Journal Pre-proof

Associations between erythrocyte membrane fatty acid compositions and biomarkers of vascular health in adults with type 1 diabetes with and without insulin resistance: a cross-sectional analysis.

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Key Messages

1. The relationship between fatty acid composition, IR, and validated parameters of vascular health in type 1 diabetes is currently unknown.
2. Specific erythrocyte membrane fatty acid compositions are strongly associated with IR and vascular outcomes in adults with T1D.
3. Identification of unfavourable erythrocyte fatty acid compositions amongst adults with T1D may permit targeted dietary intervention strategies.

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Key Messages

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2. Specific erythrocyte membrane fatty acid compositions are strongly associated with IR and vascular outcomes in adults with T1D.
3. Identification of unfavourable erythrocyte fatty acid compositions amongst adults with T1D may permit targeted dietary intervention strategies.

Purpose: The aim of this study was to assess the relationship between specific erythrocyte fatty acids levels and vascular health in type 1 diabetes (T1D) with and without insulin resistance (IR).

Methods: We analysed baseline pretreatment data in a subset of 23 patients with T1D from a previously published randomised controlled trial consisting of comprehensive erythrocyte-derived fatty acid profiles and a panel of inflammation-associated endothelial markers. Estimated glucose disposal rate was used to identify and categorise patients with IR. We utilised principal component analysis (PCA) to cluster vascular biomarkers to compute a single 'vascular signal' and employed univariate linear regression models to investigate the association with IR and fatty acid profiles.

Results: Subjects with IR displayed significantly higher levels of linoleic acid (p=0.001), lower levels of eicosapentaenoic acid (EPA) (p<0.001), lower total omega-3 polyunsaturated fatty acid (n-3PUFA) (p<0.006), and an increased n-6PUFA:n-3PUFA ratio (p=0.001). IR was associated with significantly higher linoleic acid levels, total n-6PUFA, and an increased ratio of n-6PUFA:n-3PUFA, and negatively associated with arachidonic and eicosapentaenoic acid levels, total saturated fatty acid, and total n-3PUFA. The PCA-derived vascular biomarker cluster was positively associated with linoleic acid, n-6PUFA:n-3PUFA ratio and inversely associated with EPA.

Conclusion: Specific erythrocyte membrane fatty acid compositions are associated with impaired vascular health and IR in adults with T1D. These findings suggest that IR and risk of associated complications may be influenced by specific fatty acid profiles, and thus potentially modified by the selective targeting of dietary fatty acids.

Keywords: Type 1 diabetes; vascular health; insulin resistance; erythrocyte fatty acids.

Trial Registration: ISRCTN4081115; registered 27 June 2017**.**

Declarations

Funding

This study was funded by the Nutricia Research Foundation.

Conflicts of interest/ competing interests

No conflicts of interest or competing interests relevant to this article are reported.

The RCT received ethical approval from the UK National Health Service Health Research Authority (REC Reference 17/NE/0244) and all participants gave written informed consent.

Consent for publication

Not applicable

Availability of data and material

The data that support the findings of this study are available on request from the corresponding author.

Code Availability

Not applicable

Authors contributions

LLO, OJP, and MDC designed the research. LLO, NMO, GM and RC conducted the research. RAA aided with recruitment. MDC performed statistical analysis. LLO, RC, AS-K, RAA, NMO, GM, OJP, and MDC wrote the paper. All authors read and approved the final manuscript.

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Type 1 diabetes (T1D) is associated with an increased risk of both micro and macrovascular complications [1]. The pathological processes governing the development of these complications is driven, at least in part, by insulin resistance (IR) [2-4]. IR is mediated by a chronic, low-grade, tissue-specific inflammatory response [5], much of which may be diet-induced. In adults with type 2 diabetes, saturated fatty acid (SFA) intake, specifically the fraction of palmitic acid within erythrocyte phospholipids, is associated with IR [6,7]. Conversely, polyunsaturated fatty acids, especially eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), show improvements on insulin sensitivity from experimental animal models and observational human studies [8,9]. However, not all polyunsaturated fatty acids are associated with such improvements, with recent data highlighting that high serum dihomo-γ-linolenic acid levels, an omega-6 polyunsaturated fatty acid, are associated with adiposity and IR in individuals with type 2 diabetes [10]. The divergence in effect between individual fatty acids of the same classification suggests that the individual biochemical properties of fatty acids may yield important biological effects beyond their classification [11]. This is important given current dietary recommendations typically focus on the amount and type of fat (namely saturated, monounsaturated, and polyunsaturated) but fail to differentiate between individual fatty acids within the same classification [12].

The varying biochemical properties of individual fatty acids may result in different biological effects that could promote IR, and the progression of diabetes-related vascular complications [13-15]. Yet, the relationship between dietary fatty acid composition, IR and vascular health in patients with T1D has yet to be established. In this study, we therefore adopted a novel pragmatic approach and reanalysed data from a previously published randomised controlled trial (RCT) [16] to assess the relationship between fatty acid composition, IR, and validated parameters of vascular health.

METHODS

Study design and population

We performed a cross-sectional analysis using baseline data from a previously published RCT [16] (ISRCTN registration ISRCTN40811115). The RCT received ethical approval from the UK National Health Service Health Research Authority (REC Reference 17/NE/0244) and all participants gave written informed consent. Detailed information concerning the study procedures have been published previously and are summarised below. In the

with a diagnosis of T1D >2 years on enrolment and free from diabetes-related complications.

Quantification of erythrocyte fatty acids and inflammation-associated endothelial biomarkers

Following an overnight fast, a total of 10-mL venous blood was collected, of which 4 mL was immediately analysed for the quantification of erythrocyte fatty acids and glycosylated haemoglobin (HbA1c). Erythrocyte fatty acids concentrations were determined via gas chromatography using methods previously described [17]. The remaining sample was centrifuged at 2700 x g for 10 minutes at 4°C and the resultant plasma was subsequently stored at -80°C for retrospective analysis of inflammation-associated endothelial biomarkers. A customised 7-plex human fluid-phase magnetic immunoassay (R&D Systems, Minneapolis, USA) was used for the simultaneous detection and quantification of E-selectin, intercellular adhesion molecule-1 (ICAM-1), pentraxin-3 (PTX3), P-selectin, tumor necrosis factor alpha (TNFα), vascular cell adhesion molecule-1 (VCAM-1), and vascular endothelial growth factor (VEGF) . All biochemical data were collected on a Luminex® 200™ cytometer (Luminex, Texas, USA) and analysed using specialised software (Bio-Plex Manager 6.1, Bio-Rad, California, USA) as per the manufacturer's instructions.

Other physiological variables

To assess IR, we calculated estimated glucose disposal rate (eGDR), a validated marker of IR in T1D [18-20], using the following formulae: eGDR = 19.02 – (0.22 x body mass index [kg/m^2) – (3.26 x HTN) – (0.61 x HbA1c [%]), wherein HTN is hypertension (1 = yes, 0 = no) [4]. Lower eGDR values indicated greater degrees of IR. Anthropometric measures including weight and body mass index were obtained, and percentage body fat estimated via bioelectrical impedance analysis (SC-331S, Tanita, Amsterdam, Netherlands). Blood pressure assessed via an automated oscillometric device (Intellisense HEM-907XL, Omron, Kyoto, Japan).

Statistical analyses

Data were analysed using SPSS Statistics version 25 (IBM SPSS Statistics 25, IBM Corporation, USA) and assessed for normality. Continuous variables are reported as mean±SD and categorical variables are reported as frequency (%). Differences between dichotomised variables were assessed using independent *t* tests. To assess the association between clinical parameters and fatty acid profiles we employed a Pearson correlation coefficient matrix (Figure 1). Relationships between eGDR and the inflammation-associated vascular biomarkers were

the purpose of reducing the number of dependent variables in the analyses and to optimise the vascular marker signal, principal component analysis (PCA) was employed to 'cluster' vascular biomarkers [21]. This method allows assessment of the covariance structure or interactions between vascular biomarkers, and captures the overall inflammatory state, which may otherwise be underestimated in analyses evaluating single markers [22]. All inflammation-associated vascular endothelial biomarkers were log transformed, except for E-selectin and P-selectin, , prior to conducting PCA to achieve normality. PCA was conducted with 7 vascular variables (ICAM, VCAM, E-Selectin, P-Selectin, VEGF, TNFα, and PTX3). A single principal component was retained, determined based on Eigenvalues >1 and the evaluation of scree plots. The generated PC was interpreted using variable factor loadings of ≥0.40, which measures the contribution of each variable to the PC pattern; the factor loadings were: ICAM, 0.785; VCAM, 0.613; E-Selectin, 0.898; P-Selectin, 0.651; VEGF, 0.950; TNFα 0.882, and PTX3, 0.889. The variance explained by this single PC was 67.29%. Calculated scores for each participant were used as dependent variables in linear regression analyses. Statistical significance was determined as $p<0.05$ for all analyses.

RESULTS

Baseline clinical characteristics and erythrocyte fatty acid profiles of the study population are presented in Table 1 and 2, respectively. We stratified this cohort by IR status, with an IR cut point corresponding to an eGDR <7.5. By definition, IR subjects were older (40±16 vs. 27±7 years, $p=0.029$) and presented with increased body mass index (28.13±5.65 vs. 24.14±3.18 kg/m^2, $p=0.042$), body fat% (27.85±13.44 vs. 16.79±6.56%, $p=0.018$), systolic blood pressure (137±6 vs. 123±8 mmHg, $p<0.001$), and an adverse vascular profile (all analyses $p<0.01$, [except VCAM-1] ; Table 1); HbA1c was similar between groups (7.59±1.21 vs 7.31±1.08, $p=0.555$). Subjects with IR displayed significantly higher levels of linoleic acid (13.96±2.83 vs. 11.05±1.34, $p=0.001$), lower levels of EPA (0.57±0.08 vs 0.88±0.21, $p<0.001$) and total n-3PUFA (6.71±1.20 vs 8.10±0.96, $p<0.006$), and an increased n-6PUFA:n-3PUFA ratio (25.44±3.53 vs 18.56±3.45, $p=0.001$) (Table 2).

We determined correlations between clinical parameters and fatty acid levels; a corresponding correlation matrix of clinical biomarkers versus fatty acids is shown in Figure 1. We observed strong associations between total SFA and eGDR ($p=0.020$); no associations were observed between total SFA and vascular parameters ($p>0.05$). n-3PUFA and the ratio of n-6PUFA:n-3PUFA were strongly associated with eGDR, ICAM, VEGF, E-Selectin, PTX3, and TNFα ($p<0.05$).

Figure 2 shows the unadjusted and adjusted associations of eGDR and the PCA-derived vascular biomarker cluster with erythrocyte fatty acid profiles. By utilising this approach, we have accommodated for correlations between individual vascular biomarkers, and generated a new variable (vascular biomarker cluster) which uses their combined contribution to explained variance as a scaling factor to determine the relative contribution of the overall inflammatory signal to explained variance in fatty acids. Following adjustment for confounders (age, sex and diabetes duration) eGDR was inversely associated with linoleic acid, total n-6PUFA, and the ratio of n-6PUFA:n-3PUFA, and positively associated with arachidonic acid, eicosapentaenoic acid, total SFA, and total n-3PUFA.

In unadjusted linear regression analyses, the vascular biomarker cluster was positively associated with linoleic acid, n-6PUFA:n-3PUFA ratio, and total n-6PUFA, and inversely associated with arachidonic acid, EPA, DHA, and total n-3PUFA. Following adjustment for confounders (age, sex, diabetes duration), only associations with linoleic acid, n-6PUFA:n-3PUFA ratio, and EPA remained robust.

DISCUSSION

In the present study, we examined the association between fatty acid profiles and parameters of vascular health in people with T1D with and without IR. Collectively, our findings indicate that IR and an increased inflammation-associated vascular milieu are associated with a fatty acid profile favouring n-6PUFAs. Specifically, we show that individuals with T1D presenting with concomitant IR present with a significantly higher n-6PUFA:n-3PUFA ratio with higher linoleic acid levels, and significantly lower EPA and total n-3PUFA. We also show that increased IR in T1D, determined using eGDR, is associated with increased levels of multiple n-6PUFAs, and associated with reduced levels of arachidic acid and total SFA, as well as EPA and total n-3PUFA. Moreover, higher levels of linoleic acid, an increased n-6PUFA:n-3PUFA ratio and lower EPA levels were associated with an elevated inflammatory vascular profile.

Given the established causal link between IR and inflammatory processes, the finding of elevated endothelial inflammation in individuals with pre-existing IR is unsurprising and supports previous research in individuals with and without T1D [23,8,24]. However, the present study extends this work by employing PCA to optimise the vascular marker signal to evaluate the pleiotropic and synergistic effect of our chosen biomarkers. This approach

vascular biomarkers in isolation [22].

The increased inflammatory response that triggers IR is at least in part mediated by fatty acids at several insulin-sensitive tissue sites [25]. Increased storage of saturated fatty acids trigger hypertrophy-driven adipocyte necrosis activating c-Jun N-terminal kinase and nuclear factor kappa B signalling pathways [26-28]. This process is partly mediated by saturated fatty acids activating toll-like receptor 2 and toll-like receptor 4 which initiate the aforementioned signalling pathways [29,30]. Activation of these pathways increases the secretion of pro-inflammatory cytokines and endothelial adhesion molecules that facilitate adhesion and migration of monocytes into adipocytes that further initiate pro-inflammatory mediators, creating a pro-inflammatory milieu and impairment of tissue insulin signalling [31,32]. In light of the above, and the strong associations observed between fatty acids and eGDR, and vascular markers, our data suggest that IR and vascular health in T1D are influenced by specific fatty acids profiles, raising the intriguing possibility that IR and risk of associated complications may be modifiable by the selective targeting of dietary fatty acids.

Erythrocyte concentrations of n-6PUFA were higher and n-3PUFA concentrations were lower amongst IR individuals. This finding concurs with previous research that reports higher n-3PUFA concentrations are associated with increased insulin sensitivity [33]. However, meta-analyses assessing the effects of increased dietary intake of n-3PUFA on insulin sensitivity are conflicting [34,35]. It is posited that higher erythrocyte concentrations of n-3PUFA improve IR in part by downregulating inflammatory pathways via attenuated endoplasmic reticulum stress, improved mitochondrial function (i.e. increased fatty acid β-oxidation and uncoupling), and increased motofusin-2, a mitochondrial fusion protein associated with IR [36]. Given erythrocytes are incapable of de novo phospholipid synthesis, erythrocyte fatty acid concentrations are representative of the overall milieu, which is largely dependent upon dietary intake [37]. It is important to note, however, that we have recently shown that 6-months supplementation with a daily high-dose bolus of n-3PUFA did not result in an overall improvement in inflammation-associated vascular endothelial biomarkers or glucose control in T1D [16]. Notably, we observed a degree of heterogeneity in treatment effect in response to n-3PUFA supplementation[37], which in the context of the present study, suggests supplementation may impact lipid levels but may not impact vascular health by the same margin.

individual fatty acids to vascular health differs from one phospholipid to another [46]. In the present study, the PCA-derived vascular biomarker cluster was positively associated with linoleic acid. Linoleic acid induces endothelial cell activation and the subsequent increase in inflammation-associated endothelial biomarkers via two signalling pathways: (i) phosphatidylinositol 3-kinase/ amino kinase terminal and (ii) extracellular signal regulated kinase 1/2 [38]. Additionally, the ratio of n-6PUFA:n-3PUFA was positively associated with inflammation-associated endothelial biomarkers, whereas EPA was inversely associated. This corresponds with previous findings in rodents that report that a high n-6PUFA:n-3PUFA ratio promotes inflammation and IR [39]. Furthermore, high n-3PUFA concentrations are associated with decreased inflammatory biomarkers amongst individuals with diabetes and cardiovascular disease [40]. n-3PUFAs and n-6PUFAs are metabolised by the same desaturation/elongation pathway and require the same rate-limiting enzyme, with higher n-6PUFA:n-3PUFA ratios favouring the conversion of n-6PUFA [41]. This results in n-6PUFA derived eicosanoids which are pro-inflammatory [42]. Additionally, lipid mediators derived from n-3PUFA produce resolvins protectins and maresins which possess both anti-inflammatory and pro-resolving properties [43,44]. These mechanisms may explain why linoleic acid and the ratio of n-6PUFA:n-3PUFA are positively associated with inflammation-associated endothelial biomarkers and may offer targets for modification of vascular risk in T1D [45].

Methodological considerations and future research

To the best of our knowledge, this is the first study to examine the associations between erythrocyte membrane fatty acid compositions and indices of vascular health in adults with T1D with and without IR. The strengths of this study include the comprehensive analysis of 26 individual erythrocyte fatty acids, providing a complete investigation of individual SFAs, monounsaturated fatty acids, and PUFAs, and the use of PCA to 'cluster' vascular biomarkers to accommodate the pleiotropic and synergistic relationship between individual parameters. Further, to avoid under- or overestimation of associations between fatty acid compositions and indices of vascular health we adjustment for multiple confounders (age, sex, and diabetes duration). Collectively, this allows for the comprehensive assessment of associations [47]. Owing to the cross-sectional research design of this study, we are unable to make casual inferences from our observations [48], and the effect of residual confounders not included in the statistical model cannot be excluded [49].

Conclusions

associated with IR and vascular outcomes in adults with T1D. Specifically, n-3PUFA and the ratio of n-6PUFA:n-3PUFA were strongly associated with eGDR, ICAM, VEGF, E-Selectin, PTX3, and TNFα. SFAs were also strongly associated with eGDR. Adults with T1D and IR display significantly poorer vascular health than those without IR and significantly higher and lower n-6PUFA and n-3PUFA concentrations, respectively. The findings of this study can be used to identify individuals with T1D who may have impaired vascular health subsequent to unfavourable erythrocyte fatty acid compositions, which may permit targeted dietary intervention strategies.

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Figure 1. Correlation matrix showing the interrelationships between clinical biomarkers vs. fatty acids with Pearson correlation coefficients.

Figure 2. Mean (95%CI) difference in fatty acids by 1-SD difference in (**A**) eGDR, and (**B**) PCA panel of inflammatory-associated vascular endothelial biomarkers. Estimated differences are unadjusted (grey) and adjusted (black) for age, sex, and diabetes duration. Note the two-segment x-axis.

Tables

Table 1. Clinical and biochemical variables

Table 2. Erythrocyte fatty acid profiles

Table 2. Clinical and laboratory variables

	All patients	**IR status**		
		IR	non-IR	*p* value
n	23	12	11	---
Male (%)	74	83	64	0.275[b]
Age (years)	34±14	40±16	27±7	0.029[a] *
HbA1c (%)	7.46±1.13	7.59±1.21	7.31±1.08	0.555[a]
Diabetes duration (years)	18±12	21±15	14±9	0.344[a]
BMI (kg/m^2)	26.22±4.98	28.13±5.65	24.14±3.18	0.042[a] *
Body Fat (%)	22.56±11.91	27.85±13.44	16.79±6.56	0.018[a] *
eGDR	7.14±2.38	5.21±1.47	9.25±0.88	<0.001[a] ***
Systolic BP (mm/Hg)	131±10	137±6	123±8	<0.001[a] ***
Diastolic BP (mm/Hg)	78±8	79±7	76±9	0.491[a]
Hypertension (%)	48	92	0	<0.001[b] ***
VCAM-1 (ng/mL)	773±658	833±615	708±726	0.534[a]
ICAM-1 (ng/mL)	970±600	1263±680	650±261	0.005[a] **
VEGF (pg/mL)	75.91±36.64	52.05±15.38	97.78±37.13	0.001[a] **
E-Selectin (ng/mL)	40.66±18.83	52.53±13.32	27.70±14.08	<0.001[a] ***
P-Selectin (ng/mL)	33.50±12.09	40.16±9.77	26.23±10.26	0.003 [a] ***
PTX3 (ng/mL)	2.77±2.66	4.25±2.97	1.15±0.61	<0.001[a] ***
TNFα (pg/mL)	53.21±33.94	76.10±30.39	28.24±14.29	<0.001[a] ***

Note: Metric variables are reported as mean±SD; categorical variables are reported as frequency (percentage). [a] = independent *t*-test; [b] = Fisher's Exact test. * denotes p<0.05; ** denotes p<0.01; *** denotes p<0.001. IR, insulin resistance

Fatty Acid		**All patients**	**IR Status**		
Nomenclature	Name		IR	non-IR	*p* value
Saturated Fatty Acids					
C14:0	Myristic Acid	0.31±0.12	0.33±0.11	0.29±0.12	0.250[a]
C16:0	Palmitic Acid	22.13±1.26	22.38±1.21	21.86±1.31	0.331[a]
C18:0	Stearic Acid	16.31±1.21	15.86±1.23	16.79±1.02	0.062[a]
C20:0	Arachidic Acid	0.21±0.03	0.20±0.03	0.22±0.03	0.147[a]
C22:0	Behenic Acid	0.49±0.12	0.47±0.11	0.51±0.14	0.439[a]
C24:0	Lignoceric Acid	1.22±0.23	1.17±0.28	1.28±0.22	0.299[a]
Total Saturated Fatty Acids		40.67±0.93	40.41±1.10	40.95±0.65	0.176[a]
Monounsaturated Fatty Acids					
C16:1ω7	Palmitoleic Acid	0.24±0.21	0.36±0.20	0.27±0.15	0.291[a]
C18:1ω9	Oleic Acid	16.01±1.06	15.85±0.93	16.19±1.21	0.455[a]
C20:1ω9	Gondoic Acid	0.29±0.04	0.29±0.04	0.28±0.05	0.561[a]
C24:1ω9	Nervonic Acid	1.42±0.35	1.33±0.30	1.53±0.29	0.130[a]
Total monounsaturated Fatty Acids		18.04±1.00	17.83±1.15	18.27±1.16	0.309[a]
Omega-6 Polyunsaturated Fatty Acids					
C18:2ω6	Linoleic Acid	11.98±1.98	13.96±2.83	11.05±1.34	0.002[a] **
C18:3ω6	γ-linolenic Acid	0.05±0.04	0.07±0.04	0.06±0.05	0.444[a]
C20:2ω6	Eicosadienoic Acid	0.24±0.03	0.25±0.03	0.22±0.03	0.129[a]
C20:3ω6	Dihomo-γ-linolenic Acid	1.70±0.37	1.69±0.34	1.71±0.42	0.117[a]
C20:4ω6	Arachidonic Acid	14.93±1.69	14.29±1.78	15.63±1.34	0.056[a]
C22:4ω6	Adrenic Acid	3.06±0.61	2.84±0.66	3.30±0.47	0.074[a]
C22:5ω6	Docosapentaenoic Acid	0.72±0.17	0.70±0.15	0.73±0.19	0.683[a]
Total Omega-6 Polyunsaturated Fatty Acids		33.27±1.59	33.80±1.50	32.70±1.56	0.096[a]
Omega-3 Polyunsaturated Fatty Acids					
C18:3ω3	α-linolenic Acid	0.19±0.17	0.21±0.08	0.16±0.06	0.124[a]
C20:5ω3	Eicosapentaenoic Acid	0.65±0.25	0.57±0.08	0.88±0.21	<0.001[a] ***
C22:5ω3	Docosapentaenoic Acid	2.51±0.45	2.34±0.44	2.70±0.39	0.045[a] *
C22:6ω3	Docosahexaenoic Acid	3.79±1.27	3.59±0.96	4.36±0.84	0.056[a]
Total Omega-3 Polyunsaturated Fatty Acids		7.31±1.29	6.71±1.20	8.10±0.96	0.006[a] ***
Omega-6:3 Ratio		22.82±7.35	25.44±3.53	18.56±3.45	<0.001[a] ***

Note: Metric variables are reported as mean±SD; categorical variables are reported as frequency (percentage). [a] = independent *t*-test; [b] = Fisher's Exact test. * denotes p<0.05; ** denotes p<0.01; *** denotes p<0.001. IR, insulin resistance.