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THE STUDY OF SERUM PCSK9 IN CHRONIC KIDNEY DISEASE

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Abstract

Introduction: Chronic Kidney Disease (CKD) is associated with abnormal kidney function and a progressive decline in GFR ≤ (60ml/min/1.73m^2) and irreversible reduction in nephrons. PCSK-9 (Proprotein convertase subtilisin/kexin type 9) is a new biomarker which has been observed to be raised in CKD patients.

Aim: To study relationship of serum PCSK-9 with different parameters like age, gender and Body Mass Index (BMI) in CKD patients.

Materials and methods: The study was conducted in department of Biochemistry of Dharbanga Medical College, Laheriasarai from 1st May 2018 to 31st December 2018. 60 patients suffering from CKD in between 16 years to 65 years of age of both genders were consecutively enrolled. Serum PCSK9 was estimated using Ultra-Sensitive Human PCSK9 ELISA EK1147 PicoKine^{TM} (BOSTER) and Quetelet Index (Weight in Kgs/ Height in meters^2) was used to calculate BMI. Results were statistically analysed.

Observation: Serum PCSK 9 levels didn't show any statistically relevant relationship with age. However, levels were significantly more in females (2390.05±1088.78 ng/ml) than in males (1658.10±1245.78 ng/ml). Moreover, its levels also increased as BMI increased. With BMI<25 kg/m^2 it was 1312.50 ± 1128.78 ng/ml (Mean ±SD), with BMI 25-29 kg/m^2 it increased to 2186.62 ± 1185.62 ng/ml (Mean ±SD) and with BMI >- 30 Kg/m^2 it reached 2864.47 ± 753.39 ng/ml(Mean ±SD). This rise was statistically significant (p-0.05).

Conclusion: This study concluded that serum PCSK9 was significantly higher in females suffering from CKD and with increase in BMI, its levels increase.

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Keywords: Chronic Kidney Disease (CKD), Proprotein convertase subtilisin/kexin type 9 (PCSK9), Body Mass Index (BMI), LDL Cholestrol and Ultra-Sensitive Human PCSK9 ELISA EK1147 PicoKine^{TM} (BOSTER).

1. Introduction

Chronic Kidney Disease is a debilitating illness which is pathophysiologically defined as abnormal kidney function with a progressive decline in GFR ≤ (60ml/min/1.73m^2) and with continuing of process, there is significant irreversible reduction in nephron number.[1]
Worldwide prevalence of CKD in adult population is about 11%.[2] In India the prevalence of CKD is about 17.2%.[3] In males the prevalence of CKD is about 57% whereas in females the prevalence of CKD is about 43%.[4]
PCSK-9 (Proprotein convertase subtilisin/kexin type 9) is an enzyme encoded by PCSK9 gene in humans on chromosome 1. It is the 9th member of proprotein convertase family of proteins that activate other proteins. Its cytogenetic location is 1p32.3 which is a short arm of chromosome 1 at position 32.3 and its molecular location is base pairs 55,039,548 to 55,064,853 on chromosome 1.[5]
PCSK9- was first discovered in 2003 by Seidah et al. Since then, it has become a real point of interest because it was proved that this protein plays a crucial role in LDL- cholesterol metabolism.[6]
It is a 75 Kilo-Dalton protein synthesized mainly in liver and small intestines as a 692 amino acid sequence. The autocatalytic processing occurs in an endoplasmic reticulum and forms the mature and stable protein, which is then secreted into the blood stream. Based on its protein structure, removal of the signal peptide (amino acids 1-30) produces a secreted heterodimer protein with 3 domains:

i. A pro-domain (amino acids 31-152) - it undergoes autocatalytic cleavage but continues to associate with the rest of the protein.
ii. A catalytic domain (amino acids 153-454) – it contains a proteolytic active site which is inactivated by the associated pro-domain.
iii. C-terminal domain – (455-692 residues).[7]

Half-life of PCSK-9 under normal conditions is 5 minutes. Low Density Lipoprotein Receptor (LDLR) is the major regulator for it in both humans and mice. In presence of LDLR, its half-life reduces to 2.9 minutes (50%) and in its presence its half-life increases to 3 to 10 minutes above normal. PCSK9 binding to LDLR has been described as biphasic, with a first rapid phase characterized by a half-time of 6.6 minutes, which accounts for 35% of the equilibrium binding and a second slow phase who's half-time is 94 minutes. Similarly, 25% of the PCSK9 bound to LDLR dissociates during the rapid phase with a half-time of 19 minutes, while the remaining PCSK9 dissociates slowly with a half-time of 297 minutes. Despite the rapid binding of PCSK9 and internalization of LDLR, PCSK9-mediated degradation of LDLR in vitro has only been observed after several hours. It was further shown that, at least in mice, PCSK9 remains intact in the liver for up to 4 hours after its internalization, thus suggesting that other events might be required in order to allow PCSK9-mediated degradation of LDLR (or LDLR mediated degradation of PCSK9).[8]
PCSK-9 is regulated by the SREBP through a sterol regulatory element motif in the promoter region.[9] Its main functions are neuronal differentiation, regulation of LDL metabolism, LDLR internalization and degradation in liver, production of triglyceride rich apolipoprotein-B in small intestines, regulation of expression of Very-Low Density Lipoprotein Receptors (VLDLRs) and apo-E receptor 2 in brain, binding to VLDLR and apo-E 2 and causing

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lysosomal degradation of these receptors, reducing epithelial sodium channel receptors and inhibiting them.[10]
Present study was an attempt to study relationship of serum PCSK9 with different parameters like age, gender, serum LDL cholesterol and Body Mass Index (BMI) in CKD patients.

2. Materials and methods

The study was conducted in department of Biochemistry of Dharbanga Medical College, Laheriasarai from 1st May 2018 to 31st December 2018. 60 patients suffering from CKD in between 16 years to 65 years of age of both genders were consecutively enrolled. Permission was taken from Institutional Ethical Committee. All subjects were explained about the study in their own vernacular language and written informed consent was obtained.

1. Sociodemographic data were recorded on sociodemographic proforma-1

2. Sample collection

2ml of venous blood collected from the antecubital vein after taking full antiseptic precautions. Blood was allowed to clot at room temperature and then centrifuged for 20 minutes and the serum was separated and stored at 2-8 degree centigrade and estimation was done on next day.

3. Estimation of Serum PCSK9 Level

Ultra-Sensitive Human PCSK9 ELISA EK1147 PicoKine™ (BOSTER) was used to estimate serum PCSK9. Human PCSK9 Pre-Coated ELISA (Enzyme-Linked Immunosorbent Assay) kit is a solid phase immunoassay specially designed to measure Human PCSK9 that is pre-coated with antibody specific for PCSK9. The detection antibody is a biotinylated antibody specific for PCSK9. The capture antibody is monoclonal antibody from mouse, the detection antibody is polyclonal antibody from goat. The kit contains recombinant Human PCSK9 with immunogen.

Working reagent was prepared in following steps: -

All reagents were brought at room temperature prior to use.

1. Wash buffer- given powder was dissolved in 1000ml of deionized water.
2. Biotinylated Anti-Human PCSK9 antibody -It is recommended to prepare this reagent immediately prior to use by diluting the Human PCSK9 Biotinylated antibody (100x) 1:100 with Antibody Diluent. 100 µl was prepared by adding 1 µl of Biotinylated antibody (100x) to 99 µl of Antibody Diluent for each well. Then it was mixed gently and thoroughly and used within 2 hours of generation.
3. Avidin-Biotin-Peroxidase Complex -It is recommended to prepare this reagent immediately prior to use by diluting the Avidin-Biotin Peroxidase Complex (100x) 1:100 with Avidin-Biotin-Peroxidase Diluent. 100 µl was prepared by adding 1 µl of Avidin-Biotin-Peroxidase Complex (100x) to 99 µl of Avidin-Biotin-Peroxidase Diluent for each well. Mixed gently and thoroughly and was used within 2 hours of generation.
4. Human PCSK9 Standard- It is recommended that the standards be prepared no more than 2 hours prior to performing the experiment. One 10ng of lyophilized Human PCSK9 standard has been used for each experiment. Gently the vial was spined prior to use. The standard was reconstituted to a stock concentration of 10ng/ml by using 1ml of sample diluent. Then the standard was allowed to sit for a minimum of 10 minutes with gentle agitation prior to making dilutions.

Dilution of Human PCSK9 standard was done in following steps:-

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1. First tubes were numbered from 1-8. Final Concentrations to be Tube # 1 –10000pg/ml, #2 –5000pg/ml, #3 – 2500pg/ml, #4-1250pg/ml, #5 – 625pg/ml, #6 – 312.5pg/ml, #7 – 156.25pg/ml, #8 – 0.0 (Blank).
2. For standard #1, 1000µl of undiluted standard stock solution was added to tube #1.
3. 300 µl of sample diluent was added to tubes # 2-7.
4. To generate standard #2, 300 µl of standard #1 from tube #1 was added to tube #2 to prepare a final volume of 600 µl. It was mixed properly.
5. To generate standard #3, 300 µl of standard #2 from tube #2 was added to tube #3 to prepare a final volume of 600 µl. Mixture was mixed properly.
6. Similar serial dilutions were continued from tube #4-7.
7. Tube #8 was set as a blank standard to be used with every experiment.

Standard curve for calibration was prepared as follows:-

1. All reagents and working standards were prepared as directed previously.
2. Excess microplate strips were removed from the plate frame and sealed and stored in the original packaging.
3. 100 µl of the standard, samples, or control per well were added and 100 µl of the sample diluent buffer was added into the control well (Zero well). At least two replicates of each standard, sample, or control are usually recommended.
4. It was all covered with the plate sealer provided and incubated for 120 minutes at RT (or 90 min. at 37 °C).
5. Cover was removed and liquid was discarded in the wells into an appropriate waste receptacle. Then plate was inverted on the bench top onto a paper towel and tapped to gently blot any remaining liquid. It is usually recommended that the wells are not allowed to completely dry at any time.
6. 100 µl of the prepared 1x Biotinylated Anti-Human PCSK9 antibody was added to each well.
7. Plate sealer was covered and incubated for 90 minutes at RT (or 60 minutes at 37°C).
8. Plate was washed 3 times with the 1x wash buffer.
    a. Liquid was discarded in the wells into an appropriate waste receptacle. Then plate was inverted on the benchtop onto a paper towel and tapped to gently blot any remaining liquid. It is usually recommended that the wells are not allowed to completely dry at any time.
    b. 300 µl of the 1x wash buffer was added to each assay well. (For cleaner background incubate for 60 seconds between each wash). c. Then steps a-b 2 were repeated.
9. 100 µl of the prepared 1x Avidin-Biotin-Peroxidase Complex were added into each well. Plate sealer provided was covered and incubated for 40 minutes at RT (or 30 minutes at 37°C).
10. Plate was washed 5 times with the 1x wash buffer.
    a. Liquid was discarded in the wells into an appropriate waste receptacle. Then, plate was inverted on the benchtop onto a paper towel and tapped to gently blot any remaining liquid. It is recommended that the wells are not allowed to completely dry at any time.
    b. 300 µl of the 1x wash buffer added to each assay well. (For cleaner background incubate for 60 seconds between each wash).
    c. Then steps a-b 4 were repeated.
11. 90 µl of Colour Developing Reagent added to each well. Plate sealer provided was covered and incubated in the dark for 30 minutes at RT (or 25-30 minutes at 37°C).

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(The optimal incubation time must be empirically determined. A guideline to look for is blue shading the top four standard wells, while the remaining standards remain clear.)

12. 100 µl of Stop Solution added to each well. The colour should immediately change to yellow. Reading of each well taken in ELISA reader at 450nm.
13. The O.D. absorbance has been readied with a microplate reader at 450nm.

CONTROL VALUE (ng/ml)	ABSORBANCE (450 nm)
0	-0.016
156.2	0.2654
312.5	0.5801
625	0.9279
1250	1.3675
2900	1.8526
5000	2.498

Assay procedure: For this serum was taken in spite of calibrator and its procedure is same as done in case of calibration of standard curve and value was measured by calibration curve.

4. Body Mass Index (BMI) was calculated using Quetlet Index (Weight in Kgs/ Height in meters^2).

5. Relevant Statistical calculations used were- Mean, Standard Deviation (SD), t-value, p-value. Findings were analyzed on

Observations

This study conducted on 60 patients suffering from CKD revealed following results:-

Table 1-Relationship between Serum PCSK9 and age of patients.

Age Group (Years)	No. of patients	Serum PCSK9 (ng/ml)	Mean±SD	t-value	p-value
16-20	4	1486-2993	2408.75±687.86	0.435	0.671
21-30	10	215-3298	2108.20±1289.54		
31-40	7	54-3146	2264.43±1364.62	0.980	0.338
41-50	16	65-3216	1698.38±1236.15		
51-60	18	45-3291	1897.39±1294.14	1.247	0.226
61-70	5	89-2617	1099.60±1137.44		

Above table shows that serum PCSK 9 level didn't show any relevant relation with age of patients. Its levels were maximum in age group 31-40 years (2264.43 ± 1364.62 ng/ml) and minimum in age group 41-50 years (1698.38 ± 1236.15). Which was statistically insignificant.

Table 2- Relationship between Serum PCSK9 and gender of patients.

Gender	No. of patients	Serum PCSK9 (ng/ml)	Mean±SD	t-value	p-value
Male	41	54-3216	1658.10±1245.78	2.199	0.032
Female	19	45-3298	2390.05±1088.78		

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Above table shows a statistically significant correlation between gender of patient and serum PCSK9. Levels were more in females (2390.05±1088.78 ng/ml) than in males (1658.10±1245.78 ng/ml).

Table 3- Relationship between Serum PCSK9 and BMI of patients

BMI (Kg/m^2)	Number of patients	Serum PCSK9 (ng/ml)	Mean±SD	t-value	p-value
<25	32	45-3216	1312.50±1128.78	2.321	0.025
25-29	13	89-3128	2186.62±1185.62	1.831	0.079
≥30	15	418-3298	2864.47±753.39	4.830	0.001

Above table shows statistically significant correlation between BMI of patients and serum PCSK9 levels. With increase in BMI of patients, serum PCSK9 levels also increased.

3. Discussion

Present study conducted on 60 patients suffering from CKD revealed PCSK9 didn't show any relation with increasing age. This was contrary to many past studies which reveal that as age increases serum PCSK9 also increases.[11] Possible reason behind this could be that serum PCSK9 levels might not be efficiently cleared from the circulation, since the liver contains 70% of the total body LDL-R, it is generally accepted that majority of circulating PCSK9 levels are cleared there. It is presumed that in humans the Renal failure also reduces liver LDL-R levels and consequently PCSK9 clearance. Therefore, CKD progression leads to renal failure and it may also directly decrease the LDL-R PCSK9 clearance by the kidney and thereby contribute to its high Serum concentration observed in CKD.[12]

It was observed in this study that serum PCSK9 levels were detected more in females (2390.05±1088.78 ng/ml) than in males (1658.10±1245.78 ng/ml) which was statistically significant. This finding correlates with Wen Guo et al 2015., where serum PCSK9 level was found higher in female than in males. They also found that serum PCSK9 level were higher in post-menopausal women than in pre-menopausal women and its level were positively correlated with BMI.[11] Similar results were also found by Alexis Baass et al 2009., where serum PCSK9 level were higher in females than in males which was statistically significant.[13] This could be due to the fact that serum PCSK9 is regulated by hormones like oestrogen.[14]

Present study also revealed a positive correlation with serum PCSK9 levels and BMI. In patients with BMI < 25 Kg/m^2 levels were 1312.50±1128.78 and as BMI increased to >30 Kg/m^2 levels also increased to 2864.47±753.39. Emma MR et al 2017 also found that serum level of PCSK9 were higher in obese subjects than in normal weight individuals. Short term weight loss with low fat diet did not significantly affect PCSK9 levels.[15] Similar results were found by Amy E. Levenson et al 2018. They found that obesity was associated with significantly higher levels of PCSK9 in females.[16] Reason behind this positive correlation is that atherosclerosis and endothelial damage in vessels is associated with increase in lipids like LDLs and latter leads to obesity.[17] Many studies further have reported that obesity and increased LDL lead to increase in PCSK9 and that further leads to CKD and coronary artery disease.[18-20]

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4. Conclusion

Present study successfully reported a positive relation of serum PCSK9 with BMI and female gender, however with age, a statistically significant correlation could not be derived. Study was limited in nature as sample size was only small and it was a hospital-based study. Future large-scale studies should be carried in community settings.

Strengths of study

There are a lot of studies done on serum PCSK9 as a diagnostic marker in cardi-vascular diseases and CKD. However, there is dearth of Indian literature on this subject. Present study is just an attempt to highlight its importance.

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