Estimation Of RBC Membrane And Serum Lipid Composition In Central Indian Sickle Cell Disease Population With And Without Pulmonary Hypertension.

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Abstract : Aim: This study is designed to estimate and examine the relation between the levels of RBC membrane and serum lipids in central Indian sickle cell disease population with and without Pulmonary Hypertension .Methods: This study was carried out on central Indian sickle population at the Dept.of Biochemistry at MGM medical college & M.Y. hospital, Indore. From june 2011 to October 2012. Plasma Lipid concentrations were determined in 135 Sickle cell disease (SCD) patients, out of this 65 patients had sickle cell disease with Pulmonary Hypertension (SCD-PH) and 80 normal healthy matched individuals (controls). Study group comprises of both male and females in the age group of 18-56 years. Weight , height, waist hip ratio and blood pressure were recorded. All the blood samples were analyzed to determine the serum lipid concentration and RBC membrane lipid composition. Results: The body mass index and the systolic blood pressure of SCD with Pulmonary Hypertension (27.87 ± 4.68, 128.60 ± 22.49 mmHg) and without pulmonary hypertension (25.87 ± 4.68, 125.23 ± 15.89 mmHg) were higher when compared with controls (24.67 ± 5.18, 119.15 ± 13.03 mmHg). The SCD with PH population (1.21 ± 0.07) and SCD without PH (1.09 ± 0.04) subjects showed significantly higher levels of RBC membrane cholesterol compared with controls (0.84 ±0.01). The trends of decreased serum cholesterol and normal high-density lipoprotein(HDL) levels in SCD patients were noted as compared with controls and these levels are statistically significant. The low-density lipoprotein cholesterol (LDL) was also significantly lower in SCD-PH and SCD when compared with control subjects. Interestingly serum Triglyceride levels are highly elevated in SCD-PH (208.43±.56.97) when compared with SCD (132.34±8.97) and controls (141.43±26.98). Elevated TGL concentrations are positively correlated with haemolytic markers (Lactate dehydrogenase and Total Bilirubin, ($r=0.326$ and $r=0.468, P<0.001$)). On a prospective screening of SCD population we found that around one third total population are with elevated tricuspid regurgetant jet velocity (TRJV) of 2.5m/s or higher. Conclusion: Our study data suggests that there is a relationship between RBC membrane and serum lipids in SCD population. Significant increase in RBC membrane cholesterol and decreased phospholipids in sickle cell disease play an important role in the fluidity and structural stability of the membrane and possibly in the Hemolysis and sickle shape of RBC. In pulmonary hypertension except the elevated levels of TGL remaining serum lipids are equal to the normal SCD population. This indicates the relation between TGL and vascular dysfunction in SCD. [Agnihotram G et al NJIRM 2013; 4(5) : 6-14]

Key Words: sickle cell disease, Pulmonary Hypertension, Tricupsid Regurgetanr Jet Velocity, Plasma Triglycerides, Vasoendothelial damage

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Introduction: Sickle cell disease, an autosomal recessive hemoglobinopathy and a genetically determined pathology due to an aminoacid substitution of valine for glutamic acid on the beta globin chain of hemoglobin. SCD is one of the most prevalent disorder[1], there are more than 200 million carriers of sickle cell trait worldwide, and 200 000 to 300 000 people are born annually with major hemoglobinopathies [2]. In India SCD is well spread in central Indian population specifically comprises higher amounts of aboriginal population. Pulmonary complications playing a key role in morbidity and mortality in population with sickle cell disease. There is a wide variation in the in the clinical manifestations of SCD from one effected individual to another and from haplotype to another. The red blood cell membrane is a complex mixture of lipids and proteins. Both cholesterol and hundreds of different phospholipids take part in the making and maintainace of structural core lipid bilayer of RBC membrane. These lipids form a highly dynamic bilayer in which proteins are embedded and, together with the underlying membrane skeleton, provide a strong and

flexible membrane for the red cell to perform its tasks in the circulation. Both the composition and organization of the lipids are well maintained during the life of the cell, and alterations will lead to a dysfunction of the membrane and a loss of cellular viability. The lipids move rapidly in the plane of the bilayer, but this movement is not a random process. Lowering of the cholesterol content of the membrane tends to "dissolve" the rafts, combined lipid and protein organization microdomains, which are involved in specific physiological processes such as signal transduction. Dissolved rafts leading to an altered function of the membrane, and changes in the cytosol such as an increase in calcium can lead to shedding of vesicles enriched in specific lipids and proteins [3].

In SCD population haemoglobin S denatures and forms Heinz bodies. Binding of Heinz bodies to the inner surface of the sickle cell membrane promotes the clustering and colocalization of RBC membrane proteins and loss of translipid bilayer asymmetry. Altered transbilayer phospholipid asymmetry in the plasma membrane of sickle red cells has been documented by experiments in which red cells were treated with phospholipases and by studies of the distribution of spin-labeled phospholipids in the two lipid bilayer hemileaflets [4, 5, 6] Serum lipid levels are closely related with atherosclerosis, High serum LDL and Total Cholesterol levels are positively correlated with risk of atherosclerosis and High LDL levels converts the macrophages to foam cells, which further causes the smooth muscle cells transfer from tunica media to intima and formation of atheroma in the coronary artery.

But in SCD there is a Low levels of LDL and Total Cholesterol levels also causing the cardiovascular diseases without the formation of atheromas. Therefore normal or low HDL levels with Elevated TGL levels interestingly playing a role in vascular haemolysis and cardiovascular diseases. red cells Structure of RBC is the basis to understand pathophysiology of SCD is totally around the structure of studied RBC membrane lipids are Some studies conducted in this population related to the understanding the pathophysiology and complications of SCD, in our knowledge this is the first study with the view of RBC membrane lipid component changes and pulmonary hypertension. In recent years, Pulmonary Hypertension, a proliferative vascular disease of the lung, has been recognized as a major complication and independent correlate with death among adults with SCD.

Pulmonary artery systolic pressure (PASP) can be estimated by Doppler echocardiography, utilizing the tricuspid regurgitate jet velocity (TRJV). A TRJV of 2.5–2.9 m/s is at least two standard deviations above the mean and is considered representative of borderline or mildly elevated PASP, whereas a TRV 3.0 m/s of higher, approximately three standard deviations above the mean, represents significantly elevated PASP, often meeting criteria for pulmonary arterial hypertension. Increased TRV is estimated to be present in approximately one-third of adults with SCD and is associated with early mortality[7, 8].

In the more severe cases, increased TRV is associated with histopathologic changes such as plexogenic changes and hyperplasia of the pulmonary arterial intima and media [9,10,11,12].

These histopathological changes are very similar to those seen in the arterial wall thickening of atherosclerosis. Indeed, PH and atherosclerosis share several overlapping pathophysiologic features, including vascular smooth muscle proliferation, decreased NO bioavailability, oxidant stress, endothelial dysfunction, endothelial activation, increased levels of endogenous NOS inhibitors, platelet

activation, in situ thrombosis, and accelerated renal insufficiency [13,14]. Several studies are conducted to reveal the pathophysiological and metabolic aspects of RBC in sickle patients. in our knowledge this is the first study with the view of RBC membrane lipid component changes and pulmonary hypertension in central Indian sickle cell disease population.

Materials and Methods: The objective of this multi centred case control study to reveal the relationship between RBC membrane and serum lipids sickle cell disease population and asses the correlation between the pulmonary hypertension and these lipid markers. Study group comprises of Sickle cell disease population and all the subjects in study are divided in to three groups of normal Control and subjects, Sickle cell without Pulmonary Hypertension (Group-A), Sickle cell disease with Pulmonary Hypertension (Group-B). subjects both male and female all are in 18-56 years age group, Sickle cell disease diagnosed by Cellulose gel Electrophoresis (Interlab-Genio's Electrophoresis) all subjects provided with informed consent.

Determination of RBC membrane lipid composition: Fasting venous blood samples were collected in purple colored EDTA tubes from the subjects, blood samples subjected to centrifugation 3000g for 10 minutes, Supernatant (plasma) seperated and 2ml of normal saline added to the tubes mixed and centrifuged at 5000g for 10 minutes. Supernataent was discarded and this procedure was repeated twice then 0.1ml of tris HCl buffer, pH 7.4 (isotonic) added and centrifuged for 5 minutes. This procedure was repeated three times. The clear cells obtained were suspended in hypotonic buffer and stored for 4^0 C for four hours. Afterwards the cells were again washed with hypotonic buffer and then centrifuged at 15000g and 10000g for 30 min and 20 minutes respectively, this procedure was continued until the solution became colorless. 2 drops of isotonic solution was added to the solution and it was homogenized to get the red cell membrane, this was used for the extraction of lipids.

Extraction of lipids: The red cell membrane collected was centrifuged at 15000g at 10 minutes. The supernatant was decanted and the pellet obtained was suspended in 1ml of methanol and homogenized. The solution was made up to the 5ml with the same methanol and then centrifuged at 15 minutes at same speed. The supernatant was decanted, added 14ml of chloroform to the tube and transferred in to a flatbottom flask. The content of this flask was evaporated in a fume hood. To the evaporated content, added 5 ml of chloroform-methanol mixture to dissolve the solid and the resulting solution is poured into a labeled centrifuge tube and covered with paraffin. These tubes are centrifuged at 6000 rpm and two differed layers of fluid were visible. The upper aqueous layer was aspirated and discarded while the lower layer was retained for lipid testing [15]. Aliquots of this layer were used for the estimation of cholesterol, phospholipids, and TG.

Estimation of RBC Membrane Cholesterol: Lipid extract of 0.6 ml was evaporated. Different standard concentrations ranging from 20, 40, 60, 80, 100, and 120 µg was used. Each standard of 0.05 mL was transferred from respective tubes to the tubes labelled S1 to S6 and evaporated. To these tubes (including the test) was added 3 ml of ferric acetate uranyl acetate. The blank was run simultaneously. Sulphuric acid ferrous sulphate 2 ml was added and the contents were mixed properly. The tubes were left standing for 20 minutes and their color intensity was read at 560 nm. The cholesterol concentration of the test sample was determined using a standard graph plotted [16].

Estimation of RBC Membrane Phospholipids: Lipid extract of 0.6 ml was evaporated in a fume hood. Standard phosphate solutions of five different concentrations were used. A blank was run simultaneously. To all the tubes added 1 ml of perchloric acid, 0.5 ml of 3% ammonium molybdate (freshly prepared), and 0.5 ml ascorbic acid (freshly prepared). The total volume of all the tubes was made up to 6 ml with deionised water. All tubes were kept in boiling water for 6 minutes and the blue color developed was red at 710 nm. The phospholipid concentration of the test sample was determined using a standard graph plotted [17].

Estimation of serum lipid composition: Serum lipids are estimated by using specific enzymatic methods using semi auto analyzer. Total cholesterol estimation by Cholesterol oxidase-peroxidase method (CHOD-PAP,Erba Mannheim Inc. ,end point, FREBCEMI0161), Plasma HDL is estimated by (Phosphotungstic acid precipitation and then CHOD-PAP end point method, FREBCEMM0249), Plasma TGL by (GPO-PAP method, Erba Mannheim Inc., end point, FREBCEMI0187), serum LDH is estimated through (DGKC kinetic method, Erba Mannheim Inc., FREBCEMI0163) and Total plasma Bilirubin is estimated by (Diazo method, Erba Mannheim Inc., FREBCERM0043).

Tricupsid Reguretant Jet Velocity (TRJV): Pulmonary hypertension is a potentially life-threatening complication, detected by echocardiographic evidence of elevated tricuspid regurgitant velocity (TRV). This condition has been described in adults with sickle cell disease (SCD) and other haemolytic disorders; The Philips E33 system (Philips Medical Systems, Bothell, WA) was used for all studies, which included 2D imaging, M-mode, spectral, and colour Doppler interrogation. Sonographers measured peak TRJV with the transducer placed at the cardiac apex to obtain a 4-chamber view and the ultrasound beam directed parallel to the tricuspid regurgitant jet to obtain the cleanest Doppler tracing of the velocity. Cardiologists with expertise in ECHO read all measurements, TRJV elevation was defined by a peak TRJV ≥2.5 m/sec, corresponding to a pulmonary artery systolic pressure ≥30–35 mmHg calculated by the modified Bernoulli equation $4(TRJV)^2$ and assuming a right atrial pressure of 5–10 mmHg.

Statistical Analysis: The results were expressed as Mean±SD and statistical significance at $P<0.001$. Statistics are done by SAS (v.9.0.SAS institute Inc,cary, NC, USA) software, univariate analysis is done on all three study groups through ANOVA . Correlation studies have done between the TRJV and haemolytic parameters.

Results: Thirty seven percent of population in the study are (HbAA) with normal haemoglobin adult type and sixty three percent comprised of Sickle cell disease, out of this 30% population are belongs to Group-A and remaining 32% to Group-B. estimated lipid markers in plasma are presented in (Fig-1), Mean plasma total cholesterol levels in the SCD population are significantly lower when compared with Control subjects , plasma HDL levels are normal in all study group(Control 37.67±18.56, Group-A 34.17±9.18,Group-B 35.45±12.45) and there is no statistical difference in between SCD groups also. Although Plasma LDL levels are responsible for atherosclerosis there is no increased levels are observed in SCD populations (105.86±21.5, 102.37 ±34.2), low LDL levels observed in disease group compared to controls (114.15±34.46). Elevated TGL levels are observed specifically in Group-B (208.43±56.97), Interestingly Plasma TGL levels in Group-A (132.34±8.97) are lower when compared with normal control subjects (141.43±26.98). TRJV is <2.5m/s (1.96±0.34) in

controls, Group-B have >2.5m/s and this is taken as the deciding factor for Pulmonary Hypertension. The results shown in the central Indian sickle cell disease population are given in the Table-1, which is giving a summary of analysis parameters along with statistical significance in all three groups. The Plasma Haemolytic parameters LDH and Plasma Total Bilirubin (170±70.18, 2.8±1.2 in Group- A, 190±81.56, 4.5±3.4 in Group-B) are significantly higher when compared with controls (130±56.12, 0.6±0.7).

Table-1

PARAMETER	CONTROL (N=80)	SICKLE CELL DISEASE (N=135)	
		GROUP-A (N=70)	GROUP-B (N=65)
Plasma Total Cholesterol (mg/dl)	156.23±12.76	142.45±34.2*	140.14±18.53*
Plasma HDL (mg/dl)	37.67±18.56	34.17±9.18	35.45±12.45
Plasma LDL (mg/dl)	114.15±34.46	105.86±21.5	102.37 ±34.2
Plsma TGL (mg/dl)	141.43±26.98	132.34±8.97	208.43±56.97*
Plsama PHOSPHOLIPIDS	158.34±14.5	146.34±13.6	135.35±23.6
TRJV (m/sec)	1.96±0.34	2.34±0.13	2.76±0.67*
Plasma LDH (IU/L)	130±56.12	170±70.18	190±81.56*
Plsma Total Bilirubin (mg/dl)	0.6±0.7	2.8±1.2	4.5±3.4*

Group-A: SCD without Pulmonary Hypertension, Group-B : SCD With Pulmonary Hypertension, HDL= High Density Lipoprotein, LDL = Low Density Lipoprotein, TRJV=Tricupsid regurgitation jet velocity, LDH= Lactate Dehydrogenase, * statistical significance is P<0.001

TABLE-2

Parameter	Control (N=80)	Sickle Cell Disease (N=135)	
		Group-A (N=70)	Group-B (N=65)
Membrane Cholesterol (mg/ml RBC)	0.84±0.01	1.09±0.04*	1.21±0.07*
Membrane Phospholipids (mg/ml RBC)	1.46±0.25	1.21±0.14	1.16±0.09*
Cholesterol/Phospholipds	0.56±0.04	0.91±0.02*	0.94±0.03*

Group-A: SCD without Pulmonary Hypertension, Group-B : SCD With Pulmonary Hypertension, *statistical significance is P<0.001

Table-2 shows RBC membrane Lipid markers, Membrane Cholesterol and membrane Phospholipids are estimated. High membrane cholesterol levels and low membrane phospholipids were observed in SCD population (Fig-2) when compared with normal Subjects. The TRJV levels in study group are represented in (Fig-3). The correlation between TRJV and Plasma Cholesterol is not statistically significant, except TRJV with plasma TGL correlation, remaining all lipids is negatively correlated with TRJV and not significant.

Discussion: Data obtained during this study has enables us to establish clearly that both erythrocyte membrane changes and plasma factors play a crucial role in determining the nature and extent of adhesive contact between sickle erythrocytes and vascular endothelial cells. RBC membrane is maintained throughout the life of the red cell, and

alterations in either the lipids or proteins of the membrane will lead to apoptosis during erythropoiesis or early demise of the cell in the circulation. The globin mutations that lead to hemoglobinopathies such as sickle cell disease or thalassemia have a profound effect on the RBC membrane. In this present study we observed that there is a high levels of RBC membrane cholesterol and low membrane Phospholipids in Sickle cell disease patients, compared to that there is statistically significant rise and fall in both Pulmonary Hypertension with sickle cell disease are vulnerable to oxidative modification.

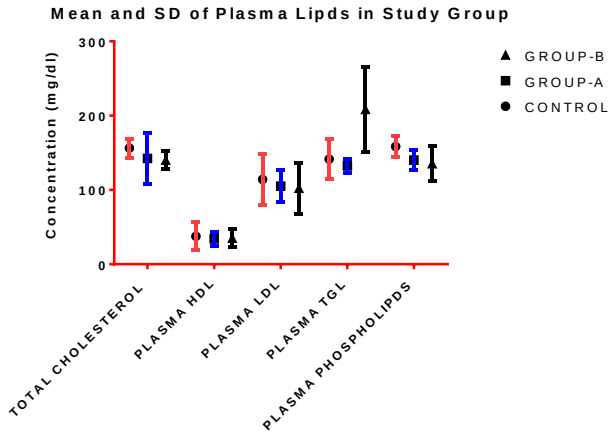

Figure-1: Mean plasma lipids are estimated in all three groups, elevated plasma TGL values are observed in SCD with Pulmonary Hypertension (Group-B), except TGL remaining all lipids are lower than normal control subjects.

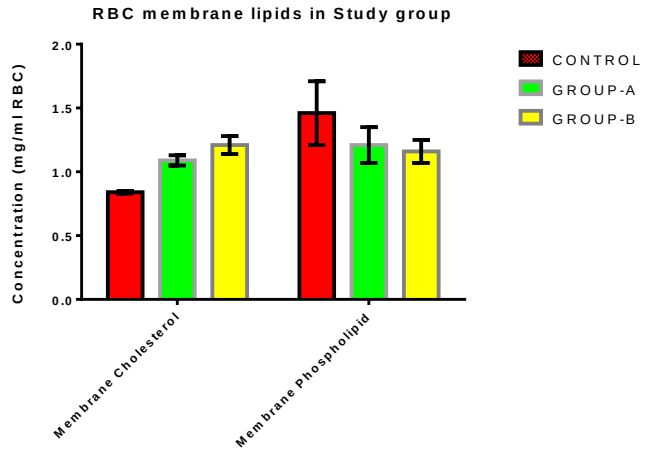

Fig-2: Mean and SD expression showing estimation of RBC membrane Cholesterol and Phospholipids in three groups, In Sickle cell disease (Group-A and Group-B) Cholesterol levels are significantly raised and Phospholipids are lower than control subjects.

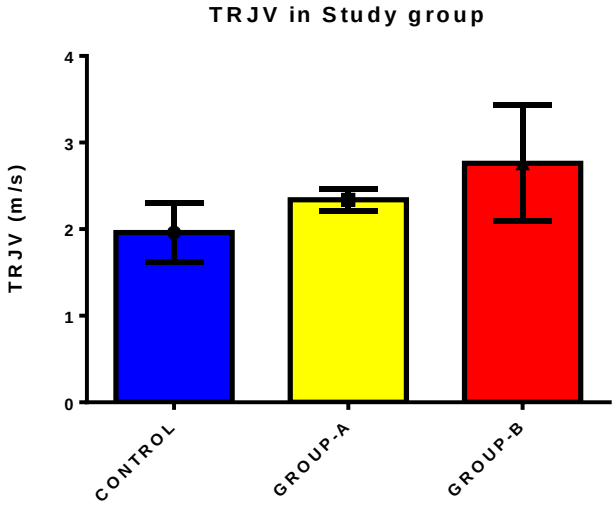

Fig-3: Tricupsid Regurgitation Jet Velocity estimation by trans thoracic echocardiography, Results expressed as Mean with SD, showing elevated TRJV (>2.5 m/s) in SCD with Pulmonary Hypertension (Group-B)

Oxidative stress is playing a pivotal role in damage to the RBC membrane; in particularly double bonds in the phospholipid acyl chains

The addition of oxygen in the apolar chains will alter the local packing of the bi layer and affect its functionality. Both oxidative stress and elevated cytosolic calcium play a role in exposure of Phospholipids. Phospholipids are maintained in the RBC inner leaflet by an ATP-dependent transporter known as the flippase. Membrane bound $Mg2^{+}$-ATPases, exclusively found in eukaryotes, seem to play a key role in the maintenance of the membrane lipid organization. This subfamily of P-type ATPases has been reported to actively translocate aminophospholipids across membranes, and several members of this family have been identified in the genome of mammals. The RBC flippase, a vanadate sensitive ATPase of approximately 110 to 120

kDa, was recently identified as two isoforms of the type 4-ATPase 8A member 1 (ATP8A1) .

The structure and mode of action of these proteins in the plasma membrane needs to be established, and it is not known whether the two isoforms have different functions. The different forms of this protein may transport amino phospholipids at different rates for different molecular species.

Hypocholesteremia and plasma LDL concentrations are significantly reduced in both the groups along with the rise in TRJV, Decreased TC and LDL-C in SCD has been documented in virtually every study that examined lipids in SCD adults [18, 19, 20, 21, 22, 23, 24] Although it might be hypothesized that SCD hypocholesterolemia results from increased cholesterol utilization during the increased erythropoiesis of SCD, cholesterol is largely conserved through the enterohepatic circulation, at least in healthy individuals, and biogenesis of new RBC membranes would likely use recycled cholesterol from the haemolysed RBCs. Westerman demonstrated that hypocholesterolemia was not due merely to increased RBC synthesis by showing that it is present in both hemolytic and non-hemolytic anemia[24]. Serum cholesterol is proportional to the hematocrit, suggesting serum cholesterol may be in equilibrium with the cholesterol reservoir of the total red cell mass. Consistent with such equilibration, tritiated cholesterol incorporated into sickled erythrocytes is rapidly exchanged with plasma lipoproteins [25]. Thus, low plasma cholesterol appears to be a consequence of anemia itself rather than increased RBC production . Although LDL-C levels are decreased in SCD patients, LDL from SCD patients is more susceptible to oxidation and cytotoxicity to endothelium [26] and an unfavourable plasma fatty acid composition has been associated with clinical severity of SCD [27]. Lipolytic generation of arachadonic acid, eicosanoids, and inflammatory molecules leading to vascular dysfunction is a well-established phenomenon [28].

RBCs do not have de novo lipid synthesis [29]. In SCD the rate of triglyceride synthesis from glycerol is elevated up to 4-fold in sickled reticulocytes [30] , but SCD patients have defects in post absorptive plasma lipoproteins and homeostasis of fatty acids [31]. Lipoproteins and albumin in plasma can contribute fatty acids to red blood cells for incorporation into membrane phospholipids , But RBC membranes are not triglyceride-rich and contributions of RBCs to plasma triglyceride levels have not been described. Vascular endothelial damage causes the raise in Hemolytic factors in serum and TRJV raised pulmonary hypertension subjects with SCD shown similar results without pulmonary hypertension SCD subjects, except in Plasma TGL concentration, up to now we are assessing the high LDL and low HDL are principal Parameters in Atherosclerosis and Cardiovascular diseases. Triglycerides also have some role in end organ failure pathophysiology of sickle cell disease.

Conclusion: Our study concluded that non invasive lipid markers are playing a prominent role in diagnosing and assessing the chronic symptoms of sickle cell disease along with invasive specific markers of end organ failure, TRJV is the prominent marker estimating now a days, along with this raise in plasma TGL is giving a good prediction in severity of Pulmonary Hypertension, the relation between oxidative stress markers and RBC membrane damage is also further need to investigate at molecular level to reveal the new insights in vasoendothelail damage mechanism of Sickle cell disease.

References:

1. Shores J, Peterson J, VanderJagt D, Glew RH. Reduced cholesterol levels in African-American

adults with sickle cell disease. J Natl Med Assoc. 2003; 95:813–817.
2. Abraham VA, Hohnson EC. Molecular disease and their medical implications. N Engl J Med 1983; 799 : 2481-2497.
3. Salzer U, Hinterdorfer P, Hunger U, Borken C, Prohaska R. Ca(++)-dependent vesicle release from erythrocytes involves stomatin-specific lipid rafts, synexin (annexin VII), and sorcin. Blood. 2002;99:2569-2577.
4. Lubin, B., D. Chiu, J. Bastacky, B. Roelofsen, and L. L. M. van Deenen.1981. Abnormalities in membrane phospholipid organization in sickled eryth- rocytes. J. Clin. Invest. 67:1643–1649.
5. Waugh, S. M., and P. S. Low. 1985. Hemichrome binding to band 3: nu- cleation of Heinz bodies on the erythrocyte membrane. Biochemistry. 24:34–39.
6. Middelkoop, E., B. H. Lubin, E. M. Bevers, J. A. F. Op den Kamp, P. Comfurius, D. T. Y. Chiu, R. F. A. Zwaal, L. L. M. van Deenen, and B. Roelof- sen. 1988. Studies on sickled erythrocytes provide evidence that the asymmetric distribution of phosphatidylserine in the red cell membrane is maintained by both ATP-dependent translocation and interaction with membrane skeletal proteins. Biochim. Biophys. Acta. 937:281–288.
7. Ataga KI, Moore CG, Jones S, Olajide O, Strayhorn D, Hinderliter A, Orringer EP. Pulmonary hypertension in patients with sickle cell disease: a longitudinal study. Br J Haematol. 2006;134:109–115.
8. Gladwin MT, Schechter AN, Ognibene FP, Coles WA, Reiter CD, Schenke WH, Csako G, Waclawiw MA, Panza JA, Cannon RO 3rd. Divergent nitric oxide bioavailability in men and women with sickle cell disease. Circulation. 2003; 107:271–278.
9. Adedeji MO, Cespedes J, Allen K, Subramony C, Hughson MD. Pulmonary thrombotic arteriopathy in patients with sickle cell disease. Arch Pathol Lab Med. 2001; 125:1436–1441.
10. Graham JK, Mosunjac M, Hanzlick RL, Mosunjac M. Sickle cell lung disease and sudden death: a retrospective/prospective study of 21 autopsy cases and literature review. Am J Forensic Med Pathol. 2007; 28:168–172
11. Haque AK, Gokhale S, Rampy BA, Adegboyega P, Duarte A, Saldana MJ. Pulmonary hypertension in sickle cell hemoglobinopathy: a clinicopathologic study of 20 cases. Hum Pathol. 2002; 33:1037–1043.
12. Manci EA, Culberson DE, Yang YM, Gardner TM, Powell R, Haynes J Jr, Shah AK, Mankad VN.Causes of death in sickle cell disease: an autopsy study. Br J Haematol. 2003; 123:359–365.
13. Kato GJ, Gladwin MT, Steinberg MH. Deconstructing sickle cell disease: reappraisal of the role of hemolysis in the development of clinical subphenotypes. Blood Rev. 2007; 21:37–47.
14. Gladwin MT, Schechter AN, Ognibene FP, Coles WA, Reiter CD, Schenke WH, Csako G, Waclawiw MA, Panza JA, Cannon RO 3rd. Divergent nitric oxide bioavailability in men and women with sickle cell disease. Circulation. 2003; 107:271–278.
15. Folch JM, Lees M, Sloane Stanley GH. 1957. A simple method for the isolation and purification of total lipids from animal tissue. J Biol Chem, 226:497–509.
16. Parekh AL, Jung DH. 1970. Cholesterol determination with ferric acetate – uranium. Anal Chem, 42:1423.
17. Rouser G, Fleischer S, Yamamoto A. 1970. Two dimensional thin layer chromatographic separation of polar lipids and determination of phos- pholipids by phosphorous analysis of spots. Lipids, 5:494–6.
18. el-Hazmi MA, Jabbar FA, Warsy AS. Cholesterol and triglyceride level in patients with sickle cell anaemia. Scand J Clin Lab Invest. 1987; 47:351–354.
19. el-Hazmi MA, Warsy AS, al-Swailem A, al-Swailem A, Bahakim H. Red cell genetic disorders and plasma lipids. J Trop Pediatr. 1995; 41:202–205.
20. Marzouki ZM, Khoja SM. Plasma and red blood cells membrane lipid concentration of sickle cell disease patients. Saudi Med J. 2003; 24:376–379.
21. Sasaki J, Waterman MR, Buchanan GR, Cottam GL. Plasma and erythrocyte lipids in sickle cell anaemia. Clin Lab Haematol. 1983; 5:35–44.
22. Shores J, Peterson J, VanderJagt D, Glew RH. Reduced cholesterol levels in African-American

adults with sickle cell disease. J Natl Med Assoc. 2003; 95:813–817.
23. Westerman MP. Hypocholesterolaemia and anaemia. Br J Haematol. 1975; 31:87–94.
24. Westerman MP, Diloy-Puray M, Streczyn M. Membrane components in the red cells of patients with sickle cell anemia. Relationship to cell aging and to irreversibility of sickling. Biochim Biophys Acta. 1979; 557:149–155.
25. Ngogang J, Mouray H, Lebreton de Vonne T, Raisonnier A. Erythrocyte and plasma cholesterol exchange in sickle cell anemia. Clin Chim Acta. 1989; 179:295–304.
26. Belcher JD, Marker PH, Geiger P, Girotti AW, Steinberg MH, Hebbel RP, Vercellotti GM. Low-density lipoprotein susceptibility to oxidation and cytotoxicity to endothelium in sickle cell anemia. J Lab Clin Med. 1999; 133:605–612.
27. Ren H, Ghebremeskel K, Okpala I, Ugochukwu CC, Crawford M, Ibegbulam O. Abnormality of erythrocyte membrane n-3 long chain polyunsaturated fatty acids in sickle cell haemoglobin C (HbSC) disease is not as remarkable as in sickle cell anaemia (HbSS). Prostaglandins Leukot Essent Fatty Acids. 2006; 74:1–6.
28. Boyanovsky BB, Webb NR. Biology of secretory phospholipase A2. Cardiovasc Drugs Ther. 2009; 23:61–72.
29. Kuypers FA. Red cell membrane lipids in hemoglobinopathies. Curr Mol Med. 2008; 8:633–638.
30. Lane TA, Ballas SK, Burka ER. Lipid synthesis in human erythroid cells: the effect of sickling. 1976; Blood. 47:189–195.
31. Buchowski MS, Swift LL, Akohoue SA, Shankar SM, Flakoll PJ, Abumrad N. Defects in postabsorptive plasma homeostasis of fatty acids in sickle cell disease. JPEN J Parenter Enteral Nutr. 2007; 31:263–268.

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