

Lipidomic profiles and their relationship to incident diabetic retinopathy in type 2 diabetes: The Fremantle Diabetes Study Phase II

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ABSTRACT

Aims: To determine whether plasma lipid profiles predict diabetic retinopathy (DR).

Methods: DR status (none, mild non-proliferative diabetic retinopathy (NPDR), moderate NPDR, severe NPDR/worse) was categorised in 762 adults with type 2 diabetes (mean age 64.5 years, 54.3% males) at baseline and Year 4/6. Ultra-performance liquid chromatography-tandem mass spectrometry generated baseline plasma lipid profiles. Multiple logistic regression identified baseline associates of new/worsening DR and incident DR. The likelihood ratio test (LRT) evaluated incremental biomarker contribution. The net reclassification improvement (NRI) was calculated.

Results: New/worsening DR occurred in 121 (16%). Of 495 without DR at baseline, 35 (7.0%) developed DR. For new/worsening DR, the inclusion of cholesterol ester (20:4), fatty acid (20:3) and lysophosphatidylglycerol (18:1) in addition to conventional risk factors (hypertension, HbA_{1c}, diabetes treatment intensity, urinary albumin:creatinine) added to the conventional model predictivity (LRT, $P = 0.00002$). The NRI gain at a 10% cut-off was 5.7% (SE 2.8%; $P = 0.043$). For incident DR, fatty acid (20:3), lysophosphatidylglycerol (18:0) and phosphatidylcholine (18:0 20:3) with HbA_{1c} improved model performance (LRT, $P = 0.00001$). The NRI gain at 5% risk cut-off for incident DR was 28.3% ($P = 0.002$).

Conclusions: This exploratory study identified five lipid biomarkers with the potential to improve DR risk prediction and the understanding of pathophysiological mechanisms.

1. Introduction

Diabetic retinopathy (DR) is a common microvascular complication of diabetes mellitus and a leading cause of blindness in adults.¹ Early diagnosis and targeted treatment are important in the prevention of vision loss due to DR, including optimal control of glycemia, hypertension and lipidemia.^{2,3} While these modifiable risk factors have some prognostic value, they are unable to reliably identify patients at future risk of DR. Regular retinal imaging is an effective method of early detection, especially when linked to machine learning models,⁴ but resources for population-based screening programmes may be inadequate in many countries.⁵ There is, therefore, a need for an easily administered baseline test that provides valid detection of individuals living with diabetes who are at risk of new or worsening DR.

Lipidomics, the comprehensive profiling of lipid species in biological

fluids, offers the characterisation of potential biomarkers and underlying mechanisms of the pathogenesis of microvascular complications of diabetes.⁶ Recent advances in large-scale lipidomics have been applied in several fields, permitting the implementation personalised predictive models to improve cost-effective screening strategies. Technological developments in lipidomics include the targeted, quantitative profiling of thousands of species using liquid chromatography-mass spectrometry with minimal sample volume,⁷ including microsamples,⁸ for affordable, quality and accessible screening in at risk populations. Lipidomics has been applied to a range of cross-sectional studies of DR.^{9–13} Predictive studies of the relationship between lipidomic markers and incident DR have been restricted to one population-based longitudinal study¹⁴ and two Mendelian randomisation studies.^{15,16} The inconsistencies between the results of these studies are likely to reflect the strong influence of environmental factors on the plasma lipidome,¹⁷ especially in older

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individuals and those with diseases such as diabetes.¹⁸ This means that studies across different populations are needed to characterise the utility of a lipidomic prognostic test for DR complicating type 2 diabetes.

In the present study, targeted lipidomic profiling spanning 20 different sub-classes was performed on plasma samples collected from representative community-based participants in the longitudinal Fremantle Diabetes Study Phase II (FDS2) at both baseline and Year 4 or 6 follow-up. The study aimed to characterise and identify circulating plasma lipid biomarkers associated with the development and progression of DR observed from serial retinal imaging.

2. Methods

2.1. Participants and approvals

Data and samples from the Fremantle Diabetes Study Phase II (FDS2), a longitudinal community-based observational study were used. Details of FDS2 have been published.¹⁹ In brief, 1551 participants with clinically diagnosed T2D were recruited from a postcode-defined urban area surrounding the port of Fremantle between 2008 and 2011. All underwent a comprehensive baseline and then biennial face-to-face assessments including an interview, detailed questionnaires, a physical assessment, and fasting biochemical tests performed in a single nationally accredited laboratory. Retinal photography was scheduled at each visit (i.e. baseline, Year 2, Year 4 and Year 6) using a Canon CR-DGi Non-Mydriatic Retinal Camera (Canon Medical Systems USA Inc., Tustin, CA, USA). The South Metropolitan Area Health Service Human Research Ethics Committee approved the FDS2 protocol (reference 07/397) and written informed consent was obtained from each participant. The present sub-study was approved by the Murdoch University Human Research Ethics Committee (reference 2022/020). The study protocol conformed to the ethical guidelines of the Declaration of Helsinki.

2.2. Retinopathy assessment

Diabetic retinopathy was assessed from retinal images according to the modified Airlie House Classification system for the Early Treatment Diabetic Retinopathy Study, as has been described previously.^{20,21} Baseline fundus photographs were assessed by a single external grader accredited by the Centre for Eye Research Australia, University of Melbourne, who was blinded to other participant data. Severity was classified under the modified Airlie House Classification as none, mild non-proliferative diabetic retinopathy (NPDR), moderate NPDR, or severe NPDR or worse.²² Level 10 was classified as no DR, levels 11–31 as mild NPDR, levels 41 and 84 as moderate NPDR, and levels 51–80 and 86 as severe NPDR or worse. For participants who did not have fundus photography performed, or whose photographs proved ungradable, further information regarding DR status was sought from hospital records, optometrists and ophthalmologists. Participants without information on DR status at baseline were excluded from analysis. Follow-up fundus photographs were assessed independently by at least one experienced ophthalmologist and categorised using the same system as the baseline images.²³ The two DR outcomes assessed were new or worsening DR and incident DR. New or worsening DR was defined as any incident DR or progression by at least one severity step (e.g. mild to moderate NPDR). Incident DR was defined as new DR in those with no DR at baseline.

2.3. Liquid chromatography-mass spectrometry lipidomic analysis

Lipidomic analysis was performed using baseline samples from FDS2 participants. Optima™ LC-MS grade acetonitrile (ACN), isopropanol (IPA) and methanol (MeOH) were obtained from Thermo Fisher Scientific (Malaga, WA, Australia) and ammonium acetate from Sigma Aldrich (North Ryde, NSW, Australia). A Milli-Q IQ 7000 water purification system (Merck Millipore) provided ultrapure LC-MS grade water.

The stable isotope labelled internal standards (SIL ISTD) used were Avanti Polar Lipids UltimateSPLASH™ ONE and SphingoSPLASH™ (Sigma-Aldrich, North Ryde, NSW, Australia).

Study plasma samples stored at -80°C were transferred on dry ice to the Australian National Phenome Centre in Perth, Australia for lipidomic analysis. Samples were randomised, thawed at 4°C and 10 μL combined with 90 μL of stable isotopically labelled internal standards diluted (information regarding the SIL ISTD concentrations is outlined in Supplementary Table S1) in isopropanol for protein precipitation and lipid extraction. Samples were chilled and centrifuged for 15 min at 14,000g before 50 μL of supernatant was transferred into a 350 μL 96-well plate for analysis (Eppendorf, Macquarie Park, NSW, Australia). A Biomek i5 liquid handling system was used for automated sample preparation (Beckman Coulter, Mount Waverley, Victoria 3149, Australia). For quality control (QC) an independent commercially available human plasma pool (BioIVT, Westbury, NY, USA) was prepared and analysed between every tenth study sample for the assessment of analytical precision.

Targeted analysis of samples was performed using an ExionLC UHPLC coupled to a QTRAP 6500+ MS (SCIEX, MA, USA) with a reversed-phase chromatographic separation method as previously described.²⁴ Separation was performed using a Waters Acquity BEH C₁₈ 1.7 μm , 2.1×100 mm column (Waters Corp., MA, USA) at 60°C . Mobile phase A consisted of water/acetonitrile/isopropanol (50/30/20, v/v/v), containing 10 mM ammonium acetate. Mobile phase B consisted of water/acetonitrile/isopropanol (1/9/90, v/v/v), also containing 10 mM ammonium acetate. The flow rate was set at 0.4 mL/min with gradient elution starting at 10% B, increasing to 45% B at 2.7 min, 53% B at 2.8 min, 60% B at 8.0 min, 80% B at 8.1 min, 100% B at 12.0 min, before returning to 10% B at 13.0 min for a 2 min re-equilibration. The resulting total cycle time was 15 min.²⁴ The injection volume was 5 μL and the sample plates were stored at 10°C in the autosampler.

The MS platform was operated with electrospray ionization (ESI) with polarity switching. Multiple reaction monitoring (MRM) mode was used to acquire a total of 1230 transitions. The identities and MRM parameters for each lipid analyte and SIL ISTD can be found in Supplementary Table S1. The mass spectrometer settings used were a capillary voltage of +5500 V for positive and -4500 V for negative ion modes. The temperature was set at 300°C , with curtain gas at 20 psi, ion source gas 1 at 40 psi, and ion source gas 2 at 60 psi.

2.4. Data pre-processing

Raw MS data files were converted from .wiff to .mzML format in MSConvert.²⁵ Chromatographic peak integration was performed using a combination of an in-house retention time finder function in R (v4.0.4)²⁶ and SkylineMS (v22.2.0.351).²⁷ Peak area integrals then underwent pre-processing in R (v4.0.4) using in-house scripts that performed the following steps: First, the data underwent a combination of missing value filters that removed any samples with >50% missing values (indicative of a failed sample e.g. extraction or LC-MS injection), followed by the removal of lipid targets with >50% missing values in the remaining samples. The remaining missing values in the dataset were considered as below the limit of detection and therefore were replaced using the minimum intensity of that analyte divided by two. This was followed by calculation of peak area response ratios of each target lipid to the corresponding SIL ISTD (detailed in Supplementary Table S1), and subsequent adjustment for concentration factor by the multiplication of each response ratio by the known concentration of each SIL ISTD. Finally, features were assessed for precision by evaluating the relative standard deviation (RSD) across the QC samples. Lipid features with an RSD% of >30% were removed from the dataset.

2.5. Statistical analysis

The computer package IBM SPSS Statistics 29 (IBM Corporation,

Armonk, NY, USA) was used for statistical analysis. Data are reported as percentage, mean $\pm$ SD, geometric mean (SD range), or, when variables are not approximately normally distributed, median [interquartile range]. Two-way comparisons were performed using Fisher's exact test for independent samples, Student's *t*-test for approximately normally distributed variables, and Mann-Whitney *U* test for non-normally distributed variables. The Benjamini-Hochberg (False Discovery Rate (FDR)) procedure was used to control the expected proportion of false discoveries.

Multiple logistic regression (backward conditional variable selection with $P < 0.050$ for entry and ≥ 0.050 for removal) was used to identify independent baseline associates of incident DR. All clinically plausible conventional variables with bivariable $P \leq 0.20$ were considered for model entry in a backward stepwise manner. These included demographic, behavioural, diabetes-related factors, blood pressure, lipid profile, antihypertensive and lipid-modifying medications, and renal function (Model 1). In the absence of significant FDR comparisons, MS-detected lipid biomarkers with Mann-Whitney *U* test $P < 0.050$ were considered for entry into each most parsimonious conventional model (Model 2). A two-tailed significance level of $P < 0.050$ was used throughout.

To determine the predicted probability of any new or worsening DR, and any incident retinopathy (excluding those with prevalent DR), during 4-years of follow-up, the linear predictor for each participant was computed using the coefficients from each final logistic regression model and the respective participant data. These were then exponentiated. The performance of each model was assessed against the observed outcome. Model calibration was determined graphically by plotting observed and predicted numbers of participants who experienced the retinopathy outcome across quintiles of risk, with the Hosmer-Lemeshow test used for goodness of fit. Model discrimination was determined by the area under the receiver operating characteristic curve (AUROC) and optimised to differentiate individuals at high risk of the retinopathy outcomes from those at low risk. The sensitivity, specificity,

positive predictive value (PPV), and negative predictive value (NPV) were determined at moderate- and high-risk cut-offs and the Youden Index (sensitivity + specificity – 1) which maximises sensitivity and specificity. Comparing two nested regression models using AUROC can lead to both the test statistic and its estimated variance being seriously biased.²⁸ Therefore, the likelihood ratio test was used as the primary test to evaluate the incremental contribution of potential new metabolomic lipid biomarker panels. For completeness, the net reclassification improvement (NRI) was calculated. This is an index that attempts to quantify how well a new model reclassifies participants - either appropriately or inappropriately - as compared to an old model but has also been demonstrated to be misleading.²⁹

3. Results

Of the 1551 FDS2 participants with type 2 diabetes, 762 (49.1%) had lipids measured on baseline samples and DR status available at both baseline and Year 4 or 6 (see Fig. 1). Those included were younger (64.5 ± 10.3 versus 66.8 ± 12.7 years at study entry, $P < 0.001$), and had a shorter diabetes duration (median [IQR] 7.0 [2.0–14.2] versus 10.9 [4.0–17.4] years, $P < 0.001$) compared to those who were excluded. There was no statistically significant difference in sex (54.3% female versus 49.6% male, $P = 0.060$). One third (252 (33.1%)) of participants had DR at baseline and 38 (15.1%) of these had moderate NPDR or worse. For the 510 participants with no DR at baseline, 35 developed DR between baseline and Year 4/6 while 15 were undetermined since they had no Year 2 visit and did not have DR at Year 4/6 (these could have had a microaneurysm between baseline and Year 4/6 which had resolved by Year 4/6). Thus 35/495 (7.1%) developed incident DR between baseline and Year 4/6.

Supplementary Table S2 summarizes the comparisons in MS-detected lipid biomarkers by any new or worsening DR between any visits between baseline and Year 4/6, and any incident retinopathy between baseline and Year 4/6, excluding those with prevalent

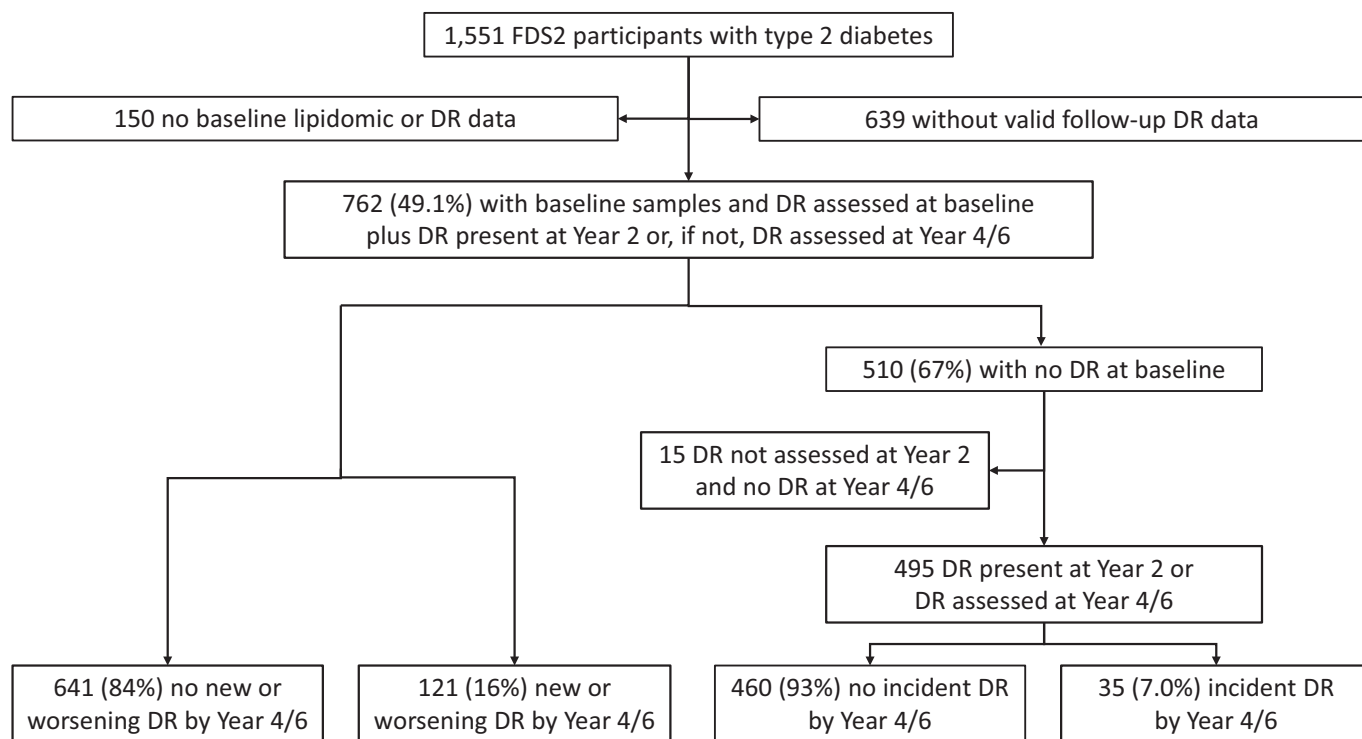


Fig. 1. The disposition of FDS Phase II participants with type 2 diabetes with baseline blood samples and lipidomic biomarkers available and assessed for diabetic retinopathy at baseline, Year 2 and Year 4/6. If participants had incident retinopathy at Year 2, it was assumed this was also present at Year 4 if they did not attend for assessment.

retinopathy. No comparisons were statistically significant using the Benjamini-Hochberg procedure, but a number were significant using the Mann-Whitney *U* test.

The baseline characteristics stratified by the two retinopathy outcomes are shown in Table 1. Any new or worsening DR was significantly associated with younger age at diabetes diagnosis and longer diabetes duration, higher HbA_{1c} and more intensive diabetes treatment, higher systolic and diastolic blood pressure, and higher urinary albumin:creatinine ratio (uACR). Any incident retinopathy was significantly associated with higher HbA_{1c} and more intensive diabetes treatment. Multiple logistic regression modelling of conventional risk factors (see Table 2) identified higher systolic blood pressure, HbA_{1c} and uACR together with more intensive diabetes treatment as independent risk factors for any new or worsening DR. Only higher HbA_{1c} independently predicted any incident retinopathy. When the natural logarithm of the MS-detected lipid biomarkers significant at the 5% level in bivariable analysis were considered for entry into the multiple logistic regression model of conventional risk factors for any new or worsening DR, ln(cholesterol ester 20:4), ln(dihomo- γ -linolenic acid), and ln(lysophosphatidylglycerol 18:1) added significantly to the conventional model. The likelihood ratio test (LRT) comparing the two models was highly significant ($P = 0.000021$) indicating that the more complex model, which included both conventional and MS-detected lipid biomarkers,

was superior. Likewise for any incident retinopathy, ln(dihomo- γ -linolenic acid), ln(lysophosphatidylglycerol 18:0), and ln(phosphatidylcholine 18:0/20:3) added significantly to the conventional model with a LRT $P = 0.000012$.

Fig. 2 shows the observed and predicted number of participants experiencing each retinopathy outcome by quintiles of risk. For any new or worsening DR, goodness of fit was better for Model 2 with the Hosmer-Lemeshow test, $P = 0.018$ for Model 1 (poor fit) and $P = 0.515$ for Model 2 (good fit). For incident retinopathy, fit was good for both models ($P = 0.191$ and 0.134 , respectively).

When the test performance of the conventional model (Model 1) was compared to the complex (conventional plus metabolomic lipid biomarkers, Model 2), for any new or worsening DR the AUROCs were 0.73 (0.68, 0.78) versus 0.76 (0.71, 0.80), $P = 0.498$ (see Table 3 and Supplementary Fig. S1). The sensitivity, specificity, PPV and NPV at the respective optimum cut offs for Youden's Index were 89%, 46%, 24% and 96% (Model 1, cut-off 12.2%) vs 75%, 66%, 30% and 93% (Model 2, cut-off 15.5%). The total NRI gain for Model 2 versus Model 1 at a moderate cut-off of 10% was 5.7% (SE 2.8%), $P = 0.043$. For any incident retinopathy, the AUROCs were 0.64 (0.54, 0.74) versus 0.76 (0.68, 0.84), $P = 0.086$ for Model 1 versus Model 2. The sensitivity, specificity, PPV and NPV at the respective optimum cut offs for Youden's Index were 63%, 66%, 12% and 96% (Model 1, cut-off 6.6%) vs 80%, 61%, 13% and

Table 1

Baseline characteristics of participants with any new or worsening DR between any visits between baseline and Yr 4/6, and with any incident retinopathy between baseline and Yr 4/6, excluding those with prevalent retinopathy.

Baseline variable	None	Any new or worsening DR	<i>P</i> -value	None	Incident DR	<i>P</i> -value
Number (%)	641 (84.1)	121 (15.9)		460 (92.9)	35 (7.1)	
Age (years)	64.6 $\pm$ 10.2	64.5 $\pm$ 11.1	0.956	64.8 $\pm$ 10.1	66.7 $\pm$ 11.7	0.276
Male (%)	53.5	58.7	0.320	48.5	51.4	0.861
Ethnic background (%):			0.469			0.877
Anglo-Celt	60.5	57.0		62.2	62.9	
Southern European	10.6	12.4		8.5	8.6	
Other European	7.5	9.1		6.5	5.7	
Asian	4.5	4.1		4.1	2.9	
Aboriginal	1.1	3.3		1.1	2.9	
Mixed/other	15.8	14.0		17.6	17.1	
Education beyond primary school (%)	91.7	94.2	0.462	91.7	100	0.097
Not fluent in English (%)	6.9	5.0	0.550	5.0	8.6	0.417
Smoking status (%):			0.476			>0.999
Never	43.4	47.1		42.4	42.9	
Ex	50.1	44.6		51.3	51.4	
Current	6.6	8.3		6.3	5.7	
Alcohol (standard drinks/day)	0.3 [0–1.5]	0.1 [0–1.2]	0.600	0.3 [0–1.5]	0.1 [0–1.2]	0.381
ABSI (m ^{11/6} /kg ^{2/3})	0.081 $\pm$ 0.005	0.081 $\pm$ 0.005	0.100	0.081 $\pm$ 0.005	0.082 $\pm$ 0.004	0.173
BMI (kg/m ²)	31.2 $\pm$ 6.0	32.0 $\pm$ 5.8	0.204	31.2 $\pm$ 6.1	31.6 $\pm$ 6.3	0.713
Supine SBP (mm Hg)	144 $\pm$ 20	150 $\pm$ 21	<0.001	143 $\pm$ 20	149 $\pm$ 20	0.054
Supine DBP (mm Hg)	80 $\pm$ 12	83 $\pm$ 12	0.020	80 $\pm$ 12	79 $\pm$ 10	0.703
Antihypertensive medications (%)	72.7	79.3	0.143	72.0	80.0	0.432
Age at diagnosis of diabetes (years)	56.4 $\pm$ 11.1	52.8 $\pm$ 12.1	0.001	57.8 $\pm$ 10.3	57.4 $\pm$ 13.4	0.887
Duration of diabetes (years at study entry)	6.0 [1.7–13.5]	11.0 [5.5–16.0]	<0.001	5.0 [1.2–11.7]	8.0 [4.3–14.2]	0.092
Diabetes treatment (%):			<0.001			0.006
Diet and exercise	31.7	8.3		35.2	14.3	
Oral $\pm$ non-insulin injectable antihyperglycaemic agents	52.7	56.2		54.1	60.0	
Insulin only	3.3	6.6		2.6	8.6	
Insulin + oral $\pm$ non-insulin injectable antihyperglycaemic agents	12.3	28.9		8.0	17.1	
HbA _{1c} (%)	6.7 [6.1–7.4]	7.3 [6.6–8.3]	<0.001	6.6 [6.1–7.3]	7.2 [6.3–8.3]	0.007
HbA _{1c} (mmol/mol)	50 [43–57]	56 [49–67]	<0.001	49 [43–56]	55 [45–67]	0.007
Total cholesterol (mmol/L)	4.3 $\pm$ 1.0	4.3 $\pm$ 1.0	0.666	4.3 $\pm$ 1.0	4.4 $\pm$ 1.1	0.656
HDL-cholesterol (mmol/L)	1.24 $\pm$ 0.32	1.22 $\pm$ 0.36	0.443	1.25 $\pm$ 0.33	1.28 $\pm$ 0.38	0.664
Serum triglycerides (mmol/L)	1.5 (0.9–2.4)	1.5 (0.9–2.5)	0.701	1.5 (0.9–2.4)	1.5 (0.9–2.6)	0.523
Statin use (%)	67.2	66.9	>0.999	67.4	71.4	0.710
Fibrate use (%)	2.0	2.5	0.729	2.2	0	>0.999
Urinary albumin:creatinine (mg/mmol)	2.6 (0.8–7.9)	4.1 (1.0–16.5)	<0.001	2.5 (0.8–7.4)	2.4 (0.9–6.9)	0.966
eGFR (CKD-EPI) categories (%):			0.612			0.603
≥ 90 mL/min/1.73m ²	41.8	46.3		43.5	34.3	
60–89 mL/min/1.73m ²	47.7	42.1		47.2	60.0	
45–59 mL/min/1.73m ²	6.2	8.3		5.9	2.9	
30–44 mL/min/1.73m ²	3.6	2.5		3.0	2.9	
<30 mL/min/1.73m ²	0.6	0.8		0.4	0	

Data are presented as percentages, mean $\pm$ SD, geometric mean (SD range) or median [interquartile range].

Table 2

Multiple logistic regression models of any new or worsening DR between any visits between baseline and Year 4/6, and any incident retinopathy between baseline and Year 4/6, excluding those with prevalent retinopathy.

	Any new or worsening DR				Any new DR			
	Model 1		Model 2		Model 1		Model 2	
	OR (95% CI)	P-value	OR (95% CI)	P-value	OR (95% CI)	P-value	OR (95% CI)	P-value
Systolic blood pressure (increase of 10 mmHg)	1.12 (1.01, 1.24)	0.032	1.12 (1.01, 1.24)	0.037				
HbA _{1c} (increase of 1% or 11 mmol/mol)	1.22 (1.04, 1.43)	0.013	1.20 (1.02, 1.41)	0.031	1.50 (1.18, 1.90)	0.001	1.43 (1.11, 1.83)	0.006
Oral/non-insulin injectable antihyperglycaemic agents	3.39 (1.69, 6.83)	<0.001	3.16 (1.56, 6.42)	0.001				
Insulin ± oral/non-insulin injectable antihyperglycaemic agents	5.77 (2.68, 12.4)	<0.001	5.59 (2.57, 12.2)	<0.001				
Ln(uACR (mg/mmol)) ^a	1.19 (1.01, 1.41)	0.040	1.19 (1.001, 1.40)	0.048				
Ln(cholesterol ester 20:4) ^a			0.39 (0.23, 0.64)	<0.001				
Ln(dihomo-γ-linolenic acid) ^a			1.83 (1.06, 3.13)	0.029			4.40 (1.73, 11.2)	0.002
Ln(lysophosphatidylglycerol 18:0) ^a							7.15 (2.44, 20.9)	<0.001
Ln(lysophosphatidylglycerol 18:1) ^a			3.37 (1.57, 7.22)	0.002				
Ln(phosphatidylcholine 18:0/20:3) ^a							0.26 (0.09, 0.79)	0.017
Log Likelihood, df, chi-square P-value	−301.3, 5, <0.001		−289.1, 8, <0.001		−121.8, 1, 0.0020		−109.0, 4, <0.001	
LRT (vs Model 1)			0.000021				0.000012	
Hosmer-Lemeshow test, P-value	0.074		0.892		0.339		0.218	
Nagelkerke R ²	0.138		0.188		0.048		0.170	

^a An increase of 1 in ln(x) equates to an increase of 2.718 in x; Model 1 is the most parsimonious model of conventional risk factors; Model 2 added the lipids in a backwards stepwise manner when their Mann-Whitney *U* test in Supplementary Table S2 was significant at the 5% level.

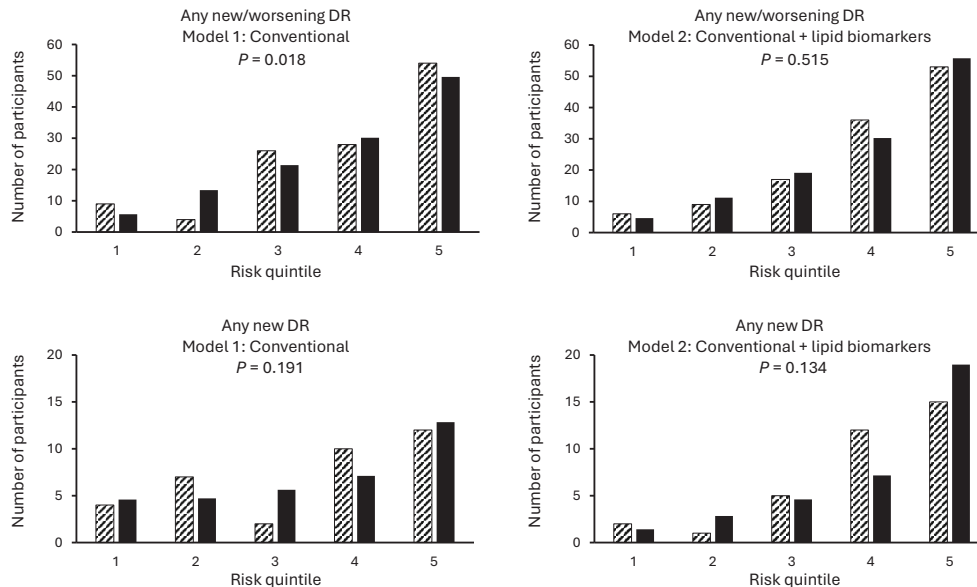


Fig. 2. Calibration plots showing the observed and predicted number of participants experiencing each retinopathy outcome by quintiles of risk. Observed numbers are shown by the hatched bars and predicted numbers by the solid bars. *P*-values are those for each Hosmer-Lemeshow test.

98% (Model 2, cut-off 5.3%). The total NRI gain for Model 2 versus Model 1 at a moderate cut-off of 5% was 28.3% (SE 9.1%), *P* = 0.002.

4. Discussion

The present data add to the emerging evidence of an association between the plasma lipidome and the future risk of DR in people with type 2 diabetes. We identified five lipid moieties that were

independently associated with the subsequent development of DR and/or its progression in well-phenotyped community-based individuals and in models incorporating conventional DR risk factors. The inclusion of the lipid parameters only modestly improved predictive models, especially for identifying individuals with at least a moderate (>10%) increased risk of newly emergent DR. Nevertheless, some of the associations could provide insights into the pathophysiological mechanisms underlying DR complicating type 2 diabetes.

Table 3

Test performance of Models 1 and 2 for predicting any new or worsening DR, and any incident DR (excluding those with prevalent DR) between baseline and Year 4/6.

	Any new or worsening DR		Any incident DR	
	Model 1	Model 2	Model 1	Model 2
Number of participants	761	761	495	495
Observed outcomes (%)	121 (15.9)	121 (15.9)	35 (7.1)	35 (7.1)
AUROC (95% CI)	0.73 (0.68, 0.78)	0.76 (0.71, 0.80)	0.64 (0.54, 0.74)	0.76 (0.68, 0.84)
Comparison of AUCs for Model 2 vs Model 1 ^a	$P = 0.498$		$P = 0.086$	
Youden's Index (YI)	0.355	0.413	0.289	0.407
At YI cut-off:	(12.2%)	(15.5%)	(6.6%)	(5.3%)
Sensitivity (%)	89.3	75.2	62.9	80.0
Specificity (%)	46.2	66.1	66.1	60.7
PPV (%)	23.8	29.5	12.4	13.4
NPV (%)	95.8	93.4	95.9	97.6
At moderate-risk cut-off (10%)*:	(10%)	(10%)	(5%)	(5%)
Sensitivity (%)	91.7	86.8	77.1	80.0
Specificity (%)	36.3	46.9	32.3	57.8
PPV (%)	21.4	23.6	8.0	12.6
NPV (%)	95.9	94.9	92.0	97.4
At high-risk cut-off (20%):	(20%)	(20%)	(10%)	(10%)
Sensitivity (%)	58.7	58.7	25.7	42.9
Specificity (%)	75.3	76.9	88.3	81.5
PPV (%)	31.0	32.4	14.3	15.0
NPV (%)	90.6	90.8	94.0	85.0
Total NRI gain (%) for Model 2 vs Model 1 at moderate risk cut-off		5.7% (SE 2.8%), $P = 0.043$		28.3% (SE 9.1%), $P = 0.002$

AUC Area under the receiver operation curve; PPV positive predictive value; NPV negative predictive value; NRI net reclassification improvement; SE standard error. Discrimination performance measures are given for moderate- and high-risk cut-offs for the outcome occurring up to and including the Year 4/6 assessment, as well as for the optimum cut-off defined by Youden's Index (sensitivity + specificity – 1). Cut-offs in parentheses.

^a A conservative test when the AUCs are nested²⁸.

In the case of the models of incident new or worsening DR, the well-recognised modifiable risk factors of blood pressure and glycaemia were independent determinants,^{2,3} while increasing therapeutic intensity (a likely surrogate for diabetes duration) and albuminuria are also established risk factors.³⁰ Cholesterol ester 20:4 (eicosatetraenoate or cholesteryl arachidonate), one of the most abundant polyunsaturated fatty acids in human plasma, was inversely associated with new or worsening DR. It is enzymatically oxidised by 15-lipoxygenase to 15-hydroxy-5,8,11,13-eicosatetraenoic acid (15-HETE)³¹ which may promote the development of DR.^{32,33} Our data suggest that there is an increase in 15-lipoxygenase activity in DR which would reduce circulating eicosatetraenoate and increase 15-HETE as has been found previously.^{33–35} Dihomo- γ -linolenic acid was positively associated with new or worsening DR. Although low levels have been cross-sectionally associated with DR,^{36,37} dihomogamma-linolenic acid has been associated with obesity and insulin resistance in type 2 diabetes, likely reflecting an excessive intake of n-6-polyunsaturated fatty acids and decreased desaturase activity converting dihomogamma-linolenic to arachidonic acid.³⁸ It has also been associated with inflammation.³⁹ Furthermore, epidemiological studies show a positive relationship between dihomogamma-linolenic acid concentrations and glycaemia,⁴⁰ which would increase the risk of DR. The positive association with lysophosphatidylglycerol 18:1 possibly represents a relationship between this lipid class and endothelial dysfunction including angiogenesis through stimulation of endothelial cell chemotaxis.⁴¹ Although the three lipid moieties were independent variables in the logistic regression model for new or worsening DR, they did not add significantly to the predictive value of the conventional risk

factors. Their inclusion was associated with a non-significant increase in AUROC of 0.03, and there were no clinically important changes in PPV and NPV at relevant risk thresholds, thus questioning their utility in a usual care context.

In the smaller group of FDS2 participants who were included in the models of new incident DR, glycaemic control was the sole recognised established independent risk factor. As with new or worsening DR, dihomogamma-linolenic acid was a positive predictor. In addition, lysophosphatidylglycerol 18:0 was strongly positively associated with incident DR. Lysophosphatidylglycerol 18:0 has been shown to be a positive biomarker for asthma through dysregulation of T cells.⁴² Of potential relevance to the present study, there is some evidence of a link between DR and other complications of diabetes and altered regulatory T cell activity.^{43,44} The inverse association between phosphatidylcholine 18:0/20:3 and incident DR could reflect a similar inverse relationship with glycaemic control in type 1 diabetes,⁴⁵ while there is evidence that phosphatidylcholines may have anti-inflammatory properties.⁴⁶

The strengths of the present study include its community-based sample and comprehensive serial assessments. A major limitation of this study is that the large number of lipid parameters evaluated inherently increased the risk of false-positive associations due to multiple testing. However, because this is one of only two studies to examine the relationship between lipidomics and incident DR, we chose to maintain a conventional level of statistical significance. This exploratory approach prioritizes the identification of novel lipid signatures over the potential omission of meaningful, yet underpowered, associations. Future external validation in other populations is thus required to confirm these findings. The plasma samples used in the present analyses were unfrozen, sealed and light protected at -80°C to maintain long term stability,⁴⁷ but we cannot exclude the possibility of degradation and transformation. There was also the possibility of recruitment bias inherent in most observational studies, with relatively healthy people with type 2 diabetes participating in FDS2. In addition, and consistent with longitudinal studies in older cohorts including those with diabetes,^{48,49} the FDS2 participants included in the present analyses were significantly younger and had a shorter diabetes duration than those who did not have valid follow-up data. At Year 4, 60% of FDS2 participants reattended for assessment and this was just over 50% by Year 6, a rate typical for longitudinal observational studies (30–70%).⁵⁰ Thus, allowing for the fact that we included Year 4 and/or Year 6 DR data for nearly 50% of the total FDS2 cohort in the present analyses, we captured data from the vast majority of those who reattended. We did not assess lipidomic biomarkers in those participants with mild/moderate NPDR who progressed to severe NPDR or worse as the numbers were insufficient for valid analysis. We did not include macular oedema grading in our analyses given that DR was assessed from retinal photography without optical coherence tomography.

5. Conclusions

The five lipid moieties identified in the present study as having a potential role in the development or progression of DR complicating type 2 diabetes have not been reported in the other longitudinal studies,^{14–16} which suggests that there may be genetic and/or lifestyle factors that influence the lipidome and its pathophysiological consequences in different populations. All the candidate lipids that we identified as independent determinants in multivariable models appear to have some plausible relationship to DR, and the presence of well-established conventional risk factors in the models attests to their validity. The inclusion of the lipid levels in our predictive models was associated with, at best, a modest contribution to identifying people with type 2 diabetes at moderate risk of new incident DR. Nevertheless, the present study should be viewed as exploratory and data from additional longitudinal studies should be used to confirm our findings.

CRediT authorship contribution statement

Timothy M.E. Davis: Writing – original draft, Resources, Funding acquisition, Conceptualization. **Jocelyn J. Drinkwater:** Writing – review & editing, Formal analysis, Data curation. **Alanah Grant-St James:** Writing – review & editing, Investigation. **Elaine Holmes:** Writing – review & editing, Resources, Conceptualization. **Wendy A. Davis:** Writing – review & editing, Formal analysis, Data curation. **Nicola Gray:** Writing – review & editing, Resources, Methodology, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

There are no other potential conflicts of interest relevant to this article.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jdiacomp.2026.109412>.

Data availability

The data reported in this study are available from the Corresponding author (TMED) on reasonable request.

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