

Hyperhomocysteinemia As A Risk Factor For Atherosclerosis In Chronic Kidney Disease

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Abstract: Background: The role of Hyperhomocysteinemia (HHomocysteine) as a risk factor for atherosclerosis in Chronic kidney disease (CKD) has gained much interest worldwide. The primary objective of the study was to ascertain the association between serum homocysteine levels and GFR. Method: This case control study was done in stage 3, 4 and 5 of CKD (cases= 63 ,controls =21) to assess the association between Serum homocysteine (S.Homocysteine), Serum creatinine (S.Cr), Glomerular Filtration Rate (GFR) and Carotid intima media thickness (CIMT). Result: Statistical analysis using ANOVA and Pearson's correlation revealed a significant association between S.Homocysteine and stages of CKD ($p=0.00$), S.Homocysteine and GFR ($p=0.00$, $rsq=0.3686$) S.Homocysteine and CIMT ($p=0.002$, $rsq=0.1429$) and CIMT and CKD ($p=0.00$). Conclusion: On the basis of these observations, it was concluded that HHomocysteine exists in CKD and that it produces atherosclerosis. Hence early screening and treatment for HHomocysteine and atherosclerosis should be done in CKD to prevent cardiovascular diseases.

Key

Words: Homocysteine (Homocysteine), Hyperhomocysteinemia (HHomocysteine), Chronic Kidney Disease (CKD), atherosclerosis, Glomerular Filtration Rate (GFR), Carotid intima media thickness (CIMT).

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Introduction: Chronic kidney disease (CKD) is an important chronic disease that affects the world¹. It is a clinical human model of accelerated atherosclerosis and there is increased cardiovascular morbidity and mortality in CKD patients². Despite the high prevalence in CKD, the traditional risk factors (old age, hypertension, diabetes mellitus, dyslipidaemia and physical inactivity) fail to entirely account for the progression of atherosclerotic diseases. Unique renal related risk factors like HHomocysteine contribute to the high risk of atherosclerosis in CKD³.

CKD patients have an excess prevalence of mild to moderate HHomocysteine (85-90%) and HHomocysteine has been independently linked to Cardiovascular diseases in CKD in many recent prospective observational studies³. This study was done with the objective of finding the correlation of Homocysteine values with renal function and extent of atherosclerosis in CKD patients.

Material and Method:

Study design: This case control study was done at Nephrology outpatient department Government Medical college Hospital, Trivandrum, Kerala, India. The 63 cases

belonged to stage 3, 4 and 5 of CKD, 21 cases in each group, belonging to either gender and of 25-65 years of age. Cases with diabetic nephropathy and proteinuria of >1 gm were excluded. CKD included those patients with kidney damage for ≥ 3 months with structural or functional abnormality or a GFR of < 60 ml/min/1.73m² for >3 months with or without kidney damage. GFR of 30-59 ml/min/1.73m² is CKD stage 3, GFR of 15-29 ml/min/1.73m² is stage 4 and GFR of <15 ml/min/1.73m² is stage 5. Age and sex matched controls were selected from healthy kidney donors attending the nephrology clinic. Those donors who volunteered to participate were free of overt CKD, Coronary artery disease, Diabetes mellitus and hypertension. The protocols were submitted to and approved by Human Ethical Committee of Medical College, Thiruvananthapuram.

MATERIALS:

- A) Blood parameters :- Blood samples were taken for S.Homocysteine (serum homocysteine), S.creatinine, blood urea nitrogen and S.albumin estimation.
- B) Glomerular Filtration Rate (GFR)
- C) Carotid Intima Media Thickness (CIMT)

1.Homocysteine estimation: Homocysteine is a thiol-containing amino acid produced by the intracellular demethylation of methionine. Homocysteine assay was done using Axis Homocysteine Eia Fhomocysteine 100 Kit. Assay principle :Axis® Homocysteine Enzyme Immunoassay (EIA) is an enzyme immunoassay for the determination of Homocysteine in blood. Protein-bound Homocysteine is reduced to free Homocysteine and enzymatically converted to S-adenosyl-L-homocysteine (SAH) in a separate procedure prior to the immunoassay. The enzyme is specific for the L-form of homocysteine, which is the only form present in the blood.

Reduction: Homocysteine, mixed disulfide and protein-bound forms of Homocysteine in the

sample are reduced to free Homocysteine by use of dithiothreitol (DTT).

Enzymatic conversion: Homocysteine in the test sample is converted to S-adenosyl-L-homocysteine by the use of SAH hydrolase and excess adenosine.

The following solid-phase enzyme immunoassay is based on competition between SAH in the sample and immobilized SAH bound to the walls of the microtitre plate for binding sites on a monoclonal anti-SAH antibody. After removal of unbound anti-SAH antibody, a secondary rabbit anti-mouse antibody labelled with the enzyme horse radish peroxidase (HRP) is added. The peroxidase activity is measured spectrophotometrically after addition of substrate, and the absorbance is inversely related to the concentration of Homocysteine in the sample.

REAGENTS

Kit components	Solution	Cap code	Component description	Volume
Reagent A	Assay bufferer	A	Phosphate bufferer,sodiumazide	54ml
Reagent B	Adenosine/DTT	B	Adenosine/dithiothreitol,citric acid	3.5ml
Reagent C	SAH-hydrolase	C	Recombinant S-adenosyl-L-homocysteinehydrolase, trisbufferer, glycerol,methylparaben	3.5ml
Reagent D	Enzyme inhibitor	D	Merthiolate,phosphate bufferer	55ml
Reagent E	Adenosine deaminase	E	Adenosine deaminase ,phosphate bufferer,sodiumazide,BSA,phenol-red dye	55ml
Reagent F	Anti-SAH antibody	F	Monoclonal mouse anti-S-adenosyl-L-homcysteineantibody,BSA, merthiolate	25ml
Reagent G	Enzyme conjugate	G	Rabbit anti mouse antibody enzyme conjugate,BSA,horseradish peroxidase,blue dye	15ml
Reagent H	Substrate solution	H	n-methyl-2-pyrrolidon,propyleneglycol	15 ml
Reagent S	Stop solution	S	0.8 M sulphuric acid	20 ml
Bufferer Wash	Wash bufferer	W	Phosphate bufferer, merthiolate, tween 20,BSA	60 ml
CAL-1 To CAL 6	Calibrators	1-6	S-Adenosylhomocysteine (2,4,8,15,30,50µmol/l) in bufferer with preservative	6×1.5 ml
Microtitre Strips	Microtitre strips		Coated with S-adenosyl-L-homocysteine	12×8wells

Additional requirements : Plastic or glass tubes for pre-treatment of samples, pipettes/ multipipettes 25 µL, 100 µL, 200 µL and 500 µL or 8 channel multipipette for 100 µLand 200 µL, Volumetric flask 50 mL and 600 mL, Incubator,

37 °C, Washer and reader (450 nm) for microtitre plates.

Preparation and Storage of Kit Components Components were refrigerated at 2 - 8 °C. All bottles were stored upright and tightly

capped. The sample pre-treatment solution was made by mixing Reagent A, B and C.

The solution which is stable for one hour had to be freshly made for each run. The Wash bufferer was diluted (1+9) with distilled water before use. The prepared wash bufferer was stable for 4 weeks when stored at room temperature (18-25 °C). Reagent D and H were stored in dark bottles to avoid exposure to light. The microtitre strips were kept dry, i.e. in the sealed bag with drying capsules, and stored refrigerated. Equilibration for a minimum of two hours was required to reach room temperature (18 - 25 °C). The strips were left in the bag during equilibration. Only the necessary number of microtitre strips were kept in the frame during the run. Unused strips were kept in the sealed bag with drying capsules. Exposure of the kit to temperatures exceeding 37 °C was avoided to prevent the denature of the enzymes.

Specimen Collection and Preparation: EDTA-plasma was used with the Axis® Homocysteine Enzyme Immunoassay (EIA). EDTA-plasma samples were put on ice immediately after drawing. EDTA plasma samples may be kept on ice for up to 6 hours prior to separation by centrifugation. Complete mixing of thawed samples was done before use. Plasma samples could be stored for 12 weeks at 2 - 8 °C, for up to 3 weeks at room temperature (18 - 25 °C).

Procedure: All solutions and microtitre strips were equilibrated to room temperature before use.

Sample pre-treatment procedure: Sample pre-treatment solution was made up no more than 1 hour prior to the start of the assay. Volume needed per 10 samples: 4.5 mL REAGENT A+0.25 mL REAGENT B+0.25 mL REAGENT C. All the Reagents were then mixed. Calibrators and samples/controls in plastic or glass tubes were diluted as follows: 25µL calibrator /sample /control+ 500µL sample pre-treatment solution were mixed well. They were incubated for 30 minutes at 37°C (The tubes were covered with parafilm during incubation). 500µL REAGENT D was added and mixed well. It was incubated

for 15 minutes at 18-25°C. 500 µL REAGENT E was added and mixed well. It was Incubated for 5 minutes at 18-25°C.

Microtitre plate procedure 25 µL diluted calibrator / sample / control was pipetted from step 4 into the wells of the SAH-coated microtitre strips. 200 µL REAGENT F was added to each well. It was incubated for 30 min at 18-25°C. The enclosed lid was used during all incubations. It was washed manually 4 times with 350µl of diluted Wash buffer (BUF WASH + purified water). After washing, the wells were emptied onto paper towels. 100 µL REAGENT G was added to each well and incubated for 20 min at 18-25°C. It was washed 4 times with 350µl of diluted Wash bufferer (BUF WASH + purified water). After washing, the wells were emptied onto paper towels. 100 µL REAGENT H was added to each well and incubated for 10 min at 18-25°C. 100 µL REAGENT S was added to each well. The wells were shaken and reading taken at 450 nm. A logistic curve fit was used for preparing the calibration curve and calculation of unknown samples.

Estimation Of Glomerular Filtration Rate (GFR): Glomerular filtration rate was calculated using the Modification of Diet in Renal study equation (MDRD) as recommended by the National Kidney Foundation Kidney Disease Outcome Quality Initiative (KDOQI). The MDRD Equation is as follows :-

$$GFR (ml/min/1.73m^2) = 170 \times (Scr)^{-0.999} \times (Age)^{-0.176} \times (\text{Serum Urea N})^{0.370} \times (\text{Alb})^{0.318} \times (0.762 \text{ if female}) \times (1.180 \text{ if black})$$

The cases with GFR of >15 to <60 ml/min/1.73 m² were selected for the study.

Carotid Intima Media Thickness Study After taking informed consent and relevant history, high resolution After taking informed consent and relevant history, high resolution ultrasonographic examination of the common carotid arteries was carried out with 6 - 12 MHz linear probe in GE VOLUSON PRO machine. Patient was placed in supine position with neck slightly extended; head was placed away from the examination site. Intima-media complex

thickness was measured at 1 - 1.5 cm proximal to the carotid bulb in longitudinal plane. The area under study had to be free of plaque. Scanning of both side arteries was performed in anteroposterior projections and to obtain a better image, sound wave was adjusted perpendicularly to the arterial surface of the posterior wall of the vessel, yielding two parallel

echogenic lines which corresponds to lumen-intima and media-adventitia interfaces. The distance between the lines was taken as the combined thickness of the intima and media (IM complex).

STATISTICAL ANALYSIS: Statistical analysis was done Using ANOVA and Pearson's correlation.

Table 1 : Mean value of variables in each group

Stage Of CKD	Age (Yrs)	Weight (Kg)	S.Creatinine (µmol/L)	GFR MI/Min/1.73m2	S.Homocysteine (µmol/L)	CIMT (Cm)
CONROLS						
Mean	46.00	70.43	79.98	86.67	11.06	.055
SD	10.43	6.98	10.26	13.70	1.72	.006
CKD 3						
Mean	41.57	63.43	168.80	39.29	25.76	.090
SD	10.98	13.39	30.73	9.59	9.33	.021
CKD 4						
Mean	45.00	51.38	287.09	21.71	38.86	.099
SD	11.70	6.54	58.35	5.16	13.49	.031
CKD 5						
Mean	45.67	52.52	744.66	8.57	49.19	.109
SD	13.89	6.87	319.21	3.59	13.54	.031

Fig 1: S.Homocysteine Values Of Cases And Controls (µmol/L)

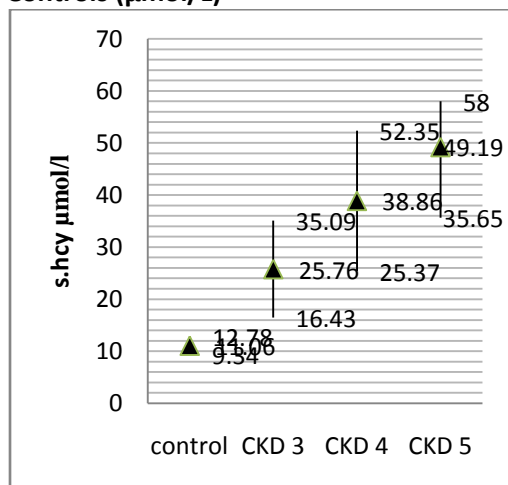


Fig 2 : Carotid Intima Media Thickness Of Cases And Controls (Cm)

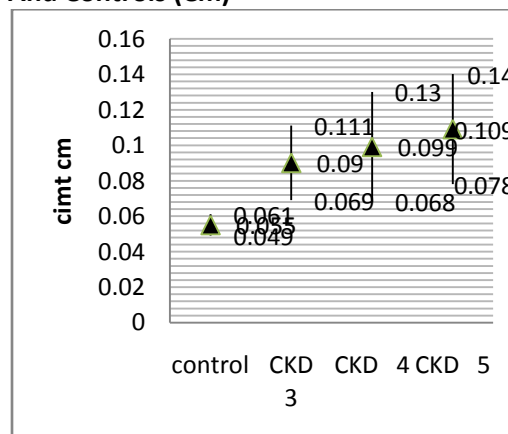


Table 2: Association Between S.Homocysteine And GFR In Cases

Model Summary

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate
1	.607 ^a	.369	.358	12.45261

a. Predictors: (Constant), GFR

ANOVA^a

Model		Sum of Squares	df	Mean Square	F	Sig.
1	Regression	5522.286	1	5522.286	35.612	.000 ^a
	Residual	9459.110	61	155.067		
	Total	14981.396	62			

a. Predictors: (Constant), GFR

b. Dependent Variable: S.HCY

Coefficients^a

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.
		B	Std. Error	Beta		
1	(Constant)	53.389	3.013		17.721	.000
	GFR	-.662	.111	-.607	-5.968	.000

a. Dependent Variable: S.HCY

Table 3: Association Of S.Homocysteine And CIMT

Model Summary

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate
1	.378 ^a	.143	.129	.02693

a. Predictors: (Constant), S.HCY

ANOVA^a

Model		Sum of Squares	df	Mean Square	F	Sig.
1	Regression	.007	1	.007	10.167	.002 ^a
	Residual	.044	61	.001		
	Total	.052	62			

a. Predictors: (Constant), S.HCY

b. Dependent Variable: CIMT

Coefficients^a

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.
		B	Std. Error	Beta		
1	(Constant)	7.300E-02	.009		8.085	.000
	S.HOMOCYSTEINE	7.014E-04	.000	.378	3.189	.002

a. Dependent Variable: CIMT

Result & Discussion: Serum Homocysteine and CKD: In this case control study , the mean value of the S. Homocysteine in the controls is found to be 11.06+1.72 µmol/L. The S. Homocysteine value increases significantly across the stages of CKD – 25.76+9.33 µmol/L in stage 3, 38.86 +13.49 µmol/L in stage 4 and 49.19 + 13.54 µmol/L in stage 5 (Table 1, Fig 1) . ANOVA of S. Homocysteine between cases and controls as well as between the groups of cases is significant at **p value = 0.000**. The above results are consistent with the

previous studies , which date from 19/7/94 , that hyperhomocysteinemia is associated with CKD. Arnadottir et al⁵, Chauveau et al⁶, Hultberg et al⁷, Cleveland clinic studies⁸, Litaoruan et al⁹, Samuelsson et al¹⁰ and Wilcken et al⁴ consistently found a significant association between hyperhomocysteinemia and CKD. Various reasons has been cited in many review articles for this significant association .

Arteriovenous studies in normal rat and human kidneys show that Homocysteine is normally filtered, reabsorbed and metabolized by the kidney. Hence only minimal levels of Homocysteine is normally excreted in urine. In CKD, the metabolism and filtration is altered, leading to HHomocysteine¹¹. Retained uremic toxins inhibit extrarenal Homocysteine metabolism by inducing transsulfuration defects¹².

Genetic polymorphisms of C677T also determine the Homocysteine levels in CKD. Certain studies report that HHomocysteine is the cause rather than the consequence of CKD¹².

Serum Homocysteine and Glomerular Filtration Rate : In this study, serum homocysteine shows a significant negative linear relation with GFR ($p=0.000$, $rsq=0.3686$) (Table 2, Fig 3). This relation is consistent with the previous studies from various parts of the world. This significant relation suggests that kidney plays an important role in plasma Homocysteine handling. GFR values estimated from Serum creatinine or calculated creatinine clearance is consistently and inversely correlated with plasma Homocysteine levels.

Certain studies suggest that the relation between S.Homocysteine and GFR is because of creatinine, from which GFR values are estimated. But, studies which estimated GFR by other Method (serum creatinine, Creatinine clearance, plasma iohexol clearance, ^{51}Cr -EDTA clearance or plasma Cystatin C) have shown that declining renal function is associated with high Homocysteine levels. This inverse relation extends from normal to End stage renal disease and to hyperfiltrating diabetic nephropathy¹¹.

Some studies suggest that HHomocysteine causes intrarenal arteriosclerosis or arterial hyalinosis, resulting in reduced renal perfusion pressure. This leads to focal or global glomerulosclerosis, tubular atrophy and interstitial fibrosis. This can also be the reason for a negative relation of S.Homocysteine with GFR^{11,12}.

Carotid Intima Media Thickness And CKD:

In the present study, the mean CIMT increase with progress in Stages of CKD. In the controls the

mean value is $0.055 \text{ cm} \pm 0.006$, $0.090 \pm 0.021 \text{ cm}$ in stage 3, $0.099 \pm 0.031 \text{ cm}$ in stage 4, $0.109 \pm 0.031 \text{ cm}$ in stage 5 (table 1, fig 2). ANOVA shows significant association between CIMT and CKD. ($p=.000$) (table 2) This association is consistent with previous studies done by Baptista et al¹³, Benedetto et al¹⁴, Bevc et al¹⁵, Kumar et al¹⁶, Ryuichi et al¹⁷ and Zoungas et al¹⁸.

The main reasons for the increased incidence of atherosclerosis in CKD are dyslipidaemia, oxidative stress, Hyperhomocysteinemia and raised markers of inflammation (CRP, fibrinogen and cytokines)².

Serum Homocysteine And Atherosclerosis. In this study, Carotid intima media thickness, a marker of atherosclerosis, shows a significant positive linear relation with homocysteine values (table 3 FIG 3) ($p=0.002$, $rsq=0.1429$). This relation is consistent with most of the previous studies.

Several mechanisms have been postulated by which Homocysteine might cause atherosclerosis and atherothrombosis:

Homocysteine metabolism generates reactive superoxide radicals which cause endothelial injury. It promotes vascular smooth muscle proliferation by stimulation of the mitogen-activated protein kinase signal transduction pathway and DNA synthesis. Homocysteine promotes adhesion between neutrophil and endothelial cells. Homocysteine oxidizes LDL and promotes the cellular uptake of modified LDL¹⁷. Homocysteine induces the expression of TDAG51 which increases apoptosis and the risk of rupture of atherosclerotic lesions by decreasing its stability²⁰.

Conclusion: Mild to moderate hyperhomocysteinemia exists in CKD and Serum homocysteine shows a significant negative correlation with GFR. Carotid intima media thickness is significantly elevated in the groups of CKD and it shows a significant positive relation with S.Homocysteine.

This suggests that HHomocysteine in CKD is atherosclerotic and early detection of atherosclerosis can be done with carotid intima media thickness study. Along with the screening and treatment of other risk factors of

atherosclerosis in CKD, hyperhomocysteinemia should also be treated. Further experimental, biochemical, genetic and prospective follow up studies are required for understanding the pathophysiology and consequences of hyperhomocysteinemia in CKD.

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