

Original Research Article

Evaluation of erythrocyte membrane lipids and proteins in renal disorders

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ABSTRACT

Background: Membrane lipids and proteins play a significant part in imparting membrane its rheological properties. These parameters are altered in diseased states. Exploring the conformational changes in renal disorders can widen our understanding of its impact on the circulatory system. This could lead to a new diagnostic parameter to study the progress of a disease.

Methods: 120 blood samples collected from 30 kidney donors, 30 stage 3-4 Chronic kidney disease (CKD) patients (group 1) and 30 stage 5 CKD patients on dialysis (pre and post dialysis) (group 2) were lysed and washed to obtain erythrocyte ghost membranes. The proteins extracted from these membranes were estimated colorimetrically using Micro BCA kit. Phospholipids were separated and quantified using HPTLC. Fatty acids and cholesterol were analysed using GCMS.

Results: The erythrocyte membrane protein profile showed lower values in group 2 participants than group 1 participants, but this difference was not significant. Distinct decreases in percentages of palmitic acid, myristic acid, stearic acid, dodecanoic acid, cholesterol, phosphatidylserine, phosphatidylcholine and phosphatidylethanolamine were observed in both groups, with the lowest values in patients undergoing dialysis. Sphingomyelin and linoleic acid did not show any such trend across groups.

Conclusions: The data is suggestive of an altered membrane structure in participants undergoing dialysis patients than the control group. This could be because of uremic toxins in the circulatory system affecting the membrane lipids. Decreased levels of essential phospholipids can impact the functions and lifespan of the erythrocytes. This could be a reason behind anaemia seen in most patients with CKD.

Keywords: Erythrocyte ghost membrane, GCMS, HPTLC, Membrane lipids, Membrane proteins

INTRODUCTION

Erythrocytes undergo repetitive elongation and compression to be able to squeeze through the capillaries during microcirculation. To be reversibly deformable, its membrane must be both fluid and flexible; it should also preserve its biconcave shape, facilitating gas exchange. Membrane fluidity is a physicochemical feature of

biomembranes that is an essential factor in modulating cell rheologic behaviour.¹⁻³

Erythrocyte membrane fluidity has been reported to be impaired in patients with chronic renal failure, especially in that undergoing maintenance haemodialysis.^{4,5} This impaired membrane fluidity could be closely related to various conditions in these patients, such as increased

blood viscosity, hyperparathyroidism, uremic toxins, osmotic trauma, anaemia, and hypersplenism.⁵

Changes in erythrocyte morphology are associated with increased serum and membrane lipids.⁶ Studies show that an intramembrane redistribution of phospholipids follows increased bilirubin levels and that externalisation of membrane lipids may facilitate haemolysis.⁷ Erythrocyte membranes show significant alterations in protein status and lipid compositions in hepatic lipid metabolism, resembling the changes occurring in oxidative stress conditions.⁸ Anaemia is one of the most common additional burdens in renal patients, significantly impacting the quality of life and also in some cases resulting in mortality.^{4,5} This results from the accumulation of uremic toxins in the bloodstream and the bone marrow's inability to produce erythropoietin. There have been reports of oxidant-antioxidant imbalance and increased free radical activity in patients with renal disorders.^{9,10} Blood loss and premature erythrocyte destruction are also common in patients with stage 5 Chronic kidney disorders (CKD) undergoing haemodialysis. These factors contribute to both metabolic and mechanical stress to erythrocytes. The erythrocyte membrane fluidity had been reported to be impaired in patients with chronic renal failure, especially in that undergoing maintenance haemodialysis.^{9,11} This impaired membrane fluidity could be closely related to these patients' various conditions such as increased blood viscosity, uremic toxins, osmotic trauma, and hypersplenism. Estimation of total membrane lipid content can provide an insight into the variation of membrane lipid content in disease states and its effect on fluidity.^{12,13}

Spectrin, a significant protein network component, covers the cytoplasmic (outer) surface of the erythrocyte membranes.¹⁴ The overall membrane shape confers a flexibility on the protein and, in turn, on the red blood cell membrane. Attached to the inner aspect of the membrane of the red blood cell are several peripheral cytoskeletal proteins that play essential roles in preserving shape and flexibility. A significant function of the protein skeleton in erythrocytes is to provide mechanical support for the membrane bilayer.^{15,16}

Cholesterol is considered a prime factor for the membrane's fluidity.^{17,18} This is because of its property to regulate the mobility of the fatty acyl chain of phospholipids, preventing the transition to the solid gel state and, at the same time, rigidifying fluid membrane by reducing the flexibility of neighbouring unsaturated acyl chains. An increase in cholesterol relative to phospholipids indicates decreased fluidity.^{17,19-21} These changes can lead to sludging of blood in the microcirculatory system, blockage of vital capillaries and set up a vicious cycle of thromboischaemic complications.^{11,17,22} Each phospholipid has its own set of fatty acids. It will be interesting to observe the association of membrane proteins with cholesterol and phospholipids, specifically the inner

membrane phospholipids such as phosphatidyl-ethanolamine and sphingomyelin.^{23,24}

The field of lipidomics is constantly evolving and expanding with precise and high throughput techniques for lipid analysis. High-performance thin-layer chromatography (HPTLC) is a simple technique for biochemical studies. Phospholipids comprise a widespread class of lipids. Separating and quantifying all of these can be done using HPTLC using a complex solvent system that can provide multiple polarities for different classes of lipids.²⁵ The recent developments in Mass spectrometry (MS) and chromatography have led to greater sensitivity and accuracy faster than other techniques.^{26,27} An extensive range of profiling technologies is now available to the lipid analyst, and their applications have proven to be diverse. Gas chromatography (GC) is widely used for fatty acid separation and quantitation. MS enables the confirmation of the analyte's identity and separation of peaks from a noisy background or coeluting peaks.²⁶⁻²⁹ GC-MS coupled together can provide an ideal technique for analysing multiple biomarkers from any biological matrix.

To be separated efficiently using GC, Fatty acids (FA) need to be derivatised into volatile Trimethylsilyl (TMS) derivatives. This derivatisation can be done in the presence of pyridine with a mixture of 1% TMS chloride, Chlorotrimethylsilane (TMCS) in N, O-Bis-(Trimethylsilyl) acetamide (BSA).^{30,31} Fatty acid derivatives thus prepared can be extracted from samples by using organic solvents like n-hexane or benzene.³² The most commonly used carrier gases for analysing fatty acid methyl esters are helium, nitrogen and hydrogen.²⁷

Colorimetric methods are fast and straightforward methods for protein quantification. Bicinchoninic acid (BCA), a sodium salt, is a water-soluble compound that forms an intense purple complex with a cuprous ion (Cu^{1+}) in an alkaline environment.³³ This reaction is the base reaction for the quantitative method for measuring the Cu^{1+} ions produced in the protein reaction with alkaline Cu^{2+} . The intense, purple-coloured BCA/copper complex is water-soluble. It exhibits a strong linear absorbance at 562 nm with increasing protein concentrations, maintaining high sensitivity and low protein-to-protein variation.³⁴

Understanding the conformational changes in the erythrocyte membrane in different disease groups will help establish the relationship of membrane fluidity with renal disorders. This data generated could help identify erythrocyte membrane fluidity as a possible biomarker.

METHODS

Study type

This study was conducted from the October 2019 to October 2021 in the hospital and research facility of Seth

GS Medical College and King Edward Memorial Hospital, Parel, Mumbai.

Ethical approval

The project was commenced only after approval from the IEC of Seth GC Medical College and King Edward Memorial Hospital. Participants were briefed about the study, their involvement and confidentiality, and written informed consent was obtained from them.

Participant selection and recruitment

This study was a prospective study, where the sample size was calculated to be a minimum of 30 for each subject category.³⁵ The study groups were- (i) 30 healthy donors (control), (ii) group 1: 30 patients with chronic kidney disorders and (iii) group 2: 30 dialysis patients.

The participants were screened based on the inclusion and exclusion criteria mentioned below. Those fulfilling the criteria were shortlisted and briefed about the study. Willing participants were informed about maintaining confidentiality, their involvement, risks, discomfort, and participation compensation in this study. Blood samples were collected only after getting a signed informed consent form. Approximately 3 ml of blood was drawn in EDTA-coated vacutainers.

Blood collection was done before and after the dialysis process from group 2. All the participants required one visit only to be involved in this study. Blood samples were collected twice from patients undergoing dialysis, once before the dialysis process and once after. The samples collected amounted to a total of 120 samples for membrane studies.

The patient's samples were collected from the department of nephrology, Seth GS Medical College and King Edward Memorial Hospital, Parel, Mumbai.

Inclusion criteria

Individuals between 18-60 years of age and both genders male and female subjects were included in all three groups.

Diagnostic criteria for different groups was as follows- (a) group 1 (dialysis patients): patients in CKD stage 4-5 with CGFR<30 ml/min; (b) group 2 (renal injury): patients in CKD stage 1-3 with CGFR<59 ml/min and increase in SCr by 0.3 mg/dl times ($\times 26.5$ l mol/l) within 48 hours; and (c) group 3 (control): patients with no history of renal disorder, screened and cleared as kidney donors with CGFR>90 ml/min.

Exclusion criteria

Patients who were paediatric, terminally ill and who were not willing to participate were excluded.

Chemicals

HPLC grade n-hexane, methanol, ethyl acetate, dichloromethane, chloroform and n-propanol, and analytical grade copper sulphate, ethanol and butanol, were purchased from Merck. Reagent grade pyridine was purchased from Qualigens. Extra pure, TMCS and BSA having 95% purity, were purchased from SRL. Phospholipid standards of L- α -phosphatidylethanolamine PE (>97% purity), L- α -phosphatidylcholine PC (>99% purity), Sphingomyelin SM (>90% purity), Cholesterol (>99% purity), 1,2-Diacyl-sn-glycerophospho-L-serine PS (>97% purity) were purchased from Sigma Aldrich.

Ghost membrane preparation

Erythrocyte ghost membranes were prepared following the method by Steck and Kant with slight modification.³² Ghost membranes were prepared using 3 ml of blood samples collected in tubes coated with EDTA. These were centrifuged at 8000 rpm for 10 min at 4°C to separate the serum and buffy coat from the erythrocytes. Cells were washed thoroughly with saline and separated by centrifugation at 5000 rpm for 10 min at 4°C. Cells lysis was done using lysis buffer of 8.3 g NH₄Cl, 1.0 g KHCO₃, 1.8 ml of 5% EDTA in 1 l distilled water, pH 7.4. Lysis was done by thoroughly vortexing and centrifuging at 14000 rpm speed for 12 min at 4°C. The ghost membranes thus obtained were washed with Phosphate buffer saline (pH 7.8). The final erythrocyte pellet was suspended and stored in PBS at -20°C.

Protein extraction

Membrane proteins were extracted by the method described by Muinao, Pal and Boruah, where both detergents Triton X-100 and Sodium dodecyl sulphate (SDS) with aprotinin as protease inhibitor.³⁷ These extracts were labelled as A and B where, part A comprises the hydrophilic proteins, which constitute the inner leaflet of the membrane bilayer, such as spectrin ankyrin etc. 10 μ l of this buffered solution was diluted using 90 μ l of PBS. Part B comprises the proteins embedded in membrane lipids, such as the integral proteins. The membrane aliquot suspended in PBS was briefly centrifuged to separate the PBS (part A) from the membrane. The ghost membrane pellet (part B) was then resuspended in 150 μ l of lysis buffer comprising 50 mM Tris, 150 mM NaCl, 1% SDS, 1% Triton X-100 and 0.2% of aprotinin at pH 7.5. Extraction was done at 4°C using an ice bath to prevent denaturation of proteins. The samples were incubated for 20 min followed by sonication for 3 \times 10 amplitude at 4°C. The samples were then centrifuged at 8000 rpm at 4°C for 20 mins. 10 μ l of the supernatant was used for protein estimation.

Protein estimation

Protein quantification was done using the Micro BCA protein assay kit by Thermo Scientific. Extracted proteins

were diluted at a 1:9 ratio using PBS solution. This dilution was done to reduce the interference of SDS and Triton 100 \times to 0.1%, nullifying their interference during colorimetric reading. 100 μ l of working reagent was added to all the wells, and the microplate was briefly shaken using a plate shaker. The 96-well plate was covered with aluminium foil and incubated at 30°C for 2 hrs. The plate was allowed to cool down after incubation, and absorbance was measured at 562 nm using spectrometer SPECTROstar Nano by BMG Labtech. The protein content of the 10 μ l aliquot of the unknown samples was determined by linear regression using the absorbance of the blanks and 100 μ l BCA standards (0.5-200 μ g/ml range). The final protein content in the 1 ml sample was calculated and expressed in percentage. The results were analysed individually and separately to understand the integral protein and cytoskeletal membrane contents. The percent content of these proteins was used for further statistical analysis.

Lipid extraction

The erythrocyte membrane lipid content was determined by extracting the lipids using the modified Bligh and Dyer method.^{26,38} The ghost membranes were thoroughly vortexed, followed by sonication in chloroform: methanol (2:1) for 30 min at 37°C. These samples were then transferred in fresh glass tubes where 2 ml of chloroform: methanol: hydrochloric acid: distilled water (2:1:1:0.5) (0.1 N methanolic HCl). The samples were thoroughly vortexed for 30 sec and centrifuged briefly at 3000 rpm. The lower layer of chloroform was collected in a fresh tube, and the previous step was repeated two more times. 1 ml of hexane was added in the remaining aqueous phase to extract the remainder lipids. This layer of hexane was also added to the chloroform collected. The organic solvent mixture collected was kept for evaporation overnight at 37°C. The use of a nitrogen evaporator was avoided to prevent lipid peroxidation. Post evaporation, dried lipids at the base of the tube were reconstituted in 0.2 ml chloroform and used for further analysis.

HPTLC of phospholipids

A standard mixture of phospholipids (PS, PE, PC and SM) ranging from 125-1250 μ g was applied and separated on TLC plates to obtain linearity of these lipids (Figure 1). Phospholipids were separated using the mobile phase of chloroform: ethyl acetate: acetone: isopropanol: ethanol: methanol: water: glacial acetic acid (30:6:6:6:16:28:6:2) up to 60 mm of the plate. The plate was dried for 5 min at 90°C in a hot air oven.

After the plate had cooled down, 10 ml charring reagent composed of 1% CuSO₄ (w/v) mixture and 8% H₃PO₄ (v/v) was carefully applied using a pipette. The plate was gently swerved side to side for approximately 60 s. Following this, the excess of charring reagent was removed by decanting. The back of the plate was cleaned with a dry tissue to remove the excess of charring reagent.

Finally, the plate was heated for 30 min at 120°C. The density of the developed bands of phospholipids was analysed by HPTLC scanner, quantified using Wincats software, and expressed in percent content. This linearity run was repeated six times to assess the method's repeatability. The linear graph thus plotted was used to calculate the concentration of phospholipids the lipid extracted from erythrocyte membranes.

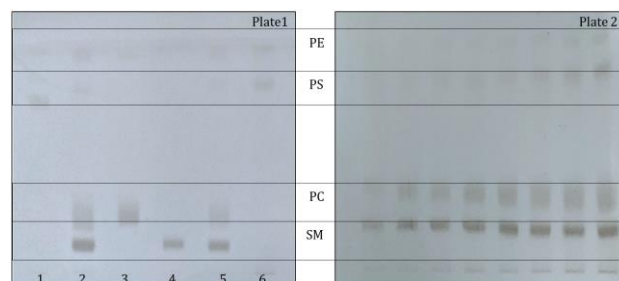


Figure 1: Plate 1- standards and standard mixture track 1 Phosphatidylserine (PS), track 2 and 5 standard mixture, track 3 Phosphatidylcholine (PC), track 4 Sphingomyelin (SM), track 6 Phosphatidylethanolamine (PE); and plate 2- linearity of standard mixture of ranging from 125 μ g to 625 μ g of PS and PE and 250 μ g to 1250 μ g of SM and PC.

5.0 μ l of the reconstituted membrane lipid extract was applied to an activated TLC plate. TLC was performed on 10 cm \times 10 cm TLC plates, pre-treated (full height) in a Camag developing chamber by Merck to remove impurities, using a mobile phase of methanol: ethyl acetate (6:4). The plates were activated for 10 min at 130°C in a hot-air oven. Samples were applied as narrow bands (6 mm) to the activated TLC plate at 8 mm from the edge using the Camag Linomat IV under a stream of nitrogen gas.

The plate was then developed in a Camag vertical developing chamber containing dichloromethane: ethyl acetate: acetone (80:16:4) till 80 mm to separate cholesterol, free fatty acids, and triglycerides from phospholipids. The plate was dried for 5 min at 90°C in a hot-air oven. The plate was cooled, and phospholipids were separated by a second run using a mobile phase of chloroform: ethyl acetate: acetone: isopropanol: ethanol: methanol: water: glacial acetic acid (30:6:6:6:16:28:6:2) up to 60 mm.

The plate was derivatised, as mentioned previously, for the standard plate. Statistics data were analysed with Microsoft Excel.

GCMS of lipids

The lipids extracted from the ghost membrane were reconstituted in 200 μ l of chloroform. 25 μ l was then collected in a fresh, clean tube and evaporated. Residual lipids were resuspended in 0.100 ml pyridine and derivatised using 100 μ l 1% TMCS prepared using BSA at

90°C for 1hr after cooling down room temperature; the derivatised products were extracted in 200 µl hexane. GC was performed using Trace 1300 of Thermofisher.

The TG-5 MS column with the following specification was used for the analysis, length- 30m, I.D- 0.25 mm, film- 0.25 µm. Helium was used as a carrier gas with 1 ml/min flow rate. Split inlet with a ratio of 10:0 with a purge flow of 5.0 ml/min. 1.0 µl of the samples were injected manually, with the inlet temperature set at 250°C. The initial column oven temperature was 100°C, which was eventually raised to 200°C, at a ramp of 10°C/min, carried ahead to 290°C, at a ramp of 20°C/min and held for 10 min, which amounted to a total run time of 20.08 min.

MS was performed using ISQLT by the ThermoScientific. Ion source temperature was 240°C with EI ionisation at 70 eV. The mass range was set at 45-800 at the scan time of 1 sec. The lipids separated and detected were expressed in their percent content, and data were analysed with Microsoft excel.

Statistical analysis

There were four sets of data generated- (a) controls, (b) test group, (3) test group 2- predialysis, and (d) test group 2 post-dialysis. These four sets of data were treated as individual data sets. The data generated by HPTLC analysis, GCMS, and protein colourimetry studies were individually screened to observe any data trend using regression analysis in 4 different test groups. The quantitative data are presented as mean (standard deviation), median (range) and standard error. The qualitative data are presented in the form of frequency and percentage. All analyses were conducted using the Microsoft excel version.

RESULTS

Protein estimation

Of the total proteins quantified, 'part A' of the protein extract, which comprised of the proteins embedded in the membrane, showed a mean of 69.1% in the control group, 75.7% in group 1 (CKD stage 3-4 patients), 66.1% before dialysis and 69.68% post-dialysis. While the percentage of proteins in 'part B' of the membrane extract showed a mean of 30.9 in the control group, 24.3% in group 1, 33.8% in group 2 pre-dialysis and 30.32% group 2 post-dialysis. Figure 2 shows the trends observed in the total protein composition in the three groups. Quantitatively, the control showed a range of about 45-89 µg of hydrophobic proteins with a range of 11-55 g of hydrophilic proteins.

Group 1 were within the range of 45-92 µg of hydrophobic proteins and 8-55 µg of hydrophilic proteins. Group 2, undergoing dialysis, was within 25-82 µg of hydrophobic proteins pre-dialysis and 30-95 µg of hydrophobic proteins post-dialysis. Group 2, undergoing dialysis, showed about 18-75 µg of hydrophilic proteins pre-dialysis and 5-70 µg of hydrophilic proteins post-dialysis.

HPTLC of phospholipids

A standard mixture comprising 500 ppm of each standard (phosphatidylcholine, phosphatidylethanolamine, sphingomyelin and phosphatidylserine) was prepared (Figure 2). This mixture was run using the previously mentioned method to obtain a linear graph. This helped in establishing a range for the phospholipids.

SM was separated closest to the point of application with the (Retention factor) Rf of 0.12 (Figure 3). The percentage of SM in the control group was 23.19%, group 1 contained 21%, group 2, pre-dialysis contained 22.39%, and post dialysis was 22.74%.

Phosphatidylcholine (PC) was separated next with the Rf of 0.27. The mean percentage of PC in the control group was 38.45%, while in Group 1 was 23.06%, and dialysis group, group 2, pre-dialysis was 26.01%, and post-dialysis was 21.18%. Phosphatidylserine (PS) was separated next with the Rf of 0.60 (Figure 3).

Mean percentage PS in control group 20.42% while the group 1 was 21.03%, and in the dialysis group, group 2, pre-dialysis showed 15.87% and post-dialysis 13.90%. Phosphatidylethanolamine (PE) was separated farthest, with the Rf of 0.75. The mean per cent in the control group was 28.95%, while group 1 was 26.42%, and the dialysis group, group 2, pre-dialysis was 27.29%, and post-dialysis was 22.44%.

GCMS of lipids

The GCMS database searched against the separated peaks. TMS derivatives of dodecanoic acid (C10), palmitic acid (C16), stearic acid (C18:0), oleic acid (C18:1), and cholesterol with Retention time (RT) of 6.6 min, 9.04 min, 9.9 min, 9.4 min, and 16.6 min, respectively (Figure 6) were observed.

These molecules, along with smaller peaks of TMS derivatives of other fatty acids, glycolic acids and a few methyl esters, were well-separated products formed during derivatisation identified based on the elucidation structural characteristics and mass profiles.

TMS ester of the acyl group in palmitic acid and other fatty acids was also found and identified. Analysis of the erythrocyte membranes' fatty acid components was performed by evaluating the fatty acid percentages and correlating the percentages of different groups, together with the sums of cholesterol, MUFA, and PUFA and some indicative ratios. There were differences in the fatty acid profiles of erythrocyte membranes derived from both groups 1 and 2 of CKD patients versus matched controls (Figure 4, Figure 5).

GCMS of TMS derivatives of palmitic acid showed a mean of 10.6% in the control group and 10.4% in group 1, group 2, undergoing dialysis showed about 7.11% pre-dialysis 5.88% post-dialysis.

Table 1: Demographic data of study participants.

Participant groups		Gender		
		Female	Male	Total
Group 1: CKD	Number	9	21	30
	%	30	70	100
Group 2: patients undergoing dialysis	Number	10	20	30
	%	33.33	66.67	100
Control	Number	18.00	12.00	30
	%	60.00	40.00	100
Total	Number	37.00	53.00	90
	%	41.11	58.89	100

Table 2: GCMS results of membrane lipids.

Lipid	Participant group	Mean %	Range	SE	
Palmitic acid	Control	10.62	3.02-18.47	0.72	
	Group 1	10.41	3.19-16.27	0.66	
	Group 2: dialysis	Pre	7.11	1.54-16.85	1.02
		Post	5.88	0.11-16.27	1.00
Linoleic acid	Control	2.55	0.17-8.08	0.46	
	Group 1	1.62	0.28-6.75	0.31	
	Group 2: dialysis	Pre	2.26	0.31-6.74	0.39
		Post	1.96	0.16-6.56	0.40
Myristic acid	Control	2.76	0.31-6.63	0.28	
	Group 1	2.66	0.24-5.56	0.30	
	Group 2: dialysis	Pre	2.35	0.67-4.33	0.20
		Post	1.41	0.13-4.03	0.20
Dodecanoic acid	Control	6.56	1.05-12.53	0.64	
	Group 1	6.14	0.24-14.6	0.81	
	Group 2: dialysis	Pre	2.48	0.18-8.21	0.45
		Post	2.45	0.24-10.37	0.54
Octadecanoic acid	Control	10.69	6.51-15.38	0.55	
	Group 1	6.12	1.05-14.79	0.67	
	Group 2: dialysis	Pre	2.15	0.13-5.73	0.31
		Post	2.31	0.07-6.44	0.32
Arachidonic acid	Control	2.29	0.13-9.22	0.46	
	Group 1	2.06	0.06-5.46	0.45	
	Group 2: dialysis	Pre	2.21	0.22-8.48	0.40
		Post	2.01	0.05-7.12	0.48
Cholesterol	Control	25.64	17.13-36.22	0.94	
	Group 1	16.93	8.64-34.03	1.20	
	Group 2: dialysis	Pre	13.95	3.74-29.7	1.30
		Post	14.64	5.83-35.20	1.30
Oleic acid	Control	3.44	0.16-9.76	0.43	
	Group 1	2.75	0.19-9.81	0.48	
	Group 2: dialysis	Pre	2.45	0.13-6.27	0.38
		Post	3.11	0.19-11.44	0.65

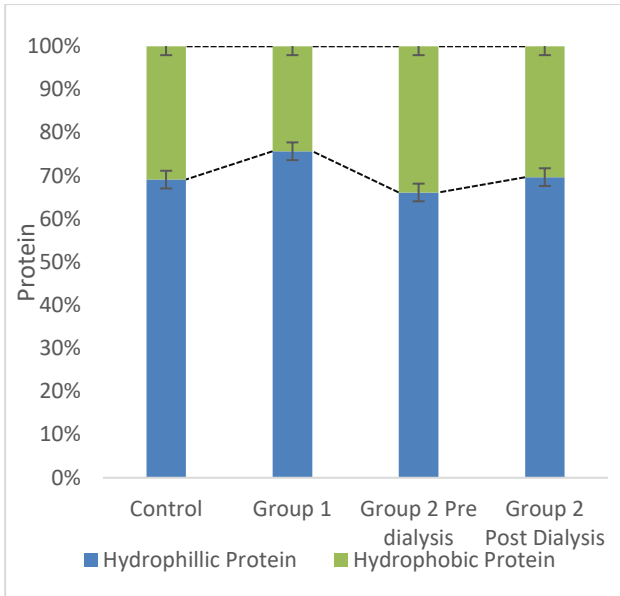


Figure 2: Percent protein composition of erythrocyte membrane in different participant groups. Each bar represents total percentage of proteins composed of hydrophilic proteins (blue) and hydrophobic proteins (green).

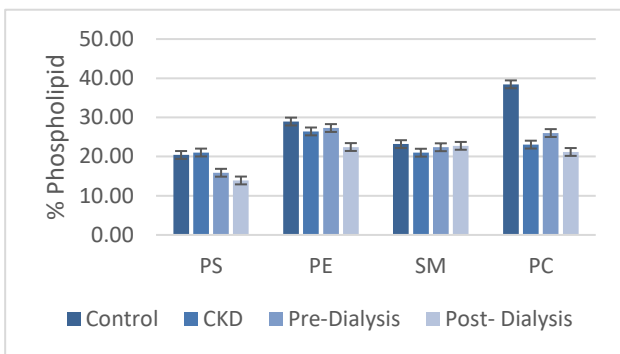


Figure 3: Percentage phospholipid composition of erythrocyte membrane in different groups by HPTLC.

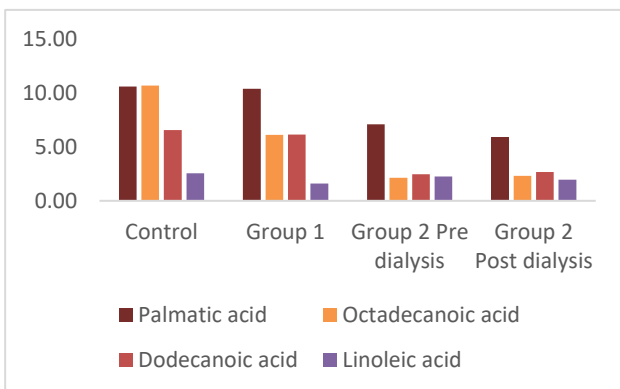


Figure 4: Mean percentage composition of palmitic acid, octadecanoic acid, dodecanoic acid and linoleic acid of erythrocyte ghost membranes across different groups of renal disorders.

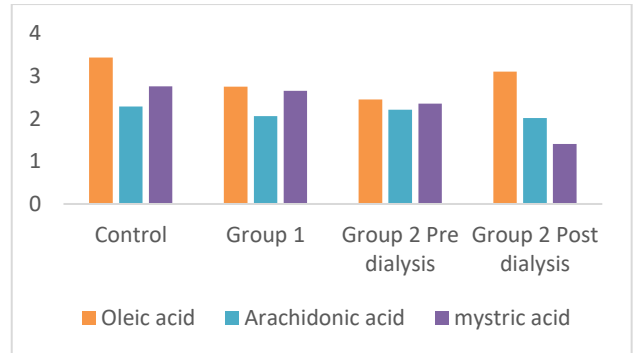


Figure 5: Mean percentage composition of oleic acid, arachidonic acid and myristic acid in erythrocyte ghost membrane across different groups of renal disorders.

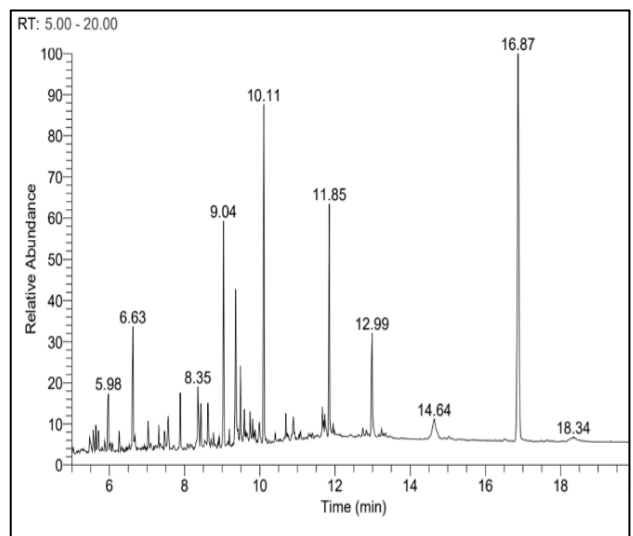


Figure 6: Chromatogram obtain after GCMS of lipid extract derivatised by 1% TMCS in BSA. TMS derivative of dodecanoic acid is detected at Rt 6.6 followed by myristic acid at 7.8, palmitic acid at 9.04, linoleic acid at 9.15, stearic acid at 9.4, octadecanoic acid at 9.9, arachidonic acid at 10.2 and cholesterol at 16.8 towards the end.

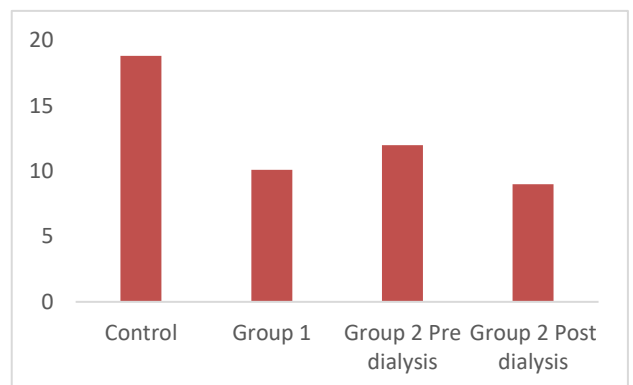


Figure 7: Mean percentage composition of cholesterol in the erythrocyte ghost membrane across different groups of renal disorders.

DISCUSSION

The composition and organisation of lipids at the cellular level is tightly regulated. So far, the analysis of blood cell lipid metabolism in human subjects has been primarily confined to variations in metabolic and vascular diseases. The functional properties of erythrocytes depend on the composition of the bilayer. Modification of lipid: protein ratio and changes in the degree of the membrane Fatty acids increase the lipid bilayer packing density and membrane microviscosity.^{1,39}

HPTLC of membrane lipids provided with distinct separation of various phospholipids and other lipids. Of these, only the phospholipids corresponding with the standards used were noted, and the percentage reported was from the total of these four phospholipids. SM did not significantly change across the groups from the data generated, while PS, PE and PC showed a distinct decrease from the control to the dialysis group. PC is a vital phospholipid comprising about 50% of total phospholipids in a healthy erythrocyte.²⁶

One of its primary roles in the bilayer is contributing as a bulking agent. It also provides an anchoring site for membrane proteins such as cytochrome C oxidase. A decrease in PC could result in the membrane losing its compact structure and lesser membrane proteins.¹⁴ PS is one of the most abundantly present anionic phospholipids present in the inner leaflet of the bilayer.¹⁶ It regulates the charge across the bilayer.

It is also a precursor to PE. Changes in PS content could impact the net membrane charge: its rigidity and membrane-protein interaction.¹⁸ Reduced PS in the membrane could also explain the reduced PE content. Both PS and PE have significant roles in chaperoning proteins to their folded state and regulating membrane charge for membrane proteins to function correctly.

The study of the membrane lipid composition showed that high stearic and oleic acid content is common to all lipid extracts. A high degree of saturation is characteristic of the sphingomyelin fraction comprising behenic, arachidic, palmitic, myristic and stearic fatty acids. Experimental results suggest that changes in the FA composition of erythrocyte membranes reduce as the severity of renal disorder increases.

The GCMS results demonstrate a significantly higher ratio between cholesterol and fatty acids, which could raise susceptibility to peroxidation. A significant increase in the percentage cholesterol content of the erythrocyte membranes of patients with renal disorders than the control group may explain the higher effect of the uremic toxins on the membrane. Erythrocytes because of their intrinsic potential for free radical generation, attributable to their remarkably high oxygen content, might be a suitable environment for cholesterol to exert its pro-oxidant reaction.¹¹ Thus, there could be a linkage of

membrane susceptibility to peroxidation in patients undergoing dialysis than with stage 3-4 CKD patients.

The erythrocyte membrane protein profile showed lower values in CKD stage 5 patients undergoing dialysis than CKD stage 3-4 patients. A trend to lower values for embedded proteins was observed in group 1 compared to the control group. Pre and post dialysis samples also showed altered membrane composition. Although changes were observed, these changes in membrane proteins were not significant. However, a trend can be obtained when associated with the changes in lipid content, specifically the saturated fatty acids. Separating these proteins and understanding the changes in their quantities by electrophoresis or other such proteomic technique can give a better insight into the effect of renal disorders on membrane protein composition.

Limitations

A larger sample size and monitoring of the erythrocyte membrane composition with the progress of disease can give a better insight into the impact of uremic toxins on membrane structure along with different renal functional parameters. Given that most of the participants from the patient group were above age of 40 years while those of control were below it, the impact of ageing on the vascular system can also be not ignored. Factors such as age, diet and stress can have a significant impact on different physiological processes.

CONCLUSION

This study shows how membrane lipid and protein compositions are altered in different stages of renal disorders. Data shows significant in membrane cholesterol and phosphatidylcholine composition of patients with CKD. This could be due to uremic toxins incorporated within lipid at the membrane surface. The process of dialysis rids the membrane surface of binding uremic toxins, reflected in a slight change in composition post-dialysis. All these might affect the phospholipid bilayer's packing properties that form the cellular membrane, altering its biochemical-biophysical characteristic by affecting the packing properties and flexibility. Unsaturated fatty acids and phospholipids of the cell membrane are essential in maintaining membrane fluidity. Changes in the physical properties of membrane bilayers alter cell metabolism, production of reactive oxygen species, and activity of membrane-associated proteins. Consequently, their structure changes dramatically during the membrane lipids oxidation, and the erythrocyte functions are disturbed. Increased lipid peroxidation may reduce hydrophobicity and result in the formation of covalent cross-links between molecules of lipid or lipids and proteins, damaging cell membranes leading to a significant impact on the lifespan of the cells. This could explain the increased cases of anaemia in renal patients. Further comparative proteomic studies are required, which help better understand the membrane proteome's

compositional changes. Integrated omics approaches and high-throughput studies in uremic blood and erythrocytes may reveal proteins, metabolites, and other factors that mediate membrane fluidity. Extending the application of metabolite, proteome, and lipidome profiling to the erythrocyte membrane is challenging to broaden the understanding of erythrocyte membrane composition renal disorders.

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