Genome-wide scan identifies novel genetic loci regulating salivary metabolite levels

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Abstract

Saliva, as a biofluid, is inexpensive and non-invasive to obtain, and provides a vital tool to investigate oral health and its interaction with systemic health conditions. There is growing interest in salivary biomarkers for systemic diseases, notably cardiovascular disease. Whereas hundreds of genetic loci have been shown to be involved in the regulation of blood metabolites leading to unprecedented insights into the pathogenesis of complex human diseases, little is known about the impact of host genetics on salivary metabolites. Here we report the first genome-wide association study exploring 476 salivary metabolites in 1,419 subjects of European ancestry from the TwinsUK cohort (discovery phase). A total of 14 salivary metabolites were significantly associated ($p<10^{-10}$) with genetic variants that mapped to 11 distinct loci, most of which replicated in the Study of Health in Pomerania (SHIP-2) cohort. Interestingly, while only a limited number of the loci that are known to regulate blood metabolites were also associated with salivary metabolites in our study, we identified several novel saliva-specific locus-metabolite associations, including associations for the *AGMAT* (with the metabolites 4-guanidinobutanoate and beta-guanidinopropanoate), *ATP13A5* (with the metabolite creatinine) and *DPYS* (with the metabolites 3-ureidopropionate and 3-ureidoisobutyrate) loci. Our study suggests that there are biological pathways which are specific to the regulation of the salivary metabolome. In addition, some of our findings may have clinical relevance, such as the utility of the pyrimidine (uracil) degradation metabolites in predicting 5-fluorouracil toxicity and the role of the agmatine pathway metabolites as biomarkers of oral health.

Introduction

Metabolic reactions pervade every aspect of human physiology, abnormalities in which underlie a plethora of human diseases[1]. Investigating the genetic underpinnings of population-wide variation of metabolites can offer novel insights into human metabolism and diseases, in addition to providing potential therapeutic targets to modulate metabolite levels. Large-scale genetic association studies have so far identified hundreds of loci that regulate the levels of metabolites in blood[2–6], and to a lesser extent in other biospecimens as well[7–9]. Previous studies have shown that genetic variants on average explain a greater proportion of trait variance for metabolites compared to what is generally observed for complex traits[3,4], highlighting the utility of metabolites as intermediate traits for dissecting the genetics of complex diseases.

Saliva is an abundantly produced biofluid, and it can be obtained in an inexpensive and non-invasive manner, without the need for healthcare professionals. It is mainly composed of water (>99%) and several other minor constituents such as mucous, digestive enzymes, cytokines, immunoglobulins, antibacterial peptides and low molecular weight metabolites[10].

Recent advances in metabolomic profiling allow quantification of hundreds of metabolites belonging to diverse biochemical pathways in large population samples[4,11]. In 2015, the Human Metabolome Database (HMDB) incorporated data on the 'salivary metabolome' which included 853 salivary metabolites that were systematically characterized using a multiplatform approach[12]. Since saliva is separated from the systemic circulation by just a thin layer of cells, which allows passive and active exchange of substances[13], it provides a reflection of not just oral health but the functioning of other organ systems as well[14]. Indeed, a number of studies have previously reported associations between oral health and systemic conditions such as cardiovascular diseases, diabetes, autoimmune diseases, mental health disorders, dementia,

74 among others[15–19]. Therefore, investigation of salivary metabolites could not only provide novel
75 biomarkers but also further our understanding of biological pathways underlying oral as well as
76 general health conditions.
77 Here we report a genome-wide association analysis (based on 1000 Genomes imputed data) for
78 476 salivary metabolites in the population-based TwinsUK study, followed by replication in the
79 population-based Study of Health in Pomerania (SHIP-2) cohort.

Material and methods

(I) Discovery phase

Study population

The discovery phase of the study was conducted in the TwinsUK cohort, an adult twin registry comprising of healthy volunteers, based at St. Thomas' Hospital in London[20]. Twins gave fully informed consent under a protocol reviewed by the St. Thomas' Hospital Local Research Ethics Committee. Subjects of European ancestry with available genotype data and for whom salivary metabolite profiling was done on a fasting state sample were included in our study (N = 1,419; mean age = 62.2 years; % females = 92.7).

Genotyping, imputation and QC

Subjects were genotyped in two different batches of approximately the same size, using two genotyping platforms from Illumina: 300K Duo and HumanHap610-Quad arrays. Whole genome imputation of the genotypes was performed using the 1000 Genomes reference haplotypes[21], further details of which are provided in Moayyeri *et al* (2013). Stringent QC measures, including minimum genotyping success rate (>95%), Hardy-Weinberg equilibrium ($p>10^{-6}$), minimum MAF (>0.5%) and imputation quality score (INFO>0.5), retained ~9.6 million variants for genome-wide analysis.

103 **Saliva sample collection**

104 Saliva samples were obtained by asking the fasted volunteer to spit as much saliva as possible
105 into an empty sterile pot over a period of 10 minutes. The saliva samples were immediately
106 refrigerated and then frozen at -80°C (usually within 4 hours of sample collection) before further
107 processing. Following that, the samples were shipped on dry ice for metabolite profiling at
108 Metabolon Inc., Durham, USA (See **Supplemental methods (I)** for further details on sample
109 processing).

110

111 **Metabolic profiling of saliva samples**

112 Metabolite concentrations in the saliva samples were estimated using Ultrahigh Performance
113 Liquid Chromatography-Tandem Mass Spectroscopy (UPLC-MS) i.e. chromatographic
114 separation, followed by full-scan mass spectroscopy, to record all detectable ions in the samples
115 (see **Supplemental methods (II)** for further details). Based on their unique ion signatures
116 (chromatographic and mass spectral), 997 distinct metabolites were identified, of which 823 had
117 known chemical identity at the time of analysis. The 823 known metabolites were broadly
118 classified into 8 metabolic groups (amino acids, peptides, carbohydrates, energy, lipids,
119 nucleotides, cofactors and vitamins, and xenobiotics) as described in the KEGG (Kyoto
120 Encyclopedia of Genes and Genomes) database[22]. The 8 metabolic groups were further
121 subdivided into 99 distinct biochemical pathways.

122 Raw metabolite values were normalised for the volume and osmolality measurement of the
123 saliva samples. The normalised metabolite values were then log-transformed, and scaled to
124 uniform mean 0 and standard deviation 1. Of the 823 known metabolites, 476 were retained for
125 analysis based on presence of measurement in more than 80% samples. For the samples with

126 missing values for these 476 metabolites, data were imputed using the run day minimum value
127 for the metabolite. The resulting imputed dataset of the 476 metabolites was used for further
128 analysis.

129

130 **Genome-wide association analysis of salivary metabolites**

131

132 ***(i) Primary genome-wide association analysis***

133 For each of the 476 metabolites, a linear mixed-model was fitted to test the association between
134 the metabolite (dependent variable) and genome-wide variants (independent variable). Age, sex
135 and time of saliva sample collection were included as covariates in the model. The score test
136 implemented in GEMMA[23], which utilises a sample kinship matrix (estimated using a subset of
137 ~500,000 variants) to account for the twin structure or relatedness in the TwinsUK data, was
138 used to assess significance of the associations. A genome-wide and metabolome-wide
139 significance cut-off of $p<10^{-10}$ (corresponding to the conventional genome-wide significance
140 threshold of $5x10^{-8}$, corrected for 476 metabolites) was used to identify significant variant-
141 metabolite associations. For each locus that was significantly associated with a metabolite, we
142 reported the variant with the lowest association p-value.

143

144 ***(ii) Testing loci identified in the primary analysis for additional metabolite***
145 ***associations***

146 Next, we focused just on the loci that were identified in the primary stage of association testing
147 to look for additional variant-metabolite associations for those loci. For each locus that was
148 identified in the primary analysis, we clumped all variants located within a 100 Mb block and

149 with LD (r^2) > 0.2, and checked for additional metabolite associations at a significance threshold
150 of $p<10^{-6}$ (corresponding to p=0.05, corrected for 476 metabolites and a prior assumption of
151 about 100 associated loci).

152

153 ***(iii) Testing the significantly associated loci using metabolite data from other***
154 ***biospecimens***

155 For each locus-metabolite association, we further assessed the most significant variant-
156 metabolite pair by using measurements for the respective metabolite in serum[4] and faecal
157 samples[7] of the TwinsUK subjects (provided the metabolite was measured in that biospecimen).
158 We tested only those serum and faecal samples that overlapped with the ones in saliva and were
159 collected within 5 years of the saliva samples (in order to ensure that, for a given individual,
160 samples from the different biospecimens being tested were obtained within a certain period of
161 one another). Association testing for serum and faecal metabolites was done using an identical
162 model to that described for the analysis of salivary metabolites.

163

164 ***(iv) Conditional analysis for the significantly associated loci***

165 ***(a) Detection of secondary association signals***

166 We used approximate conditional analysis, as implemented on GCTA[24], to test whether any of
167 the associated loci had multiple distinct i.e. secondary association signals, at a "locus-wide"
168 significance threshold of $p<10^{-5}$. For each associated locus, all variants that surpassed the study-
169 wide significance threshold ($p<10^{-10}$) were conditioned on the most significantly associated
170 variant at that locus (using the association summary statistics). For the conditional analysis, we

171 used genotype data from the complete TwinsUK dataset (N=5,654) to model LD patterns
172 between variants.

173 ***(b) Adjustment for periodontal disease status***

174 Since salivary metabolites are known to be associated with periodontal disease (PD)[11], for each
175 locus-metabolite association, we additionally adjusted the most significant variant-metabolite
176 pair for PD status. Self-reported gingival bleeding, and a history of gum disease or tooth mobility
177 were used as indicators of PD in TwinsUK[25] (270 PD cases; 1,083 controls).

178

179 **Expression quantitative trait locus (eQTL) analysis**

180 We used the version 7 data release of the Genotype-Tissue Expression (GTEx) project (accessed
181 15 April 2019), which was based on RNA-Seq data obtained from 48 non-diseased tissue sites
182 across ~1,000 individuals, to test whether the most significant variant at each associated locus
183 had an eQTL effect on transcripts located within a 1 Mb window of the variant.

184

185 **Annotation of associations using reference databases**

186 We searched the NHGRI GWAS catalogue (accessed 15 April 2019) for previous disease
187 associations for the significantly associated loci that were identified in our study. We also
188 searched the OMIM database (accessed 15 April 2019) to check the candidate genes at the
189 associated loci for a causal link with inborn errors of metabolism. Moreover, we also queried the
190 HMDB[12] and KEGG[22] databases to identify biochemical pathways and known disease
191 associations for the associated metabolites.

192

193 **(II) Replication phase**

194 The replication phase was performed in the Study of Health in Pomerania (SHIP-2), a
195 population-based study comprising of European ancestry subjects, conducted in the northeastern
196 area of Germany. Further details of SHIP-2, including cohort details, genotyping and imputation,
197 and saliva sample collection are provided in **Supplemental methods (III)**. Metabolic profiling
198 for SHIP-2 saliva samples (N=1,000) was performed using an identical process to that described
199 for TwinsUK.

200 Since the method of saliva sample collection in SHIP-2 (chewing on a piece of cotton) meant
201 that the sample thus obtained represented stimulated saliva, normalisation of the metabolite
202 measurements for sample osmolality was not considered necessary. The fact that the salivary
203 osmolality values in SHIP-2 had a much narrower distribution compared to that in TwinsUK
204 verified our rationale (**Figure S1**).

205 For each locus-metabolite association that was identified in the discovery phase, we tested the
206 most significantly associated variant using a linear regression model was fitted on R (version
207 3.5.2). Covariates used in the association model were similar to those used for the discovery
208 phase.

209

210 **(III) Testing the significantly associated salivary metabolites with phenotypes**
211 **of interest**

212 We wanted to test how salivary metabolites that were regulated by genetic loci related to relevant
213 phenotypes. For that, we selected the metabolites that were uniquely associated in saliva i.e. the
214 ones for which a genetic association had not been previously reported in blood, and tested them
215 with phenotypes (diseases / traits / adverse drug effects) relating to the metabolite or its

216 associated biochemical pathway. We obtained the relevant phenotype information from the
217 TwinsUK database, selecting one twin per pair (N=1,426). The phenotype association analysis
218 was performed on R (version 3.5.2) by fitting a linear regression model to test the association
219 between the salivary metabolite and the disease / trait / adverse drug effect (adjusted for age and
220 sex).

221 # Results

222

223 ## Identification of novel genetic loci regulating salivary metabolite levels

224 Primary genome-wide discovery analysis in TwinsUK identified 13 metabolites that were
225 significantly associated with genetic loci after correcting for multiple testing ($p<10^{-10}$).
226 Furthermore, when we narrowed our analysis to just the loci that were identified in the primary
227 stage, one additional metabolite was found associated ($p<10^{-6}$). Consequently, a total of 14
228 distinct locus-metabolite associations (hereafter, referred to as 'mQTLs') were identified in the
229 discovery phase of our study. The set of significantly associated variants mapped to 11 distinct
230 genetic loci, which have been referred to by the name(s) of the overlapping or the nearest gene(s)
231 (**Figure 1**). Three of those loci (*AGMAT, SLC2A9* and *DPYS*) were associated with two
232 metabolites each (**Table 1**). In all three instances, the two metabolites regulated by the same
233 locus were correlated (Pearson's r^2 for the metabolite pairs ranged between 0.42 - 0.84) (**Figure**
234 **S2**). On the other hand, none of the 14 metabolites that was associated in our study had more
235 than one significant locus. Quantile-quantile (QQ) plots for the significantly associated
236 metabolites are provided in **Figure S3**.

237 Of the 11 genetic loci that were identified in the discovery phase, four of them (*SLC2A9,*
238 *DMGDH, FADS2* and *ACADS*) have previously been reported in association with blood
239 metabolites[2,4,5]; the remaining seven loci were novel i.e. there were no previously known
240 associations for them with metabolites in blood or any other biospecimens.

241 The *AGMAT* locus, one of the novel loci identified, was associated with the metabolites 4-
242 guanidinobutanoate and beta-guanidinopropanoate. These metabolites are generated as
243 intermediate products in the polyamine synthesis pathway, the main site of action for the enzyme

244 agmatinase (encoded by the *AGMAT* gene)[26]. The most significantly associated variants for the
245 two metabolites i.e. rs10927806 and rs6690813, respectively, are in high LD with one another
246 (r^2=0.99), suggesting a shared underlying genetic regulation for the two metabolites by the
247 *AGMAT* locus. Likewise, the association between the *ATP13A5* locus and creatinine (a widely
248 used measure of renal function), observed in our study, has also not been reported previously.
249 Another interesting novel association that we identified pertained to the metabolism of the
250 pyrimidine uracil - the *DPYS* locus (encodes for the enzyme dihydropyrimidinase, involved in
251 uracil degradation) was associated with the metabolites 3-ureidopropionate and 3-
252 ureidoisobutyrate (breakdown products of uracil metabolism). The novel association for the
253 *TYMS/ENOSF1* locus with ribonate is also intriguing, since it has previously been shown that
254 ribonate is one of the substrates for the catalytic activity of reverse thymidylate synthase (rTS),
255 the protein product of *ENOSF1*[27]. Therefore, it appears that *ENOSF1*, which is the source of anti-
256 sense RNA of *TYMS,* is probably the functional gene mediating the observed association of the
257 *TYMS/ENOSF1* locus with salivary ribonate. The associations of the *SLC2A9* locus with
258 allantoin, the *ABO* locus with N-acetylglucosamine/N-acetylgalactosamine, the *UGCG* locus
259 with glycosyl-N-stearoyl-sphinganine, and the *MGP* locus with gamma-carboxyglutamate were
260 the remaining mQTLs observed in our study that have not been reported previously.
261 Of the 14 distinct mQTLs that were identified in our study, it was possible to test the most
262 significant variant-metabolite pair for nine mQTLs in both serum and faecal metabolite data in
263 TwinsUK (metabolites corresponding to the remaining five mQTLs were not present in the
264 serum and faecal metabolite datasets). Of them, the associations for the *ATP13A5* and *DPYS* loci
265 did not replicate in serum ($p>0.05$); while, none of the associations, barring the one for the
266 *ACADS* locus, replicated in the faecal data ($p>0.05$) (**Table S1**). Thus, a comparison across all

267 three biospecimens (for the significantly associated salivary metabolites which were also
268 measured in serum and faecal samples in TwinsUK) demonstrates that the effects of the
269 *ATP13A5* and *DPYS* loci were specific to saliva (**Figure S4**).
270 For none of the mQTLs that was identified, did we find any additional independent association
271 signals after conditioning for the most significant variant at the locus (conditional $p>10^{-5}$ for all
272 variants tested at each locus), a finding which was verified by the regional association plots
273 (**Figure S5**). The observation that a single genetic association signal underlies each of the
274 significantly associated metabolites might partly be due to our lack of power to detect secondary
275 signals at these loci.
276 Moreover, the strength of association for the most significant variant-metabolite pair did not
277 change much on adjusting for PD status, for any of the mQTLs (**Table S2**). Hence, it does not
278 appear that the presence of underlying PD has a significant effect on the associations observed in
279 our study.
280 For 8 of the 11 associated loci, it was observed that the most significant variant demonstrated an
281 eQTL effect on at least one transcript in one of the tissues in the GTEx database (no significant
282 eQTL effects were observed for the *ATP13A5*, *SLCA2A9* and *ABO* loci) (**Table S3**). In case of 7
283 of those 8 loci (except *DPYS*), the significant eQTL effect was observed for the overlapping or
284 the nearest gene transcript; and for 5 of those 7 loci (*AGMAT*, *FADS2*, *DMGDH*, *TYMS/ENOSF1*
285 and *UGCG*), the eQTL effect was observed in one of the gut-related tissues. While eQTL data
286 for transcripts assayed in the minor salivary glands was available for a small number of donors
287 (N=97) in the GTEx database, none of the significant eQTL effects that we observed was in the
288 salivary tissue.
289

290 **Replication of the discovery phase findings**

291 In SHIP-2, we could attempt replication for 9 of the 14 mQTLs that were identified in the
292 discovery phase (metabolites corresponding to the remaining five mQTLs were not measured in
293 SHIP-2). In the initial replication analysis, which was performed using salivary metabolite data
294 that was not normalised for sample osmolality, 8 of the 9 discovery phase associations were
295 replicated ($p<0.05$), with the direction of effect consistent with that observed in TwinsUK (**Table**
296 **2**). The association between the *ABO* locus and N-acetylglucosamine/N-acetylgalactosamine was
297 the only finding from the discovery phase that did not replicate in SHIP-2. When we repeated the
298 replication analysis in SHIP-2 with metabolite data that was normalised for the sample
299 osmolality, the strength of all the associations was comparatively reduced (**Table S4**).

300

301 **Phenotypic associations for salivary metabolites**

302 Majority of the loci that were associated in our study have been reported in relation with GWAS
303 traits, inborn errors of metabolism and / or clinically relevant biochemical pathways (**Table 3**).
304 We further tested the salivary metabolites associated with the *DPYS*, *AGMAT* and *ATP13A5* loci
305 in relation with specific phenotypes using information available in the TwinsUK database (**Table**
306 **4**).

307 The *AGMAT*-associated metabolites (4-guanidinobutanoate and beta-guanidinopropanoate) are
308 generated in the polyamine synthesis pathway (https://www.genome.jp/kegg-
309 bin/show_pathway?hsa00330). This pathway also produces the compound putrescine, which has
310 been implicated in poor oral health and foul breath[28,29]. Consequently, we evaluated the
311 significance of the *AGMAT*-associated metabolites in oral health by testing them with PD status -
312 the levels of both 4-guanidinobutanoate and beta-guanidinopropanoate were significantly higher

313 in PD cases compared to controls (p=0.0003 and p=0.0006, respectively). Since eGFR (estimated
314 glomerular filtration rate) is calculated on the basis of serum creatinine, and these two commonly
315 used measures of renal function are negatively correlated, we wanted to investigate the
316 association between salivary creatinine and eGFR. We observed a similar strong negative
317 relationship between salivary creatinine and eGFR ($p=1.6 \times 10^{-11}$), which was indicative of a
318 homeostasis between creatinine concentrations in serum and saliva. Furthermore, creatinine is
319 also known to be a marker of muscle mass and strength[30]. We, therefore, tested salivary
320 creatinine in relation with grip strength (a measure of muscle strength), which suggested a
321 positive correlation between them (p=0.01).
322 Apart from these findings, the remaining phenotypic associations that we tested were largely
323 negative, as follows:
324 The enzyme agmatinase (encoded by the *AGMAT* gene), which acts on the substrate agmatine,
325 has been implicated in the pathophysiology of mood disorders[31]. Moreover, studies have also
326 proposed agmatine as a novel neuromodulator[32]. We, therefore, tested the *AGMAT*-associated
327 metabolites in relation with a diagnosis of clinical depression or anxiety disorder, and responses
328 (on a ordinal scale) to questions in the Hospital Anxiety and Depression Scale (HADS)
329 questionnaire[33]. But, neither analysis showed any significant association (**Table S5**).
330 *ATP13A5*, the locus that was associated with salivary creatinine, belongs to the family of
331 ATPases that regulate the activity of HMG-CoA reductase[34], the main site of action of the
332 cholesterol-lowering class of drugs called statins. Statins are known to cause muscle dysfunction
333 (myopathy) in a small fraction of patients[35]. Given that *ATP13A5* and statins both act in the same
334 biochemical pathway, we assessed whether salivary creatinine is also associated with statin

335 usage, and could therefore be used as a biomarker for statin-induced myopathy. There was,
336 however, no association between salivary creatinine and statin usage (p=0.22).
337 5-Fluorouracil (5-FU) is a pyrimidine analogue that is a commonly used anticancer drug. It is
338 eliminated from the body via the pyrimidine degradation pathway, hence variants in genes
339 coding for the pyrimidine degradation enzymes (for instance, *DPYS*) are known to be associated
340 with the development of 5-FU toxicity[36], which mainly manifests as gastrointestinal side effects.
341 Since we could not directly assess the *DPYS*-associated metabolites (3-ureidopropionate and 3-
342 ureidoisobutyrate) in relation to the gastrointestinal side effects of 5-FU toxicity, we tested the
343 metabolites with a commonly observed phenotype of gastrointestinal dysfunction, irritable bowel
344 syndrome or IBS (ascertained using the ROME-III questionnaire[37]). For both metabolites, we
345 observed that the levels were not significantly different in IBS "cases" compared to "controls"
346 (p>0.05, for both metabolites). However, this negative finding does not negate the possibility
347 that these metabolites could be of clinical use in predicting 5-FU toxicity.

348 # Discussion

349

350 Here we report a genome-wide association analysis of 476 metabolites measured in saliva
351 samples of healthy population-based studies of European descent. We identified a total of 11
352 distinct genetic loci that regulate the level of 14 salivary metabolites, of which three loci were
353 associated with more than one metabolite each.

354
355 The fact that saliva is reflective of the concentration of biochemicals in blood forms the basis for
356 certain clinical applications of saliva that others have proposed previously such as therapeutic
357 monitoring of drugs[38], cortisol measurement[39] and renal function monitoring[40]. Using salivary
358 metabolite data, we replicated the associations for certain well-established genetic loci that are
359 known to regulate the level of blood metabolites. Thus, our findings add further credence to the
360 notion that, as biofluids, a certain degree of homeostasis exists between blood and saliva.

361
362 Additionally, we identified some novel associations in our study, which have expanded our
363 knowledge of genetic influences on human metabolites. In particular, the association between the
364 *DPYS* locus and pyrimidine metabolites is intriguing because of its clinical relevance. Mutations
365 in the pyrimidine catabolism genes such as *DPYD* (encodes dihydropyrimidine dehydrogenase)
366 and *DPYS* (encodes dihydropyrimidinase) have been linked to inborn errors of metabolism[41,42]
367 [MIM:274270 and 222748, respectively] as well as development of severe toxicity to the
368 chemotherapeutic agent 5-FU[36,43,44]. Studies have previously demonstrated the applicability of
369 salivary measurement of certain pyrimidine pathway metabolites (uracil and dihydrouracil) for
370 evaluating 5-FU toxicity due to deficient *DPYD* activity[45]. In our study, variants in the *DPYS*
371 gene correlated with the levels of specific pyrimidine metabolites (3-ureidopropionate and 3-

372 ureidoisobutyrate). Therefore, studies to explore the utility of salivary measurements of these
373 metabolites as non-invasive tools for predicting 5-FU toxicity resulting from mutations that
374 affect *DPYS* activity are warranted. While we could not test 3-ureidopropionate and 3-
375 ureidoisobutyrate in relation to the gastrointestinal side effects of 5-FU toxicity, we did not find
376 any association between these metabolites and a phenotype relevant to gut dysfunction (IBS
377 phenotype). Similarly, the observed association between salivary ribonate and the *ENOSF1*
378 locus, overexpression of which has been shown to cause resistance of tumour cell lines to 5-
379 FU[27], was further indicative of the possible role of saliva as a tool for drug monitoring. The other
380 novel finding of note was the association between the *AGMAT* locus and the metabolites 4-
381 guanidinobutanoate and beta-guanidinopropanoate. The *AGMAT*-associated metabolites are
382 produced in the polyamine synthesis pathway, which also generates the compound putrescine
383 that has been implicated in oral health. Moreover, in our study, the *AGMAT*-associated
384 metabolites were correlated with PD status, a disease related to poor oral health. Together, these
385 findings suggest that the *AGMAT*-associated metabolites might serve as potential biomarkers for
386 oral health. On the other hand, though the agmatine pathway has been previously implicated in
387 mood disorders, we did not find any association between the *AGMAT*-associated metabolites and
388 either symptoms of or a prior diagnosis of clinical depression or anxiety disorder. Thus, evidence
389 based on the *AGMAT*-associated metabolites does not lend support to the hypothesis regarding a
390 potential link between pathways involved in maintaining oral health and regulation of mood[15,18].
391 There were a few other novel associations of interest in our study, such as the metabolite
392 gamma-carboxyglutamate (associated with the *MGP* locus, which is known to cause abnormal
393 vascular calcification in patients with cardiovascular disease[46]), for which we could not assess

394 the clinical significance since specific phenotypic information was not available in sufficient
395 numbers in those with salivary metabolite data.
396
397 Given that we identified some novel genetic associations for salivary metabolites i.e. genetic loci
398 that had not been reported in association with blood metabolites, it is indicative of the presence
399 of regulatory pathways that are specific to the salivary metabolome. For the novel genetic loci, in
400 most cases, the overlapping or the nearest gene transcript was expressed in one or more gut-
401 related tissues, including salivary glands. However, for these loci, we did not find much evidence
402 for cis-eQTL effects specific to salivary or other gut tissues, whereby we could attribute their
403 association with the respective salivary metabolite(s) to transcriptional regulation of overlapping
404 / neighbouring genes. Interestingly, there is growing evidence to suggest that the human
405 metabolome is a reflection of an interaction between the host and the gut microbiome[7,47,48] – it
406 will, therefore, be worth exploring whether the salivary metabolites that were associated in our
407 study correlate with the composition of the salivary microbiome. Attempting to elucidate, in this
408 manner, biological processes that might mediate the observed associations between genetic loci
409 and salivary metabolites will help broaden our understanding of the underlying pathways.
410
411 In summary, our study has provided an initial map of genetic loci that regulate the salivary
412 metabolome. Based on what has been observed for other complex human traits, future studies
413 with larger sample sizes are expected to uncover additional genetic loci with much smaller
414 effects on salivary metabolites. Furthermore, our study also offered insights into hitherto
415 unknown biological pathways involved in maintaining the levels of salivary metabolites. While
416 we did explore, to an extent, the clinical relevance of a few salivary metabolites of interest, a
417 more comprehensive analysis with a wider range of phenotypic domains will help broaden our

418 understanding of the relationship between the salivary metabolome and systemic health
419 conditions.

Supplemental Data

Supplemental data includes supplemental materials and methods, five supplemental tables and six supplemental figures.

Author Contributions

Conceived and designed the experiments: C.J.S., C.M., T.D.S., G.K., R.P.M., M.V.M, J.R., U.V.

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Wrote the manuscript: A.N., C.J.S., C.M., K.S., R.P.M.

All authors revised the manuscript

Declaration of Interests

R.P.M. is an employee of Metabolon, Inc. and, as such, has affiliations with or financial involvement with Metabolon, Inc.

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448 the Federal State of Mecklenburg-West Pomerania (SHIP; http://www.medizin.uni-
449 greifswald.de/cm).

450 **Web Resources**

451 1000 Genomes project: http://www.internationalgenome.org/

452 Metabolon: https://www.metabolon.com/

453 GEMMA: http://www.xzlab.org/software.html

454 KEGG: https://www.genome.jp/kegg/

455 GCTA: http://cnsgenomics.com/software/gcta/#COJO

456 GTEx: https://gtexportal.org/home/

457 NHGRI GWAS catalogue: https://www.ebi.ac.uk/gwas/

458 OMIM: http://omim.org/

459 HMDB: http://www.hmdb.ca/

460 LocusZoom: http://locuszoom.org/

References

1. DeBerardinis, R.J., and Thompson, C.B. (2012). Cellular metabolism and disease: what do metabolic outliers teach us? Cell *148*, 1132–1144.

2. Illig, T., Gieger, C., Zhai, G., Romisch-Margl, W., Wang-Sattler, R., Prehn, C., Altmaier, E., Kastenmuller, G., Kato, B.S., Mewes, H.-W., et al. (2010). A genome-wide perspective of genetic variation in human metabolism. Nat. Genet. *42*, 137–141.

3. Kettunen, J., Tukiainen, T., Sarin, A.-P., Ortega-Alonso, A., Tikkanen, E., Lyytikainen, L.-P., Kangas, A.J., Soininen, P., Wurtz, P., Silander, K., et al. (2012). Genome-wide association study identifies multiple loci influencing human serum metabolite levels. Nat. Genet. *44*, 269–276.

4. Shin, S.-Y., Fauman, E.B., Petersen, A.-K., Krumsiek, J., Santos, R., Huang, J., Arnold, M., Erte, I., Forgetta, V., Yang, T.-P., et al. (2014). An atlas of genetic influences on human blood metabolites. Nat. Genet. *46*, 543–550.

5. Long, T., Hicks, M., Yu, H.-C., Biggs, W.H., Kirkness, E.F., Menni, C., Zierer, J., Small, K.S., Mangino, M., Messier, H., et al. (2017). Whole-genome sequencing identifies common-to-rare variants associated with human blood metabolites. Nat. Genet. *49*, 568–578.

6. Gieger, C., Geistlinger, L., Altmaier, E., Hrabe de Angelis, M., Kronenberg, F., Meitinger, T., Mewes, H.-W., Wichmann, H.-E., Weinberger, K.M., Adamski, J., et al. (2008). Genetics meets metabolomics: a genome-wide association study of metabolite profiles in human serum. PLoS Genet. *4*, e1000282.

7. Zierer, J., Jackson, M.A., Kastenmuller, G., Mangino, M., Long, T., Telenti, A., Mohney, R.P., Small, K.S., Bell, J.T., Steves, C.J., et al. (2018). The fecal metabolome as a functional readout of the gut microbiome. Nat. Genet. *50*, 790–795.

8. Suhre, K., Wallaschofski, H., Raffler, J., Friedrich, N., Haring, R., Michael, K., Wasner, C., Krebs, A., Kronenberg, F., Chang, D., et al. (2011). A genome-wide association study of metabolic traits in human urine. Nat. Genet. *43*, 565–569.

9. Luykx, J.J., Bakker, S.C., Lentjes, E., Neeleman, M., Strengman, E., Mentink, L., DeYoung, J., de Jong, S., Sul, J.H., Eskin, E., et al. (2014). Genome-wide association study of monoamine metabolite levels in human cerebrospinal fluid. Mol. Psychiatry *19*, 228–234.

10. Soini, H.A., Klouckova, I., Wiesler, D., Oberzaucher, E., Grammer, K., Dixon, S.J., Xu, Y., Brereton, R.G., Penn, D.J., and Novotny, M. V (2010). Analysis of volatile organic compounds in human saliva by a static sorptive extraction method and gas chromatography-mass spectrometry. J. Chem. Ecol. *36*, 1035–1042.

11. Barnes, V.M., Kennedy, A.D., Panagakos, F., Devizio, W., Trivedi, H.M., Jonsson, T., Guo, L., Cervi, S., and Scannapieco, F.A. (2014). Global metabolomic analysis of human saliva and plasma from healthy and diabetic subjects, with and without periodontal disease. PLoS One *9*, e105181.

12. Wishart, D.S., Feunang, Y.D., Marcu, A., Guo, A.C., Liang, K., Vazquez-Fresno, R., Sajed, T., Johnson, D., Li, C., Karu, N., et al. (2018). HMDB 4.0: the human metabolome database for 2018. Nucleic Acids Res. *46*, D608–D617.

13. Spielmann, N., and Wong, D.T. (2011). Saliva: diagnostics and therapeutic perspectives. Oral Dis. *17*, 345–354.

14. Chiappin, S., Antonelli, G., Gatti, R., and De Palo, E.F. (2007). Saliva specimen: a new laboratory tool for diagnostic and basic investigation. Clin. Chim. Acta. *383*, 30–40.

15. Kisely, S., Baghaie, H., Lalloo, R., Siskind, D., and Johnson, N.W. (2015). A systematic review and meta-analysis of the association between poor oral health and severe mental illness. Psychosom. Med. *77*, 83–92.

16. Kaufman, E., and Lamster, I.B. (2002). The diagnostic applications of saliva--a review. Crit. Rev. Oral Biol. Med. *13*, 197–212.

17. de Oliveira, C., Watt, R., and Hamer, M. (2010). Toothbrushing, inflammation, and risk of cardiovascular disease: results from Scottish Health Survey. BMJ *340*, c2451.

18. O'Neil, A., Berk, M., Venugopal, K., Kim, S.-W., Williams, L.J., and Jacka, F.N. (2014). The association between poor dental health and depression: findings from a large-scale, population-based study (the NHANES study). Gen. Hosp. Psychiatry *36*, 266–270.

19. Noble, J.M., Borrell, L.N., Papapanou, P.N., Elkind, M.S. V, Scarmeas, N., and Wright, C.B. (2009). Periodontitis is associated with cognitive impairment among older adults: analysis of NHANES-III. J. Neurol. Neurosurg. Psychiatry *80*, 1206–1211.

20. Moayyeri, A., Hammond, C.J., Valdes, A.M., and Spector, T.D. (2013). Cohort Profile: TwinsUK and healthy ageing twin study. Int. J. Epidemiol. *42*, 76–85.

21. Auton, A., Brooks, L.D., Durbin, R.M., Garrison, E.P., Kang, H.M., Korbel, J.O., Marchini, J.L., McCarthy, S., McVean, G.A., and Abecasis, G.R. (2015). A global reference for human genetic variation. Nature *526*, 68–74.

515 22. Kanehisa, M., Goto, S., Sato, Y., Furumichi, M., and Tanabe, M. (2012). KEGG for integration and
516 interpretation of large-scale molecular data sets. Nucleic Acids Res. *40*, D109–D114.
517 23. Zhou, X., and Stephens, M. (2012). Genome-wide efficient mixed-model analysis for association studies. Nat.
518 Genet. *44*, 821–824.
519 24. Yang, J., Ferreira, T., Morris, A.P., Medland, S.E., Madden, P.A.F., Heath, A.C., Martin, N.G., Montgomery,
520 G.W., Weedon, M.N., Loos, R.J., et al. (2012). Conditional and joint multiple-SNP analysis of GWAS summary
521 statistics identifies additional variants influencing complex traits. Nat. Genet. *44*, 369–375, S1–S3.
522 25. Kurushima, Y., Tsai, P.-C., Castillo-Fernandez, J., Couto Alves, A., El-Sayed Moustafa, J.S., Le Roy, C.,
523 Spector, T.D., Ide, M., Hughes, F.J., Small, K.S., et al. (2019). Epigenetic findings in periodontitis in UK twins: a
524 cross-sectional study. Clin. Epigenetics *11*, 27.
525 26. Dudkowska, M., Lai, J., Gardini, G., Stachurska, A., Grzelakowska-Sztabert, B., Colombatto, S., and
526 Manteuffel-Cymborowska, M. (2003). Agmatine modulates the in vivo biosynthesis and interconversion of
527 polyamines and cell proliferation. Biochim. Biophys. Acta *1619*, 159–166.
528 27. Wichelecki, D.J., Froese, D.S., Kopec, J., Muniz, J.R.C., Yue, W.W., and Gerlt, J.A. (2014). Enzymatic and
529 structural characterization of rTSgamma provides insights into the function of rTSbeta. Biochemistry *53*, 2732–
530 2738.
531 28. Goldberg, S., Kozlovsky, A., Gordon, D., Gelernter, I., Sintov, A., and Rosenberg, M. (1994). Cadaverine as a
532 putative component of oral malodor. J. Dent. Res. *73*, 1168–1172.
533 29. Porter, S.R., and Scully, C. (2006). Oral malodour (halitosis). BMJ *333*, 632–635.
534 30. Patel, S.S., Molnar, M.Z., Tayek, J.A., Ix, J.H., Noori, N., Benner, D., Heymsfield, S., Kopple, J.D., Kovesdy,
535 C.P., and Kalantar-Zadeh, K. (2013). Serum creatinine as a marker of muscle mass in chronic kidney disease: results
536 of a cross-sectional study and review of literature. J. Cachexia. Sarcopenia Muscle *4*, 19–29.
537 31. Bernstein, H.-G., Stich, C., Jager, K., Dobrowolny, H., Wick, M., Steiner, J., Veh, R., Bogerts, B., and Laube, G.
538 (2012). Agmatinase, an inactivator of the putative endogenous antidepressant agmatine, is strongly upregulated in
539 hippocampal interneurons of subjects with mood disorders. Neuropharmacology *62*, 237–246.
540 32. Regunathan, S. (2006). Agmatine: biological role and therapeutic potentials in morphine analgesia and
541 dependence. AAPS J. *8*, E479–E484.
542 33. Stern, A.F. (2014). The hospital anxiety and depression scale. Occup. Med. (Lond). *64*, 393–394.
543 34. Roitelman, J., and Simoni, R.D. (1992). Distinct sterol and nonsterol signals for the regulated degradation of 3-
544 hydroxy-3-methylglutaryl-CoA reductase. J. Biol. Chem. *267*, 25264–25273.
545 35. Sathasivam, S., and Lecky, B. (2008). Statin induced myopathy. BMJ *337*, a2286.
546 36. Akai, F., Hosono, H., Hirasawa, N., and Hiratsuka, M. (2015). Novel single nucleotide polymorphisms of the
547 dihydropyrimidinase gene (DPYS) in Japanese individuals. Drug Metab. Pharmacokinet. *30*, 127–129.
548 37. Longstreth, G.F., Thompson, W.G., Chey, W.D., Houghton, L.A., Mearin, F., and Spiller, R.C. (2006).
549 Functional bowel disorders. Gastroenterology *130*, 1480–1491.
550 38. Drobitch, R.K., and Svensson, C.K. (1992). Therapeutic drug monitoring in saliva. An update. Clin.
551 Pharmacokinet. *23*, 365–379.
552 39. Aardal-Eriksson, E., Karlberg, B.E., and Holm, A.C. (1998). Salivary cortisol--an alternative to serum cortisol
553 determinations in dynamic function tests. Clin. Chem. Lab. Med. *36*, 215–222.
554 40. Goll, R.D., and Mookerjee, B.K. (1978). Correlation of biochemical parameters in serum and saliva in chronic
555 azotemic patients and patients on chronic hemodialysis. J. Dial. *2*, 344–399.
556 41. Hamajima, N., Kouwaki, M., Vreken, P., Matsuda, K., Sumi, S., Imaeda, M., Ohba, S., Kidouchi, K., Nonaka,
557 M., Sasaki, M., et al. (1998). Dihydropyrimidinase deficiency: structural organization, chromosomal localization,
558 and mutation analysis of the human dihydropyrimidinase gene. Am. J. Hum. Genet. *63*, 717–726.
559 42. Berger, R., Stoker-de Vries, S.A., Wadman, S.K., Duran, M., Beemer, F.A., de Bree, P.K., Weits-Binnerts, J.J.,
560 Penders, T.J., and van der Woude, J.K. (1984). Dihydropyrimidine dehydrogenase deficiency leading to thymine-
561 uraciluria. An inborn error of pyrimidine metabolism. Clin. Chim. Acta. *141*, 227–234.
562 43. Wei, X., McLeod, H.L., McMurrough, J., Gonzalez, F.J., and Fernandez-Salguero, P. (1996). Molecular basis of
563 the human dihydropyrimidine dehydrogenase deficiency and 5-fluorouracil toxicity. J. Clin. Invest. *98*, 610–615.
564 44. van Kuilenburg, A.B., Haasjes, J., Richel, D.J., Zoetekouw, L., Van Lenthe, H., De Abreu, R.A., Maring, J.G.,
565 Vreken, P., and van Gennip, A.H. (2000). Clinical implications of dihydropyrimidine dehydrogenase (DPD)
566 deficiency in patients with severe 5-fluorouracil-associated toxicity: identification of new mutations in the DPD
567 gene. Clin. Cancer Res. *6*, 4705–4712.
568 45. Neto, O.V., Raymundo, S., Franzoi, M.A., do Carmo Artmann, A., Tegner, M., Muller, V.V., Hahn, R.Z., Alves,
569 G.V., Schwartsmann, G., Linden, R., et al. (2018). DPD functional tests in plasma, fresh saliva and dried saliva
570 samples as predictors of 5-fluorouracil exposure and occurrence of drug-related severe toxicity. Clin. Biochem. *56*,

571 18–25.
572 46. Mizobuchi, M., Towler, D., and Slatopolsky, E. (2009). Vascular calcification: the killer of patients with chronic
573 kidney disease. J. Am. Soc. Nephrol. *20*, 1453–1464.
574 47. Chen, M.X., Wang, S.-Y., Kuo, C.-H., and Tsai, I.-L. (2019). Metabolome analysis for investigating host-gut
575 microbiota interactions. J. Formos. Med. Assoc. *118 Suppl 1*, S10–S22.
576 48. Peisl, B.Y.L., Schymanski, E.L., and Wilmes, P. (2018). Dark matter in host-microbiome metabolomics:
577 Tackling the unknowns-A review. Anal. Chim. Acta *1037*, 13–27.
578 49. Alfonso, P., Navascues, J., Navarro, S., Medina, P., Bolado-Carrancio, A., Andreu, V., Irun, P., Rodriguez-Rey,
579 J.C., Pocovi, M., Espana, F., et al. (2013). Characterization of variants in the glucosylceramide synthase gene and
580 their association with type 1 Gaucher disease severity. Hum. Mutat. *34*, 1396–1403.
581 50. Hirano, H., Ezura, Y., Ishiyama, N., Yamaguchi, M., Nasu, I., Yoshida, H., Suzuki, T., Hosoi, T., and Emi, M.
582 (2003). Association of natural tooth loss with genetic variation at the human matrix Gla protein locus in elderly
583 women. J. Hum. Genet. *48*, 288–292.
584

585 # Figure Titles and Legends

586

587 **Figure 1: Manhattan plot illustrating the findings of the discovery phase (TwinsUK) of the**
588 **genome-wide association study for salivary metabolites (mGWAS)**

589 The red horizontal line demarcates the study-wide significance threshold of 10^{-10}. Eleven loci
590 surpassed the study-wide significance threshold. The loci are referred to by the name(s) of the
591 overlapping or the nearest gene(s).

592

593 **Figure S1: Comparison of the distribution of saliva sample osmolality in TwinsUK and**
594 **SHIP-2**

595 The distribution of saliva sample osmolality observed in SHIP-2 was much narrower in
596 comparison to TwinsUK, indicative of the fact that, in contrast to TwinsUK, the saliva samples
597 in SHIP-2 represented stimulated saliva.

598

599 **Figure S2: Plots demonstrating the correlation between the pairs of metabolites that were**
600 **associated with the same locus in the discovery phase**

601 The figure demonstrates the correlation between the pairs of metabolites which were regulated
602 by the same locus, as follows (i-iii): beta-guanidinopropanoate and 4-guanidinobutanoate
603 (*AGMAT*); urate and allantoin (*SLC2A9*); 3-ureidopropionate and 3-ureidoisobutyrate (*DPYS*).

604

605 **Figure S3: Quantile-quantile (QQ) plots of the genome-wide association analysis for the 14**
606 **salivary metabolites that were significantly associated in the discovery phase**

607 The figure demonstrates the QQ plots for the significantly associated salivary metabolites, as
608 follows (i-xiv): 4-guanidinobutanoate; beta-guanidinopropanoate; creatinine; urate; allantoin;
609 dimethylglycine; 3-ureidopropionate; 3-ureidoisobutyrate; N-acetylglucosamine/N-
610 acetylgalactosamine; glycosyl-N-stearoyl-sphinganine; 1-(1-enyl-palmitoyl)-2-arachidonoyl-
611 GPC; ethylmalonate; gamma-carboxyglutamte; ribonate.

612

613 **Figure S4: A comparison of the effect of the index variant (from the salivary mGWAS)**
614 **using measurements of the corresponding metabolite in faecal, serum and saliva samples of**
615 **the TwinsUK subjects**

616 The plots demonstrate a comparison of the effect of the index variant (from the salivary
617 mGWAS) for the significantly associated salivary metabolites that were measured in all three
618 biospecimens (faeces, serum and saliva), as follows (i-vii): 4-guanidinobutanoate; creatinine;
619 urate; dimethylglycine; 3-ureidopropionate; ethylmalonate; ribonate.
620
621 **Figure S5: Regional association plots for the genetic loci that were significantly associated**
622 **with a salivary metabolite in the discovery phase**
623 Regional association plots were created using the LocusZoom tool for the locus-metabolite
624 associations (mQTLs) that were identified in the discovery phase, as follows (i-xiii): *AGMAT*
625 and 4-guanidinobutanoate; *AGMAT* and beta-guanidinopropanoate; *ATP13A5* and creatinine;
626 *SLC2A9* and urate; *SLC2A9* and allantoin; *DMGDH* and dimethylglycine; *DPYS* and 3-
627 ureidopropinoate; *DPYS* and 3-ureidoisobutyrate; *ABO* and N-acetylglucosamine/N-
628 acetylgalactosamine; *UGCG* and glycosyl-N-stearoyl-sphinganine; *FADS2* and 1-(1-enyl-
629 palmitoyl)-2-arachidonoyl-GPC; *ACADS* and ethylmalonate; *TYMS/ENOSF1* and ribonate
630 *The regional association plot for the *MGP* locus has not been included since LD information for
631 variants at that locus was not well characterised in the 1000 Genomes dataset.
632
633 **Figure S6: Boxplots demonstrating the distribution of the significantly associated salivary**
634 **metabolites, stratified by the genotypes of the corresponding index variant**
635 The 14 salivary metabolites that were identified in the discovery phase of our study mapped to
636 11 distinct genetic loci: of which, three loci (*AGMAT*, *SLC2A9* and *DPYS*) were associated with
637 two metabolites each; and the remaining eight loci were each associated with one metabolite.
638 The plots demonstrate the distribution of the significantly associated salivary metabolite with
639 respect to the genotypes of the corresponding index variant (for each index variant, the number
640 of observations for each genotype is provided), as follows (i-xiv): 4-guanidinobutanoate; beta-
641 guanidinopropanoate; creatinine; urate; allantoin; dimethylglycine; 3-ureidopropionate; 3-
642 ureidoisobutyrate; N-acetylglucosamine/N-acetylgalactosamine; glycosyl-N-stearoyl-
643 sphinganine; 1-(1-enyl-palmitoyl)-2-arachidonoyl-GPC; ethylmalonate; gamma-
644 carboxyglutamte; ribonate.

Tables

Locus	Index variant	Chr	Position (build 37 bp)	Biochemical (Metabolite)	EA	EAF	Beta	SE	P-value
AGMAT	rs10927806	1	15,909,239	4-guanidinobutanoate	C	0.45	0.252	0.037	4.4×10^{-11}
	rs6690813	1	15,861,073	beta-guanidinopropanoate	C	0.45	0.708	0.034	4.6×10^{-66}
ATP13A5	rs55918334	3	193,086,314	creatinine	G	0.53	0.305	0.039	3.9×10^{-14}
SLC2A9	rs13129697	4	9,926,967	urate	T	0.72	0.337	0.042	1.3×10^{-14}
	*rs7675964	4	9,941,434	allantoin	C	0.73	0.239	0.043	4.1×10^{-8}
DMGDH	rs248386	5	78,330,227	dimethylglycine	C	0.81	-0.349	0.048	1.1×10^{-12}
DPYS	rs80274300	8	105,470,882	3-ureidopropionate	C	0.81	-0.764	0.043	8.1×10^{-54}
	rs80274300	8	105,470,882	3-ureidoisobutyrate	C	0.81	-0.482	0.048	5.8×10^{-22}
ABO	rs201298979	9	136,145,424	N-acetylglucosamine/ N-acetylgalactosamine	AC	0.77	-0.314	0.046	2.6×10^{-11}
UGCG	rs10981098	9	114,591,623	glycosyl-N-stearoyl-sphinganine	T	0.79	-0.308	0.046	4.6×10^{-11}
FADS2	rs174564	11	61,588,305	1-(1-enyl-palmitoyl)-2-arachidonoyl-GPC	A	0.67	0.324	0.039	1.9×10^{-15}
ACADS	rs34673751	12	121,171,891	ethylmalonate	G	0.74	-0.539	0.041	5.1×10^{-34}
MGP	rs5796614	12	15,053,094	gamma-carboxyglutamate	G	0.6	-0.29	0.038	1.9×10^{-13}
TYMS/ ENOSF1	rs2790	18	673,086	ribonate	A	0.81	0.325	0.049	5.8×10^{-11}

Table 1: Summary of genetic loci that were significantly associated with salivary metabolite(s) in the discovery phase (TwinsUK)

(Chr = Chromosome; EA = Effect Allele; EAF = Effect Allele Frequency; SE = Standard Error)

For the primary stage of association analysis in the discovery phase (TwinsUK), locus-metabolite associations (mQTLs) were identified using a genome-wide and metabolome-wide significance threshold of $p<10^{-10}$.

*This mQTL was identified when the analysis was restricted to only the loci that were identified in the primary stage of association testing (significance threshold of $p<10^{-6}$)

Locus	Chr	Index variant	Biochemical (Metabolite)	EA	Beta	SE	P-value
AGMAT	1	rs10927806	4-guanidinobutanoate	C	0.115	0.044	8.9×10^{-3}
ATP13A5	3	rs55918334	creatinine	G	0.239	0.04	2.8×10^{-9}
SLC2A9	4	rs13129697	urate	T	0.576	0.045	7.7×10^{-35}
	4	rs7675964	allantoin	C	0.209	0.049	2.3×10^{-5}
DMGDH	5	rs248386	dimethylglycine	C	-0.336	0.055	1.4×10^{-9}
DPYS	8	rs80274300	3-ureidopropionate	C	-1.035	0.049	3.1×10^{-82}
ABO¶	9	rs9411378	N-acetylglucosamine/ N-acetylgalactosamine	A	0.032	0.048	0.5
ACADS	12	rs34673751	ethylmalonate	G	-0.132	0.049	7.3×10^{-3}
TYMS/ ENOSF1	18	rs2790	ribonate	A	0.137	0.055	0.013

Table 2: Summary of the discovery phase associations that were tested in the replication study (SHIP-2)

(Chr = Chromosome; EA = Effect Allele; SE = Standard Error)

¶Since information for the index variant (rs201298979) at the *ABO* locus was not available in the replication study, a proxy variant (rs9411378) that was in high LD (r^2=0.95) with the index variant was used for the replication analysis.

Replication in SHIP-2 could be attempted for 9 of the 14 mQTLs that were identified in the discovery phase (metabolites corresponding to the remaining five mQTLs were not measured in SHIP-2). For each of these nine mQTLs, the most significant variant-metabolite pair identified in the discovery phase was tested in SHIP-2 using salivary metabolite data that was not normalised for osmolality (since SHIP-2 saliva samples represented stimulated saliva).

Locus	Biochemical (Metabolite)	Metabolite super-pathway[1]	Metabolite sub-pathway[1]	Known GWAS disease associations for the locus[2]	Known OMIM phenotype for the locus[3]	Clinical significance of the locus, the metabolite or the biochemical pathway involved
AGMAT	4-guanidinobutanoate	Amino acid	Guanidino and Acetamido metabolism	Glomerular filtration rate, alcoholic chronic pancreatitis	-	Putrescine, a compound that is generated in the pathway which involves the *AGMAT* gene i.e. polyamine synthesis pathway, has been implicated in bad breath / oral health[28,29]
	beta-guanidinopropanoate	Xenobiotics	Plant (food) component			
ATP13A5	creatinine	Amino acid	Creatine metabolism	-	Kufor-Rakeb syndrome	The *ATP13A5* gene encodes the family of proteins that regulate the activity of HMG-CoA reductase[34], the rate-limiting enzyme in cholesterol synthesis
SLC2A9	urate	Nucleotide	Hypo(Xanthine) / Inosine (purine) metabolism	Gout	Renal hypouricemia type 2	The *SLC2A9* gene encodes a carrier protein that is involved in urate transport in the proximal convoluted tubules of kidneys
	allantoin	Nucleotide	Hypo(Xanthine) / Inosine (purine) metabolism			
DMGDH	dimethylglycine	Amino acid	Glycine, Serine and Threonine metabolism	-	Dimethylglycine dehydrogenase deficiency	The *DMGDH* gene encodes an enzyme (dimethylglycine dehydrogenase), which catalyses the conversion of dimethylglycine to sarcosine
DPYS	3-ureidopropionate	Nucleotide	Uracil (pyrimidine) metabolism	Paget's disease	Dihydropyrimidinuria	Variants in the *DPYS* gene, which encodes an enzyme in the pyrimidine degradation pathway, have been associated with 5-fluorouracil toxicity[36]
	3-ureidoisobutyrate	Nucleotide	Uracil (pyrimidine) metabolism			
ABO	N-acetylglucosamine/ N-acetylgalactosamine	Carbohydrate	Aminosugar metabolism	Gastric carcinoma, stroke, venous thromboembolism, ovarian cancer, malaria, blood cholesterol level, type 2 diabetes	-	The *ABO* gene encodes a protein with glycosyltransferase activity, which forms the basis of the ABO blood group system
UGCG	glycosyl-N-stearoyl-sphinganine	Lipid	Ceramide metabolism	-	-	The *UGCG* gene is involved in glycosphingolipid synthesis, defects in which are known to cause Gaucher's disease[49], a lysosomal storage disorder
FADS2	1-(1-enyl-palmitoyl)-2-arachidonoyl-GPC	Lipid	Plasmalogen	ADHD, blood cholesterol level	-	The *FADS2* gene encodes an enzyme (fatty acid desaturase) which catalyses the rate-limiting step in the desaturation of polyunsaturated fatty acids (PUFA)
ACADS	ethylmalonate	Amino acid	Leucine, Isoleucine and Valine metabolism	-	Ethylmalonic aciduria	The *ACADS* gene encodes the acyl-CoA dehydrogenase enzyme, which catalyses the initial step in the fatty acid of oxidation pathway
MGP	gamma-carboxyglutamate	Amino acid	Glutamate metabolism	-	Keutel syndrome	The *MGP* gene, a regulator of physiologic tissue calcification, is associated with calcification of vasculature in patients with cardiovascular disease[46]. The *MGP* gene has also been associated with natural tooth loss in elderly women[50]
TYMS/ ENOSF1	ribonate	Carbohydrate	Pentose metabolism	Hypertension	-	The *TYMS* gene encodes the enzyme thymidylate synthase, the main site of action of 5-fluorouracil. The *ENOSF1* gene encodes the antisense RNA of *TYMS*

Table 3: Annotation of the genetic loci and the corresponding metabolites that were significantly associated in the discovery phase

[1]obtained from the KEGG database

[2]accessed from the NHGRI GWAS catalogue

[3]accessed from the OMIM database

The mQTLs that were identified in the discovery phase were annotated for the metabolite pathway, known disease associations for the locus from the GWAS and the OMIM databases, and the clinical significance of the locus, the metabolite or the biochemical pathway involved.

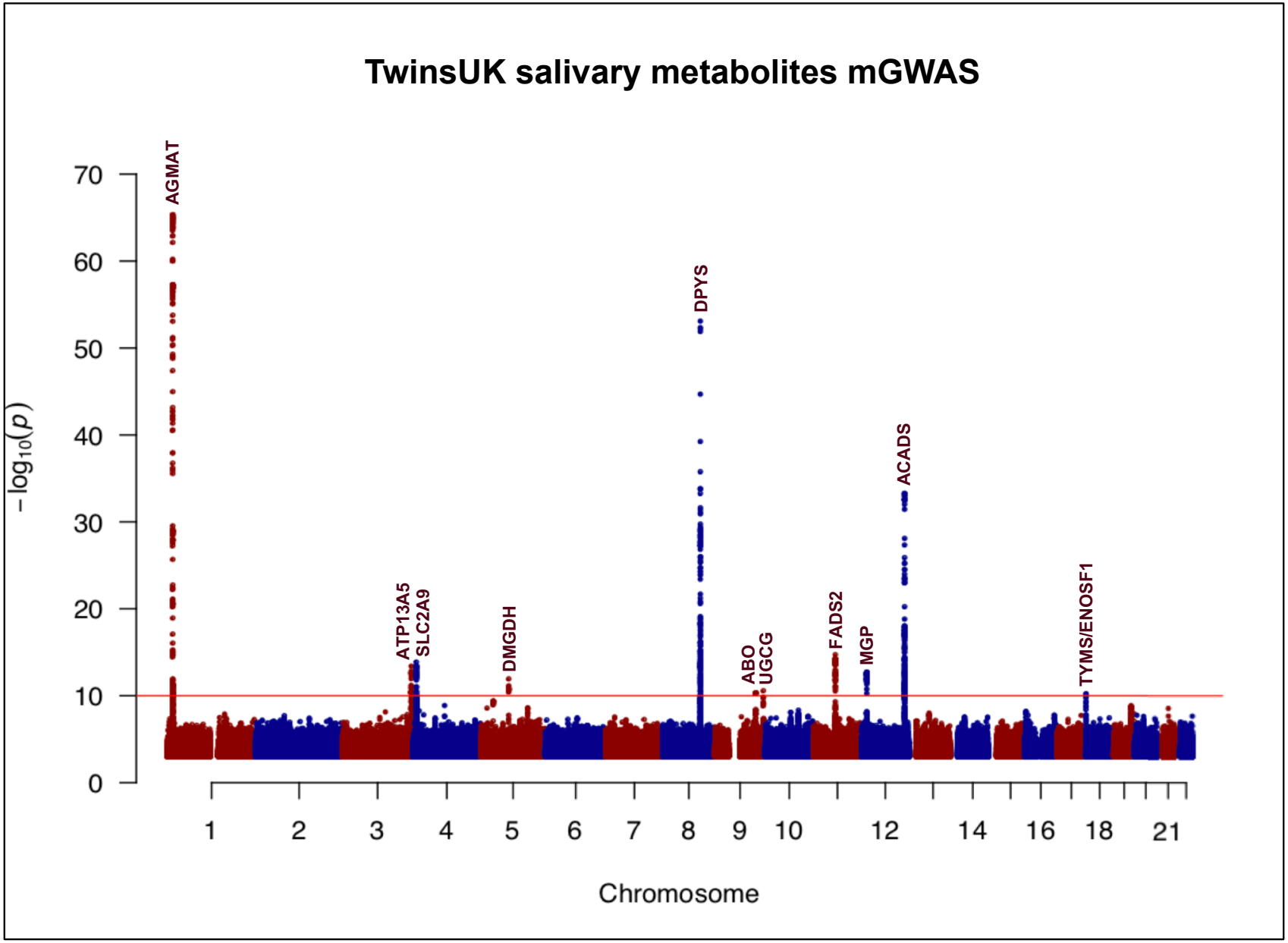
Locus	Chr	Biochemical (Metabolite)	Phenotypic association(s) tested
AGMAT	1	4-guanidinobutanoate	1. Periodontal disease 2. Clinical depression or anxiety disorder
		beta-guanidinopropanoate	
ATP13A5	3	creatinine	1. eGFR (measure of renal function) 2. Grip strength (measure of muscle strength) 3. Statin usage
DPYS	8	3-ureidopropionate	1. Irritable bowel syndrome (based on the ROME-III criteria[36])
		3-ureidoisobutyrate	

Table 4: Summary of the phenotypes that were tested in relation with specific salivary metabolites

(Chr = Chromosome; eGFR = effective Glomerular Filtration Rate)

The metabolites that were uniquely associated in saliva i.e. the ones for which a genetic association had not been previously reported in blood, were tested with phenotypes (diseases / traits / adverse drug effects) relating to the metabolite or its associated biochemical pathway, using information from the TwinsUK database.

Figure 1


TwinsUK salivary metabolites mGWAS
AGMAT
ATP13A5
SLC2A9
DMGDH
DPYS
ABO
UGCG
FADS2
MGP
ACADS
TYMS/ENOSF1
70
60
50
40
30
20
10
0
$-\log_{10}(p)$
1
2
3
4
5
6
7
8
9
10
12
14
16
18
21
Chromosome