RESEARCH PAPER

Unmasking the Membrane: Insights into Red Blood Cell's Surface Properties and Deviation in Liver Diseases

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Introduction

Erythrocytes/ Red blood cells (RBCs) are specialized anucleate cells that play a vital role in oxygen transport and microcirculatory flow. They are flexible biconcave discs that lack a nucleus and other organelles, which maximizes their surface area for gas exchange and allows them to deform as they traverse narrow capillaries. Mature human RBCs typically survive about 120 days in circulation and are continuously produced in the bone marrow under the control of erythropoietin [1-2]. Their function is closely tied to membrane integrity and its surface characteristics. Research on alteration in biophysical properties of RBC membranes have been extensively done on diseases such as sickle cell anemia, malaria or non-pathological conditions like exercise etc. but not much in liver diseases [3].

Chronic Liver Diseases such as cirrhosis, hepatitis, and Non-Alcoholic Fatty Liver Disease (NAFLD) are frequently associated with systemic complications including metabolic dysfunction, inflammation, oxidative stress which contributes to alterations in RBC morphology. Anaemia affects approximately two-thirds of individuals with advanced chronic liver disease, which is linked to poorer clinical outcomes, including increased risks of hepatic decompensation, hospital admissions, liver failure and both liver-specific and overall mortality [4].

The liver plays a vital role in lipid metabolism, detoxification, and inflammatory regulation which directly or indirectly affect RBC membranes

representing an additional and under-recognised cause of anaemia [5]. These aberrant RBCs, called acanthocytes or also referred to as spur cells, lose their characteristic biconcave shape, showing irregular cell surface projections that are enlarged distally. Such projections increase RBC susceptibility to trapping and destruction by the spleen. Spur cell anemia has been recently recognised as a predictive factor of early mortality in patients with cirrhosis [6].

These morphological and biochemical alterations are connected to altered surface properties of RBC membrane. Understanding the connection of liver diseases associated metabolic changes with RBC membrane's surface properties is essential due to their clinical and research significance. Although anemia and hemolysis are well-documented complications of liver diseases, very few studies offer a comprehensive analysis of how hepatic dysfunction influences RBC surface architecture. Synthesis of the available data is thus critical to bridge this gap and guide future research and clinical practice. This review aspires to illuminate the multifaceted alterations in RBC membrane surface properties within the context of liver diseases and associated pathophysiology by using available data. It also offers a comprehensive exploration of their detection methods and clinical significance.

Methods

Defined research question: Despite decent number of individual studies, a unified review compiling the diverse and scattered findings on altered RBC membrane surface properties in liver disorders remains absent. This review aims to provide an integrated, single-source reference encompassing all available data in this field."

Comprehensive Search Strategy We conducted a search on Google scholar database for published original and review articles, book chapter using various combinations of the following search terms "red blood cell membrane" / "erythrocyte membrane", Spur Cell Anemia/ "liver disease" /"cirrhosis" / "hepatitis" / "NAFLD" /Alcoholic liver disease, "surface charge"/ "zeta potential"/"membrane fluidity"/"lipid peroxidation"/ "membrane deformability"/ Membrane elasticity / "Oxidative Stress" /"Membrane topography" for studies published from January 1 1980 to July, 2025, and manually searched the references of selected articles for additional relevant articles. A total of 120 records were retrieved.

Inclusion-Exclusion Criteria & Article Selection: We considered all papers that reported normal RBC architecture, RBC membrane's physiochemical alteration or surface properties changes in various liver diseases. Suitable articles were selected in two stages. First, the title and abstract of each article was screened independently by two authors. If there was consensus that an article was not suitable for inclusion based on the title and/or abstract, the article was excluded. Next, the full-text articles were screened independently by two authors and included if both authors agreed; if needed, the article was discussed with a third author until consensus was reached. The authors were not blinded with respect to the article's authors or the journal in which it was published. Given the limited number of publications regarding this subject, no search restrictions were applied with respect to study design, number of cases reported, and patient age. Articles on only humans published in English were used.

Data extraction and synthesis were carried out independently by two reviewers. The extracted data included key parameters such as author and publication year, study design, the normal erythrocyte architecture data, type of liver disease studied, the sample size and characteristics of the population involved, the reported biophysical and biochemical alterations in RBCs, and the analytical techniques employed to assess these changes. Given the considerable heterogeneity in study designs, populations, and methodologies across the included studies, a narrative synthesis approach was adopted to summarize and interpret the findings [Figure-1]

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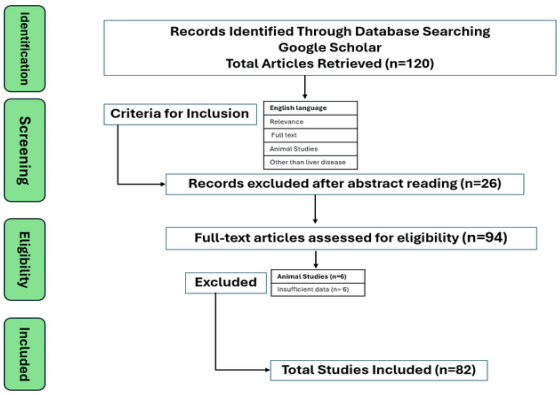

Figure 1: Data collection strategy flow chart for Narrative Review

Normal Structure and Function of RBC Membrane

The RBC membrane is a highly specialized and dynamic structure composed of a lipid bilayer, membrane proteins, and an underlying cytoskeletal network. The lipid bilayer, enriched with phospholipids (PLs) such as phosphatidylcholine (PC), sphingomyelin, phosphatidylethanolamine (PE), and phosphatidylserine (PS), along with cholesterol

(CHOL), provides structural integrity and fluidity to the membrane [7]. PC (≈ 40%) and PE (≈ 30%) are the most abundant molecules in the membrane followed, by SM (≈ 9%). PC is mainly present in outer leaflet while PE are located in inner leaflet and cholesterol can diffuse between the two leaflets. Type of fatty acids present in these molecules are very important to characterize mechanical and biophysical properties of RBC membrane. About 40% of the tails are unsaturated, 21% have one or two double bonds, and 29% have more than three double bonds [8]. Distribution of head groups, the degree of saturation of the fatty acid tails and the concentration of cholesterol affects lipid organization [3].

Integral membrane proteins like band 3 and glycophorin-A facilitate ion transport, while peripheral proteins such as sceptrin, ankyrin, and actin form a supportive cytoskeletal mesh that maintains the biconcave shape and mechanical resilience of which is a unique property of RBC membrane compared to other cell membranes [9]. The RBC's cytoskeleton is a two-dimensional structure. It is formed by triangularly arranged spectrin filaments parallel to the cytoplasmic membrane. The cytoskeleton is anchored to the cytoplasmic membrane through tethering sites that are formed by two macromolecular complexes of membrane proteins, the ankyrin-based complex, and the 4.1R-based complex. Glycophorin A contributes significantly to the negative surface charge through sialic acid residues, preventing cell aggregation and enabling proper circulation [10-11]. Figure-2

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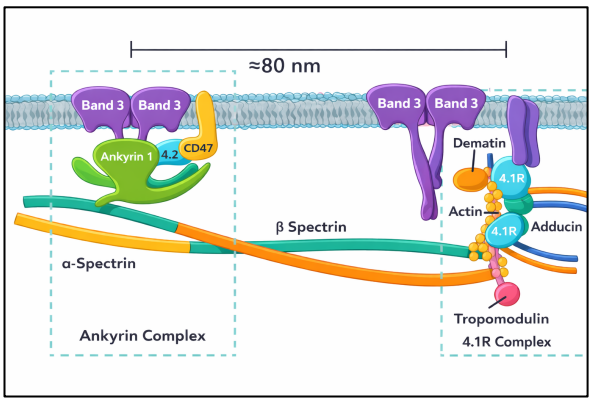

Figure 2: The cytoplasmic membrane and associated cytoskeleton

Disruptions in any of these structural components can compromise cell function, reduce deformability, and lead to premature senescence or clearance [12-13].

Recent study also highlights the importance of lipid-protein interactions in maintaining membrane flexibility and functioning where membrane proteins can be regulated by specific interactions with its lipid constituents [14]. However, the biomechanical intricacies governing the interactions between the lipid bilayer and the cytoskeleton-mediated through the anchoring points of transmembrane proteins-remain largely elusive [15].

Surface Properties of RBC Membrane: RBC membranes exhibit specific surface characteristics that govern how these cells interact within the bloodstream and contribute to normal physiological processes. These surface properties include structural, electrical, mechanical, and biochemical features that are vital for maintaining cellular deformability, survival, and functional circulation[2]. Each of these surface characteristics contributes to the overall biophysical behaviour of RBCs. Disruption in any domain, such as through oxidative damage or metabolic imbalance, can cascade into broader dysfunction, emphasizing the need for integrated analysis when assessing RBC health in systemic diseases like liver disorders. Changes in RBC membranes during liver disease might affect how well these cells perform, how flexible they are, or how long they survive.

Here we describe these properties and their alterations in various liver diseases-

1. Electrostatic Surface Charge (Zeta Potential):

Zeta potential is an electrochemical characteristic that reflects electrical potential at the shear plane of the cell surface, influenced by the charged molecules exposed on the membrane. RBCs possess a negatively charged membrane, which attracts a tightly bound layer of cations from the surrounding medium. This layer is further enveloped by a diffuse, cloud-like region composed of both cations and anions [12,16]. Figure-3

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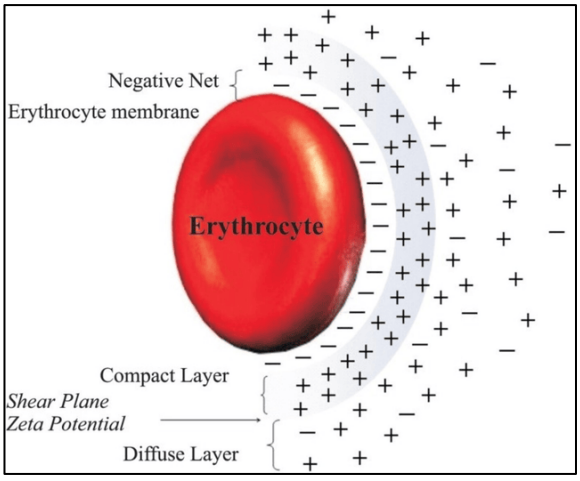

Figure 3: Zeta potential of erythrocyte membrane

(Note: This figure has been adapted from an open-access article Fernandes HP, 2011 [13] distributed

The net negative charge of RBC membrane originates from sialic acid-rich glycoproteins, such as glycophorin A. This negative surface potential prevents spontaneous aggregation of RBCs and ensures smooth movement through the microvasculature. Zeta potential measurements offer a quantifiable index of this electrostatic repulsion [17].

RBCs zeta potential can be evaluated using two distinct yet complementary methodologies. One approach involves quantifying the force acting on a silica bead bound to a single RBC under the influence of an applied electric field. The alternative, more traditional method estimates zeta potential by analysing the RBC's terminal velocity following its release from an optical trapping system [18]. These techniques do not provide information on the spatial distribution of charge or the contribution of individual membrane components, which may require complementary biochemical or imaging approaches. A more negative zeta potential indicates a higher surface charge density. Changes in membrane charge have been linked to various pathologies including malaria infection, pre-eclampsia, liver disease, diabetes, and oxidative stress, where membrane composition is altered [19- 21].

Surface charge alteration in Liver diseases:

No study directly measured zeta potential of RBC membranes in liver diseases but few studies have indirectly assessed changes in surface charge by examining related membrane properties. Freikman I, 2008 treated normal RBCs with oxidants (t-butylhydroxyperoxide and H2O2 and observed that negatively charged PS was decreased by treatment with oxidants and treatment with vitamin C and N-acetyl cysteine antioxidants increased the PS content in the membrane for which they used Nuclear magnetic resonance (NMR) spectroscopy [22].

Jewell SA, 2012 used the dye di-8-ANEPPS to measure changes in membrane dipole potential ($\psi(d)$) of human RBCs under oxidative stress. They treated cells with cumene hydroperoxide and H_2O_2, and monitored $\psi(d)$, cell diameter, and membrane composition using Raman micro spectrometry [23]. The results showed that oxidants caused altered lipid order and protein crosslinking (spectrin-haemoglobin complex) thus membrane stiffening. Tokumasu F, 2012 treated RBCs with neuraminidase which cleaves sialic acids and decreased RBC zeta potential from approximately -15.7 mV to -6.06 mV, demonstrating the key role of sialic acid in maintaining surface negativity [21]. We can conclude that oxidative stress impacts RBC zeta potential by damaging membrane lipids via peroxidation, stripping away negatively charged sialic acids and PS, and cross-linking structural proteins-resulting in reduced net negative surface charge and lowered zeta potential. As no study to date has directly measured the zeta potential of red blood cell membranes specifically in liver disease patients, highlighting a significant research gap and presenting valuable scope for future investigations.

2. Membrane Flexibility and Fluidity: Membrane fluidity is a physiochemical property which reflects the lateral mobility of lipid and protein molecules in the bilayer, and is essential for RBCs to deform while navigating narrow capillaries. RBCs are known for their high deformability. The cells are constantly exposed to mechanical stress as they pass through the vascular system. It is remarkable that the RBCs can pass through constrictions that are much smaller than their own diameter [24]. During that flow, RBCs experience four main types of membrane deformation: shear stress, compression, tension, and bending. These dynamic mechanical transformations are regulated by membrane lipid composition, cholesterol content, and oxidative status. Membrane fluidity depends on interactions between polyunsaturated lipids and cholesterol. Although cholesterol typically stiffens membranes, this effect lessens with increasing lipid unsaturation [25]. Reduced fluidity impairs RBC deformability and increases hemolytic risk, potentially contributing to microvascular complications [2, 26]. These structural and mechanical characteristics of RBC membranes have been extensively studied using a wide range of biophysical techniques. To assess deformability and elasticity, several established methods are employed. For population-based or high-throughput analysis, microfluidic platforms offer a robust means to examine RBC deformability by forcing cells through narrow constrictions and measuring transit times and shape deformations [27]. Ektacytometry is a laser diffraction viscometer, is widely used technique that measures the elongation index of RBCs under controlled fluid shear stress where RBCs are placed into the solutions of varying osmolality, offering insights into the average deformability of cell populations [28]. Micropipette aspiration is a single cell technique that provides quantitative data on membrane elasticity by applying suction through a micropipette to deform the cell, allowing calculation of mechanical parameters such as the shear modulus, area compression modulus, surface viscosity, and bending rigidity [29].

Another powerful tool is optical tweezers, which use highly focused laser beams to trap and stretch RBCs or silica beads attached to them. This method enables precise measurement of the force-extension response and mechanical compliance of individual cells providing insights into membrane elasticity and cytoskeletal integrity [30]. Similarly, optical and magnetic twisting cytometry involves the use of magnetic or optically manipulated beads bound to the RBC surface. The application of oscillatory torque allows for the determination of both elastic storage and viscous loss moduli, capturing the viscoelastic behaviour of the cell membrane over a range of frequencies [31].

To evaluate membrane fluidity, techniques based on fluorescence and resonance are frequently utilized. Fluorescence anisotropy and time-resolved fluorescence spectroscopy employ lipid-soluble fluorophores or molecular rotors to probe membrane micro viscosity and the lateral and rotational mobility of lipids [32]. Electron spin resonance (ESR), using spin-labelled probes, provides data on lipid dynamics by measuring rotational correlation times, while deuterium nuclear magnetic resonance uses deuterated lipids to calculate membrane order parameters, offering direct insight into the fluidity of the lipid bilayer [33]. Another biophysical technique is the Langmuir-Blodgett (LB) trough. In this method, lipids extracted from RBC membranes are spread on a water subphase to form a monolayer, which is then compressed using movable barriers to generate surface pressure-area (π-A) isotherms. These isotherms provide information on lipid packing, phase transitions, molecular area per lipid, and collapse pressure, which reflect the fluidity, elasticity, and stability of the membrane lipids [34-35]. The Langmuir-Blodgett trough is not only limited to lipid-only systems but now days is optimized to characterize membrane proteins-lipid interactions [36].

Together, these diverse biophysical approaches provide a comprehensive understanding of the mechanical and structural behaviour of RBC membranes. Each method targets specific aspects of membrane mechanics; from lipid bilayer fluidity at the molecular level to whole-cell deformability under hydrodynamic forces-thereby offering valuable insights into RBC function and dysfunction in health and disease.

Membrane Fluidity Alteration in Liver Disorders

Owen JS, in 1982 observed spur cell in blood of many patients and studied the RBC membrane of hepatitis and cirrhosis patients with various severity. For which they used hydrophobic fluorescent probe diphenylhexatriene in fluorescence polarization technique. They affiliated the decreased fluidity of erythrocytes with as a consequence of increased CL/PL ratio where increased cholesterol mainly affected the ratio. PL composition and protein abnormalities had negligible impact on membrane fluidity [37]. Malik P, 2002 & Goel A, 2008 observed acanthocytes and spur cell anemia in alcoholic liver cirrhosis patients. The pathogenesis of spur cell anemia was due to changes in serum lipids that affected the lipid composition, increased membrane cholesterol and altered fluidity of erythrocyte membranes [38-39]. Arendt BM, 2013 reported decreased PC/PE ratios in the erythrocyte membranes of patients with NAFLD and non-alcoholic steatohepatitis (NASH) compared to healthy controls. Here the lowered PC/PE was because of lower PC but PE in erythrocytes was not different among the groups [40].

Similar to the above findings, study by Papadopoulos C, 2020 explored how inflammation and metabolic disturbance induced by hepatitis C impact the lipid composition of erythrocyte membranes. Using thin-layer chromatography, they measured membrane cholesterol and various PL levels in patients with hepatitis C and advanced fibrosis, both before and after antiviral therapy. Prior to treatment, elevated PE levels and reduced PC/PE and (Cholesterol/PE) ratios were observed. These alterations normalized following therapy. Systemic inflammation may have led to selective depletion of membrane PC without affecting PE potentially via hydrolysis to lysophosphatidylcholine independent of secretory phospholipase A2 activity. The post-treatment increase in PC/PE ratio may reflect resolution of hepatic inflammation [41].

Maksane S, 2021 also observed that increased Cholesterol/PL ratio exacerbate the reduction in RBC membrane fluidity in mild and moderate liver cirrhosis patients [42]. Forsyth AM, 2012 employed a microfluidic flow-focusing device to elucidate the relationship between erythrocyte membrane cholesterol content, its deformability and ATP release. The in vitro experiment demonstrated that reducing membrane cholesterol enhanced RBC deformability and ATP release. Furthermore, simvastatin treatment increased cell deformability by acting directly on the membrane and also elevated ATP release in cholesterol-enriched cells [43]. For role of fatty acids in membrane fluidity, Owen JS, 1982 observed the higher content of palmitic acid and lower content of arachidonic and stearic acid but the impact of this

altered fatty acid composition on fluidity was not significant [37]. Another case-control study by Mouillot T, 2020 examined fatty acid profiles in erythrocyte membranes of cirrhotic patients with (n=349) and without (n=550) Hepato-Cellular Carcinoma (HCC). Using gas chromatography, researchers found that relative ratio of saturated FAs (39%): monounsaturated FAs1(4%): Polyunsaturates (46%) was similar in both groups. However, elevated levels of odd-chain saturated FAs (C15:0 + C17:0), eicosenoic acid, linoleic acid and eicosadienoic acid were associated with increased HCC risk. Conversely, lower levels of stearic acid and arachidonic acid were observed in HCC cases [44].

Indirect studies on diabetics have reported that a shift toward saturated fatty acids and diminished unsaturated PLs in RBC membranes promote membrane stiffening by close packing and increased intermolecular van der Waal forces [45-46]. This can be explained by the theory given by Baral S, 2020 which suggest that the flip-flop rate of cholesterol between two leaflets of plasma membrane is regulated by type of fatty acids present in PLs. Presence of polyunsaturated lipids substantially increased flip-flop rates, thus decreases the stiffening effect of cholesterol [47]. Oxidative stress is another key driver of membrane rigidity in hepatic disorders. Chronic liver diseases, including cirrhosis, NAFLD, HCC, hepatitis, alcoholic liver diseases are associated with increased production of reactive oxygen species (O2•-, H_2O_2, NO_2•, HO•) which affects not only liver but other extrahepatic tissues. It can peroxidize cellular membrane lipids, proteins and induce cross-linking of membrane proteins thus promote premature RBC senescence or clearance by the spleen [48-51].

Evaluation of the redox and oxidative status of RBC membranes involves measuring oxidative damage markers and antioxidant defences. Common biochemical assays include quantification of lipid peroxidation products such as malondialdehyde (MDA) using the thiobarbituric acid reactive substances (TBARS) assay, and assessment of protein oxidation via carbonyl content determination. The glutathione (GSH/GSSG) ratio is frequently measured by enzymatic recycling assays to evaluate intracellular redox balance [52]. Electron paramagnetic resonance (EPR) spectroscopy can directly detect free radical species, providing detailed insights into oxidative stress [53]. Geetha A, 2007 study revealed that cirrhotic patients showed elevated lipid peroxides-hydroperoxides, and nitric oxide, along with increased methemoglobin levels. Antioxidant enzymes (except glutathione peroxidase) and membrane-bound ATPase activities were decreased. The C/PL ratio in RBC membranes was significantly altered, and osmotic fragility was increased [54]. Allard JP, 2007 studied NASH patients and experienced significant depletion of liver n-3 and n-6 PUFAs and higher oxidative stress, despite no changes in dietary fatty acid intake [55].

Maturu P, 2010 investigated the effects of chronic alcohol consumption on erythrocyte membrane properties and enzyme activity by comparing chronic alcoholic with non-alcoholic controls. Measurements included NO levels, membrane lipid composition, oxidative damage markers, membrane fluidity, and Na^+/K^+-ATPase enzyme activity. Results showed that chronic alcoholics exhibited elevated NO levels in plasma and RBCs, which correlated with increased oxidative stress, including higher lipid peroxidation and protein carbonylation, along with a reduction in membrane protein sulfhydryl groups. This oxidative damage was accompanied by an increased CHOL/PL ratio and reduced membrane fluidity [56]. Tutino V, 2024 evaluated the fatty acid profile and oxidative damage indices in erythrocyte membranes of overweight and obese subjects with metabolic dysfunction-associated hepatic steatosis. The study found arachidonic acid content and peroxidation index of RBC membrane of obese subjects was significantly higher than overweight subjects indicating increased oxidative damage [57].

In contrary to above observations, one study specifically investigated structural changes in erythrocyte membranes of patients with alcohol-induced liver cirrhosis stage c, focusing on conformation of membrane cytoskeleton proteins mainly spectrin actin complex, physical state of peripheral proteins and membrane lipid fluidity using EPR and spectrophotometric methods. The concentration of TBARS was also measured as an indicator of lipid peroxidation. The study also observed structural modifications in spectrin actin complex and increased membrane lipid fluidity in severe cirrhotic patients. These changes were accompanied by elevated TBARS levels, suggesting oxidative stress-induced conformational alterations in membrane proteins, increased lipid peroxidation and disrupted protein-lipid interactions contributing to enhanced membrane fluidity [58]. Similarly, Maksane S, 2015, using Langmuir-Blodgett monolayer techniques, found increased membrane compressibility and reduced hysteresis in patients with advanced (Child-Pugh C) liver cirrhosis, signifying enhanced membrane fluidity [42].

Collectively, these studies suggest that oxidative stress-driven lipid and protein alterations in liver disease compromise RBC membrane architecture and function, potentially affecting the fluidity and impairing deformability.

Topography and Mechanical Rigidity:

RBC membrane topography refers to the surface architecture and nanoscale morphological features of the erythrocyte membrane, including curvature, asymmetry, shape, roughness, protrusions, invaginations and distribution of membrane proteins which determine its mechanical stability. The topography is shaped by several key determinants that work in concert to maintain RBC structure and functionality. One of the primary factors is the lipid composition of the membrane, including the types and distribution of phospholipids and cholesterol. Beneath the lipid bilayer lies the cytoskeletal network, composed mainly of spectrin, actin, and associated proteins, which provides mechanical support and helps maintain the biconcave shape of the cell. Additionally, transmembrane proteins such as Band 3 and glycophorins play a critical role as their clustering, conformational changes, and interactions with the cytoskeleton can significantly alter the surface contour. The hydration status and cell volume also impact topography, as osmotic changes can cause the cell to swell or shrink, modifying the membrane's appearance. This topography can be visualized using high-resolution imaging techniques like Atomic Force Microscopy (AFM) and Scanning Electron Microscopy (SEM). AFM provides high-resolution surface imaging of single cell and quantitative measurements of membrane stiffness at the nanoscale. It uses a cantilever with a sharp tip to scan the RBC surface, generating three-dimensional topographical maps and force-distance curves that quantify mechanical properties such as Young's modulus [59]. It can give information about spikes; bulges formed on membrane and their link with cytoskeleton [60]. Additionally, SEM offers detailed and direct visualization of membrane morphology, cell interaction, cell deformation and variations of intercellular distances but does not directly quantify mechanical properties [28].

Topography of RBC Membranes in Liver Diseases:

Advanced imaging and biophysical techniques such as AFM and SEM have been employed to characterize topographical alteration in liver diseases. Oxidative stress, membrane lipid alterations, and cytoskeletal disruptions, altered cytosolic viscosity significantly impair RBC membrane architecture.

Leo M, 2020 investigated the topographical alterations in RBC membrane of a cirrhotic patient with spur cell anemia before and after liver transplant. AFM analysis showed that pre-transplant cells exhibited increased stiffness, elevated Young's modulus, and heightened energy dissipation which indicate impaired viscoelasticity. After transplantation, stiffness and hysteresis decreased and membrane elasticity approached physiological levels. SEM analysis further supports these observations as before the transplant, SEM images revealed acanthocytes with spike-like processes of different sizes on the entire cell membrane together with agglutinated cells [61]. Figure-3 depicts the acanthocytes of a liver cirrhosis patient.

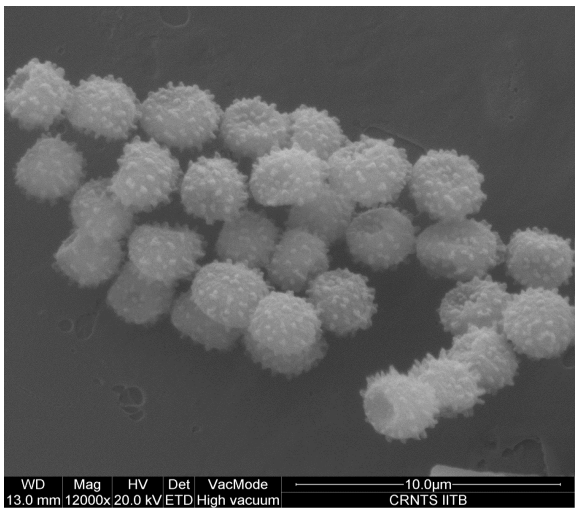

Figure 3: SEM micrograph of erythrocytes obtained from the cirrhotic patient

Their observation was in accordance to Takashimizu S, 2000, who observed 10 % acanthocytes in alcoholic liver disease patients and Maksane S, 2015, also used SEM to visualize the morphological alterations of RBCs in liver disease. The SEM images revealed notable changes in the erythrocyte surface, including loss of the typical smooth biconcave disc shape and appearance of irregularities such as membrane roughness and macrovesicle formation [42, 62].

Leo M, 2020 also reported qualitative alterations in the erythrocyte cytoskeleton using Alexa Fluor 568 phalloidin, a dye with high specificity for F-actin. Compared to controls, erythrocytes from the cirrhotic patient exhibited lower fluorescence intensity relative to the background, indicating reduced F-actin expression and a disrupted cytoskeletal network [61].

4. Lipid and Protein: Structure and Organization: The lipid organization within the membrane is largely affected by the distribution of head groups, the degree of saturation of the fatty acid tails and the concentration

of cholesterol which can diffuse between both leaflets of the membrane. The molecules are asymmetrically distributed between the two leaflets [2, 47]. Saturated fatty acids have straight chains without double bonds, allowing tight packing in membranes. In contrast, cis double bonds in unsaturated fatty acids, such as in palmitoleic and oleic acids, introduce a bend of ~133°, preventing close packing. This structural bend increases the cross-sectional area (A) of the hydrocarbon chain region in phospholipids. Replacing saturated chains with unsaturated ones expands area A by ~15%, increasing interchain distance. Consequently, reduced van der Waals forces between chains lead to enhanced membrane flexibility and decreased rigidity [46, 63].

Cholesterol is preferably located in areas with saturated lipid tails where it straightens the lipid tails and leads to a reduced area per lipid [64]. These cholesterol-rich patches are referred to as rafts, which are a manifestation of the ordered lipid phase (lo). These rafts are characterized by enhanced molecular order, reduced fluidity and are speculated to be relevant for cell signalling events [65]. Alterations in lipid asymmetry or protein structure can impact membrane flexibility, charge distribution, and intracellular signalling pathways [66].

The membrane's mechanical integrity is also determined by the horizontal linkages between spectrin-spectrin dimers and spectrin, actin, and protein 4.1R in the junctional complex of the spectrin-based membrane skeleton. If these horizontal linkages are defective, it can lead to loss of surface area, decrease in the membrane's mechanical integrity [67-68]. However, to date, very few studies have been identified that specifically characterize the alterations of these proteins in the context of liver diseases.

Fluorescence microscopy allows visualization of the spatial distribution of specific lipids and proteins using fluorescently labelled probes or antibodies. It enables detailed analysis of membrane architecture, including molecular orientation, curvature, and protein conformation. Use of fluorescent probes allows visualization of membrane protein in their native environment and study of its dynamics as well as functional dynamics of lipids. Accurate detection of protein orientation requires that the fluorescent probe be rigidly bound to the protein to prevent independent rotation that may distort polarization signals [69-70].

Total Internal Reflection Fluorescence (TIRF) Microscopy can be used to study lipid rafts and has the capacity to show the adsorption of proteins and peptides to lipids in supported lipid bilayers [71].

Fluorescence Recovery After Photobleaching (FRAP) provide insights into how membrane heterogeneity affects diffusion of proteins into the membrane and to characterize the factors that can influence the membrane organization, like cholesterol [72]. Fluorescence Resonance Energy Transfer (FRET) can be used to study protein-protein interactions and impact of cholesterol on lipid order [73-74]. Mass spectrometry-based lipidomics and proteomics enable detailed compositional analysis of membrane lipids based on charge, size, masses and shapes and proteins, revealing changes in lipid species and post-translational modifications [75]. Other techniques such as X-ray diffraction, X-ray crystallography, nuclear magnetic resonance, electron spin resonance have become standard methods to investigate membrane structure on molecular length scales; allows study of conformational properties of proteins [76].

Organizational changes in lipids and proteins in liver diseases:

One in vitro study by Stott BM, 2008 used fluorescence spectroscopy with three probes-Laurdan (for membrane packing), 1,6-Diphenyl-1,3,5-hexatriene (for lipid order), and MC540 (for lipid spacing) to examine cholesterol's effects on erythrocyte membranes where cholesterol levels were varied using methyl-β-cyclodextrin and generated a phase map showing how cholesterol alters membrane fluidity, order, and lipid organization. They observed that in normal conditions, the membrane shows well-ordered lipid regions with moderate fluidity and tight lipid packing. When cholesterol is depleted, the membrane becomes more disordered and fluid, though lipid spacing may remain unchanged. At moderate cholesterol levels (~30%), the membrane exhibits high fluidity and increased lipid spacing, indicating a transition to a more loosely packed state. A dominant intermediate phase was also identified, showing reduced lipid order but lower fluidity and tighter spacing than in severely cholesterol-depleted states. These findings highlight cholesterol's crucial role in maintaining membrane structure and stability [77].

In the study by Maksane S, 2015, evaluated the packing behaviour and compressibility of lipid extracts from RBC membranes in liver disease. They reported that early-stage cirrhosis was associated with increased membrane rigidity, while advanced (Child-Pugh C) cirrhosis showed a reversal to increased fluidity and compressibility. These ultrastructural disruptions are indicative of compromised membrane integrity and correlate with biophysical changes observed in the Langmuir-Blodgett monolayer experiments. Such

morphological aberrations likely contribute to impaired RBC deformability and increased fragility seen in liver disease patients [42].

Leo M, 2020 studied the connection between RBCs mechanical, cytoskeletal and lipid membrane alterations with confocal fluorescence microscopy experiments on cirrhotic patient. They characterised RBC membrane lipid domains using Laurdan fluorophore to map changes in the membrane structure for control and cirrhotic patients. They observed that in the cirrhotic patient, the liquid disordered lipid domains diminish, corelating with an excess of cholesterol, which induces a rigidification of fluid membrane lipid domains at a physiological temperature [61]. The decrease of lipid domains with lower Generalised Polarisation indicates about solid ordered lipid layer which is consistent with an excess of cholesterol, which induces a rigidification of fluid membrane lipid domains at a physiological temperature [77-78].

They also attempted for validation of these novel erythrocyte-based mechanical biomarkers for their usefulness in disease diagnosis and therapy monitoring by comparing the changes after liver transplant. The results showed that liver transplantation not only contributes to restoring the proper RBC morphology, but it also induces recovery of the physiological viscous behaviour of cells, further stressing the relevance of viscous and dissipative forces in determining the RBC biomechanical response.

The oxidation of lipids is associated with the oxidation of protein and crosslinking to cytoskeleton and membrane proteins [79-80]. Band 3 is a major integral-anion exchanger protein of the erythrocyte membrane that anchors the lipid bilayer to the cytoskeleton through interactions with ankyrin. Oxidative modifications of Band 3 disrupt these interactions, compromising membrane stability, flexibility, and overall function [9]. Increased erythrocytes tyrosine kinases activity causes tyrosine phosphorylation in the cytoplasmic domain of the band 3 protein. This phosphorylation mediates interactions with ankyrin, leading to membrane destabilization [81]. In liver diseases, RBC membranes undergo significant structural and protein organizational changes due to oxidative stress and metabolic dysregulation occurs. We could not find any other study which evaluated detailed structural and organizational changes in Band-3 protein in any liver diseases.

The cytoskeletal proteins, including spectrin and ankyrin, are also notably affected. Gwoździński L, 2011 observed oxidative stress induced and structural and conformational modifications in spectrin actin complex and in severe cirrhotic patients [58]. Surface glycoprotein changes, particularly desialylation of glycophorin A, facilitates its fixation and increases glycophorin A content in RBC membrane and contribute to decreased surface charge [82].

This narrative review focusing on RBC membrane surface properties in liver diseases is both timely and necessary. RBC membrane surface properties undergo significant alterations in liver diseases, including reduced membrane fluidity (increased in severe liver disorders), surface charge, altered membrane lipid protein organization and increased oxidative modifications. These changes may have diagnostic, prognostic, and therapeutic implications. Parameters such as RBC deformability, surface charge, and oxidative stress markers can serve as accessible and cost-effective biomarkers for tracking liver disease progression and identifying associated complications. Recognizing potentially reversible modifications in RBC surface properties opens avenues for the development of targeted interventions, such as antioxidant therapies or agents that stabilize the membrane. These strategies could help alleviate liver disease-associated anemia and improve microcirculatory dynamics. More rigorous, standardized research is needed to validate their clinical application.

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