

1 **Plasma metabolites related to the consumption of different types of dairy products and**
2 **their association with new-onset type 2 diabetes: analyses in the Fenland and EPIC-**
3 **Norfolk Studies, United Kingdom**

4 *Short title: Dairy, metabolites and type 2 diabetes incidence*

5 Eirini Trichia¹, Albert Koulman¹, Isobel D. Stewart¹, Soren Brage¹, Simon J. Griffin¹, Julian
6 L. Griffin², Kay-Tee Khaw¹, Claudia Langenberg¹, Nicholas J. Wareham¹, Fumiaki Imamura^{1*},
7 Nita G. Forouhi^{1*}

8 **Equal contribution*

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10 ¹MRC Epidemiology Unit, Institute of Metabolic Science, University of Cambridge School
11 of Clinical Medicine, Cambridge, United Kingdom

12 ²Department of Biochemistry, University of Cambridge, Cambridge, United Kingdom

13 **Correspondence:**

14 Professor Nita G Forouhi and Dr Fumiaki Imamura
15 Medical Research Council Epidemiology Unit
16 University of Cambridge School of Clinical Medicine
17 Box 285 Institute of Metabolic Science
18 Cambridge Biomedical Campus
19 CB20QQ, Cambridge, UK

20 Fax: +44(0)12233 30316

21 Telephone: +44(0) 1223 30315

22 Email: nita.forouhi@mrc-epid.cam.ac.uk , fumiaki.imamura@mrc-epid.cam.ac.uk

23 **Keywords:** dairy, diabetes, metabolites, metabolomics, biomarkers

24 List of abbreviations: AUC: Area under the curve; CI: Confidence interval; HR: Hazard ratio;
25 NRI: Net reclassification improvement; OCSFA: Odd-chain saturated fatty acids; T2D: Type
26 2 diabetes
27

28

29 **Abstract**

30 Scope: To identify metabolites associated with the habitual consumption of dairy products
31 and investigate their associations with type 2 diabetes (T2D) risk.

32 Methods and results: Metabolomics assays were conducted in the Fenland (n=10,281) and
33 EPIC-Norfolk (n=1,440) studies. Using 82 metabolites present in both datasets we developed
34 metabolite scores to classify self-reported consumption of milk, yogurt, cheese, butter, and
35 total dairy in a subset (Fenland Study; n=6,035). We evaluated internal and external validity
36 of the scores in subsets (Fenland, n=4,246; EPIC-Norfolk, n=1,440). We assessed
37 associations between each metabolite score and T2D incidence in EPIC-Norfolk case-cohort
38 (n=641 T2D cases; 16,350 person-years). The scores classified low and high consumers for
39 all dairy types with internal validity, and milk, butter, and total dairy with external validity.
40 Metabolite scores for dairy types with external validity were associated with lower incident
41 T2D: hazard ratios (95% confidence interval) per SD: milk 0.71 (0.65, 0.77); butter 0.62
42 (0.57, 0.68); total dairy 0.66 (0.60, 0.72). These associations persisted after adjustment for
43 known dairy-fat biomarkers.

44 Conclusion: Metabolite scores identified habitual consumers of milk, butter and total dairy
45 products, and were associated with lower T2D risk. The current findings hold promise for
46 identifying objective indicators of the physiological response to dairy consumption.

47

48 **Introduction**

49 Meta-analyses of prospective cohort studies have reported diverse associations between the
50 consumption of dairy products and type 2 diabetes (T2D): inverse associations for yogurt^[1]
51 and butter,^[2] generally neutral associations for milk (total and full-fat) and high-fat dairy
52 products and inconsistent, null or inverse associations for other dairy types e.g. low-fat milk,
53 low-fat dairy products and cheese.^[1] The objective measurement and use of specific
54 biomarkers of different types of dairy consumption could offer a complementary approach to
55 subjective dietary assessment to help further elucidate the link between dairy products and
56 health outcomes.

57 Biomarkers for the consumption of total and full-fat dairy products have previously been
58 identified to include odd-chain saturated fatty acids (OCSFAs: pentadecanoate, C15:0; and
59 heptadecanoate, C17:0), and *trans*-palmitoleate (C16:1n7t).^[3] The use of metabolomics to
60 identify metabolites associated with dairy consumption might further contribute to the
61 objective assessment of the physiological response to dairy consumption. A few studies have
62 aimed to identify novel candidate dairy biomarkers using metabolomics,^[4, 5] but limited
63 evidence to date has precluded their establishment as dairy biomarkers. Issues include the
64 lack of specificity of the identified biomarkers to individual dairy types,^[6, 7] limited
65 generalisability to a general population because of inclusion of patient populations^[8] or those
66 with high dairy consumption,^[7] and use of diverse biological samples and populations
67 limiting comparability of results.^[5-8]

68 We hypothesized that a combination of selected metabolites could classify response to low
69 and high consumption of different dairy types and be associated with T2D risk adding to the
70 evidence from studies on self-reported consumption and fatty acid biomarkers. We address
71 these hypotheses by evaluating plasma metabolomics profiles in two independent,

72 geographically-similar, population-based cohorts in the United Kingdom. We specifically
73 aimed to develop and assess the validity of metabolite scores that could identify consumers of
74 milk, yogurt, cheese, butter, and total dairy. We also evaluated utility of each metabolite
75 score as a biomarker in comparison to the known fatty acid biomarkers.

76 **Experimental section**

77 *Study design and populations*

78 This study included two populations (**Figure 1**): the Fenland Study and the European
79 Prospective Investigation into Cancer and Nutrition, Norfolk (EPIC-Norfolk) case-cohort
80 study. Briefly, we evaluated participants in the Fenland Study to derive metabolite scores
81 from metabolomics so that scores could classify between low and high consumption of each
82 dairy type, to assess internal validity of each score, and to assess their utility additional to the
83 dairy fat biomarkers. External validity of the metabolite scores was assessed similarly in
84 EPIC-Norfolk with and without the dairy fat biomarkers accounted for. In EPIC-Norfolk we
85 further assessed associations of the metabolite scores with incident T2D.

86 The Fenland Study is a cohort study, which started in 2005 and recruited 12,435 adults born
87 between 1950 and 1975. Recruitment via general practices and baseline assessment were
88 carried out in Cambridge, Ely and Wisbech, Cambridgeshire, United Kingdom.^[9, 10] The
89 study was approved by the Cambridge Local Ethics Committee. All participants provided
90 written informed consent. The current sample included 10,281 participants after excluding
91 participants with no metabolomics data (n=1,751) or more than 50% of the metabolites
92 missing (n=186), pregnant women (n=3), or participants recording no dietary data (n=14), or
93 implausible energy- intake (<800 or >4,000 kcal/day among men; <500 or >3,500 kcal/day
94 among women; n=200). The remaining participants were divided into a derivation set
95 (n=6,035) and a validation set (n=4,246). We selected the derivation set as the set without

96 data on plasma phospholipid fatty acids (details in the section below on fatty acid
97 measurements). The validation set comprised cohort subset randomly selected for plasma
98 phospholipid fatty acid assays that provided data on OCSFAs (C15:0, C17:0), and C16:1n7t.
99 We evaluated participants in the validation set to assess the utility of the metabolite scores
100 additional to the fatty acids.

101 The EPIC-Norfolk Study (DOI:10.22025/2019.10.105.00004) is a cohort study with baseline
102 measurements between 1993 and 1997 among 25,639 adults recruited through general
103 practices to attend clinical and dietary assessments in Norfolk, United Kingdom.^[11] All
104 participants provided written informed consent and the study was approved by the Norwich
105 Local Ethics Committee (REC Ref: 98CN01). In the current research we evaluated data from
106 a T2D case-cohort study nested within the EPIC-Norfolk Study, with 1,503 participants (673
107 incident T2D cases) to examine biomarker-T2D associations. After exclusion of participants
108 with no dietary data (n=44), implausible energy intake (as described above; n=17), or more
109 than 50% of the metabolites missing (n=2), the current analysis included 1,440 participants
110 (641 incident T2D cases). In the analysis involving fatty acids, we further excluded
111 participants with no fatty acid measurements (n=848) leaving 592 participants (356 incident
112 T2D cases).

113 *Metabolite and fatty acid measurement*

114 In the Fenland Study, targeted metabolomics profiling of fasting blood plasma samples was
115 performed with a commercial kit (Absolute IDQ p180 kit, BIOCRATES Life Sciences AG,
116 Innsbruck, Austria).^[12, 13] Lipids and acylcarnitines were measured using flow injection
117 analysis MS (AB SCIEX 5500 Qtrap mass spectrometer, Sciex Ltd, Warrington, UK) in
118 positive ionization mode and hexose in negative ionization mode. This method provided
119 relative concentrations. Amino acids and biogenic amines were measured with UPLC-MS

120 [ultra-performance LC (Acquity UPLC, Waters Ltd, Manchester, UK), coupled to the same
121 spectrometer as above]. With the UPLC-MS analysis internal standards and calibration lines
122 were used to provide absolute concentrations. A total of 187 metabolites were measured.
123 After quality control checks, 13 metabolites were excluded, leaving 174 metabolites, which
124 consisted of amino acids (n=22), biogenic amines (n=12), acylcarnitines (n=40),
125 phosphatidylcholines (PCs;n=74), lysophosphatidylcholines (LPCs;n=14), sphingomyelins
126 (SMs;n=11) and hexose.

127 In the EPIC-Norfolk Study, untargeted metabolomics profiling was performed in non-fasting
128 plasma samples collected at baseline from 1,503 participants in the T2D case-cohort study
129 nested within the EPIC Norfolk Study (DiscoveryHD4[®] platform, Metabolon, Inc.).^[12, 13]
130 After quality control procedures, 940 metabolites were assessed, of which 602 were
131 annotated; relative concentrations were measured for all metabolite signals.

132 Of the two different metabolomics platforms used in the Fenland Study and the EPIC Norfolk
133 Study, we identified 82 metabolites present in both datasets (Supplemental Methods) and re-
134 annotated them in concordance with Biochemical Nomenclature^[14] (Supplemental Methods,
135 Supplemental Table 1).

136 *Fatty acids assays*

137 Fatty acids of the plasma phospholipid fraction were measured in 4,791 participants in the
138 Fenland Study and in 592 participants in the EPIC-Norfolk T2D case-cohort study using GC
139 as previously described (Supplementary Methods).^[15] The fatty acid measurements were
140 expressed as relative concentrations (mol%). Relative concentrations of C15:0, C17:0 and
141 C16:1n7t from the Fenland Study and C15:0 and C17:0 from the EPIC-Norfolk Study
142 (C16:1n7t not available) were evaluated as covariates when developing metabolite scores

143 from metabolites and also combined with metabolites to develop a score based on both
144 metabolites and the fatty acids.

145 *Dietary assessment*

146 In both studies, diet was assessed with a 130-item semi-quantitative food frequency
147 questionnaire to estimate habitual dietary intakes over the past year. Participants were asked
148 to choose one of the nine frequencies of dairy consumption ranging from “never or less than
149 once/month” to “6 times per day” and provide more details on the type and amount of milk
150 consumed. Questionnaire data were processed using in-house software.^[16] The questionnaire
151 validity was assessed against 7-day food diaries in the EPIC-Norfolk Study.^[17] Correlations
152 between estimates from the questionnaires and the 7-day diaries were 0.56 for milk, 0.57 for
153 yogurt, 0.33 for cheese, and 0.54 for butter.

154 *Diabetes case ascertainment*

155 Cases of incident T2D in the EPIC-Norfolk Study occurring until 31 July 2006 were
156 ascertained using a combination of information from self-report and independent health
157 records. Within the cohort, information was collected prospectively on self-report of
158 physician diagnosed diabetes or use of diabetes drugs. Data were also collected from record
159 linkage with general practices or local hospital records, hospital admissions, and mortality
160 data from the UK Office of National Statistics. To minimize misclassification of case status,
161 only cases verified by another internal (e.g. drug use) or external (record linkage) source were
162 included.

163 *Statistical analysis*

164 Data preparation

165 Because of the skewed distributions and the frequency structure of the reported dairy
166 consumption, we classified dairy consumption into low and high consumers for milk, yogurt,
167 cheese, butter and total dairy products. Milk consumption was classified into <1 serving/day
168 and ≥ 2 servings/day. Yogurt, cheese and butter consumption were classified into <1
169 serving/week and ≥ 1 serving/day. Total dairy consumption was classified into <1 serving/day
170 and ≥ 3 servings/day. We derived the energy densities of milk and total dairy products per
171 2000 kcal/day and used the two variables as continuous.

172 Metabolomics and fatty acid data were log-transformed, mean-centered, and standardized to
173 units of SD. Due to the low missingness ($<3\%$ of participants for any covariate), we imputed
174 missing information on covariates using single imputation.^[18]

175 Derivation of models classifying dairy consumption

176 In the derivation set we developed regression models classifying the consumption of
177 individual dairy types by 82 metabolites that were common to the two studies, Fenland and
178 EPIC-Norfolk (Figure 1). We fitted an elastic-net penalized logistic regression model
179 regressing each of binary dairy variables on the 82 metabolites selected to derive estimates
180 robust against high multicollinearity between the metabolites, with a low prediction error
181 among common regression-based methods.^[19]

182 We developed five regression models with different sets of covariates for each dairy type
183 (Supplemental Table 2). The primary model included 82 metabolites controlling for the
184 covariates [age, sex, test site, smoking status, physical activity, lipid-lowering drugs,
185 hormone replacement therapy (in women), and BMI].

186 We created metabolite scores from the sum of the metabolite concentrations weighted by the
187 coefficients estimated from elastic-net regression. We selected metabolites with regression

188 coefficients outside the range of $\text{mean} \pm 2 \times \text{SD}$ of all the 82 coefficients. The scores derived
189 based on selected metabolites were determined post hoc because the preliminary analysis
190 including all the metabolites produced scores apparently sensitive to random noise because of
191 the small magnitude of the regression coefficients of many metabolites.

192 Internal and external validation analyses

193 Each metabolite score was tested for whether it could classify high and low consumers of
194 each dairy type (subtypes and total) in the two validation datasets (Fenland and EPIC-
195 Norfolk) (Figure 1). We fitted standard (no penalization) multivariable-adjusted logistic
196 regression models including each metabolite score as a single predictor, each of the binary
197 dairy variables as a dependent variable, and potential confounders selected as above. As the
198 primary metric for the ability to classify high and low dairy consumption, we estimated the
199 area under the curve (AUC). We also estimated the net reclassification improvement (NRI).
200 We used the likelihood ratio test to compare different nested logistic regression models.

201 We further evaluated whether each metabolite score would improve the classification ability
202 over and above the plasma phospholipid fatty acids OCSFAs and C16:1n7t, well-recognized
203 biomarkers of dairy fat intake.^[3] We fitted three logistic regression models, with the same
204 covariates as above, to classify dairy consumption (Supplemental Table 2). The independent
205 variables were (1) OCSFAs, C16:1n7t; (2) the metabolite score; and (3) the metabolite score
206 and the three fatty acids.

207 Prospective analysis for incident T2D

208 Using metabolite scores classifying between low and high dairy consumption in the external
209 validation, we evaluated the associations between the metabolite scores and T2D incidence in
210 the nested case-cohort subset of the EPIC-Norfolk Study. We fitted Prentice-weighted Cox

211 proportional hazards models to estimate hazard ratios (HR) and their confidence intervals
212 (CI). We adjusted HR estimates for age, sex, educational level, socio-economic status, family
213 history of T2D, smoking, physical activity, lipid-lowering drugs, anti-hypertensive drugs,
214 hormone-replacement therapy (in women), dietary supplement use, dietary variables
215 (continuous variables of intakes of total energy, fruit, vegetables, cereals, red meat, processed
216 meat, margarine, sweet snacks, sugar-sweetened beverages, coffee, tea, and alcoholic
217 beverages), and BMI. The proportional-hazards assumption was tested with Schoenfeld
218 residuals.

219 Secondary and post-hoc analyses

220 We used two secondary approaches for the derivation of the metabolite scores. First, we used
221 the initial internal validation set as a derivation set to also include the two OCSFAs, in
222 addition to the 82 matched metabolites in the derivation set (internal absolute validity not
223 possible to assess). Second, we included all the 174 metabolites from the targeted platform of
224 the derivation set (external validation not possible). As per the Transparent Reporting of a
225 multivariable prediction model for Individual Prognosis Or Diagnosis guidelines,^[20] we
226 repeatedly analyzed the derivation set using models without penalization. The energy
227 densities of milk and total dairy consumption were used as secondary outcomes in all
228 analyses, for which we estimated R^2 as a performance measure, modelling multivariable-
229 adjusted linear regression.

230 To assess the sensitivity of the primary results to the criterion to select top metabolites based
231 on the $\text{mean} \pm 2 \times \text{SD}$ of 82 coefficients, we changed the criterion to the $\text{mean} \pm 1 \times \text{SD}$. We also
232 created a score, retaining all the metabolites. To assess the specificity of the metabolite scores
233 to the dairy types, we examined the associations of each score with consumption of the dairy
234 types that the score was not derived for, and with 17 other food groups in the external

235 validation set. We also performed Cox regression for T2D using metabolite scores not
236 significantly predicting dairy consumption in the external validation. We conducted two post-
237 hoc analyses. First because the two studies used different metabolomics platforms, we
238 assessed whether or not the metabolite variance-covariance matrices were similar between
239 the two studies using an asymptotic chi-square test ^[21]. Second, we assessed the performance
240 of the scores, when we removed from them the metabolite which most consistently
241 contributed the most to the scores.

242 Analyses were performed with Stata 14.2 (College Station, TX, StataCorp LP, 2015) except
243 for the development and validation of models, which were performed in Python 3.6.3 Jupyter
244 Notebooks ^[22] using the Scikit Learn module v0.19.1 ^[23] for elastic-net regression and
245 estimation of metrics and the Statsmodels v0.9.0 module for standard logistic and linear
246 regression analyses.

247 **Results**

248 The distributions of socio-demographic, lifestyle and dietary factors were similar in the
249 derivation and internal validation sets in the Fenland Study but with some variation between
250 the Fenland Study and the external validation set from the EPIC Norfolk Study (**Table 1**). For
251 example, EPIC Norfolk included a 10-year older population, with a larger proportion taking
252 antihypertensive medication and hormone-replacement therapy.

253 *Derivation, internal and external validation of metabolite scores classifying dairy* 254 *consumption*

255 Among 82 metabolites available in the Fenland Study and the EPIC Norfolk Study, 11
256 metabolites were associated with consumption status of at least one dairy type (**Figure 2**)
257 after selecting metabolites with coefficients outside the $\text{mean} \pm 2 \times \text{SD}$ of the distribution.

258 Hydroxy-sphingomyelin (SM-OH) C14:1 [representing its isobaric compounds

259 SM(d18:1/C15:0) and SM(d16:1/C17:0)], appeared to classify consumption of all the dairy
260 types similarly. Other signals classifying dairy consumption included SM C16:1 (milk and
261 total dairy products), and lyso-phosphatidylcholine a C17:0 (LPC a C17:0; for cheese, butter
262 and total dairy products). *Cis*- and *trans*-hydroxy proline (OH-Pro) were negatively
263 associated with yogurt, and *trans*-OH-Pro was negatively associated with cheese
264 consumption. After adjustment for other dietary factors, of the top metabolite signals
265 mentioned above, SM-OH C14:1 [SM(d18:1/C15:0, d16:1/C17:0)] and LPC a C17:0
266 classified dairy consumption (data not shown).

267 In the internal validation set, adding the metabolite score to the model with non-metabolite
268 covariates (reference) increased the AUCs: from 0.59 to 0.80 for total dairy products , 0.59 to
269 0.72 for butter, 0.59 to 0.64 for milk , 0.67 to 0.73 for cheese , and 0.67 to 0.69 for yogurt
270 (Supplemental Figure 1, Supplemental Tables 3-4).

271 In the external validation using 11 metabolites, the metabolite scores better classified
272 consumers of milk (AUC increased from 0.59 to 0.60, NRI=0.25, $p<0.001$). For butter and
273 total dairy, AUC increased from 0.61 to 0.62 (NRI=0.14, $p= 0.02$) and from 0.59 to 0.68
274 (NRI=0.33, $p=0.008$), respectively. The metabolite scores for yogurt and cheese did not show
275 improvement ($p=0.09$ and 0.22 , respectively) (**Table 2**).

276 *Utility of the metabolite scores over the use of fatty acids as dietary markers*

277 OCSFAs and C16:1n7t classified high dairy consumers (Supplementary Table 4). Further
278 addition of each metabolite score increased the classification ability in the internal validation
279 (Supplementary Tables 3 and 4). In the external validation, derivations of metabolite scores
280 with and without OCSFAs showed similar performance to the model without metabolites
281 resulting in increases in the AUCs mainly for butter and total dairy products (Table 2).

282 Further addition of the metabolite score to the models increased the classification ability in
283 the internal validation, but not in the external validation (Supplemental Table 3 and 4).

284 *Associations of metabolite scores classifying dairy consumption with T2D incidence*

285 During 16,360 person-years of follow-up, each of the metabolite scores for milk, butter, and
286 total dairy was inversely associated with T2D incidence (**Table 3**). Multivariable-adjusted
287 models (not adjusted for BMI) showed a 29% lower T2D incidence [hazard ratio (HR) =0.71,
288 95% confidence interval (CI): 0.65, 0.77] per 1 SD of the metabolite score classifying milk
289 consumption. One SD of the metabolite scores for butter and total dairy consumption was
290 associated with 38% [0.62 (0.57, 0.68)] and 34% [0.66 (0.60, 0.72)] lower T2D incidence,
291 respectively (Table 3). After adjustment for BMI and then OCSFAs, the associations for the
292 metabolite scores remained significant (Table 3). By contrast, these negative associations
293 were not seen for the metabolite scores for yogurt and cheese consumption, which did not
294 show evidence of external validity as above mentioned (Supplemental Table 5).

295 *Secondary analyses*

296 When we repeated the derivation analyses in Fenland including both 82 metabolites and
297 OCSFAs, 12 metabolites appeared to classify consumers of one or more dairy types (C15:0,
298 two acylcarnitines, five PCs, and two SMs) (Figure 2, Supplemental Figures 2-4). Internal
299 validation of the metabolite scores for milk and total dairy products as continuous variables
300 and when using a total of 174 metabolites in the Fenland Study, gave similar results. The
301 variance-covariance patterns of 82 metabolites or the top 11 metabolites selected for one or
302 more of the metabolite scores differed significantly between the two cohorts (p for the matrix
303 homogeneity <0.0001; Supplemental Figure 5). When removing SM-OH C14:1
304 [SM(d18:1/C15:0, d16:1/C17:0)], which was the most consistent top metabolite, from the

305 scores, there was a smaller improvement in the classification ability of the scores, but still
306 significant for butter and total dairy products (Supplemental Table 6).

307 When selecting metabolites if they had coefficients outside $\text{mean} \pm 1 \times \text{SD}$, a smaller
308 improvement in the classification ability of the scores was observed compared to the main
309 scores (Supplemental Table 6). Each metabolite score showed null or heterogeneous
310 associations with consumption of dietary components in EPIC-Norfolk (Supplemental Table
311 7). Secondary analyses with different analytic strategies to derive metabolite scores yielded
312 consistent associations with incident T2D overall with those from the primary analysis
313 (Supplemental Table 8).

314 **Discussion**

315 In analyses conducted using two independent studies we identified sets of metabolites to
316 classify physiological response between low and high habitual consumption of milk, butter
317 and total dairy products. These metabolite scores with evidence of external validity were
318 associated with lower T2D incidence.

319 *Findings in context of other evidence*

320 The associations between dairy-related metabolites and T2D that we observed were
321 consistent with those from meta-analyses evaluating self-reported consumption of butter^[2]
322 and total dairy products.^[1] Meta-analyses of milk reported null associations with T2D risk,^[1]
323 which might mean that biological pathways other than the pathway potentially reflected in
324 our results might differentially link milk to T2D.

325 The findings for metabolite scores classifying milk, butter and total dairy consumption were
326 consistent with previous evidence of inverse associations of SM-OH C14:1,^[24, 25] LPC a
327 C17:0,^[24-26] PC ae C34:1,^[24] and OCSFAs,^[27] with T2D risk or other glycemic outcomes. A
328 study with similar methodology to ours, but a different set of metabolites, observed inverse

329 associations of metabolite scores predicting total dairy, milk, yoghurt, and cheese
330 consumption with T2D risk in their derivation set, but only for total dairy and milk in their
331 external validation set,^[5] partly consistent with our findings. Notably, the external validity in
332 that study was also weak for yoghurt and cheese ^[5] consistent with our finding. Heterogeneity
333 and variability across fermented dairy products over time may have resulted in weak
334 comparability in the metabolome between the two independent cohorts that collected dietary
335 data more than 10 years apart on average^[5]

336 Of the metabolites we identified for classification of dairy consumers, SM-OH C14:1
337 [SM(d18:1/C15:0, d16:1/C17:0)] ^[6, 28] was observed to be associated with dairy consumption
338 in two other cohorts providing support that this metabolite may serve as a potential dairy
339 biomarker. When we included the OCSFAs in the metabolite set of our derivation analysis,
340 C15:0 was one of the top signals for the classification of butter consumption, which confirms
341 previous evidence.^[3] We did not identify C17:0 ^[29] as a dairy biomarker when we evaluated it
342 together with the metabolites. Failure to do so may have been due to high correlations
343 between C17:0 and other metabolites e.g. LPC a C17:0, which predicted high-fat dairy
344 consumption overall in a randomized controlled trial.^[30] As demonstrated by Münger et al.,
345 only a few novel metabolites have been identified for dairy products with insufficient
346 validation, but studies that have explored this, including randomized controlled trials, have
347 identified OCSFAs from the phospholipids fraction or as intact phospholipid or
348 sphingomyelins.^[31] Future work will be essential to profile lipid species, such as
349 phospholipids, sphingomyelins and cholesteryl esters, which contain OCSFAs and ruminant
350 trans-FAs.

351 *Biological interpretation*

352 From our metabolomics profiling, mainly lipids were identified to classify physiological
353 response between low and high dairy consumption. Among such metabolites, SM-OH C14:1
354 appeared to robustly classify different dairy products with different analytical approaches. As
355 mentioned, this lipid is an isobaric molecule of SM(d18:1/C15:0) and SM(d16:1/C17:0),
356 which contain OCSFAs. This means that these molecules have the same mass, and they
357 would need a more sensitive platform to distinguish between them. Presence of such isobaric
358 compounds, as well as high correlations between different food groups in our study highlight
359 the challenges in identifying molecules specific to different dairy types. LPC a C17:0 was
360 also identified as one of the top metabolite signals for classification. This metabolite contains
361 the OCSFA C17:0, a candidate biomarker of dairy fat. A similar explanation about OCSFAs
362 could be given for the observed associations of LPC 14:0 (C14:0) and PC ae 34:1 (C14:0 and
363 C15:0). The inverse associations observed for some metabolites, e.g. hydroxy-proline
364 isomers, might reflect more indirect pathways and are thus more challenging to interpret.

365 The association of circulating OCSFAs and C16:1n7t with lower incidence of T2D has been
366 previously reported.^[27] Self-reported butter consumption has also been previously associated
367 with lower T2D risk.^[2] Thus, the study of the potential effect of dairy fat on the T2D risk and
368 its biological mechanisms are worthy of future investigation.

369 Strengths and limitations

370 The key strength of our study is the development of metabolite scores with multiple
371 metabolites classifying dairy consumption in analyses including both internal and external
372 validation in two independent large population-based studies. Despite the 10-year difference
373 in the participant recruitment, we found evidence of external validity for scores of total dairy,
374 milk and butter (temporal generalisability), but not yogurt and cheese. Although a previous
375 study followed similar methodology to ours,^[5] our availability of plasma phospholipid fatty

376 acids and metabolomics additionally allowed us to compare the known biomarkers and new
377 data-driven metabolite biomarkers. However, we had a limited ability to derive biomarkers
378 for variation of specific dairy consumption because of the inherent correlations between dairy
379 consumption, consumption of other foods, and behavioral factors. Rather, we should interpret
380 that we detected metabolomics markers for habitual dairy consumption within overall diets
381 and in the context of other behavioral factors. Although the correlation matrices of top
382 metabolites between the two studies significantly differed, we were still able to find evidence
383 of external validity in the metabolite scores for milk, butter and total dairy products. The
384 metabolites we evaluated may not fully capture the metabolome in the blood or in other
385 potentially relevant tissues. Finally, our studies were based largely on populations of white
386 British background, so our results may not be generalizable to other ethnic groups or
387 populations with different dairy consumption patterns e.g. populations with high lactose
388 intolerance.

389 Conclusion

390 In this study, we identified metabolite scores discriminating between physiological response
391 to high and low consumers of milk, butter and total dairy products with internal and external
392 validity. Analysis using the metabolite scores supported the association of those dairy
393 products with a lower T2D risk. In contrast, this study did not identify any sets of metabolites
394 that classify yogurt or cheese consumption with external validity in our study. Our
395 methodological approach and findings should stimulate further observational studies and
396 trials including markers from other assays, biological samples e.g. gut microbiome in diverse
397 populations to better understand how consumption of a certain dietary product may influence
398 cardio-metabolic risk.

399 **Author contributions:** NJW, CL, SB, SJG, KTK, and NGF designed research; NGF, FI, and
400 ET conceived the research question; AK and JG designed and managed laboratory analyses;
401 AK managed and provided laboratory data; ET and FI analyzed data; ET, FI and NGF
402 prepared the initial draft of the manuscript which all authors contributed to improve, and all
403 authors read and approved the final version. ET and NGF had primary responsibility for final
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426 None

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Figure legends

Figure 1. Flow diagram of the inclusion process of participants in the derivation, internal and external validation sets (the Fenland Study and the EPIC Norfolk Study in the United Kingdom)

Figure 2. Relative strengths of associations of metabolites with consumption status of each dairy type: a derivation analysis in the Fenland Study (n=6,035). Two panels display the metabolites selected if each adjusted coefficient (per 1-SD of each metabolite) was greater than mean+2×SD (red) or lower than mean-2×SD (blue) calculated from all the coefficients of 82 metabolites (left) and 82 metabolites + 2 OCSFAs (right). The coefficients were adjusted for age, sex, test site, smoking status, physical activity, lipid lowering drugs, hormone-replacement therapy, and BMI in the elastic-net penalized regression models. Refer to Supplemental Methods for metabolite annotation. LPC: lyso-phosphatidylcholines, OCSFAs: odd-chain saturated fatty acids; PC: Phosphatidylcholines, Pro: proline, SM-OH: hydroxyl-sphingomyelins, SM-OH C14:1: isobaric molecule of SM(d18:1/C15:0) and SM(d16:1/C17:0)

Table 1. Participant characteristics in the Fenland study (2005-2015) and the EPIC Norfolk diabetes case-cohort study (1993-1997), the United Kingdom^a

Characteristics ^b	Fenland Study				EPIC Norfolk Study	
	Derivation		Internal validation ^c		for external validation ^d	
	n=6,035		n=4,246		n=825	
Age, years	48.9	7.4	47.8	7.3	59.0	9.4
Sex, women %	53.2		54.5		58.1	
Educational level, medium % ^a	45.9		45.9		38.8	
high %	34.4		33.7		13.9	
Smoking, former, % ^a	33.9		32.3		39.0	
current, %	11.7		12.8		13.0	
Physical activity						
energy expenditure, kj/kg/d	53.7	22.1	54.4	22.4	Na ^e	
moderately active or active, % ^a	Na ^e		Na ^e		43.4	
BMI, kg/m ²	26.9	4.8	26.8	4.7	26.1	3.7
Lipid-lowering drugs, % ^a	4.3		3.5		1.3	
Antihypertensive drugs, %	7.2		7.2		34.2	
Hormone replacement therapy, % among women	2.9		2.8		19.2	
Dairy products^a						
Milk < 1 serving/d, %	38.8		37.8		22.3	
≥ 2 serving/d, %	27.0		27.1		40.2	
Yogurt, < 1 serving/wk, %	35.6		36.6		48.7	
≥ 1 serving/d, %	13.1		12.6		8.4	
Cheese, < 1 serving/wk, %	27.9		28.0		22.7	
≥ 1 serving/d, %	8.1		9.0		8.6	
Butter, < 1 serving/wk, %	51.9		53.9		62.2	
≥ 1 serving/d, %	17.8		18.3		18.4	
Total dairy products, < 1 servings/d	6.7		7.0		3.9	
≥ 3 servings/d	39.1		39.4		44.1	

Milk, % energy	1.5	1.0	1.6	1.0	1.8	0.9
Total dairy products, % energy	3.0	1.4	3.0	1.4	3.1	1.4
Non-dairy dietary factors						
Dietary supplements, %	41.0		42.4		50.8	
Energy intake, kcal/d	1,924	571	1,939	579	2,013	558
Fruits, g/d	240.6	203.3	244.8	198.7	248.8	197.8
Vegetables, g/d	258.1	143.2	253.4	135.5	239.2	118.2
Cereals, g/d	169.3	100.6	169.2	96.4	157.4	84.4
Red meat, g/d	74.2	46.7	72.9	48.4	63.4	46.0
Processed meat, g/d	31.7	26.9	32.3	29.3	29.2	24.3
Fish, g/d	42.9	33.4	43.2	35.4	38.1	25.5
Sugar-sweetened beverages, g/d	43.2	95.1	39.9	84.7	35.0	73.6
Alcoholic beverages, g/d	151.2	249.5	149.7	238.1	248.8	202.6

^{a)}The mean and SD are presented for continuous variables and column percentages are presented for categorical variables. Categories for 'low' or 'no' status were omitted for education, physical activity, smoking, medications, and dietary supplement; and for mid-categories of dairy consumption (neither low nor high).

^{b)} Missing values for each variable were < 3% in the Fenland Study and <2% in the EPIC Norfolk Study.

^{c)} The internal validation was the set that had been previously randomly selected for measurement of blood fatty acids.

^{d)} The characteristics of the random sub-cohort from the EPIC case-cohort study are presented (The longitudinal analysis evaluated 1,440 participants in total, including 641 T2D incident cases).

^{e)} na, not applicable. Physical activity was objectively assessed in the Fenland study and expressed as a continuous variable for physical activity energy expenditure. Physical activity levels were assessed with a questionnaire in the EPIC Norfolk study and categorised into four categories.

EPIC, European Investigation into Cancer and Nutrition; T2D, type 2 diabetes

Table 2. Classification ability for consumers and non-consumers of different dairy products: external validation analysis of the metabolite scores derived from the Fenland Study and tested in the EPIC-Norfolk Study, United Kingdom^a

Components of the metabolite scores ^b	Dairy type	N	N metabolites	AUC before/after adding		NRI	P_{NRI} ^d
				the metabolite score ^c			
				before	after		
Selected metabolites ^e	Milk	900	3	0.59	0.60	0.25	<0.001
	Yogurt	875	2	0.69	0.70	0.14	0.093
	Cheese	475	4	0.65	0.66	0.08	0.22
	Butter	1,193	5	0.61	0.62	0.14	0.019
	Total dairy	687	4	0.59	0.68	0.33	0.008
Selected metabolites + fatty acids ^e	Milk	362	2	0.61	0.65	0.25	0.012
	Yogurt	391	5	0.72	0.72	0.07	0.34
	Cheese	194	3	0.70	0.70	-0.06	0.63
	Butter	515	5	0.62	0.66	0.26	0.004
	Total dairy	282	3	0.61	0.77	0.76	0.001

^a) All the statistics were adjusted for socio-demographic and lifestyle factors include age, sex, test site, smoking status, physical activity, lipid lowering drugs, hormone-replacement therapy and BMI

^b) The set of metabolites used in the analysis to derive the metabolite scores in the Fenland Study

^c) "before" refers to the model including covariates only; and "after", the model including covariates and the metabolite score

^d) P values for the metabolite score for the classification of consumers and non-consumers.

^e) Selected metabolites from 82 metabolites or from 82 metabolites + fatty acids if the regression coefficients were outside the $\text{mean} \pm 2 \times \text{SD}$.

AUC: area under the curve of a receiver operating characteristics; EPIC: European Investigation into Cancer and Nutrition; NRI: net reclassification improvement

Table 3. Associations of metabolite scores classifying dairy consumption with T2D incidence: nested case-cohort analysis of the EPIC Norfolk study^a

Metabolite score for each dairy type ^b	Model	HR	95% CI
Milk	Socio-demographic + family history of T2D ^c	0.67	0.63, 0.73
	+Smoking, physical activity, drugs ^d	0.67	0.62, 0.73
	+ Diet ^e	0.71	0.65, 0.77
	+ BMI	0.75	0.69, 0.82
	+ OCSFAs	0.71	0.61, 0.83
Butter	Socio-demographic + family history of T2D ^c	0.59	0.54, 0.64
	+Smoking, physical activity, drugs ^d	0.62	0.57, 0.67
	+ Diet ^e	0.62	0.57, 0.68
	+ BMI	0.60	0.54, 0.65
	+ OCSFAs	0.53	0.46, 0.62
Total dairy	Socio-demographic + family history of T2D ^c	0.63	0.58, 0.67
	+Smoking, physical activity, drugs ^d	0.64	0.59, 0.69
	+ Diet ^e	0.66	0.60, 0.72
	+ BMI	0.74	0.67, 0.80
	+ OCSFAs	0.68	0.58, 0.79

^a) Based on analysis of 641 cases of T2D and 16,360 person-years of follow-up, HRs and 95% CIs were estimated per one SD difference in each metabolite score. All the associations were significant ($p < 0.001$) except for the metabolite score for milk, which included all the 82 metabolites after adjustment for BMI.

^b) Metabolite scores generated from the total sample of the incident diabetes case-cohort study ($n=1,440$). Selected metabolites were the top metabolites defined as those with absolute values of elastic net coefficients $> \text{mean} + 2 \times \text{SD}$.

^c) Adjusted for age (continuous in years), sex, educational level (low, medium, high), socio-economic status (low, medium, high) and family history of T2D (yes, no)

^d) Additionally adjusted for smoking (never, former, current), physical activity (inactive, moderately inactive, moderately active, active), lipid-lowering drugs (yes, no), anti-hypertensive drugs (yes, no), hormone-replacement therapy (yes, no, men)

^{e)} Additionally adjusted for total energy intake (kcal/day), dietary supplement use (yes, no), consumption (g/day) of fruit, vegetables, total cereals, red meat, processed meat, fish, margarine, sweet snacks, sugar-sweetened beverages, coffee, tea, alcoholic beverages

CI: confidence interval; EPIC: European Investigation into Cancer and Nutrition; HR: hazard ratio;

OCSFA: odd-chain saturated fatty acids; T2D: type 2 diabetes