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Full Length Article

Red blood cell membrane cholesterol in type 2 diabetes mellitus

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ABSTRACT

Type II diabetes mellitus (T2DM) affects an estimate of 450 million individuals. Hence, there remains an urgent need to explore the use of novel biomarkers with the aim of preventing and managing cardiovascular risk among these individuals. Hallmarks of this condition are lipid and glucose dyshomeostasis which are accompanied by a prothrombotic phenotype; these pose as eminent links between T2DM and cardiovascular disease. Diabetic dyslipidemia affects not only plasma lipid profiles but extends further into the haematological system, modulating the cholesterol concentration of erythrocyte membranes. Elevations in this biomarker implicate cell biophysics and contribute to an increased cardiovascular risk. Here we investigate the variation of membrane cholesterol levels in RBCs, as well as the degree of hypercoagubility between healthy and type II diabetic subjects. Furthermore, we provide an adapted method to assess erythrocyte membrane cholesterol levels using a quantitative and qualitative approach. In conclusion, individuals with type II diabetes exhibit elevated erythrocyte membrane cholesterol levels which may act as a prominent link between the diabetic state and subsequent cardiovascular complications. Ultimately, this erythrocyte parameter exhibits applicative biomarker potential and may provide clinical utility in terms of disease monitoring and prognostics.

1. Introduction

Type II diabetes mellitus (T2DM) affects an estimate of 450 million individuals of which 75% will likely experience a fatal cardiovascular event [1,2]. Considering that cardiovascular disease (CVD) is the leading cause of global mortality and that the prevalence of T2DM is on the rise [1], there remains an emphasis on exploring and utilizing various biomarkers with the aim of preventing and managing CVD among diabetic patients. T2DM is associated with the circulation of dysregulated inflammatory markers which drives chronic and systemic inflammation [3–7]. Intimately related to this immune dysfunction is an exaggerated thrombotic state characterized by hypercoagubility, hypofibrinolysis and aberrations in clotting machinery [7–9][10,11]. In addition to this proinflammatory and prothrombotic phenotype, an eminent link between T2DM and cardiovascular disease is lipid dyshomeostasis, which is denoted by elevated triglyceride levels and an altered lipoprotein profile [12]. This metabolic imbalance – termed diabetic dyslipidemia – affects not only plasma lipid profiles, but extends further into the haematological system.

Cholesterol is a ubiquitous sterol and significant element of lipoproteins that serves as a precursor for hormone and bile synthesis. Relevant to this paper, cholesterol also constitutes an essential component of cell membranes and functions to condense phospholipid packaging and increase membrane integrity [13]. Although a vital constituent, membrane cholesterol (MC) may surpass concentrations that hinder the pliability of cells by increasing the viscosity of membranes [14]. Alternative to plasma lipids, the cholesterol content of red blood cells (RBCs) – which is modulated by long-term lipoprotein concentrations [15] – has been investigated and poses as a marker with potential to assess cardiovascular dysfunction [16]. In terms of hemorheology (blood flow within a vessel), RBCs with elevated MC levels exhibit impaired biophysical functionality and thus contribute to further cardiovascular risk in T2DM [17].

RBC MC exists in the form of free cholesterol, whereas intracellular and plasma cholesterol is predominantly esterified [13,18,19]. This presents with clinical significance in the case of atherogenesis. RBCs accumulate in atherosclerotic plaques via fibrous cap displacement [20] and intraplaque haemorrhages [21]. After the translocation from circulation to the intimal space, subsequent intraplaque degradation results in the release of free cholesterol. Since free cholesterol within a necrotic lipid core composes roughly 25% of total plaque lipids [22], it seems feasible that RBCs – which contain the highest concentration of MC among any cell type – are a major source of this cholesterol load within atherosclerosis [15]. Supporting the premise, researchers have discovered fractions of RBC membranes are contained within plaques of deceased coronary artery disease (CAD) patients [21]. Tziakas et al.

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[23] investigated the association of RBC MC with unstable and stable CAD; their results depicted higher concentrations in patients with unstable CAD thereby suggesting the use of RBC MC as a marker for disease progression and more specifically, plaque instability [23]. Similar experiments also concluded that RBC MC is associated with the severity of CAD [16,24]. Hence, elevations RBC MC concentrations past normal ranges is associated with plaque instability [25]. This emphasizes the potential of utilizing RBC membrane cholesterol as a biomarker to evaluate cardiovascular risk. Noticing an imbalance within RBC MC dynamics before overt coronary artery disease manifests may be clinically beneficial in predicting and preventing cardiovascular dysfunction as well as hindering atherogenesis. Thus, RBC MC may be utilized as a predictive marker for atherosclerotic cardiovascular disease.

Although studies revolving around this topic are somewhat scarce, the data that exists highlights its clinical utility and proposes the implementation of RBC MC as a biomarker for cardiovascular dysfunction. Further investigation and the development of techniques to study membrane concentrations are thus emphasized. The aim of this paper is therefore to depict variation in MC levels among healthy and T2DM individuals and assess the influence of exogenous cholesterol on the morphology of haematological cells and coagulation dynamics. Furthermore, we present this as a preliminary study for a novel approach for qualitatively assessing RBC MC.

2. Methods and materials

2.1. Ethical statement

Ethical clearance was obtained from the Health Research Ethics Committee (HREC) of Stellenbosch University (ethics number: HREC1-2018-6399). Prior to blood collection, all study participants were informed of the dynamics of the study and signed a written consent form for sample use and data publication. All participants received a unique number that was used to ensure confidentiality throughout the study. Investigators involved in this study strictly adhered to the Declaration of Helsinki and were certified in Good Clinical Practice.

2.2. Study design and study population

This study was cross-sectional in design and in collaboration with specialist physicians, who provided whole blood (WB) from T2DM patients. Whole blood from healthy controls were collected by a Health Professions Council of South Africa (HPCSA) registered Medical Biological Scientist and phlebotomist (MW: 0010782) at the Department of Physiological Sciences, Stellenbosch University. This study included twenty-two healthy and twenty-four type II diabetic individuals. Diabetic individuals with CVD were diagnosed according to the Society for Endocrinology, Metabolism and Diabetes of South Africa (SEMSDA) guidelines. The exclusion criteria for the healthy population included infection, chronic disease, smoking and the use of anti-inflammatory, chronic or hormone replacement drugs. The same exclusion criteria are applied to T2DM subjects. Additionally, these individuals had to have been diagnosed with the disorder for over three months prior to the study. We randomly identified healthy and T2DM individuals, for this analysis, from samples received in our laboratory. The selection process for the heathy individuals only relied on the absence of the conditions mentioned in the exclusion criteria, but also that they were normoglycemic as noted from their HBA1c levels. The T2DM individuals only had to be diagnosed with T2DM > 3 months prior to this study and their HBA1c should still reflect hyperglycemia. The T2DM statin therapy was also negated.

2.3. Collection and preparation of whole blood (WB) and preparation of samples from healthy controls and T2DM patients

Whole-blood from heathy controls and T2DM patients were collected using sterile techniques in sodium citrate and ethylenediaminetetraacetic acid (EDTA) tubes, and left to stand at room temperature for 30 min. Citrated WB was analysed on the same day of collection (see methods below). To obtain haematocrit, the citrate tubes were centrifuged at 3000 ×g for 10 min at room temperature. The supernatant containing the plasma as well as the buffy coat were removed and haematocrit collected and stored at −80 °C in Eppendorf microcentrifuge tubes for future analysis. EDTA whole-blood was analysed by an accredited Pathology Laboratory (Pathcare: Stellenbosch, South Africa (PracNum: 5200539)) for glycosylated haemoglobin (HbA1c) and total plasma cholesterol levels. The glycosylated haemoglobin and total cholesterol results are included in our manuscript.

2.4. Thromboelastography (TEG)

In order to study clot kinetics and dynamics, TEG analysis was performed on both control and T2DM freshly collected whole-blood samples. 340 μL of fresh whole-blood was carefully pipetted into disposable TEG cups and then followed by the addition of 20 μL of 0.2 mol/L calcium chloride ($CaCl_2$). $CaCl_2$ functions to commence clotting by supplying coagulation reactions with the cofactor calcium, which was inhibited by sodium citrate in the collection tube. Once $CaCl_2$ was added, the automated test was swiftly initiated. All tests were performed on the Thromboelastograph 5000 Hemostasis Analyzer System (Haemonetics S.A. Signy-Avenex, Switzerland) and utilized according to the manufacturer's protocol. In total, seven TEG parameters were measured (see Table 1).

2.5. Scanning electron microscopy

10 μL of fresh whole blood was used to make a smear on a glass cover slip. After allowing 5 min to pass by in order to allow the cells to

Table 1
TEG parameters and their subjective description, units and standard ranges.

TEG parameter	Description	Units of measurement	Normal ranges
R-value	Time of latency from start of test to initial fibrin formation (amplitude of 2 mm); i.e. initiation time	Minutes	9–27 min
K-value	Time taken to achieve a certain level of clot strength (amplitude of 20 mm); i.e. amplification	Minutes	2–9 min
Alpha angle (AA)	The angle measures the speed at which fibrin build up and cross linking takes place; i.e. thrombin burst	Degrees	22–58°
Maximum amplitude (MA)	Maximum strength/stiffness of clot; i.e. overall stability of the clot	Millimetres	44–64 mm
Maximum rate of thrombus generation (MRTG)	Maximum rate of thrombus generation using G, where G is the elastic modulus strength of the thrombus in dynes per cm − 2	$dyn{\cdot}cm^{-2}{\cdot}s^{-1}$	0–10 $dyn{\cdot}cm^{-2}{\cdot}s^{-1}$
Time to maximum rate of thrombus generation (TMRTG)	The time interval observed before the maximum speed of the clot growth	Minutes	5–23 min
Total thrombus generation (TTG)	The clot strength: the amount of total resistance generated during clot formation (total area under the velocity curve during clot growth)	$dyn{\cdot}cm^{-2}$	251–1041 $dyn{\cdot}cm^{-2}$

adhere to the glass surface, the slip was lifted using tweezers and translocated to a 24-well plate. 4% paraformaldehyde – enough to immerse the cover slip – is used to fix the samples for 30 min. After wash steps, 2–3 drops of osmium tetroxide – a potent fixative optimal for SEM – was added to sample. The samples were then dehydrated with increasing concentrations of ethanol – 30%, 50%, 70%, 90% and 100% – each for 3 min. After removing the last of the 100% ethanol, hexamethyldisilazane (HMDS) was utilized as the terminal dehydrating agent. Three drops of HMDS were added to each sample. Lastly, slips were removed from each well and placed onto a glass microscope slide ready for subsequent carbon coating and SEM analysis.

2.6. Free cholesterol assay

In order to obtain concentration values of cholesterol in haematocrit samples, a commercial cholesterol/cholesterol ester quantification assay kit was purchased from Abcam (ab65359). The kit and protocol had to be slightly modified and certain components of the assay negated. Firstly, the kit is not customized to haematocrit samples which are particularly dense in cell volume. Thus, sequential volumes of haematocrit – 20 μL, 50 μL, 100 μL and 200 μL – were tested to discover the optimal amount of sample required to suit the standard curve. 100 μL was chosen as the optimal value to suit this assay kit. Additionally, due to the viscosity of haematocrit solutions, this volume of haematocrit was diluted with PBS and then subjected to sonication at 10 mm amplitude for 10 s instead of the kits instructed homogenization procedure using a microhomogenizer. As previously stated, free cholesterol predominates in cell membranes whereas cholesteryl esters localize intracellularly or within plasma; thus, the inclusion of cholesterol ester measurement in the assay must be ignored. This is done by excluding cholesteryl esterase – a component of the kit which is necessary to convert cholesteryl esters into free cholesterol – from the assay procedure. Thus, by excluding cholesteryl esters in the assay procedure, we negate any cross reactivity and thus measurement of cholesteryl esters. (As per the kit, cholesterol esterase is required to convert any present cholesteryl esters within the sample to cholesterol in order to be quantified by the assay).

Within microcentrifuge tubes, 100 μL of haematocrit was diluted 2× with PBS in order to prevent excessive agglutination and aggregation of red blood cells. 200 μL of chloroform:isopropanol:NP-40 (7:11:0.1) – a lipid-extracting solution – was used to isolate lipids from haematocrit samples. In order to lyse and homogenize the sample for the optimal extraction of cholesterol, samples were sonicated twice for 10 s. To separate cell debris from the isolated lipids, samples were centrifuged at 3000 *g* for 10 min. The supernatant which contains cholesterol and other lipids was carefully removed with a Pasteur pipette and transferred into a new microcentrifuge tube. The remaining pellet was discarded. The microcentrifuge tubes containing the extracted lipids were left open to air dry at room temperature for 30 min in order to allow chloroform to evaporate. Isolated lipids were dissolved with 50 μL of PBS and then vortexed for a few seconds. 50 μL of diluted sample as well as 50 μL of cholesterol standard was carefully transferred to reaction and standard curve wells, respectively. 50 μL of reaction enzyme mix – devoid of cholesterol esterase – was then added to reaction and standard curve wells and left to incubate in 37 °C for 60 min. After incubation, the plate was analysed using a Tecan Spark® fluorometer with excitation and emission values of 535 nm and 587 nm, respectively.

2.7. Confocal microscopy: using filipin to visualize free cholesterol

As the majority of cholesterol in the membrane is in its free form, probes that have specificity for free cholesterol need to be utilized. Filipin is an intrinsically fluorescent, antifungal antibiotic that was first isolated in 1955 from the bacterium species *Streptomyces filipenesis* [26,27]. It binds free cholesterol with great affinity, whilst negating any association with other forms of cholesterol [28]. Relating to this study, RBCs are devoid of intracellular organelles [29]. This is advantageous over other cell types as intracellular cholesterol-containing membranes can interfere with the fluorescent emittance from the area of interest. It should be noted that filipin rapidly bleaches following laser excitation and should thus be swiftly, but precisely imaged.

A kit from Abcam (ab133116) was purchased but not utilized as specified by the supplier. Three out of five reagents of the kit were negated and excluded from the assay. Thus, the following adapted protocol utilizes only filipin and the accompanying cholesterol detection buffer provided by the manufacturers. As the kit is not specified for red blood cell samples, 10 μL of haematocrit was found to be a complimentary value to the kit.

The original method is a validated method, that is known to be reproducible, accurate and consistent. We optimized the filipin concentration and number of RBCs, to determine the optimal haematocrit dilution for the corresponding concentration of filipin. After thawing haematocrit samples, 10 μL of isolated RBCs was diluted 20× in PBS. 10 μL of diluted haematocrit was then placed onto a circular region on a glass slide bordered with a histological wax pen and left to stand for 5 min. The smear-section was washed with PBS once after which fixation with paraformaldehyde (PFA) proceeds for 10 min. PBS was used to wash the sample slides three times for 5 min each. 100 μL of filipin working solution – containing a filipin concentration of 20 μL/mL – was then pipetted onto the smears in a dark room and left to incubate for an hour. Lastly, a wash step with PBS removes any residual probe that may interfere with cholesterol-filipin-emitted fluorescence. Finally, a glass cover slip was placed on the slide which is then ready for imaging. Filipin is preferentially excited at 340–380 nm and emitted at 385–470 nm, but can also be imaged with a 405 nm laser, parallel to the generalised DAPI (4′,6-diamidino-2-phenylindole) setting.

2.8. Statistical analysis

All statistical analyses were carried out on GraphPad Prism version 7. Data were analysed for normality with the Shapiro-Wilk test. Normally distributed data was analysed with unpaired *t*-tests whereas Mann-Whitney tests were used for nonparametric data. Data is expressed as the mean ± standard error of the mean and deemed significant when $p\text{-value} < 0.05$.

3. Results

Table 2 shows the demographics of both the T2DM and the healthy (control) sample. This table includes age, gender, HbA1c values and results from a lipogram test indicating total cholesterol. Most of the population of T2DM subjects (71%) were on lipid lowering medication (Simvastatin™, Atorvastatin™, Bezafibrate™); this accounts for the lower total cholesterol concentrations in the T2DM group's plasma when compared to the controls. The difference between the two groups' HbA1c results ($p < 0.0001$) indicates the severity of glucose dysregulation in T2DM.

TEG results of both control and T2DM whole-blood samples are represented in Table 3. Significance was found in all parameters

Table 2
Control and T2DM demographics. Data represented as mean ± SD. (HbA1c – glycated haemoglobin test).

Parameter	Control	T2DM
Age	48,79 ± 14,03	63,65 ± 13,06
Gender	Male (n = 10); Female (n = 12)	Male (n = 11); Female (n = 13)
Total cholesterol (mmol/L)	4,47 ± 0,96	3,61 ± 1,05
HBA1c (%)	5,43 ± 0,42	9,33 ± 1,82

Table 3
Thromboelastography results of whole-blood from healthy and T2DM individuals with the corresponding significance ($p < 0.05$ = *; $p < 0.01$ = **; $p < 0.001$ = ***). Data are represented as mean ± SEM.

TEG parameters	Control	T2DM	Significance
R-value	7,833 ± 0,4781	5,9 ± 0,3752	**
K-value	2,979 ± 0,2462	2,058 ± 0,1539	**
Alpha angle	60,67 ± 1,85	68,75 ± 1,245	***
MA	57 ± 1,671	63,93 ± 2,324	**
MRTG	5,232 ± 0,4625	9,603 ± 1,063	***
TMRTG	11,68 ± 0,6782	9,132 ± 0,4787	**
TTG	701,8 ± 44,35	1024 ± 91,73	**

assessed. In comparison to the controls, the R-value, K-value and TMRTG of the T2DM individuals indicated significantly faster rates of clot formation and kinetics. As inferred from AA, MA, MRTG and TTG results, the T2DM group exhibited larger and stiffer clots than the control group. Ultimately, these differences indicate a greater propensity for clot formation and hypercoagubility within T2DM, as well as a possible hypofibrinolytic state (although we did not measure this) as clot strength, size and thus persistence was elevated.

Scanning electron microscopy (SEM) analyses of whole-blood from control and T2DM participants are illustrated in Fig. 1. Morphologically, the control samples have RBCs that are typical of a healthy individual (Fig. 1A–B). Associated control platelets appear to be ‘non-activated’ as inferred from their round shape and lack of spreading; the apparent pseudopodia formation is likely due to contact activation during sample preparation. In contrast, the images of T2DM whole-blood smears depict pathological RBCs and platelet morphology indicative of oxidative stress and inflammation (Fig. 1C–F). T2DM RBCs are aberrant in shape and display extensive eryptosis (programmed RBC cell death). Additionally, T2DM platelets, which are particularly denser in population as opposed to control samples, are hyper-activated and exhibit significant spreading as indicated by the arrows (Fig. 1E). In terms of qualitative quantitation, the healthy individuals contained an almost full panel of morphologically normal RBCs whilst more than half of the T2DM RBCs were pathological which emphasizes the extent of physiological insult originating from the diabetic phenotype. This has been discovered before and well elaborated.

Results obtained from the free cholesterol assay are represented in Fig. 2. With a slightly altered protocol adapted from the manufacturer's standard operating procedure, free cholesterol levels have been successfully determined using a commercial assay kit. Control and T2DM haematocrit samples measured with a mean free cholesterol concentration of 0.217 μg/well and 0.332 μg/well, respectively. The results exhibit a statistical significance of $p = 0.046$ (*) with the T2DM group possessing greater levels of free cholesterol – an anticipated result. The R-values for the standard curves of which the above data was derived from are $R^2 = 0.9969$ and $R^2 = 0.9518$.

Confocal images of RBCs successfully stained with filipin (Fig. 3) depict a clear distinction in fluorescent intensity between both groups. Control RBCs exhibit a modest level of free cholesterol as inferred by the magnitude of fluorescence whilst the T2DM group emit fluorescent intensities substantially greater in comparison. The distribution of cholesterol within control RBC membranes is generally sparse with few regions expressing relatively denser concentrations. Conversely, the free cholesterol in T2DM RBC membranes is much denser with numerous intensely fluorescent regions, as depicted in magnified images (Fig. 3C–D). Thus, filipin is a useful probe (and one of very few) for assessing free cholesterol levels in an efficient manner. In this experiment, as a negative control, filipin was excluded in the samples in order to determine if there is auto-fluorescence of RBCs or other signal interference. No interference was observed. Our T2DM sample acted as our positive control, as addition of cholesterol or cholesteryl esters to healthy RBCs would not necessarily result in incorporation of the molecules into the double phospholipid membrane.

4. Discussion

Our results point to the fact that our T2DM sample shows hypercoagulability and a morphological blood phenotype that is indicative of pathology resulting from inflammation and oxidative stress. This is in accord with previous studies [6,30–36]. Elevations in RBC MC are associated with perturbations in microcirculation, plaque instability and the severity of CAD [16,37]. As T2DM is associated with dyslipidemia, it seems sensible that RBC cholesterol homeostasis will be implicated and may act as a link between diabetic pathology and cardiovascular comorbidities; Fig. 4 summarizes the link between the RBC MC, the T2DM phenotype and subsequent cardiovascular risk.

As all of our T2DM samples were on lipid-lowering medication (statins); plasma total cholesterol concentrations (Table 2) were subsequently quite low compared to our control sample. Research suggests that drug therapies may have an ameliorative impact on RBC MC; this was shown by Tziakas and co-workers in an experiment that used the same quantitative kit as this study [38]. These authors revealed that statin therapy reduces MC concentrations to ‘stable’ levels in patients suffering from acute coronary syndrome. However, this rectification of RBC MC concentrations during statin therapy was only reflected after roughly six months [15,38]. This, along with our contradictory lipogram and RBC MC results, is in disagreement with the thinking that RBC MC is in acute equilibrium with plasma cholesterol sources [39]. It appears that MC levels of RBCs are modulated by biochemical and enzymatic processes that discerns a regulated mechanism as opposed to simple equilibrium with plasma cholesterol [14,15,17,24,40]. If this wasn't the case, then the RBC MC values of the T2DM would be lower than the controls, as is the total cholesterol values of plasma (Table 2). Since clinical instability following a cardiovascular event lasts several months, RBC MC may – via mechanisms that will be mentioned – account for part of this instability. Thus, considering that statins do have an effect on RBC MC (whether it may be indirectly and over a long period of time), this may account for the modest degree of significance exhibited between our two sample groups (p = *). Additionally, cyclodextrin-related compounds have also been shown to alter cholesterol levels within membranes of cells [41]. Thus, these drugs may provide clinical utility by reducing RBC MC and subsequent cardiovascular risk.

Our T2DM sample exhibited significantly higher RBC MC levels, than the control group, as determined by the quantitative assay and qualitative assessment. Our confocal (filipin) results also correlated to this finding. Furthermore, SEM analysis of WB confirms the presence of a significant percentage of RBCs that show membrane changes and eryptosis; this is in contrast to the morphological phenotype of RBCs in the healthy group which expresses an almost full panel of normal RBCs This finding is consistent with other studies that have also utilized this mode of morphological analysis for T2DM WB [7,31,34,35]. Other studies have different techniques (blood flow and aggregation, atomic force microscopy and flow cytometry) to show RBC structural changes, including eryptosis in T2DM [42–48]. Relevantly, this highlights the extent of impact that diabetic pathology exerts on circulating RBCs. Impaired functionality and biophysical operation of RBCs resulting from this membrane alteration will contribute to further circulatory detriment in T2DM. Increased RBC membrane viscosity is directly proportional to MC [37] and has implications on their rheological properties. RBCs possess a relevant biophysical attribute called deformability – the reversible change in cell geometry and structure – that enables RBCs, which are typically 8 μm in diameter, to traverse microvessels with a lumen as narrow as 3 μm [49]. A reduced capacity of RBCs to deform, alongside hypercoagulation, causes perturbations in microvascular blood flow and in serious cases, vessel occlusion [50]. Thus, pertaining to RBC deformability and subsequent hemorheology, increased MC diminishes the deformable capabilities of these cells which lead to microcirculatory defects.

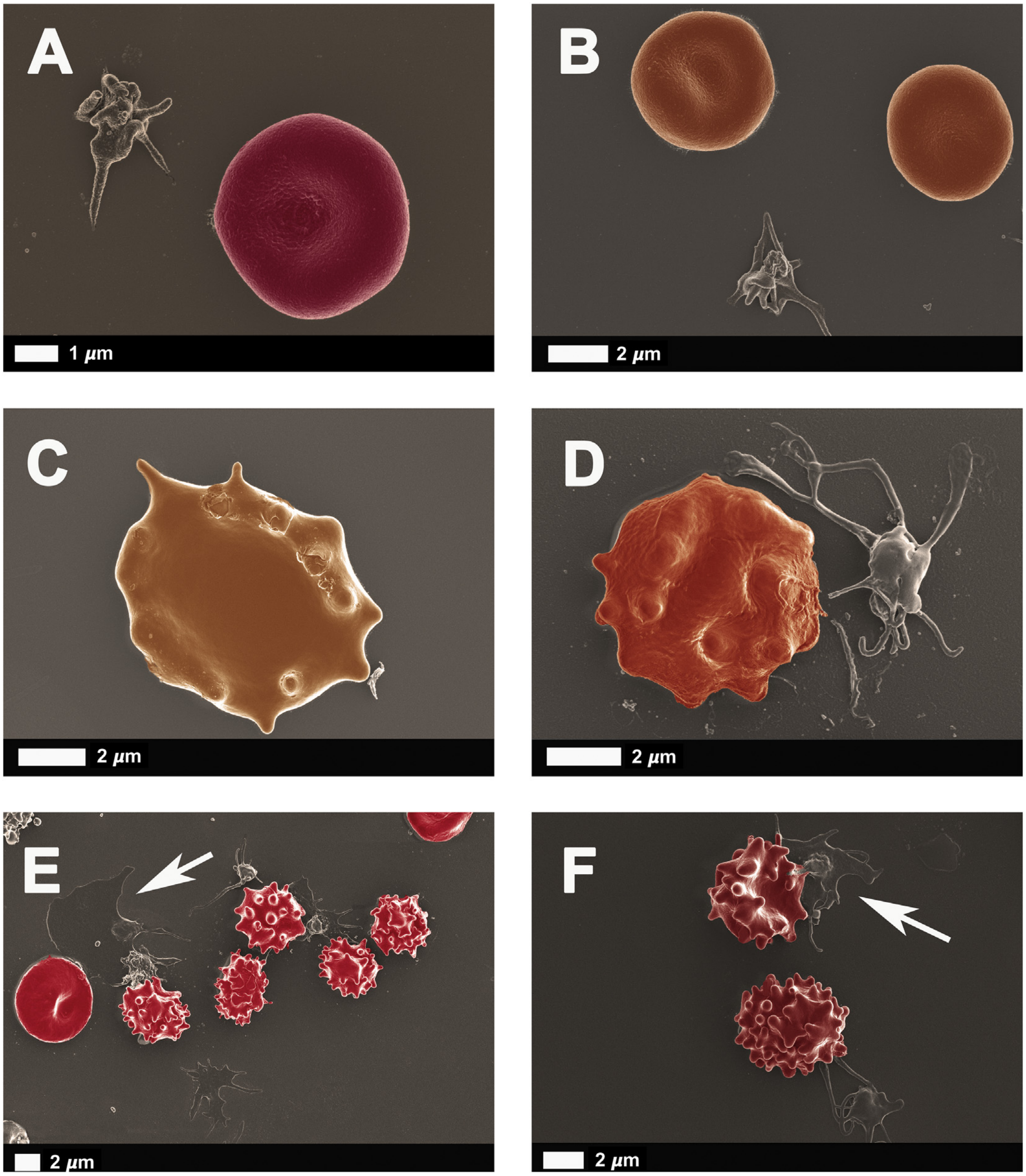

Fig. 1. Scanning electron micrographs of control and T2DM whole-blood smears. A–B) RBCs and platelets typical of a healthy individual. C–F) Eryptotic, abnormal RBCs and hyperactivated platelets of T2DM individuals. White arrows show hyperactivated platelets.

In the context of membrane transport, exaggerated RBC MC reduces O_2 permeability and rate of gaseous [39] thereby posing as a determining factor for apparent tissue hypoxia in T2DM and other cardiovascular-related pathologies. Therefore, manipulating levels of MC may improve tissue oxygenation in patients suffering from hypoxia or ischemic conditions. This reduction in membrane transport with a concurrent increase in MC is not only limited to gases, but also sodium, potassium and phosphate [51–53]. Imbalances in these ions contribute to osmotic fragility which increases the propensity of programmed cell death in RBCs (eryptosis) – a commonality within T2DM [54].

RBCs also utilize nitric oxide (NO) to enable vasodilation for optimal blood flow [55]. However, just as gases and ions, membrane cholesterol is inversely associated with nitric oxide diffusion [56]. Hence, elevated MC contributes to defective hemorheology and potentially localized hypertension via reduced NO availability. Additionally, certain RBC membrane proteins require subjective concentrations of cholesterol in order for optimal functioning [57]. Elevations in MC inhibit the activity of Na^+/K^+ ATPase (sodium/potassium adenosine triphosphate) pump which may induce deficits in energy among RBCs. Inadequate ATP levels prompt improper NO handling and reduces ATP-induced deformability. Relevantly, the Na^+/K^+ ATP pump is aberrant in function in T2DM with a decreased activity positively associated with disease severity [58]. The aforementioned implicative mechanisms resulting from elevated RBC MC are responsible for RBC and hemorheological dysfunction in this context. The goal is thus to prevent the manifestation of these defects by reducing MC levels.

Although the measurement of plasma cholesterol levels in the form of various lipoproteins remain the clinical gold-standard for lipid analysis in patients with T2DM, further investigation into diabetic dyslipidemia is vital in order to reduce the burden imposed by T2DM and associated CVD. The complimentary techniques presented aims to make assessment of this physiological parameter more efficient and specific. Here we present data reflecting variations in MC among control and T2DM RBCs, as well as the outcome of exogenous cholesterol on clot dynamics, whole-blood morphology and RBC MC concentrations. Our

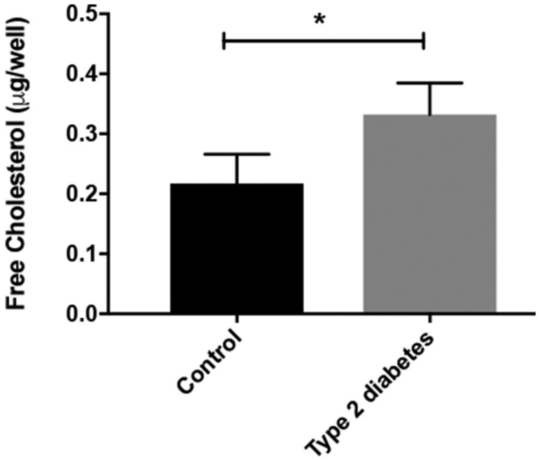

Fig. 2. Free cholesterol concentration in 20 control vs 20 T2DM RBC membranes using haematocrit samples. Values are represented in μg/well. p-Value = 0.046 ($p < 0.05$ = *).

results show consistent significance, even thought our sample numbers were low (22 healthy vs 24 T2DM individuals). Future research could expand on the study number and the selection criteria within groups (e.g. stratify according to HbA1c, CRP levels and levels of LDL and HDL). However, this paper brings to light the role of MC as a possible novel biomarker in T2DM, and more specifically, cardiovascular pathology and will hopefully pave way for further optimization and clinical implementation.

Data sharing

All raw data is available: https://1drv.ms/f/s!AgoCOmY3bkKHiYYjRj585tVE7GHhQQ

Authors' contributions

JMN wrote the paper; EP: study leader and corresponding author.

Competing interests

The authors declare that they have no competing interests to declare.

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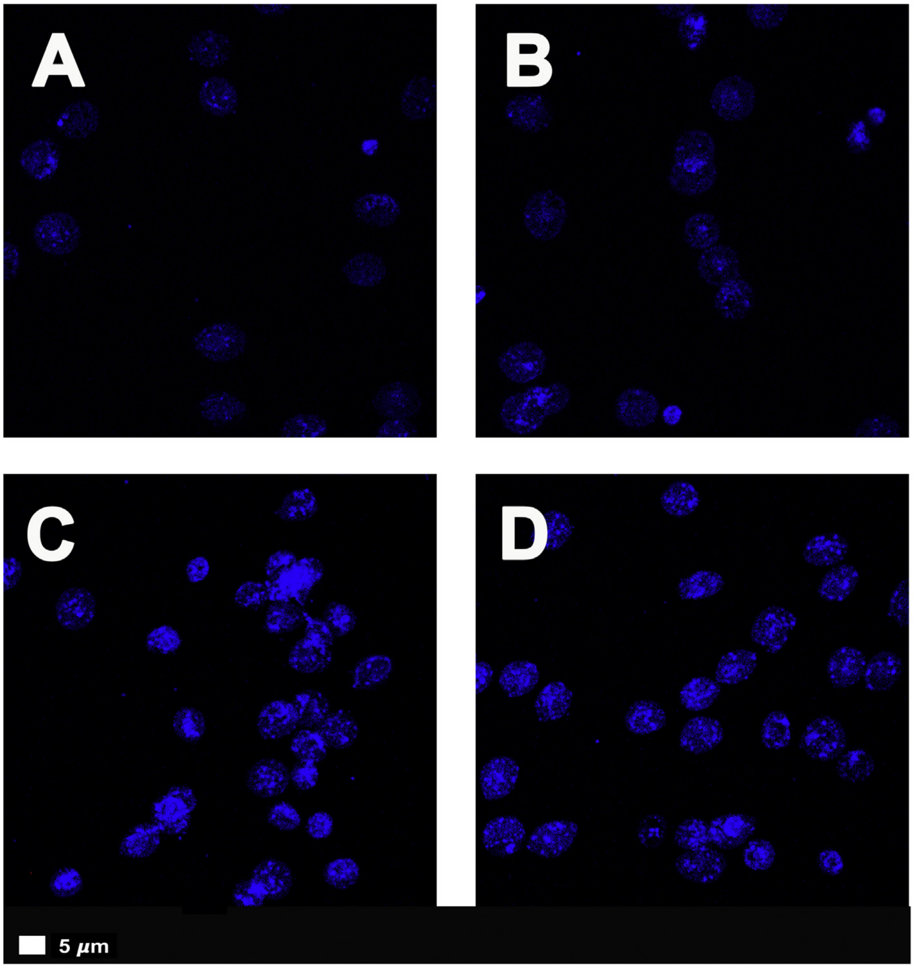

Fig. 3. Confocal microscopy images displaying cholesterol concentration and localisation within control and diabetes filipin-stained RBC membranes. Control (A and B) and T2DM (C and D) RBCs reflecting free cholesterol levels. As a negative control, filipin was excluded in the samples in order to determine if there is autofluorescence of RBCs or other signal interference (at the particular chosen wavelength). No interference was observed.

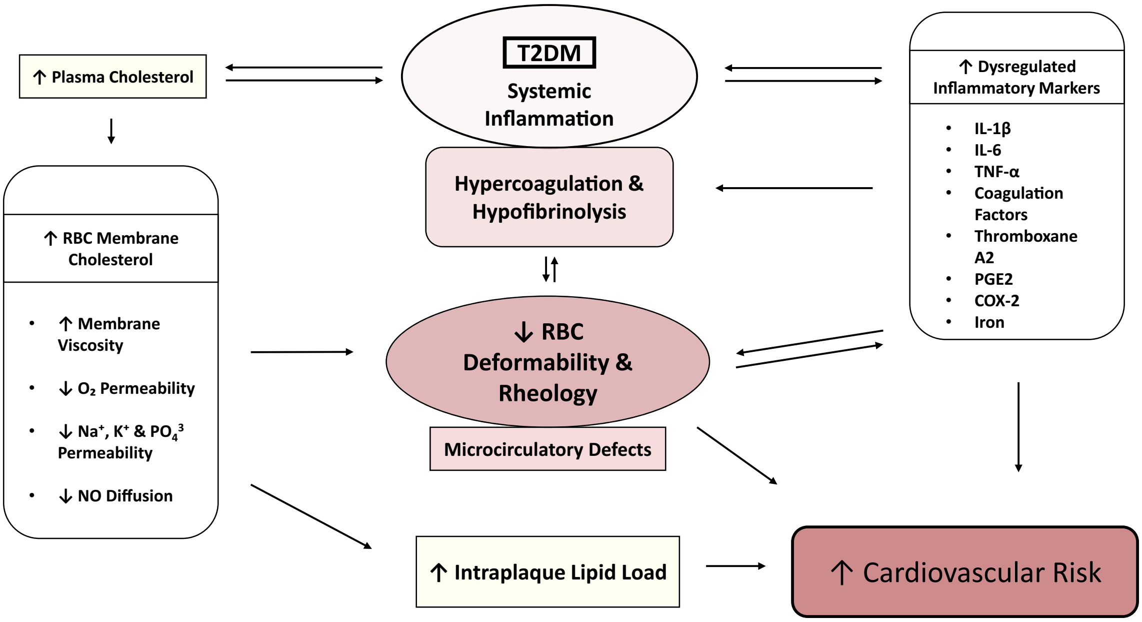

Fig. 4. Summary diagram of RBC MC and the link between T2DM and CVD. (IL –Interleukin; TNF – Tumour Necrosis Factor; PGE – Prostaglandin E; COX – Cyclooxygenase; NO – Nitric Oxide).

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