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Erythrocyte membrane fluidity: A novel biomarker of residual cardiovascular risk in type 2 diabetes

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Abstract

Aims: Improving the composition of circulating fatty acids (FA) leads to a reduction in cardiovascular diseases (CVD) in high-risk individuals. The membrane fluidity of red blood cells (RBC), which reflects circulating FA status, may be a valid biomarker of cardiovascular (CV) risk in type 2 diabetes (T2D).

Methods: Red blood cell membrane fluidity, quantified as general polarization (GP), was assessed in 234 subjects with T2D, 86 with prior major CVD. Based on GP distribution, a cut-off of .445 was used to divide the study cohort into two groups: the first with higher GP, called GEL, and the second, defined as lower GP (LGP). Lipidomic analysis was performed to evaluate FA composition of RBC membranes.

Results: Although with comparable CV risk factors, the LGP group had a greater percentage of patients with major CVD than the GEL group (40% vs 24%, respectively, $p < .05$). Moreover, in a logistic regression analysis, a lower GP value was independently associated with the presence of macrovascular complications. Lipidomic analysis showed a clear shift of LGP membranes towards a pro-inflammatory condition due to higher content of arachidonic acid and increased omega 6/omega 3 index.

Giada Bianchetti and Chiara Maria Assunta Cefalo contributed equally to this work.

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Conclusions: Increased membrane fluidity is associated with a higher CV risk in subjects with T2D. If confirmed in prospective studies, membrane fluidity could be a new biomarker for residual CV risk assessment in T2D.

KEYWORDS

cardiovascular disease, cardiovascular risk assessment, fatty acids, fluorescence microscopy, machine-learning, membrane fluidity, type 2 diabetes

1 | INTRODUCTION

Cardiovascular disease is a common comorbidity in type 2 diabetes.[1] The coexistence of several risk factors, such as long-term hyperglycaemia, atherogenic lipid profile, hypertension, obesity and microalbuminuria confer individuals with type 2 diabetes a significantly greater cardiovascular (CV) risk compared to the general population.[2,3] Moreover, despite intensive treatment to reduce major CV risk factors,[4,5] subjects with diabetes still have a residual incidence of cardiovascular events compared to those without diabetes. Furthermore, well-known CVD prediction scores, such as UK Prospective Diabetes Study (UKPDS) score[6] or the Systemic Coronary Risk Evaluation (SCORE),[7] have consistently failed in identifying subjects at greater risk of developing CVD within diabetic populations.[8]

Hypertriglyceridemia is one of the major factors proposed to explain residual cardiovascular risk in type 2 diabetes.[9,10] However, although observational and clinical trial data support the association between elevated triglyceride levels and CV event risk,[11] studies investigating different triglyceride-lowering interventions, including fibric acid derivatives, niacin and omega-3 fish oils, have shown controversial effects on CV outcomes.[12–15] The REDUCE-IT (Reduction of Cardiovascular Events with Icosapent Ethyl-Intervention Trial) demonstrated that addition of 4 g/day purified eicosapentaenoic acid (icosapent ethyl) in high-risk patients, with triglyceride levels of 135–499 mg/dL and optimized statin treatment, significantly reduced CV events versus placebo, and more importantly, this benefit was seen regardless of baseline and on-treatment triglyceride levels,[15] suggesting that observed CV risk reduction could be driven by improvement in fatty acid composition rather than triglyceride reduction per se. Recently, Sherrat et al.[16] proposed a different effect of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) on plasma membrane fluidity as a possible explanation for the contrasting results obtained by the REDUCE-it trial compared to the STRENGTH trial, in which combined therapy with EPA and DHA failed to reduce the primary composite cardiovascular endpoint.

Using a combined approach, we have already demonstrated that changes in the quality and quantity of circulating fatty acids (FA) could have a direct impact on erythrocyte membrane fluidity (determined by phospholipid composition) in rat insulinoma-derived INS-1E beta-cell line as well as in circulating cells from a lipoprotein-deficient genetic mouse model.[17,18] Further, we reported changes in viscoelastic properties of circulating red blood cell (RBC) membranes in patients with type 2 diabetes using atomic force microscopy,[19] and demonstrated different membrane micro-polarity states, mostly driven by lipid content, in subjects with diabetes compared to healthy subjects.[20,21] However, it is still unclear whether membrane fluidity changes could reflect an increased CV risk in type 2 diabetes subjects. We believe that the determination of phase behaviour and submicrometric physical state of erythrocyte membranes, which reflect changes in membrane FA composition (determined by dietary, environmental and cellular conditions), may allow us to investigate previously neglected risk factors for the development of cardiovascular complications in type 2 diabetes. Therefore, to assess whether RBC membrane fluidity could be a sensitive marker to distinguish subjects with type 2 diabetes and low cardiovascular risk from those with very high cardiovascular risk, we firstly investigated differences in RBC membrane fluidity in subjects with diabetes with or without prior CV events and then compared the efficacy of membrane fluidity assessment to the UKPDS score.

2 | MATERIALS AND METHODS

2.1 | Study design

Patients with type 2 diabetes with or without prior CV events were enrolled and underwent metabolic evaluations. Blood samples were collected from each participant for assessment of erythrocyte membrane fluidity and membrane FA composition. An additional group, including subjects with neither diabetes nor cardiovascular disease, was included in the study and underwent the same procedures.

2.2 | Study subjects

Two hundred and thirty-four patients with type 2 diabetes attending our outpatient clinic at the Centre for Endocrine and Metabolic Diseases at Policlinico 'A. Gemelli'—(Rome, Italy) were recruited for the study. Only patients with fasting C-peptide >1 ng/mL, stable metabolic control (defined as stable HbA1c for at least 6 months) and medical therapy (including all anti-hyperglycemic treatments and lipid-lowering agents) for at least 6 months prior to the screening visit were included in the study. Exclusion criteria were: type 1 diabetes (as assessed by medical history), history of diabetic ketoacidosis or hyperosmolar nonketotic coma, severe uncontrolled hyperglycaemia (defined as HbA1c >10% [≥86 mmol/mol]), asthma, uncontrolled blood pressure, symptomatic tachy- or bradyarrhythmia, chronic pancreatitis, pancreatic surgery, acute diseases, moderate/severe hepatic disease, severe chronic kidney disease (CKD-EPI eGFR <30 mL/min per 1.73 m^2), uncompensated hyper/hypothyroidism, recent or ongoing infections, pregnancy, inability or unwillingness to provide informed consent. Of the 234 recruited subjects with diabetes, 86 had a history of major cardiovascular events (any of the following: ischemic heart disease, documented myocardial infarction, percutaneous coronary intervention, coronary artery bypass grafting, objective findings of coronary stenosis (>50%) in at least 2 arteries, documented ischemic stroke, carotid stenting or endarterectomy, peripheral vascular disease). A healthy control group with neither diabetes nor cardiovascular events ($n = 32$) and matching the same exclusion criteria as the type 2 diabetic subjects was recruited from euthyroid patients attending the outpatient clinic for thyroid goitre to establish the mean and confidence intervals of membrane fluidity values as a reference frame for the assessment of values recorded in patients with type 2 diabetes. A study flowchart describing enrolment can be found in the supplementary data (Figure S1).

The study protocol was approved by the hospital ethical committee (Università Cattolica del Sacro Cuore), and all participants provided written informed consent before any study-specific procedures, in accordance with the principles of the Helsinki Declaration.

2.3 | Metabolic evaluation

After an overnight fast, all participants underwent complete medical assessment, comprehensive of ongoing therapies and anthropometric measurements including body mass index (BMI), waist circumference, systolic blood pressure (SBP), diastolic blood pressure (DBP) and heart rate were collected. A self-reported questionnaire was used to assess smoking habit, and individuals were classified as never smokers, ex-smokers or current smokers. A venous blood sample was drawn for metabolic and biochemical analyses (including HbA1c, triglycerides, HDL and total cholesterol levels). The UKPDS Risk Engine was utilized to estimate CV risk in all type 2 diabetes subjects without previous CV events as previously reported.[6]

2.4 | Sample preparation for confocal analysis

Blood samples, collected in heparinized tubes, were diluted in NaCl to reach a final concentration of 1:1000, seeded in a multi-well (ibidi, GmBH) and labelled with Laurdan 1 μM, as previously reported.[21]

2.5 | Image acquisition, pre-processing and analysis

Erythrocytes were then imaged through a confocal microscopy. The multi-wells were placed on an inverted confocal microscope (Nikon A1 MP) equipped with an on-stage incubator (T = 37°C, %5 CO_2, OKOLAB), and images were obtained using a 60× objective (NA 1.4) under 402 nm excitation for Laurdan. Two emission channels (filter cubes 450/50 nm and 525/50 nm) allowed the measurement of fluidity outcomes. A GaAsP detector collected 16-bit, unsigned images at .25 ms dwell time. Line averaging (×2) was used during image acquisition to minimize background noise. Images were acquired with an optical magnification of 2.89×. The resolution of acquired images was 1024 × 1024 pixels (16 fps—.025 ms dwell time). A minimal number of three images per sample were taken (~150 erythrocytes).

2.6 | Calculation of the generalized polarization index

As a normalized ratio of the intensity at the two emission wavelength regions, the generalized polarization (GP) provides a measure of membrane order, in the range between −1 (fluid crystalline) and +1 (gel). The GP is defined as

$$GP = \frac{I_{(425-475)} - GI_{(500-550)}}{I_{(425-475)} + GI_{(500-550)}} \quad (1)$$

It was calculated for each pixel using the two Laurdan intensity images ($I_{(425-475)}$ and $I_{(500-550)}$) with the ratiometric image processor program.[20] The calibration factor G

was obtained from the GP values of solutions of Laurdan in DMSO. The G factor has ~2% variation across the imaging area. GP images (as eight-bit unsigned images) were pseudo-coloured in ImageJ. Background values (defined as intensities below 7% of the maximum intensity) were set to zero (Figure S2).

2.7 | Erythrocyte membrane fatty acid analysis

Erythrocyte membrane fatty acid analysis was performed for 20 subjects with diabetes, 10 with no cardiovascular events and 10 with history of cardiovascular events, randomly selected among the enrolled subjects. The results of membrane fatty acid composition were compared with six samples derived from healthy subjects without diabetes. Blood samples (approximately 1 mL) were collected in vacutainer tubes containing ethylenediaminetetraacetic acid (EDTA). The whole blood in EDTA was centrifuged (4000 rpm for 5 min at 4°C) to create the two layers of plasma and cellular content, and the vials were transferred to a robotic platform to continue the automatized work-up, approved as the method MEM_LIP_1 by the UNI CEI EN ISO/EIC 17025:2018, available at the Lipidomic Laboratory #1836L of the Lipinutragen company. The subsequent steps of plasma removal, RBC selection for isolating the mature cell fraction, verified by the density,[22] and cell diameter using the cell counter (Sceptre 2.0, EMD Millipore), followed by lipid extraction and lipid transesterification to fatty acid methyl esters (FAMEs) were carried out by robotics. The automation included cell lysis, isolation of the membrane pellets, phospholipid extraction from pellets using the Bligh and Dyer method,[23] transesterification to FAMEs by treatment with a potassium hydroxide (KOH)/methyl alcohol (MeOH) solution (.5 mol/L) for 10 min at room temperature and extraction using hexane (2 mL). GC analysis of the final FAMEs mixture was run on the Agilent 6850 Network GC System, equipped with a fused silica capillary column Agilent DB23 (60 m × .25 mm × .25 μm) and a flame ionization detector. Identification and quantification of each fatty acid were conducted through calibrated procedures that are part of the MEM_LIP_1 method. Commercially available standards and a library of trans isomers of monounsaturated fatty acid (MUFA) and polyunsaturated fatty acids (PUFA) were used as standards showing the optimal separation of all fatty acids and their geometrical and positional isomers. The amount of each FA was calculated as a quantitative percentage of the total FA content (relative quantitative %), more than 97% of the GC peaks being recognized with appropriate standards.

2.8 | Statistics

First, we performed a Shapiro–Wilk test of normality for each level of GP variable. The relation of GP values with presence or absence of cardiovascular events was explored by stratifying the study population according to tertiles of GP; in order to account for gender differences in GP values, allocation into tertiles was based on the sex-specific distribution of the available values. The χ^2 test was used to compare variables across tertiles.

Subsequently, we confirmed bimodal distribution of GP value by performing an unsupervised clustering analysis with Orange version 3.31.1 (https://orangedatamining.com/) and R version 4.1.2. Briefly, based on the distance matrix, the clustering algorithm identifies the closest observations (i.e. subjects with similar GP) and iteratively merges them within the same cluster until all clusters are merged. Differences among clusters were determined by conducting a Kruskal–Wallis test for data that were not normally distributed. Normal data distribution was assessed by visual inspection, variance comparison and Shapiro–Wilk's test. Subsequently, pairwise Wilcox test was performed for post-hoc comparisons. For normally distributed variables, a one-way ANOVA with Tukey post-hoc analysis was conducted. Values of $p < .05$ were considered statistically significant for both analyses. The method used for missing data was complete-case analysis since statistical packages excluded individuals with any missing value. All these statistical analyses were performed with SPSS version 28.0.

Moreover, to validate membrane fluidity as a marker of residual cardiovascular risk in patients with type 2 diabetes, we performed a logistic regression analysis, using GP as a categorical predictor variable, set equal to 1 for patients in the high fluidity (high-risk) group (LGP) and equal to 0 for patients in the low fluidity (low risk) group (GEL), together with all other risk factors included in the UKPDS risk score calculation i.e. age, sex, duration of diabetes, HbA1c, total cholesterol, HDL, systolic blood pressure, smoking habit and history of atrial fibrillation. The binary outcome was defined by the presence or absence of previous cardiovascular events (=1 if previous MCV, =0 if not). To obtain a statistical power of .8 with a two-tailed alpha of .05 in a multivariate logistic regression model with presence of CV event as the dependent variable and Hba1C, age, duration of diabetes, smoking habits, sex, atrial fibrillation, SBP, total cholesterol and HDL levels as the independent variables, we enrolled 100 patients with CV events and 100 patients without CV events, following the rule of 10 events for 1 independent predictor.

From the evaluation of coefficients, we calculated the odds ratio, representing the ratio of the odds of an event

occurring in one group compared to the odds of the event occurring in another group, for each predictor variable as:

$$odds\ ratio = e^{coeff}$$

where *coeff* is the coefficient of the predictor variables.

Further, the ability of the two models, UKPDS + GP and UKPDS, respectively, to identify individuals with higher cardiovascular risk was assessed by the area under the receiver operating characteristic (ROC) curve.

Logistic regression analysis was performed using Python libraries.

3 | RESULTS

3.1 | GP: distribution and cut-off value settings in population with type 2 diabetes

We found that GP values decreased with increasing fluidity. When testing for normality, the data showed a significant bimodal distribution at the level of .445 GP value (Figure S3). Moreover, stratifying the study cohort according to GP tertiles, we found that in the highest tertile (GP >.429) diabetic subjects with prior CV event showed a significantly lower GP value (mean ± SD = .447 ± .015) compared to those without prior CV (mean ± SD = .455 ± .017) (Table S1). No differences were found in the other cardiometabolic risk factors between these two groups except for lower total cholesterol levels in subjects with prior CV event compared to those without prior CV events, due to the greater use of lipid-lowering treatment. In Table S1, we report the general characteristics of the study cohort stratified according to GP tertiles. On the basis of the selected GP cut-off value, given by the mean GP value of the third tertile, we were able to divide the study cohort into two groups: a group including subjects with a GP value over the defined cut-off ($n = 54$), characterized by less fluid erythrocyte membranes, and a group including subjects with a GP value under the defined cut-off ($n = 180$), with more fluid erythrocyte membranes.

3.2 | Hierarchical clustering analysis

An unsupervised approach was applied, to confirm the existence of different type 2 diabetes subgroups on the basis of erythrocyte membrane fluidity. The result is a hierarchical classification tree, as represented in the Appendix S1 (Figure 1A). The clustering was performed using the method of Ward, which has proved to outperform other hierarchical methods in producing homogeneous and interpretable clusters.[24] Three clusters were isolated from subjects with diabetes (Figure 1B): a cluster FLUID characterized by low GP (mean ± SD = .372 ± .026); a cluster INT characterized by intermediate GP (mean ± SD = .412 ± .013); and a cluster GEL characterized by high GP (mean ± SD = .461 ± .012). The high GP subgroup (GEL), defined according to cluster analysis, was superimposable on the high GP group defined according to the bimodal distribution of GPs. Therefore, the GEL group ($n = 54$) was compared with the rest of the diabetes population in which the two other GP subgroups (INT and FLUID) were merged to form the low GP (LGP) group ($n = 148$).

3.3 | Description of the type 2 diabetes subgroups

As reported in Table 1, the LGP group was characterized by higher fluidity (mean ± SD = .392 ± .017), compared to the GEL group (mean ± SD = .454 ± .017, $p < .01$), but had superimposable distribution of age, gender, duration of diabetes, HbA1c, BMI and lipid profile (total cholesterol, RBC cholesterol, TG, HDL), creatinine, red blood cell count and haemoglobin concentration and white blood cell count. Further, no differences were found between the two groups in smoking habits, treatment for dyslipidaemia (STATIN/OMEGA3), hypoglycaemic therapy, anti-hypertensive, anti-platelet and anti-coagulant therapies (Table 1). Additional information on percentage of hypoglycaemic treatments (Table S2) and anti-hypertensive drug classes (Table S3) is reported in the supplementary data. Finally, the UKPDS risk score, calculated for subjects with diabetes and without prior CVD, was comparable between the GEL and LGP groups (Table 1).

General polarization distribution of CTRL group ($n = 32$) was =.440 ± .038 (mean ± SD). As expected, the CTRL group differed from GEL and LGP in terms of glycaemic status (HbA1c) and lipid profile given the treatment with statins (Table 1).

Interestingly, the percentage of patients affected by macrovascular complications in the GEL and LGP groups differed significantly (GEL: 24.07%; LGP: 40.56%). Based on the data of Figure 2 and Table 1, we can infer that if the patient belongs to the LGP cluster, the odds of developing a macrovascular complication increase by 1.68 times ($p = .045$).

3.4 | Logistic regression analysis

The results of the logistic regression analysis performed to test membrane fluidity as a marker of cardiovascular risk in patients with type 2 diabetes are reported in Table 2.

In addition to the residual risk, identified by the GP variable, all risk factors included in the UKPDS calculation, namely age, sex, duration of diabetes, atrial fibrillation,

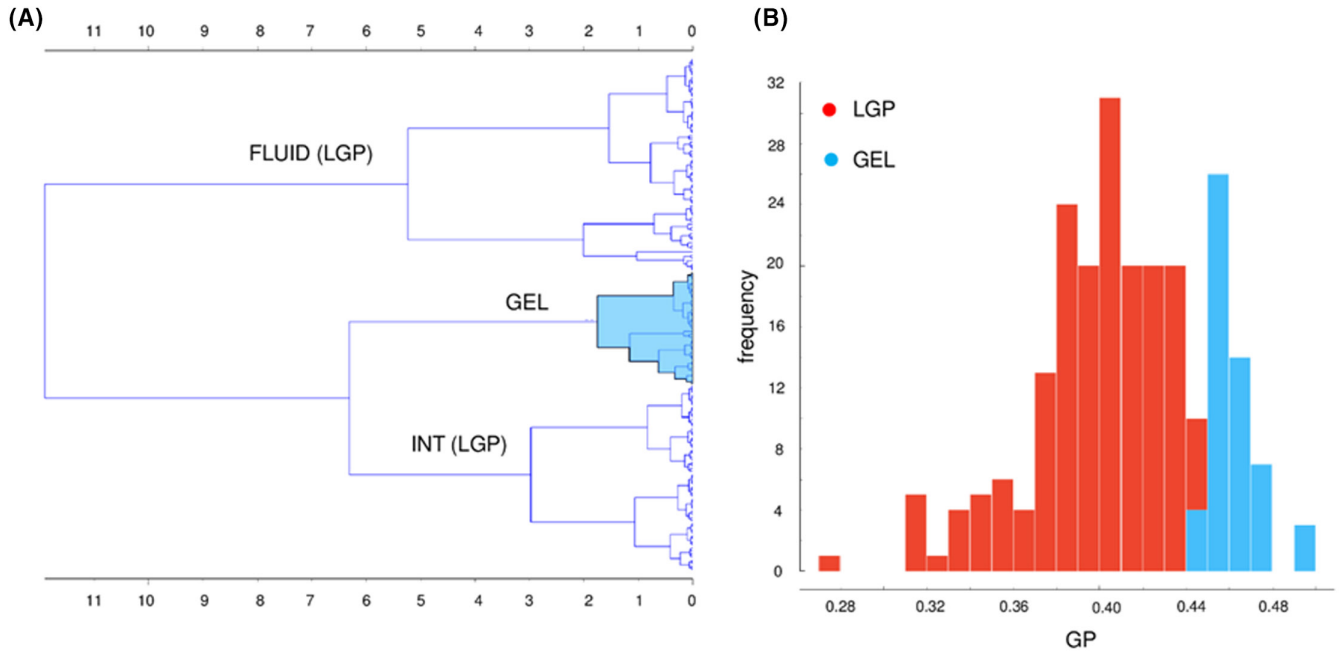

FIGURE 1 Unsupervised hierarchical clustering method. (A) The dendrogram corresponding to the matrix of distances used for hierarchical cluster analysis. Here, three clusters were identified: GEL, in light blue, and the FLUID and INT, respectively, further grouped in the LGP group. In (B), the distribution (frequency, on the *y*-axis) is represented as a function of the GP index (on the *x*-axis), which constitutes the parameter used for clustering patients: LGP (low GP) are represented in red, while GEL (high GP) are represented in light blue.

Hba1c, systolic blood pressure, smoking habit, total cholesterol and HDL, were used as input parameters for the logistic model. In Table 2, variable coefficients found to be significant, respectively: age (coeff = .0426 CI 95% = .009–.0076, *p*-value = .013), sex (coeff = .8727 CI 95% = .211–1.534, *p*-value = .01), total cholesterol (coeff = −.0156, CI 95% = −.025 to −.007, *p*-value = .001) and the GP risk variable (coeff = .7903, CI 95% = .050–1.530, *p*-value = .036) are highlighted in red. Besides this, evaluating the performance of both UKPDS + GP and UKPDS model, we observed that when the GP variable is considered, classification accuracy increased by 2%, with values of 73.1% for UKPDS + GP and 71.4% for UKPDS, respectively.

The same improvement was also found when considering the ROC (receiver operating characteristic). As reported in Figure 3, our results revealed that the UKPDS + GP model was better able to identify patients with or without MCV, compared to the model without GP variable (AUC = .769 for UKPDS + GP and = .754 for UKPDS, respectively).

3.5 | Erythrocyte membrane fatty acids profile

We compared erythrocyte FA profiles among groups in a sample of 26 patients ($n = 6$ CTRL, $n = 10$ GEL and $n = 10$ LGP). Complete RBC membrane FA composition of the three study groups is shown in the supplementary data (Table S4). Although we found no differences in total amount of polyunsaturated fatty acid (PUFA) among the groups, the percentage of arachidonic acid (AA, 20:4), linoleic acid (LA, 18:2) and the total percentage of omega-6 FA differed significantly among the three groups (Figure 4A–C). Specifically, LA mean value was lower in both GEL and LGP membranes compared to CTRL group, while we observed a significantly higher amount of AA in erythrocyte membranes of the LGP group compared to GEL. As regards the total omega-6 FA amount, we found it to be the highest in the LGP group, but similar values were found in the GEL and CTRL groups. There were no changes in total omega-3 FA (including well-studied EPA and DHA) among the three groups, though we detected a 15% decrease in the LGP group (not reaching statistical significance, $p = .13$). These changes in FA composition of LGP membranes reflect an altered omega-6/omega-3 ratio, (considered a pro-inflammatory risk index), which increased in the LGP group with respect to GEL ($p = .041$) (Figure 4D). Moreover, the omega-3/PUFA index (PUFA balance) showed a 22% decrease in LGP group compared to GEL ($p = .041$) (Figure 4E). Conversely, the GEL group had the same value of omega-3/PUFA and omega-6/omega-3 indexes as the CTRL group (Figure 4D,E).

TABLE 1 General characteristics of type 2 diabetes subjects divided into two groups on the basis of defined GP cut-off.

Combined—All patients	GEL $n=54$ Mean (SD)	LGP $n=180$ Mean (SD)	*p*-Value
Age (y)	64.74 (9.09)	64.53 (9.26)	Ns
Sex—no (M%)	63 (7)	57 (4)	Ns
BMI (Kg/m^2)	29.03 (4.60)	28.57 (5.46)	Ns
HBA1C (%)	6.57 (.83)	6.79 (.92)	Ns
Diabetes duration (y)	7.10 (7.38)	8.25 (9.54)	Ns
TOTAL CHOL (mg/dL)	160.17 (36.02)	157.55 (36.40)	Ns
LDL (mg/dL)	85.41 (28.96)	82.95 (29.99)	Ns
HDL (mg/dL)	47.74 (10.15)	48.23 (11.64)	Ns
TG (mg/dL)	129.43 (56.73)	135.40 (67.19)	Ns
Creatinine (mg/dL)	.97 (.28)	.94 (.58)	Ns
RBC count ($\times 10^{12}$/L)	4.9 (.9)	4.8 (1.3)	Ns
Haemoglobin (mg/dL)	13.8 (1.4)	14.1 (1.4)	Ns
White blood cell count ($\times 10^9$)	7.12 (1.2)	7.68 (1.7)	Ns
Systolic blood pressure (mmHg)	125.89 (12.49)	126.84 (13.45)	Ns
Smoke—no (%)	15 (5)	15 (3)	Ns
Statin—no (%)	67 (6)	70 (3)	Ns
Omega3—no (%)	20 (5)	24 (3)	Ns
Anti-hypertensive therapy—no (%)	10 (1.5)	6 (1)	Ns
Anti-platelet therapy (%)	9.9 (4.5)	38.2 (6)	Ns
Anti-coagulant therapy (%)	1.3 (.2)	7.3 (1)	Ns
Oral hypoglycaemic therapy (%)	79 (6)	71 (4)	Ns
Insulin therapy (%)	21 (8)	29 (7)	Ns
Macrovascular event—no. (%)	**24 (6)**	**41 (4)**	**.049**
UKPDS CHD RISK (w/o Major CV Event $n_{GEL}=41$; $n_l=107$)	17.22 (9.98)	17.22 (12.56)	Ns

Note: Mean ± SD and *p*-value of variables resulted significantly different between GEL and LGP groups are highlighted in bold.

Abbreviations: GEL, subjects with diabetes and less fluid membranes; LGP, subjects with diabetes and more fluid membranes.

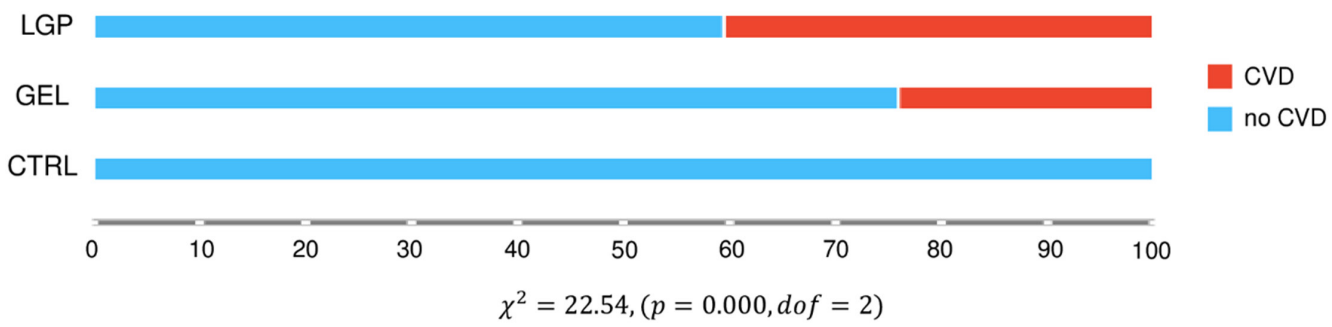

FIGURE 2 Percentage of patients with prior CVD in the three study groups. The graph shows the fraction of patients with (in red) or without (in blue) CVD, respectively, belonging to the different identified groups (LGP and GEL). A control group, constituted by healthy subjects, is reported.

4 | DISCUSSION

Over the years, researchers have attempted to identify those patients with diabetes who are more prone to developing cardiovascular complications, in order to personalize therapeutic approaches, with very poor results.[25] Here, we propose the assessment of erythrocyte membrane fluidity as a possible biomarker for

TABLE 2 Logistic regression analysis of study cohort.

	UKPDS + GP			UKPDS		
Variable	**Coeff**	**CI [.025–.975]**	***p*-Value**	**Coeff**	**CI [.025–.975]**	***p*-Value**
Age	.0426	**.009–.076**	**.013**	**.0421**	**.009–.075**	**.013**
Sex	**.8727**	**.211–1.534**	**.01**	**.8268**	**.174–1.480**	**.013**
Duration of diabetes	.0265	−.008–.061	.128	.0253	−.009–.059	.143
Atrial fibrillation	.2713	−1.026–1.568	.682	.4370	−.845–1719	.504
HBA1C	.0469	−.274–.368	.775	.0987	−.217–.415	.541
Systolic blood pressure	−.0120	−.031–.007	.206	−.0102	−.029–.008	.272
Smoking habit	−.0304	−.879–.818	.944	−.0032	−.836–.830	.994
Total cholesterol	−.0156	**−.025 to −.007**	**.001**	**−.0151**	**−.024 to −.006**	**.001**
HDL	−.0231	−.053–.007	.133	−.0225	−.052–.007	.137
GP	.7903	**.050–1.530**	**.036**			
Classification accuracy	.731	.675–.786		.714	.658–.769	
AUC	.769	.707, .832		.754	.685–.816	

Note: The coefficients and CI 95% and *p*-value of the variables found to be significant are highlighted in bold.

Abbreviations: GP, generalized polarization; UKPDS, UK Prospective Diabetes Study.

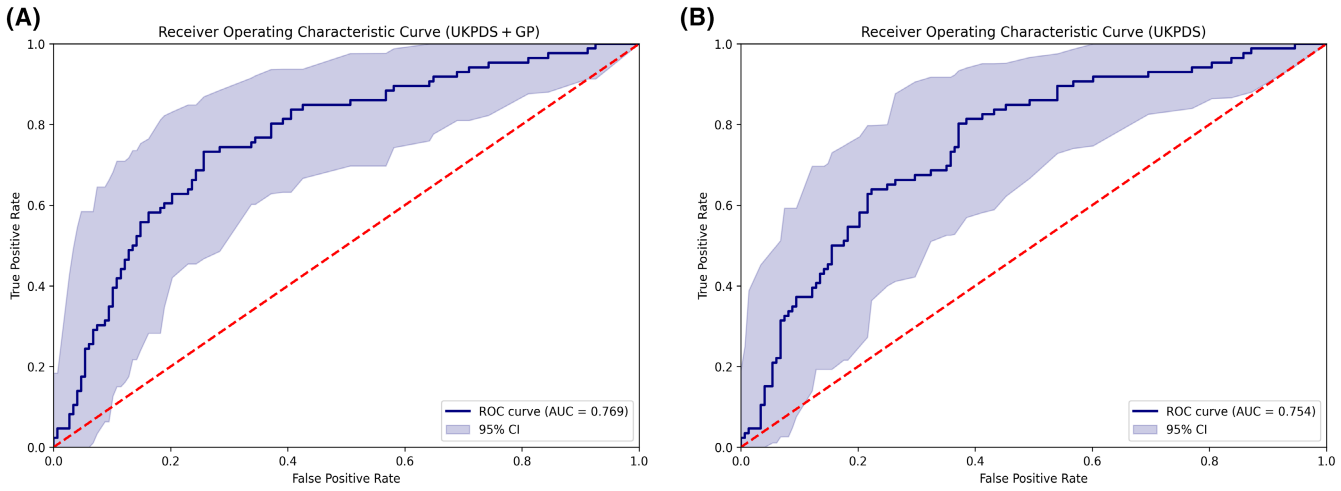

FIGURE 3 ROC curves comparing the accuracy of the two models, UKPDS + GP (panel A) and UKPDS (panel B), respectively, in identifying individuals with higher cardiovascular risk in our type 2 diabetes population. ROC, receiver operating characteristic; UKPDS = UK Perspective type 2 Diabetes Study.

the more accurate prediction of cardiovascular risk in type 2 diabetes. We found that in the group with more fluid membranes (LGP group), although characterized by same average age, BMI and cardiovascular risk factors, according to the UKPDS risk score, there was a higher percentage of subjects with macrovascular complications (60% higher compared to GEL, $p = .049$). Moreover, in a logistic regression analysis, high membrane fluidity was independently associated with the presence of macrovascular complications. Prior evidence has demonstrated that the measurement of the physical state of the membrane could be useful in monitoring diabetes progression since it reflects the state of a complex network of regulatory processes influenced by the disease,[21] However, until now, no study has correlated membrane fluidity changes to increased risk of cardiovascular outcomes in populations with diabetes. Interestingly, GP was found to be independent from known CV risk factors, suggesting that membrane fluidity, strictly dependent on FFA content and here assessed by a high-resolution bioimaging method, could be an indirect measurement of residual cardiovascular risk, currently undeterminable in people with diabetes.

Consistent with a body of evidence suggesting that higher circulating FA levels, mainly derived from the diet, are linked to changes in the lipid profile of cellular

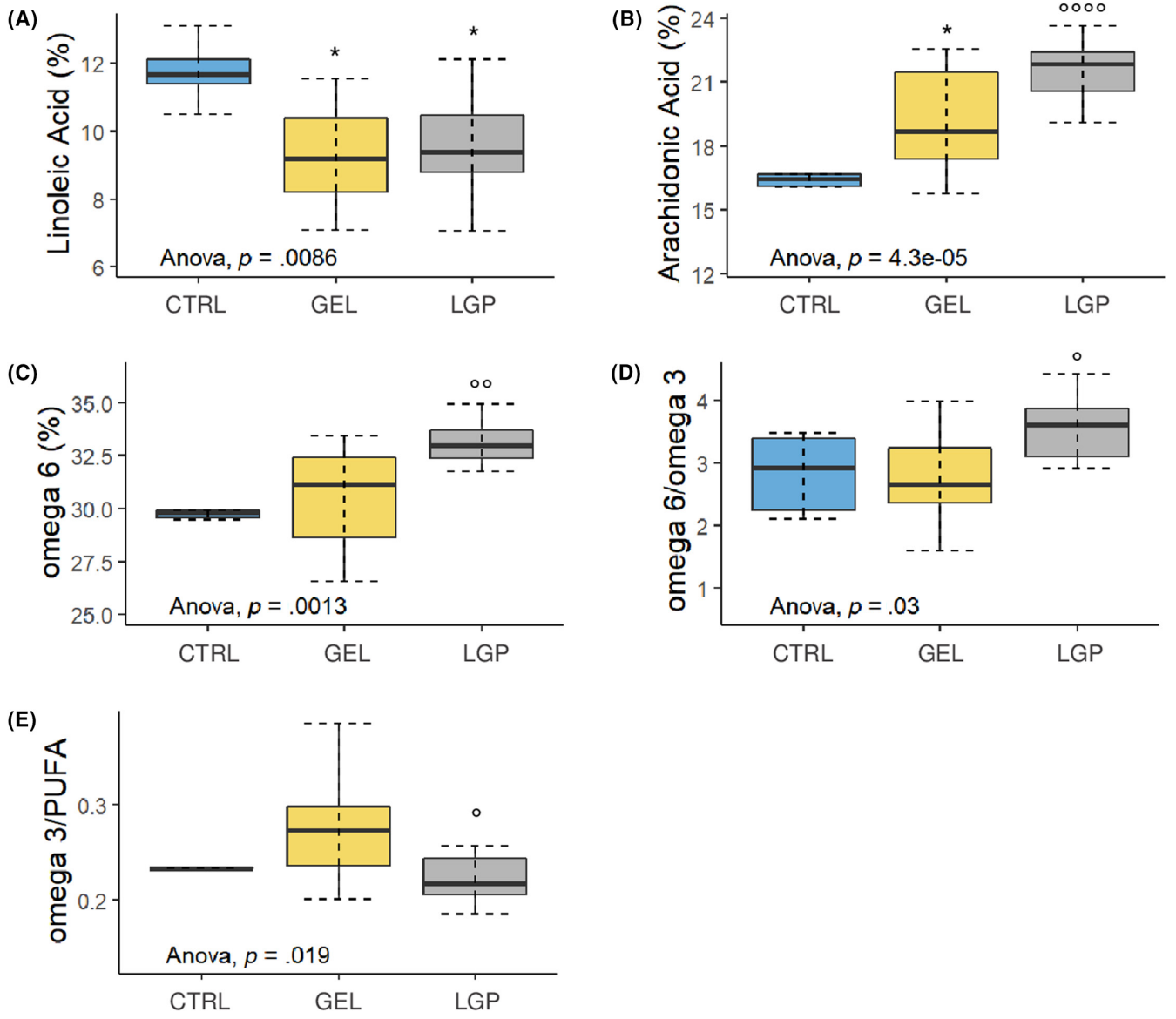

FIGURE 4 Representative PUFA levels and lipidomic indexes. Levels of linoleic acid (A), arachidonic acid (B), total content of omega 6 (C), inflammatory risk index omega 6/omega 3 (D) and omega 3/PUFA ratio (E) obtained from the fatty acid-based red blood cell membrane lipidomic analysis are shown in the box plots for CTRL (in blue), GEL (in yellow) and LGP (in grey) clusters, respectively. FA content is reported as a fraction of the total RBC membrane composition (on the *y*-axis). *p*-Values obtained from the ANOVA are indicated along with the box plots, and post-hoc comparisons are reported (*stands for *p*-value < .05 vs CTRL; °stands for *p*-value < .05 vs GEL; °°stands for *p*-value < .01 vs GEL; °°°°stands for *p*-value < .0001 vs GEL).

membranes,[26,27] the higher fluidity observed in the LGP membranes compared to GEL ones could be ascribed to the alteration in the quantity and quality of PUFA, driven by diet and/or altered enzymatic activity of desaturases and elongases, which can directly affect membrane fluidity by increasing PUFA abundance and quality. Studying FA composition of both LGP and GEL membranes, we found an increase in omega-6 metabolites and a decrease in omega-3 FA in LGP membranes compared to GEL membranes. This resulted in an increase in the omega-6/omega-3 index and a decrease in the omega-3/PUFA index, which is more sensitive in detecting an alteration in the metabolic fluxes of PUFA.[36] Given this imbalance between omega-6 and omega-3, we can argue that LGP membranes exhibited a clear shift towards a pro-inflammatory condition. In fact, while omega-6 AA has been shown to be a precursor of inflammatory mediators,[28] a reduction in omega-3 FA content could account for impaired release of pro-resolving mediators, called resolvins.[29,30] A higher ratio of omega-6/omega-3 FA, as found in today's Western diets, has been reported to promote the pathogenesis of cardiovascular disease, increasing platelet aggregation,[31] atherogenesis and vasoconstriction.[32] Moreover, recently, a large prospective cohort found a strong association

between the ratio of circulating omega-6/omega-3 PUFA and risk of CVD mortality.[33] Diets enriched with omega-3 alfa-linoleic acid (ALA), mostly derived from nuts and seeds, have been shown to significantly reduce cardiovascular events in a high-risk population in primary prevention.[34] Further, intake of omega-3 EPA has shown a greater effect in reducing CV outcomes compared to placebo in a RCT conducted on subjects with and without known prior CVD.[15]

In this context, inflammation may play a key role in identifying patients with type 2 diabetes and a higher residual risk, despite correction of all known risk factors (hyperglycaemia, atherogenic lipid profile, hypertension). The increase in pro-inflammatory molecules is a common element in both cardiovascular conditions and diabetes,[28,35] and several studies have demonstrated that plasma inflammatory markers, such as C-reactive protein, correlate with increased omega-6 and/or decreased omega-3 content in erythrocyte membranes.[36,37]

Although our study represents a novelty in the field of cardiovascular risk and diabetes, it also has some potential limitations. First, the cross-sectional design of the study prevents the establishment of a causal relationship between cardiovascular events and membrane fluidity, and therefore, longitudinal investigations are needed to confirm whether patients in the LGP group have a higher risk of developing macrovascular complications. Second, the lack of data on dietary habits, which could contribute to the differences seen in erythrocyte profiles between groups, did not allow us to adjust variables according to enrolled subjects' fatty acid supplementation/consumption. Finally, our study is an observational, nonrandomized study recruiting patients with diabetes at a referral university hospital, and therefore, the results of this study may not be extendable to the general population.

However, from our results and the knowledge gathered so far, it is evident that membrane-based biophysical measurements and molecular diagnostics can be a comprehensive and useful approach to stratify patients with type 2 diabetes and create personalized treatments. Moreover, these data may help shed light on the mechanisms linking increased intake of $n-3$ polyunsaturated fatty acids, whether through diet[34,38] or as dietary supplements,[15] and the improvement of cardiovascular outcomes in high-risk subjects. Further, the current results may be clinically relevant in view of a personalized approach to type 2 diabetes regarding nutritional recommendations based on the specific FA needs. Membrane fluidity measurement, reflecting this imbalance, could be included in protocols to create personalized nutritional strategies and treatments. According to our results, individuals in the LGP group could benefit from a higher intake of omega-3 FA, to promote a better balance between omega-6 and omega-3 pathways and reduce inflammatory precursors. Obviously, intervention studies are needed to demonstrate that dietary measures and/or supplements, aimed at increasing the omega-3 content in cell membranes, can improve membrane fluidity and therefore reduce CV risk in patients with type 2 diabetes.

5 | CONCLUSIONS

Our results suggest that erythrocyte membrane fluidity is a possible biomarker for assessing residual cardiovascular risk in subjects with type 2 diabetes mellitus. The proposed assay provides a low-cost and effective system based on UV excitation to analyse membrane properties. General polarization, which quantifies membrane fluidity, was found to be independent of the known cardiovascular risk factors but correlated to changes in plasma membrane fatty acid composition. After successful validation in a well-planned longitudinal study, our system could improve the decisional process in terms of prevention and treatment, opening possible future frontiers for the formulation of personalized dietary protocols aimed at reducing CV risk in the population with diabetes in both primary and secondary prevention.

AUTHOR CONTRIBUTIONS

GM and AG designed the study and wrote the manuscript. GB, CMAC and TM collected the data. GB, CF and AS, performed the measurements. GB, CMAC, CF, AS, CS, AA, TM, MV PMF, GR analysed and interpreted the data. All authors reviewed and edited the manuscript and gave the final approval of the version to be published.GM is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. All authors have read and approved the final manuscript. We thank Serena Rotunno (Università Cattolica del Sacro Cuore) for assistance with the editing and language revision.

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CONFLICT OF INTEREST STATEMENT

None.

DATA AVAILABILITY STATEMENT

The datasets generated during and/or analysed in the current study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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