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DNA methylation of tumour necrosis factor (TNF) alpha gene is associated with specific blood fatty acid levels in a gender-specific manner

Bethan Hussey[1,2] | **Richard P. Steel**[2,4] | **Boakye Gyimah**[1,3] | **James C. Reynolds**[1,3] | **Ian M. Taylor**[2] | **Martin R. Lindley**[1,2] | **Sarabjit Mastana**[1,2]

[1]Translational Chemical Biology (TCB) Research Group, Loughborough, University, Loughborough, UK

[2]School of Sport, Exercise and Health Sciences, Loughborough University, Loughborough, UK

[3]Department of Chemistry, Loughborough University, Loughborough, UK

[4]School of Social Sciences, Nottingham Trent University, Nottingham, UK

Correspondence
Martin R. Lindley and Sarabjit Mastana, Translational Chemical Biology (TCB) Research Group, Loughborough, University, Loughborough, UK.
Email: m.r.lindley@lboro.ac.uk; s.s.mastana@lboro.ac.uk

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Abstract

Background: Fatty acids, specifically polyunsaturated fatty acids (PUFAs) play an important role in inflammation and its resolution, however, their interaction with the epigenome is relatively unexplored. Here we investigate the relationship between circulating blood fatty acids and the DNA methylation of the cytokine encoding gene tumour necrosis factor (*TNF*, OMIM 191160).

Methods: Using a cross-sectional study approach, we collected blood samples from adults (*N*=88 (30 males, 58 females); 18–74 years old) for DNA methylation pyrosequencing analysis at four sites in *TNF* exon 1 and gas-chromatography mass-spectrometry analysis of the fatty acid profile of dried blood spots (DBS).

Results: Methylation levels of *TNF* exon 1 are significantly correlated with specific fatty acids in a gender-specific manner. In the males the PUFAs Docosahexaenoic Acid (DHA) and Arachidonic Acid (AA) were positively associated with *TNF* methylation, as was the saturated fatty acid (SFA) Stearic Acid; in contrast, mono-unsaturated fatty acids (MUFAs) had a negative association. In the females, omega-6 PUFA γ-Linolenic acid (GLA) was negatively correlated with *TNF* methylation; Adrenic acid and Eicosadienoic Acid were positively correlated with *TNF* methylation.

Conclusion: These results suggest that one way that fatty acids interact with the inflammation is through altered methylation profiles of cytokine genes; thus, providing potential therapeutic targets for nutritional and health interventions.

KEYWORDS
arachidonic acid, DNA methylation, docosahexaenoic Acid, dry blood spots, inflammation, *TNF*

1 | INTRODUCTION

The global trend towards the Western diet has altered the quantity and composition of dietary fat (Popkin, 2006). This change is significant because consuming the right sort of fat is essential for good health (de Oliveira Otto et al., 2012). Dietary fatty acids, including saturated fatty acids (SFAs), mono-unsaturated fatty acids (MUFAs), omega 3 (ω-3) polyunsaturated fatty acids (PUFAs) and omega 6 (ω-6) PUFA, are reported as having differing effects upon health (Estruch

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et al., 2006; González-Becerra et al., 2019; Innes & Calder, 2018; Lunn, 2007; Mozaffarian et al., 2010). For example, the replacement of SFAs with PUFAs is associated with a lower incidence of coronary heart disease (CHD), furthermore, consuming MUFAs rather than SFAs has a protective effect for CHD, diabetes and cancer (Lunn, 2007; Mozaffarian et al., 2010).

The nutritional components of diet, including fatty acids, ultimately contribute to the cell environment. Cells respond to their environment through changes in epigenetic mechanisms which include histone modifications, micro RNAs, chromatin remodelling and DNA methylation (Feinberg, 2018; Hussey et al., 2017; Joseph et al., 2016; Liu et al., 2019). Some of these responses result in alterations to, and errors within, the epigenome and are implicated in diseases such as cancer, CHD, and inflammatory bowel disease (Barnett et al., 2010; Ehrlich, 2019; Feinberg, 2018; Jones & Baylin, 2007; Muka et al., 2016).

A common component of these diseases is inflammation and while inflammation is meant to be transient, under certain conditions acute inflammation can become chronic (Nathan & Ding, 2010). Excess chronic inflammation is an important aetiologic factor across a range of disease states including but not limited to cardiovascular disease and diabetes (Dandona et al., 2004), neurologic diseases, for example Alzheimer (Perry et al., 2007), and cancer (Grivennikov et al., 2010).

Fatty acids are involved with this inflammatory process on multiple levels, including the production of pro- and anti-inflammatory eicosanoids, alterations in cell signalling and interactions with gene expression (Calder, 2012a). It is the epigenetic effects of fatty acids, specifically with regards to DNA methylation, in which we are interested here.

Interactions between fatty acids and DNA methylation have been the subject of limited research utilising both candidate gene and genome-wide DNA methylation association studies. These studies have identified relationships between dietary fatty acids and biologically significant pathways related to inflammation (Aslibekyan et al., 2014; Cui et al., 2016; Haghighi et al., 2015; Hermsdorff et al., 2013; Hussey et al., 2017; Ma et al., 2016; Rahbar et al., 2018; Voisin et al., 2015). In a genome-wide DNA methylation association study of an adolescent population, fatty acid ratios (including (MUFA+PUFA)/SFA and PUFA/SFA) in the diet showed significant enrichment of pathways linked to nuclear factor kappa B (NFκB), peroxisome proliferator-activated receptor (PPARα), leptin (LEP) and interleukin (IL)-6 (Voisin et al., 2015). Using a 24-hour dietary recall, the authors were only capturing the short-term dietary effects of the fatty acids. By measuring the biochemical ω-3 PUFA status of blood, a separate study considered longer-term dietary habits, incorporation of ω-3 PUFAs into the blood and the endogenous production of fatty acids by measuring nitrogen stable isotope ratio of blood as an indicator of ω-3 PUFA status (Aslibekyan et al., 2014). This research in an Alaskan (Yup'ik) cohort identified 27 significant differentially methylated regions (DMRs). DMRs were found in genes implicated in disease and inflammation, including helicase-like transcription factor (*HLTF*), actin alpha 2 smooth muscle (*ACTA2*), fas cell surface death receptor (*FAS*), neuron navigator 1 (*NAV1*), C-C motif chemokine ligand 17 (*CCL17*) and aryl-hydrocarbon receptor repressor (*AHRR*) (Aslibekyan et al., 2014). Additionally, the majority of sites (78%) had increased methylation with higher ω-3 status, suggesting that increased levels of ω-3 PUFA may improve epigenomic stability.

In candidate gene approaches, the methylation in the fatty acid desaturase 1 and 2 (*FADS1*, and *FADS2*) genes were negatively associated with ω-6 PUFAs (Cui et al., 2016; Rahbar et al., 2018). Some selected inflammation-related genes have been studied; ω-6 PUFA intake contributed to a regression model explaining ~45% of the variation of CpG sites within the tumour necrosis factor (*TNF*) promoter (Hermsdorff et al., 2013). Also, a CpG site (cg01770232) within the *IL6* gene showed a negative association with total ω-3 PUFA, with a dependency on a single nucleotide polymorphism (SNP) within the same gene (Ma et al., 2016).

As shown above, the research into the relationship between fatty acids and DNA methylation is limited; therefore, further research is warranted on specific fatty acids and genes involved in inflammation. The research presented here focuses specifically on the effect of methylation of the gene encoding the circulating inflammatory cytokine; tumour necrosis factor alpha (TNFα).

TNFα is produced predominantly in monocytes and macrophages; it is an essential mediator in inflammation and in the regulation of multiple other genes, including but not limited to transcription factors, cytokines, growth factors, receptors, cell adhesion molecules and inflammatory mediators (Vilček & Lee, 1991). Transcriptional control of the *TNF* gene (OMIM 191160) occurs partly through epigenetic mechanisms (Campión et al., 2009; Cordero et al., 2011; El Gazzar et al., 2008; Falvo et al., 2010; Gowers et al., 2011; Sullivan et al., 2007;. Furthermore, DNA methylation levels within the promoter and exon 1 regions of the *TNF* gene are associated with plasma TNFα cytokine levels (Hermsdorff et al., 2013). The DNA methylation profile of *TNF* has been associated with diseases including Crohn's disease, post-traumatic stress disorder (PTSD) and age-related inflammation, as well as truncal fat, adipose tissue and diet (Campión et al., 2009; Cordero et al., 2011; El Gazzar et al., 2008; García-Escobar et al., 2017; Gowers et al., 2011; Nimmo et al., 2012; Perfilyev et al., 2017; Pieper et al., 2008). Using a targeted candidate gene approach, we have sought to establish whether there is a relationship between blood fatty acid levels and the DNA methylation profile within the first exon of the *TNF* gene (Figure 1).

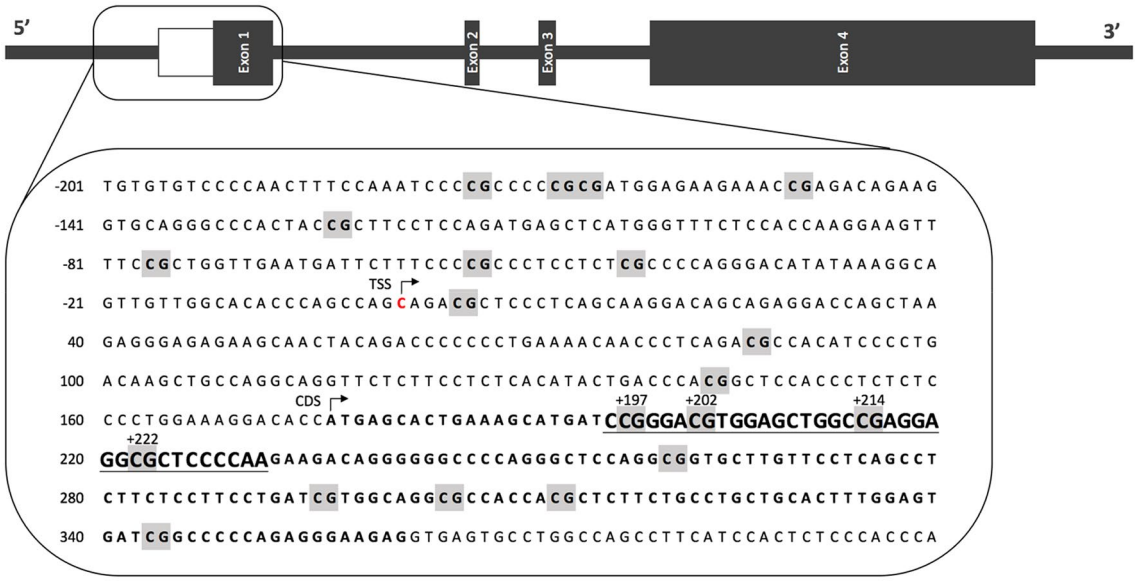


FIGURE 1 Genomic structure of TNF (MIM 191160) gene. Sequence shown for promotor and exon 1 with Cytosine-Guanine dinucleotides (CpG) sites indicated. The sequence analysed via pyrosequencing is underlined and covers four CpG sites +197 (CpG1), +202 (CpG2), +214 (CpG3) and +222 (CpG4) base pairs from the Transcription Start Site (TSS). Coding DNA Sequence (CDS). Exon 1 displayed in bold

2 | METHODS

2.1 | Ethical compliance and participants

Ethical clearance was granted by the Loughborough University ethical approvals committee, and all participants provided written informed consent. Adults (N=88 (30 males and 58 females); 18–74 years) were recruited from the local community by word of mouth, advertisements, emails and social media. Participants were free of blood-borne viruses; there were no other exclusion criteria.

2.2 | Study visit

Participants attended the laboratory for one session. After obtaining written consent, anthropometric measurements of height and weight were recorded. A finger prick capillary blood sample was collected into three 300 µL EDTA coated microvette tubes for blood cell count and DNA extraction. Four dried blood spots (DBS) were collected for fatty acid analysis.

2.3 | Fatty acid analysis

DBS were collected on Whatman cards (903 Protein saver, Sigma-Aldrich) pre-treated with butylated hydroxytoluene to stabilise the blood fatty acids for up to eight weeks at room temperature (Metherel et al., 2013). A 30 mm^2 circle from each DBS was punched from the card, weighed and frozen at −80°C. Gas chromatography mass-spectrometry (GC-MS) was used to analyse the fatty acid composition of the DBS. Briefly, the 30 mm^2 punched samples were derivatised using acetyl chloride and methanol (70°C for 60 minutes) to produce fatty acid methyl esters (FAMEs) and reconstituted in hexane containing 10 µg/ml of internal standard (heptadecanoic acid methyl ester) prior to GC-MS analysis. FAMEs were characterised by electron ionization (EI) in positive ion full scan mode and their identities confirmed both by their fragmentation pattern matching to the NIST-10 mass spectral library and retention time matching to a Supelco 37-component FAMES certified reference standard. Fatty acid quantities were determined based on their relative abundance to the internal standard. Fatty acids are presented as percentage of each fatty acid relative to the total fatty acid within each sample (%TFA).

2.4 | DNA extraction and bisulfite conversion

One aliquot of the capillary blood was immediately used for cell counting on a Beckman coulter counter with further aliquots frozen at −80°C for later DNA analysis. DNA was extracted from the frozen capillary blood and bisulfite converted using EpiTect Fast LyseAll Bisulfite conversion kit (Qiagen) using the manufacturer's protocol. Briefly, the blood sample was lysed, and proteins denatured, the resulting pellet was resuspended in PBS and added directly into the bisulfite reaction. The DNA was sodium bisulfite treated and subjected to two cycles of denaturing at 95°C for 5 minutes and incubation at 60°C for 20 minutes. Bisulfite converted DNA was desulphonated and purified using MinElute spin columns. Successful bisulfite

conversion was assessed using a bisulfite converted DNA specific dispensation during the pyrosequencing, as displayed on a pyrogram in Figure 2 highlighted in orange.

2.5 | Pyrosequencing

The methylation of four Cytosine-Guanine dinucleotides (CpG) sites (+197, +202, +214 and +222 base pairs from the transcription start site) within Exon 1 of the *TNF* were measured using pyrosequencing (PyroMark Q48 Autoprep System, Qiagen). The extracted bisulfite converted DNA was amplified using the PyroMark PCR kit (Qiagen) in a Veriti thermocycler (Applied Biosystems) using primers (Forward PCR Primer 5′-GGAAAGGATATTATGAGTAT TGAAAGTATG-3′; Reverse PCR Primer 5′-biotin-CTAA AACCCCCCTATCTTCTTAAA-3′ and Sequencing Primer 5′-ATTATGAGTATTGAAAGTATGAT-3′).

PCR product was used for CpG quantification with the PyroMark Q48 Autoprep (Qiagen) using Advanced CpG Reagents (Qiagen) in accordance with the manufacturer's protocol. The assay covered the methylation sites +197, +202, +214 and +222 base pairs from the transcription start site of *TNF*. In this manuscript, these sites are designated as CpG1 to CpG4 for data analysis and inferences. The percentage methylation of the four CpG sites was calculated within the software (PyroMark Q48 Autoprep 2.4.1 Software, Qiagen) and the methylation percentages were exported for further analysis.

2.6 | Statistical analysis

All statistical analysis was performed using IBM SPSS Statistics software (SPSS version 23). The data distribution was assessed for normality by Shapiro–Wilk's test ($p > .05$). Group differences between gender were assessed using an independent sample *t*-test or a Mann–Whitney-U test depending on normal distribution of the data. Differences between gender for categorical data were assessed using chi-square.

A key consideration in DNA methylation studies is the composition of the white blood cells (WBC) from which the DNA is extracted. The DNA methylation values are, therefore, presented as both raw and adjusted for the cell heterogeneity of the capillary blood. The method presented by Jones et al., (2015) was used to adjust DNA methylation values to account for the white blood cell composition. The adjusted figure is a sum of the mean methylation for the site and the unstandardized residual from a linear regression between DNA methylation (dependant variable) and the individual white blood cell counts (independent variables).

Spearman's correlation analysis was used to assess the relationship between the methylation (raw and adjusted) percentage for the four sites studied and the fatty acids identified within the blood. A probability value lower than 0.05 was considered significant.

3 | RESULTS

3.1 | Sample demographic

Eighty-eight adults (Male n = 30 and Female n = 58) were recruited, details of the sample demographic are shown in Table 1. The age ranged from 18 to 74 years and there was a positive skew towards younger individuals with only four individuals above the age of 60 years. A small number of individuals were current smokers (n=7), and almost a third (n = 24) had considered themselves smokers at some

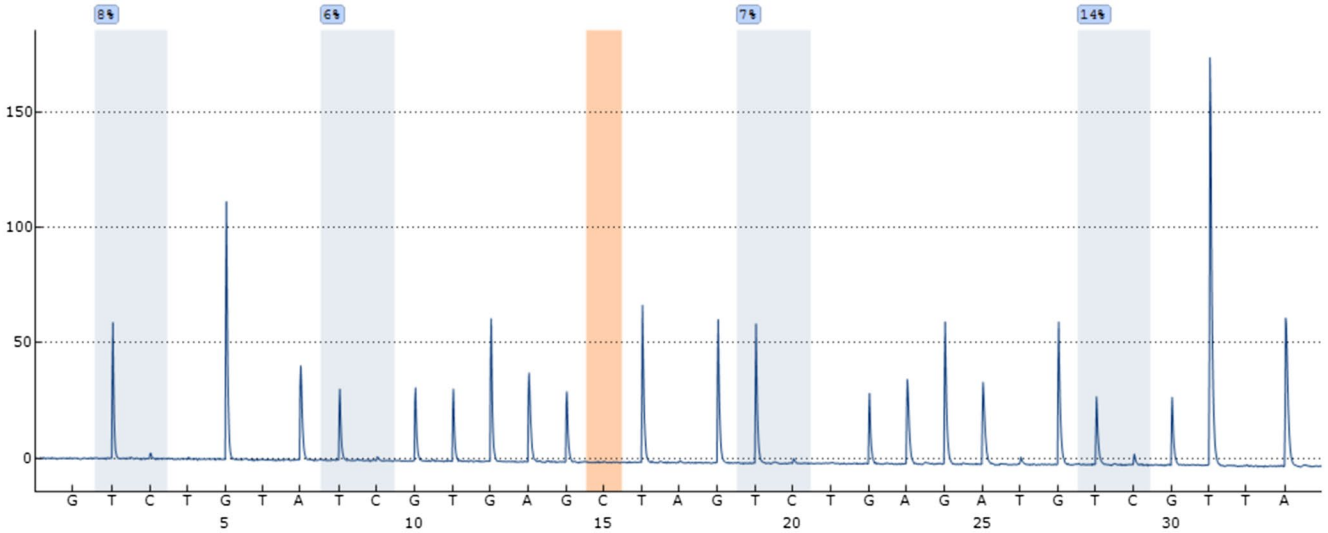


FIGURE 2 Example pyrogram for TNF exon 1 assay covering Cytosine-Guanine dinucleotides (CpG) sites +197, +202, +214 and +222 from the transcription start site. Dispensation 15 served as a bisulfite conversion control

point during their life. Height and weight differed between the genders, with male participants significantly taller and heavier than the female participants. In addition, a significant difference in total white blood cell count and neutrophil cell count was observed between males and females, therefore adjustment of DNA methylation values for cell composition was calculated both as a whole sample and separately split by gender.

3.2 | DNA methylation of TNF

The average methylation of the four sites studied was 8.71%. DNA methylation was not significantly different between males and females (Table 2) and this remained non-significant after adjustment for white blood cell composition (data not shown) despite the significant difference in white blood cell count between the genders. No associations were observed between *TNF* methylation of the four sites and BMI ($p > 0.443$).

3.3 | Fatty acid profile of peripheral blood

A total of 25 fatty acids were identified from the DBS, presented in Table 3 as a percentage of total fatty acid identified. The omega-3 polyunsaturated (ω-3 PUFA) Docosapentaenoic acid (DPA) was significantly different between genders ($p < 0.05$); all other fatty acids identified did not show any difference between the genders (Table 3). A ratio of 3.04 was

TABLE 1 Demographic characteristics of the sample population

	All participants (n=88)	Males (n = 30)	Females (n = 58)	*p*
Age (years)[a]	25 (22–41)	30 (23–47)	24 (21–39)	0.210
Height (cm)[b,d]	170.3 ± 8.7	177.3 ± 6.7	166.6 ± 7.2	**<0.001**
Weight (kg)[a,d]	70.2 (59.9–80.1)	80.6 (75.5–88.9)	63.3 (56.6–73.0)	**<0.001**
BMI[a,d]	23.9 (21.9–26.5)	24.9 (22.7–27.3)	22.9 (21.2–26.0)	0.071
Current smoker (n, %)[c]	7 (8.0%)	4 (13.3%)	3 (5.2%)	0.180
Ever Smoked (n, %)[c]	24 (27.3%)	10 (33.3%)	14 (24.1%)	0.359
Total white blood cells (10^9/L)[a,e]	6.69 ± 1.40	6.19 ± 1.12	6.94 ± 1.47	**0.018**
Neutrophils (10^9/L)[a,e]	3.95 ± 1.09	3.61 ± 0.90	4.13 ± 1.15	**0.039**
Lymphocyte (10^9/L)[a,e]	1.99 ± 0.55	1.87 ± 0.33	2.06 ± 0.63	0.132
Monocytes (10^9/L)[a,e]	0.48 ± 0.12	0.47 ± 0.11	0.48 ± 0.12	0.928
Eosinophils (10^9/L)[a,e]	0.18 ± 0.11	0.15 ± 0.07	0.19 ± 0.12	0.115
Basophils (10^9/L)[a,e]	0.06 ± 0.02	0.06 ± 0.02	0.06 ± 0.02	0.808
Large immature cells (10^9/L)[a,e]	0.04 ± 0.03	0.03 ± 0.02	0.04 ± 0.03	0.181

Bold values are statistically significant.

[a]Data were non-normally distributed, and data presented as median (lower quartile-upper quartile); differences between groups assessed by Man–Whitney-U difference test.

[b]Data were normally distributed and presented as mean ± standard deviation; differences between groups assessed by independent samples *t*-test.

[c]Categorical data, differences between groups assessed by Pearson chi-square.

[d]Height and weight data missing for two female participants, (total n=86, female n=56).

[e]Blood cell count data were not available for 3 participants (total n=85, males n=29, females n=56).

TABLE 2 DNA methylation percentages for four Cytosine-Guanine dinucleotides (CpG) sites (+197, +202, +214 and +222 base pairs from Transcription Start Site) located within exon 1 of the TNF gene (encoding cytokine Tumour Necrosis Factor alpha, TNFα)

DNA methylation (%)	All (n = 88)	Males (n = 30)	Females (n = 58)	*p*
CpG 1 (+197)	8.79 ± 2.28	8.56 ± 2.04	8.90 ± 2.40	0.511
CpG 2 (+202)	6.49 ± 1.97	6.32 ± 2.06	6.58 ± 1.94	0.568
CpG 3 (+214)	7.27 ± 2.07	7.15 ± 2.30	7.34 ± 1.96	0.679
CpG 4 (+222)	12.29 ± 2.93	12.01 ± 3.15	12.44 ± 2.82	0.520
CpG average	8.71 ± 2.14	8.51 ± 2.25	8.81 ± 2.10	0.532

Note: Data were normally distributed and presented as percentage methylation (%) mean ±standard deviation; differences between groups assessed by independent samples *t*-test.

Abbreviations: CpG, Cytosine-Guanine dinucleotides.

observed for ω-6 to ω-3 PUFAs. There was no significant difference in fatty acid ratios between the genders (Table 4).

3.4 | Relationship between blood fatty acid and TNF DNA methylation

The relationship between capillary blood fatty acid levels and DNA methylation of *TNF* at the four sites studied in exon 1 appeared to be highly dependent upon the gender of the cohort. Consequently, examining the results of the cohort as a whole could lead to misleading conclusions, and hence the following analysis considers the results according to gender. We also found that the level of correlation varied with adjustments for white blood cell composition (Figure 3).

In males, the ω-3 PUFA Docosahexaenoic Acid (DHA) was positively correlated with CpG methylation of *TNF* at all the CpG sites studied ($r > 0.38$, $p < 0.041$). Post-adjustment

TABLE 3 Fatty acid profile of dried blood spots

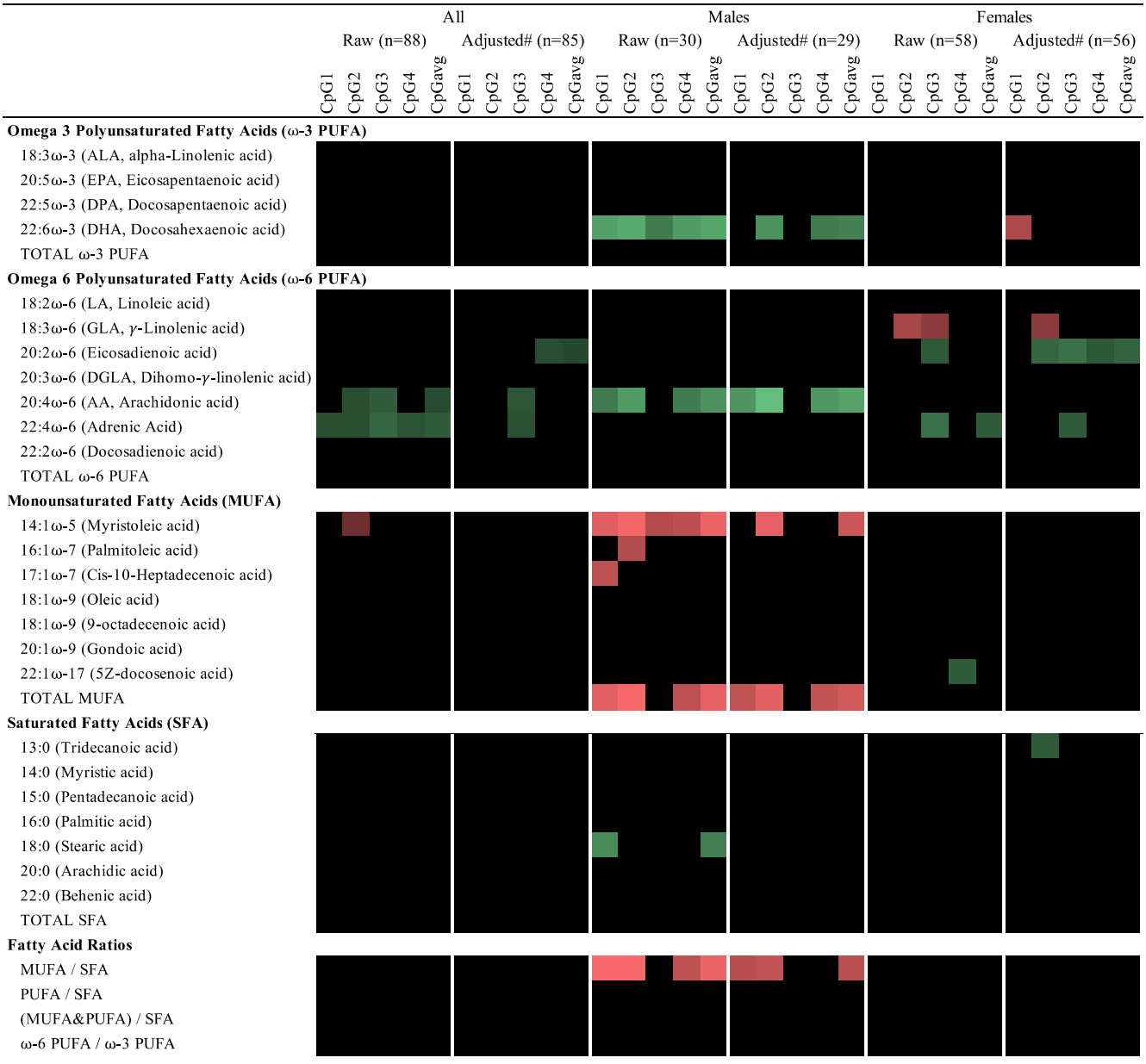
	All	Males	Females	
	(n = 88)	(n = 30)	(n = 58)	*p*
Omega 3 polyunsaturated fatty acids (ω−3 PUFA)				
18:3ω−3 (ALA, alpha-Linolenic acid)	5.31 ± 1.10	5.31 ± 1.13	5.32 ± 1.09	0.975
20:5ω−3 (EPA, Eicosapentaenoic acid)	0.56 ± 0.68	0.47 ± 0.22	0.60 ± 0.82	0.399
22:5ω−3 (DPA, Docosapentaenoic acid)	0.58 ± 0.16	0.63 ± 0.14	0.56 ± 0.16	**0.050**
22:6ω−3 (DHA, Docosahexaenoic acid)	1.25 ± 0.48	1.22 ± 0.47	1.27 ± 0.49	0.643
Total ω−3 PUFA	7.71 ± 1.51	7.63 ± 1.36	7.75 ± 1.59	0.728
Omega 6 polyunsaturated fatty acids (ω−6 PUFA)				
18:2ω−6 (LA, Linoleic acid)	13.81 ± 1.62	13.69 ± 1.62	13.86 ± 1.62	0.641
18:3ω−6 (GLA, γ-Linolenic acid)	1.15 ± 0.44	1.09 ± 0.42	1.18 ± 0.46	0.328
20:3ω−6 (DGLA, Dihomo-γ-linolenic acid)	0.80 ± 0.24	0.79 ± 0.24	0.80 ± 0.25	0.850
20:4ω−6 (AA, Arachidonic acid)	6.39 ± 1.42	6.54 ± 1.49	6.32 ± 1.39	0.491
22:4ω−6 (Adrenic Acid)	0.41 ± 0.12	0.43 ± 0.13	0.40 ± 0.12	0.193
20:2ω−6 (Eicosadienoic acid)	0.14 ± 0.06	0.14 ± 0.06	0.13 ± 0.05	0.409
22:2ω−6 (Docosadienoic acid)	0.10 ± 0.03	0.10 ± 0.03	0.10 ± 0.03	0.977
Total ω−6 PUFA	22.79 ± 2.67	22.79 ± 2.79	22.80 ± 2.64	0.986
Monounsaturated fatty acids (MUFA)				
14:1ω−5 (Myristoleic acid)	14.38 ± 2.14	14.06 ± 2.42	14.54 ± 1.98	0.319
16:1ω−7 (Palmitoleic acid)	1.08 ± 0.27	1.05 ± 0.23	1.10 ± 0.30	0.427
17:1ω−7 (Cis−10-Heptadecenoicacid)	2.44 ± 0.72	2.33 ± 0.86	2.49 ± 0.64	0.326
22:1ω−17 (5Z-docosenoic acid)	0.61 ± 0.13	0.60 ± 0.12	0.61 ± 0.14	0.687
18:1ω−9 (Oleic acid)	2.84 ± 0.97	2.87 ± 0.99	2.82 ± 0.96	0.812
18:1ω−9 (9-octadecenoic acid)	0.74 ± 0.38	0.71 ± 0.36	0.76 ± 0.39	0.559
20:1ω−9 (Gondoic acid)	0.38 ± 0.10	0.38 ± 0.09	0.39 ± 0.11	0.663
Total MUFA	22.47 ± 3.21	22.00 ± 3.53	22.71 ± 3.04	0.327
Saturated fatty acids (SFA)				
13:0 (Tridecanoic acid)	0.08 ± 0.04	0.08 ± 0.04	0.08 ± 0.04	0.447
14:0 (Myristic acid)	3.13 ± 1.19	3.25 ± 1.22	3.08 ± 1.18	0.528
15:0 (Pentadecanoic acid)	0.89 ± 0.27	0.95 ± 0.33	0.86 ± 0.22	0.146
16:0 (Palmitic acid)	22.82 ± 2.13	23.01 ± 2.00	22.72 ± 2.21	0.540
18:0 (Stearic acid)	19.20 ± 1.36	19.44 ± 1.36	19.07 ± 1.35	0.235
20:0 (Arachidic acid)	0.49 ± 0.30	0.45 ± 0.28	0.51 ± 0.32	0.391
22:0 (Behenic acid)	0.43 ± 0.12	0.41 ± 0.13	0.44 ± 0.11	0.410
Total SFA	47.03 ± 2.83	47.59 ± 2.70	46.75 ± 2.88	0.188

Note: Bold values are statistically significant.

Data were presented as percentage total fatty acids (%TFA), mean ±standard deviation; differences between groups assessed by independent samples *t*-test.

TABLE 4 Fatty acid ratios from dried blood spots

Fatty acid ratios	All (n = 88)	Males (n = 30)	Females (n = 58)	*p*
MUFA/SFA	0.48 ± 0.08	0.47 ± 0.09	0.49 ± 0.08	0.199
PUFA/SFA	0.65 ± 0.10	0.64 ± 0.08	0.66 ± 0.10	0.482
(MUFA+PUFA)/SFA	1.13 ± 0.12	1.11 ± 0.12	1.15 ± 0.13	0.159
ω−6 PUFA/ω−3 PUFA	3.04 ± 0.50	3.06 ± 0.56	3.02 ± 0.48	0.740



Significant ($p<0.05$) Spearman's correlation coefficients between the four cytosine-guanine dinucleotide (CpG) sites (+197, +202, +214 and +222 base pairs from Transcription Start Site) of *TNF* gene and fatty acids detected in blood; average methylation of the four sites (CpGavg). See key for correlation scale. # CpG methylation percentage adjusted for white blood cell population.

FIGURE 3 Spearman correlation coefficients heat map between the DNA methylation of four Cytosine-Guanine dinucleotides (CpG) sites located in exon 1 of the Tumor Necrosis Factor (TNF) gene and dried blood spot fatty acid levels measured as a percentage of total fatty acids

the correlation between methylation and DHA remained significant for CpG2 ($r = 0.44$, $p = 0.016$) and CpG4 ($r = 0.47$, $p = 0.045$) but was no longer significant at CpG1 or CPG3 (Figure 3). The ω-6 PUFA Arachidonic Acid (AA) was also found to be positively correlated ($r > 0.33$, $p < 0.043$, CpG2, $r = 0.47$, $p = 0.009$) with methylation at all sites except CpG3, with stronger correlations observed post-adjustment (CpG2, $r = 0.57$, $p = 0.001$). The results for the male cohort also indicated positive correlation of methylation at CpG1 ($r = 0.42$, $p = 0.020$) and CpG_{avg} ($r = 0.39$, $p = 0.036$) with SFA Stearic Acid. This correlation was no longer significant post adjustment (CpG1, $r = 0.32$, $p = 0.092$). The male cohort had a negative correlation between the ω-5 MUFA myristoleic acid and methylation across all sites pre-adjustment ($r < -0.38$, $p < 0.047$), correlation only remained with CpG2 ($r = -0.47$, $p = 0.011$) and CpG_{avg} ($r = -0.42$, $p = 0.024$) post-adjustment (Figure 3).

No correlations were observed between ω-3 PUFA's and CpG methylation pre-adjustment among females. Post-adjustment a negative correlation between ω-3 PUFA DHA and methylation of CpG1 was observed ($r = -0.35$, $p = 0.008$) (Figure 3). A negative correlation was also observed in respect of the ω-6 PUFA γ-Linolenic acid (GLA) with methylation at CpG2 ($r = -0.34$, $p = 0.010$) and CpG3 ($r = -0.29$, $p = 0.030$) pre-adjustment. Post adjustment the correlation only remained at CpG2 ($r = -0.28$, $p = 0.036$). In contrast the ω-6 PUFAs adrenic acid ($r = 0.34$, $p = 0.008$) and eicosadienoic acid ($r = 0.27$, $p = 0.039$) were positively correlated with methylation of CpG3 pre-adjustment. Post-adjustment these correlations remained significant, and in addition eicosadienoic acid was also found to be positively correlated with methylation at CpG2 ($r = 0.31$, $p = 0.020$) and CpG4 ($r = 0.27$, $p = 0.048$). Additional positive correlations were observed for the MUFA 5Z-docosenoic acid with methylation of CpG4 ($r = 0.28$, $p = 0.035$) pre-adjustment and for the SFA tridecanoic acid with methylation of CpG2 ($r = 0.27$, $p = 0.041$) post-adjustment.

4 | DISCUSSION

The data we report here demonstrates that there are significant relationships between specific blood fatty acids and DNA methylation of four CpG sites in exon 1 of the cytokine encoding gene *TNF*. Importantly, the amplitude and direction of associations differed according to gender.

The CpG sites investigated have previously been associated with TNFα mRNA and circulating TNFα (Hermsdorff et al., 2013; Marques-Rocha et al., 2016). The average methylation levels observed in the current study (8.7%) are comparable to those previously observed (Hermsdorff et al., 2013), with a similar profile where the fourth CpG site had slightly higher methylation (12.3%). Multiple sites within the *TNF* gene have been shown to be associated with environmental stimuli and biomarkers for phenotypes (Campión et al., 2009; Cordero et al., 2011; El Gazzar et al., 2008; Gowers et al., 2011; Nimmo et al., 2012; Pieper et al., 2008). Additionally, the methylation of these sites has been shown to be a potential biomarker of the adiposity status of an individual (Marques-Rocha et al., 2016). Although gene expression was not measured in the current study, we can hypothesise that the fatty acids with a positive relationship with methylation at these sites will have differential gene expression. This may provide an anti-inflammatory effect through reduced TNFα cytokine expression; and vice versa for those with a negative relationship.

We found that ω-6 PUFA, AA was positively correlated with *TNF* methylation in males, but not females, however, its elongated form adrenic acid was correlated in females. This is an interesting finding given that these two fatty acids only differ in length by two carbon atoms. In addition, we found a positive relationship at all sites between ω-3 PUFA DHA and *TNF* methylation also only in males. By contrast, females did not show any significant association with DHA until adjusted for the WBC population, when CpG1 showed a significant negative correlation. The differences between the associations observed may be the result of the efficiency in which males and females metabolise fatty acids (Lohner et al., 2013). An alternative explanation could involve the metabolism of fatty acids to eicosanoids, which have also been shown to differ between males and females (Pace et al., 2017). It is also important to consider that the gender differences could be the result of differential regulation of *TNF* between males and females; previous research into *TNF* promotor methylation, in a weight loss response study, also demonstrated gender-specific results (Campión et al., 2009).

One of the ways in which fatty acids have been found to modulate inflammation is altered cytokine expression (Calder, 2003, 2012b; Rangel-Huerta et al., 2012). ω-3 PUFA supplementation and *in vitro* studies reveal decreased production of TNFα in LPS-stimulated cells when treated with DHA (Weldon et al., 2007). Despite ω-6 PUFAs and their inflammatory mediators being predominantly pro-inflammatory (Calder, 2006), AA-derived eicosanoid 2-series prostaglandins also have a potent anti-inflammatory effect through the inhibition of TNFα production (Miles et al., 2002). As increased methylation of exon 1 is associated with lower levels of TNFα mRNA (Hermsdorff et al., 2013); our research suggests that higher levels of ω-6 PUFAs AA and adrenic acid, as well as the ω-3 PUFA DHA, result in higher methylation of *TNF*. Alterations to the methylation profiles of *TNF* could therefore be one mechanism through which specific fatty acids interact with inflammation.

Previous work carried out on the same CpG sites found an association with ω-6 PUFA intake, but in the opposite direction, we observed (Hermsdorff et al., 2013). It is possible that this discrepancy arises from the different methods employed for the measurement of the ω-6 PUFA's. Hermsdorff *et al* presented ω-6 dietary intake, rather than the biochemical measurement of blood fatty acids used here. Multiple factors impact upon the fats within blood, including the proportion of other dietary fats and components (Katan et al., 1994), endogenous synthesis of fatty acids and the individual's genetic makeup (Porenta et al., 2013). These factors and the inherent disadvantages of food frequency questionnaires (Prentice et al., 2011) compared to biochemical measurements may explain the differences in results.

In comparison to the ω-6 PUFA relationship we observed, the MUFA myristoleic acid was found to have a weak negative correlation with *TNF* methylation at CpG2. Males had a significant negative association between *TNF* methylation and myristoleic acid, as well as total MUFA; no relationship was observed in the female cohort. MUFAs are generally considered the healthier option to SFAs (Schwingshackl & Hoffmann, 2014), however, we observed a negative association between MUFA/SFA ratio and DNA methylation. No other fatty acid ratios showed significant relationships. The differing effects of ω-6 PUFAs and MUFAs on the methylome has been shown in an *in vitro* monocyte model (Silva-Martínez et al., 2016). AA had a trend towards global hypermethylation and the MUFA Oleic (OA) had a (weaker) hypomethylating effect.

The four CpG sites are located close to NFKB transcription binding sites and therefore alterations in their methylation may impact gene transcription. However, it is unclear if the absolute percentage differences in methylation levels of *TNF* observed in this data set are substantial enough to be biologically significant, and this warrants further investigation.

There are methodological considerations for DNA sources used within epigenetic research due to the specificity of the epigenetic makeup of each individual cell. For example, there is a greater difference in methylation between tissues from the same individual than there are from the same tissue from multiple individuals (Byun et al., 2009). In this research we used blood, which is a tissue heterogenous in cell composition. Nevertheless, there are benefits in using blood as a source of DNA, primarily it contains WBC which play key roles within the inflammatory process. To combat the heterogeneity of the cells and to minimise WBC composition as a confounding factor, the methylation results have been presented both as raw and as adjusted for WBC composition using a regression-based method (Jones et al., 2015). There was some change in the relationships observed between the fatty acids and *TNF* methylation once adjusted for WBCs, therefore the data for pre and post-adjustment is presented.

Unlike the wide range of PUFA status that the Yup'ik study utilised, our cohort displayed a small range in ω-3 PUFAs. Additionally, the ω-6/ω-3 PUFA ratio was low in comparison to that expected for a Western population (Simopoulos, 2016). Future research should investigate whether interventions could alter the methylation profile of the *TNF* gene to one that is associated with a healthier phenotype. For example, could alter the fatty acid composition of blood through dietary supplementation increase the methylation percentage of *TNF* to give a protective effect against prolonged inflammation, a characteristic of obesity and chronic disease?

In conclusion, we have shown that the levels of specific blood fatty acids are associated with the epigenetic signatures of *TNF* exon 1 and, therefore, have the potential to alter the regulation of TNFα expression in WBCs. Although not all methylation levels were associated with the fatty acids measured, some positive and some negative associations were observed. Further research into how fatty acids interact with the epigenetic control of cytokines may provide a therapeutic avenue for treatment of chronic inflammation and while cell culture and animal models play an important role limitations inherent in these models suggest that metabolic, genomic and epigenomic data from human clinical trials should be the focus when setting dietary recommendations for fats and fatty acids (Fritsche, 2015). Additionally, our results suggest that subsequent research must include the analysis of results separately between genders in order to not miss important findings that may be gender specific.

ETHICS APPROVAL

Ethical clearance was granted from the Loughborough University Ethics Approvals (Human Participants) Sub-Committee (R16-P074).

CONSENT TO PARTICIPATE

All participants provided written informed consent.

CONSENT FOR PUBLICATION

All participants agreed their data to be included in the publications.

CONFLICT OF INTEREST

The authors have declared no conflict of interest.

AUTHORS' CONTRIBUTIONS

All authors have directly contributed to the design, collection of data, analysis and writeup of this paper.

DATA AVAILABILITY STATEMENT

Collated data used in tables and figures available on request.

ORCID

Bethan Hussey http://orcid.org/0000-0002-1827-5489
Richard P. Steel https://orcid.org/0000-0001-5633-3259
James C. Reynolds https://orcid.org/0000-0003-1176-3030
Ian M. Taylor https://orcid.org/0000-0001-6291-4025
Martin R. Lindley https://orcid.org/0000-0001-7686-9421
Sarabjit Mastana https://orcid.org/0000-0002-9553-4886

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