squared=1.389, p=0.23, OR=2.04 (95% CI=0.61 -6.75) for both the SNPs. Odd's ratio confirmed that the heterozygous dominant CC genotype was at higher risk of KSD with low AHSG levels and showed low eGFR values. The negative correlation between AHSG levels and eGFR (R=-0.2581 p=0.07) provides substantial evidence for the renal protective role of AHSG. Serum uric acid and creatinine were significantly higher in patients with kidney stones compared to controls. (Table 1). A correlation analysis was performed between serum AHSG levels and other biochemical parameters. A significant positive correlation was observed between AHSG level and calcium (r=0.377, p=0.012) (fig 3). In contrast, eGFR and Phosphorous exhibits a negative correlation, and all other correlations were statistically insignificant. [eGFR and AHSG (r = -0.070, P=0.661); Uric acid and AHSG(r = 0.126, p =0.413); Phosphorous and AHSG (r = -0.070, p =0.6); Albumin and AHSG (r =0.203,p =0.177)][fig 4-7].

AHSG is one of the numerous non-collagenous proteins involved in osteogenesis and is known to have a strong affinity for calcium ions [13,14]. It has been demonstrated that AHSG prevents calcium phosphate crystals from growing and forming [15-17]. By generating a fetuin-mineral complex, hydroxyapatite crystals appear to be absorbed by AHSG, preventing its deposition and inhibiting non-bone calcification [18-20]. AHSG has been proposed as a predictor of poor prognosis in persons with acute atherosclerosis and patients receiving hemodialysis. Rats' heart, lungs, kidneys, and skin have all shown extensive calcification in response to low serum AHSG levels [3]. Additionally, it stops the calcification of vascular smooth muscle cells [20]. According to Cai et al., this protein may be important in the malfunction of mineral deposition or other mechanisms that lead to defective mineralization [21]. It may also be involved in preventing bone mineralization on the outer surface. On the other hand, it was shown by Umekawa and Nishio that the AHSG protein is insufficient to stop the development of hydroxyapatite crystals [22,23].

Our study results agree with the study by Mehrsai et al [24]. A study by Bassey et al suggested that AHSG may play a role in the aetiology of deteriorating renal function through mediating body mass index, uric acid, diabetes mellitus, and hypertetrahydroglobulinemia. Reduced serum AHSG levels observed in renal stone patients in the present study may explain the contribution. AHSG's renoprotective activity is supported by the present study's decreased AHSG levels, considerably decreased eGFR, and negative correlation between AHSG and eGFR. AHSG functions as an acute phase reactant in the extracellular space to reduce inflammatory responses because it is an acute phase anti-inflammatory protein. As a result, AHSG levels in patients with early renal disease may be normal or slightly raised. Pro-inflammatory cytokines, such as CRP, however, downregulate or block AHSG production when the inflammatory process is extended, weakening the protective impact of AHSG [25]. This could account for the low levels of circulating AHSG seen in CKD. Serum AHSG concentrations have not been extensively studied in relation to kidney stone disease, but they are correlated with a common kind of moderate osteogenesis imperfecta, but they are lowered in end-stage renal disease and during acute inflammation [26,27,3]. In the current study, AHSG's positive association with calcium and negative correlation with phosphorus highlight its function in stone formation. Additionally, AHSG controls ectopic calcification, which may increase the risk of cardiovascular mortality [28-30]. AHSG generally prevents the precipitation of hydroxyapatite from calcium and phosphate supersaturated solutions in vitro. Through the solubilization of calcium phosphate salt, AHSG has been postulated as a protective agent and is a determinant of serum phosphate [31]. Additionally, increases in AHSG levels could be an indication of this illness since AHSG is a marker of both an inflammatory and nutritional state. A second unintentional calcification process that causes major morbidity is urolithiasis; >80% of kidney stones include calcium. Citrate, glycosaminoglycans, the Tamm-Horsfall protein, osteopontin, and other substances are urinary factors that prevent calcification [4]. Recently, it was discovered that exosomal and urine AHSG levels considerably rose following cisplatin-induced tubule injury, which took place before to an increase in serum creatinine. As a result, urine AHSG levels could serve as a biomarker to predict structural renal injury [32].

Independently of other traditional promoters and inhibitors of urine crystallisation, AHSG concentrations were lower in patients with urolithiasis. These results might be explained by the AHSG-mineral complex seen in urine that has been saturated with crystallisation nuclei. We believe that the main benefit of assessing AHSG in comparison to using conventional markers of recurrent urolithiasis is a better risk prediction of recurrent urolithiasis. The study's limitation is the small sample size. However, it could be viewed as a pilot study, and more research could be done to better understand AHSG's function in kidney stone illness.

4. Conclusions

The allelic frequencies of c.742C>T and c.766C>G amongst the participants with and without renal stones did not differ significantly. However, the negative correlation between AHSG levels and eGFR confirms the renoprotective role of AHSG. Lower AHSG levels in patients with renal stone disease suggest a protective role of AHSG. There was no association between the AHSG gene polymorphisms and their expression (AHSG levels).

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