1 **Physiological and environmental parameters associated with mass**

2 **spectrometry-based salivary metabolomic profiles**

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4   **Abbreviated title:** Parameters associated with salivary metabolomic profiles

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ABSTRACT

Mass spectrometry-based metabolomic methods enable simultaneous profiling of hundreds of salivary metabolites, and may be useful to diagnose a wide range of diseases using saliva. However, few studies have evaluated the effects of physiological or environmental factors on salivary metabolomic profiles. Therefore, we used capillary electrophoresis-mass spectrometry to analyze saliva metabolite profiles in 155 subjects with reasonable oral hygiene, and examined the effects of physiological and environmental factors on the metabolite profiles. Overall, 257 metabolites were identified and quantified. The global profiles and individual metabolites were evaluated by principle component analysis and univariate tests, respectively. Collection method, collection time, sex, body mass index, and smoking affected the global metabolite profiles. However, age also contributed to the bias in sex and collection time. The profiles were relatively unaffected by other parameters, such as alcohol consumption and smoking, tooth brushing, or the use of medications or nutritional supplements. Temporomandibular joint disorders had relatively greater effects on salivary metabolites than other dental abnormalities (e.g., stomatitis, tooth alignment, and dental caries). These findings

1 **provide further insight into the diversity and stability of salivary metabolomic**

2 **profiles, as well as the generalizability of disease-specific biomarkers.**

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4 **Key Words:** capillary electrophoresis; metabolomics; mass spectrometry; oral hygiene;

5 saliva; temporomandibular joint disorders

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1 Introduction

Saliva is a readily accessible and informative biofluid that is ideal for the early detection of many diseases, including periodontitis, hepatitis, human immunodeficiency virus, viral hepatitis, and cancer (Lee et al., 2009). It can also be used to monitor the concentrations of various drugs, including marijuana, cocaine, and phencyclidine (Schramm et al., 1992). Advantages of using saliva as a diagnostic tool are that it is easy and noninvasive to collect, associated with less discomfort than venipuncture, and does not entail privacy issues, unlike urine collection (Lee and Wong, 2009). Consequently, *omics* technologies, including transcriptomics and proteomics, have been used for high-throughput identification of disease-associated salivary biomarkers, to better understand the complex biology of the oral cavity, and facilitate the diagnosis of diseases in and remote from the oral cavity (Grant, 2012, Spielmann and Wong, 2011).

Metabolomic profiles lying downstream of the regulatory information derived from other *omics* data are thought to directly reflect physical phenotypes. They also provide a new opportunity to use saliva for the diagnosis of diseases, including periodontal diseases, leukoplakia, and oral and systemic cancers (Aimetti et al., 2011, Barnes et al., 2011, Sugimoto et al., 2010, Wei et al., 2010, Yan et al., 2008). However, salivary metabolomic profiles may be affected by physiological, pathological, and

1 environmental factors, independently of a disease. Thus, the pioneering metabolomics

2 studies used [1]H-nuclear magnetic resonance (NMR) to evaluate the associations

3 between salivary metabolites and parameters of interest, including saliva collection

4 method (stimulated and unstimulated), sex, smoking, time of collection (i.e., diurnal

5 variation), body mass index (BMI), blood pressure, and dietary intake (Silwood et al.,

6 2002, Walsh et al., 2006, Takeda et al., 2009). More recently, profiling technologies

7 have switched to hyphenated mass spectrometry (MS), which allows simultaneous

8 quantification of a larger number and a wider range of metabolites compared with

9 NMR-based methods (Barnes et al., 2011, Sugimoto et al., 2010). However, to our

10 knowledge, the effects of physiological, pathological, and environmental factors on the

11 diversity and stability of MS-based salivary metabolomic profiles have not been

12 reported.

13   In this study, we used capillary electrophoresis-time-of-flight mass spectrometry

14 (CE-TOFMS) (Soga et al., 2006) to detect and quantify charged metabolites in saliva

15 obtained from healthy young subjects with reasonable oral hygiene (i.e., the subjects

16 were without periodontal disease or gingival inflammation at least). We focused on

17 females in this study because of the complexity of endogenous metabolism attributable

18 to female hormones, although we also investigated sex-specific differences as a pilot

1 study. The profiles were analyzed according to relevant physiological and

2 environmental factors to better understand the metabolomic characteristics of saliva.

3

2. Materials and Methods

2.1. Subjects and saliva collection

6 This study was approved by the Ethics Committee of the Kanagawa Dental College.

7 Volunteers were recruited from Kanagawa Dental College and Yokohama School of

8 Dental Technology. Written, informed consent was obtained from all patients and from

9 volunteers who agreed to provide saliva samples. Physiological, clinical, and lifestyle

10 characteristics were recorded by questionnaires. Oral cavity disorders included

11 stomatitis, glossalgia, xerostomia, and bruxism. The number of dental caries, presence

12 of periodontitis, and temporomandibular joint disorder (TMD) were also determined.

13 No patient with periodontal disease or glossalgia was included. The comparative study

14 design is presented in Fig. 1.

15   Saliva was collected at the same time in each subject (11:40 and 16:00), after

16 fasting for ≥ 1 h ($n = 104$) after breakfast or lunch. The mean ± standard deviation (SD)

17 age was 22.4 ± 4.4 years (range, 19–41 years) for the subjects whose saliva were

18 collected at morning ($n = 86$, including 4 with missing values) and 21.0 ± 5.0 years

1 (range, 18–27 years) for subjects whose saliva was collected in the afternoon ($n = 18$).

2 Saliva samples were collected by the absorbent method using salivettes (Sarstedt Co.

3 Ltd., Nümbrecht, Germany). Eighteen females provided saliva samples using both the

4 absorbent and the passive drool methods. During collection, the tube was kept on ice,

5 and the samples were immediately stored at –80°C. Only saliva samples used for

6 comparisons between sex (24 females and 27 males) were collected at 16:00 on another

7 day. The mean ± SD ages of these males and females were 21.8 ± 5.6 years (range,

8 18–43 years; $n = 24$) and 23.7 ± 5.0 years (range, 18–36 years; $n = 27$), respectively.

9

10 **2.2. Metabolomic analysis**

11 Frozen saliva was thawed and centrifuged through a 5-kDa cutoff filter (Millipore,

12 Bedford, MA) at 9,100×g for at least 2.5 h at 4°C to remove macromolecules. Five

13 microliters of Milli-Q water containing internal standards (2 mmol/l each of methionine

14 sulfone, 2-[N-morpholino]-ethanesulfonic acid, D-camphor-10-sulfonic acid,

15 3-aminopyrrolidine, and trimesate) was added to 45 μL of the filtrate and mixed.

16 CE-TOFMS was used to simultaneously quantify charged metabolites in the positive

17 and negative modes. Metabolites between 50 and 1,000 *m/z* were detected and matched

18 with compounds in our standard library. The instrumental parameters and measurement

1   conditions are described in the Supplementary Material and Methods.

2

3   **2.3. Data analysis**

4   Raw data were analyzed using our proprietary system, which detected all of the possible

5   peaks, eliminated noise and redundant features, and generated the aligned data matrix

6   (Sugimoto et al., 2010). Metabolite identification was conducted by matching the *m/z*

7   values and the normalized migration times with those of our standard compounds. The

8   concentrations of individual metabolites were calculated using internal and external

9   standards, a typical workflow for CE-TOFMS data processing (Sugimoto et al., 2012b).

10   Principal component analysis (PCA) was used to assess the diversity of the

11   metabolomic profiles. Different numbers of individuals and metabolites were used in

12   each PCA analysis. For example, to analyze the effects of smoking among saliva

13   collected at morning ($n = 86$), samples from nonsmokers ($n = 70$) and smokers ($n = 14$)

14   were used; the saliva samples with missing values for the parameter ($n = 2$) were

15   eliminated. The metabolites whose concentrations were 0 in all saliva samples were also

16   eliminated. Therefore, the number of metabolites included in the PCA differed

17   depending on the comparison.

18   Wilcoxon matched paired test and Wilcoxon's test were used to analyze saliva

9

1 collection method and the other characteristics, respectively. Values of $P < 0.05$ were

2 considered statistically significant. To account for multiple testing, we calculated the

3 q-value, a false discovery rate, for all P-values (Storey and Tibshirani, 2003, Broadhurst

4 and Kell, 2006). Data analyses and visualization were conducted using R-software

5 (2.14.0), JMP (9.0.2, SAS Institute, Cary, NC), and GraphPad Prism (Intuitive Software

6 for Science, San Diego, CA).

7

8 3. Results and Discussion

9 3.1. Overview of metabolomic profiles

10 CE-TOFMS detected and identified a total of 257 types of charged metabolite, with a

11 mean ± SD of 141 ± 16 metabolites in each saliva sample. The PCA score plots are

12 presented in Fig. 2 and Supplementary Fig. S1. Statistical significance of the difference

13 between principal components 1 and 2 (PC1 and PC2), and the metabolites showing

14 significant differences in concentrations are summarized in Table 1. Because many

15 metabolites were significantly different between the samples collected in the morning

16 and afternoon, and between samples collected using salivettes and the passive drool

17 method, the associations of metabolomic profiles with other features were only

18 evaluated using samples collected in the morning with a salivette. For individual

1 analyses, the subjects were selected as appropriate; for example tooth brushing was

2 analyzed in subjects who ate breakfast and did/did not brush their teeth (Fig. 1). The

3 metabolites showing differences at $P < 0.05$ are listed in Supplementary Tables S1–S16.

4

5 **3.2. Saliva collection**

6 The collection method had a marked effect on metabolite profiles, as many metabolites

7 showed significant differences between the two collection methods (Supplementary

8 Table S1). PCA revealed that the first and second PC values were significantly different

9 between saliva collected using salivettes and the passive drool method ($P = 0.0451$ and

10 $P = 0.0009$) (Fig. 3a, Table 1). Most of the metabolites were widely distributed along

11 the first PC axis of the loading plots with positive values (right side) without prominent

12 clustering, although some metabolites, including urea, mucate, and riboflavin, had

13 negative values (left side) (Fig. 3b). In contrast, along the second PC axis,

14 approximately half of the metabolites had positive (upper side) and half had negative

15 values (lower side) (Fig. 3b). As expected, the metabolites with the largest and smallest

16 PC values showed opposite trends; for example, the concentrations of asparagine and

17 agmatine were higher when collected using salivettes or the passive drool method,

18 respectively (Fig. 3c).

1   In a similarly designed study, Kozaki et al. found that melatonin concentrations

2   were lower in saliva collected using a cotton swab compared with passively collected

3   saliva (Kozaki et al., 2011). Because there is some physical contact between the cotton

4   swab and the mouth, it is likely that collection with a cotton swab weakly stimulates the

5   salivary gland. The concentrations of stimulated salivary metabolites were generally

6   lower than those of unstimulated salivary metabolites (Takeda et al., 2009). However,

7   that study used NMR and investigated only a few metabolites, whereas the metabolomic

8   profiles observed here showed more complicated changes. Aberrant lipid and organic

9   acid profiles have also been reported in saliva stimulated by chewing (Neyraud et al.,

10   2011). Collectively, these results indicate that adsorption of metabolites to a cotton swab

11   complicates the differences in metabolites observed here.

12   There were no significant differences in PC values between each collection time

13   (Table 1). Except for two outliers, the metabolites in saliva collected in the afternoon

14   congregated at the intersection of the PC1 and PC2 axes, unlike those of saliva collected

15   in the morning (Supplementary Fig. S1a). Unlike the collection method, the amino acids,

16   except for glutamic acids, were not significantly different between the two collection

17   times (Supplementary Table S2). Previous studies have already demonstrated diurnal

18   changes in the salivary metabolites of individual subjects (Bertram et al., 2009, Cooke

1   et al., 2003). It was previously reported that the concentrations of amines, such as

2   putrescine and cadaverine, were decreased in the afternoon (Cooke et al., 2003), but this

3   was not observed in our profiles. One limitation of our study is that we compared saliva

4   samples collected at two different times and from different individuals. Additionally, we

5   did record the time each subject woke in the morning. Because we already know that the

6   metabolomic profiles in blood show diurnal variation (Kasukawa et al. 2012, Minami et

7   al., 2009), we should collect saliva samples from the same individuals and more

8   frequently. Nevertheless, it is clear that salivary metabolomic profiles are sensitive to

9   sampling time. Furthermore, inconsistences in sample collection may result in

10 systematic bias in the metabolite profiles.

11

12 **3.3. Physiological parameters**

13 The effects of physiological parameters, including sex, BMI, and menstruation, were

14 evaluated. The comparison between females ($n = 24$) and males ($n = 27$) showed clearly

15 separated clusters in the PCA score plots (Fig. 2a) and significant differences in both

16 PC1 and PC2 (Table 1). Notably, the concentrations of most of the amino acids were

17 higher in females, including leucine, isoleucine, proline, and methionine (fold-change >

18 5.0) (Supplementary Table S3). As discussed in earlier NMR-based metabolomics

1   studies (Bertram et al., 2009, Takeda et al., 2009), the differences in blood metabolite

2   concentrations between males and females are at least partly due to estrogen-related

3   metabolism. Some differences in gene expression profiles in the parotid glands have

4   also been reported between males and females (Srivastava et al., 2008), which may also

5   contribute to the differences noted in our study.

6   Saliva from subjects with a low BMI (< 18.73 kg/m^2) showed significant

7   differences in PC2 ($P = 0.077$) with eight upregulated metabolites and 14

8   downregulated metabolites as compared with subjects with a BMI ≥ 18.73 kg/m^2)

9   (Table 1, Fig. 2b). By contrast, saliva from subjects with a BMI ≥ 21.00 kg/m^2 showed

10   no significant differences in PCs, and only four metabolites were downregulated as

11   compared with subjects with a BMI < 21.00 kg/m^2 (Supplementary Tables S4 and S5).

12   The effects of BMI were much smaller than the diurnal variations in NMR-based

13   profiles (Bertram et al., 2009). A total of 22 metabolites reached statistical significance

14   at $P < 0.05$ and $q < 0.5$ in comparisons of saliva from subjects with BMI < 18.73 kg/m^2

15   versus subjects with BMI ≥ 18.73 kg/m^2.

16   Saliva collected from women during menstruation would be expected to show

17   differences in female hormones. However, no significant difference was observed in the

18   PC values. Only two metabolites, 4-amino-3-hydroxybutyrate ($P < 0.05$ and $q < 0.5$)

1   and lipoamide ($P < 0.05$ and $q \geq 0.5$), were significantly affected by menstruation (Table

2   1, Supplementary Fig. S1b, Supplementary Table S6). Changes in amino acid and

3   creatinine levels in blood and urea in the luteal phase have been reported (Wallace et al.,

4   2010). However, these changes were not observed in our study, suggesting that salivary

5   metabolites are relatively unaffected by menstruation.

6

7   **3.4. Diet and tooth brushing**

8   The metabolite profiles of subjects who did/did not eat breakfast and of subjects who

9   did/did not brush their teeth showed no significant differences in their PC values (Table

10   1, Fig. 2c, Supplementary Fig. S1c). Only two amino acids, leucine and valine, were

11   significantly increased, while serine was significantly decreased by consuming breakfast

12   ($P < 0.05$, $q < 0.5$) (Supplementary Table S7). These changes in the amino acid profile,

13   which were more pronounced compared with the effects of other factors, were

14   inconsistent with the results of the following two studies. The salivary amino acid

15   profiles were relatively constant, unlike the blood and urine levels after consumption of

16   a high-protein meal (Brand et al., 1997). The increased amino acid concentrations

17   observed after the intake of an amino acid supplement decreased to basal levels within

18   10 min (Nakamura et al., 2010). The unchanged concentrations of putrescine ($P = 0.59$)

1 and cadavarine (P = 0.76) measured after tooth brushing after breakfast were

2 inconsistent with the time-course analysis of saliva metabolites (Cooke et al., 2003).

3 These studies compared multiple saliva samples from individual subjects to eliminate

4 inter-individual differences. Further studies are needed to confirm our observations in

5 another cohort of subjects under controlled dietary conditions, including timing and

6 meal content, which were not considered in the current study.

7

8 **3.5. Lifestyle habits**

9 Smoking had a significant effect on PC2, although visual inspection of the score plots

10 showed no clear clusters (Table 1, Fig. 2d). As expected, saliva nicotine concentrations

11 were much higher in smokers than in non-smokers (Supplementary Table S9).

12　　Purine metabolites, such as hypoxanthine and guanosine monophosphate (GMP),

13 were also significantly increased in smokers (Supplementary Table S9). It was reported

14 that hypoxanthine guanine phosphoribosyltransferase (HGPRT; 2.4.2.8) was markedly

15 decreased in blood from smokers compared with non-smokers (Chang et al., 2005). This

16 enzyme converts hypoxanthine to inosine monophosphate (IMP) and guanine to GMP.

17 Therefore, increases in hypoxanthine/IMP and guanine/GMP are expected if salivary

18 enzyme activity reflects that in blood. IMP was not detected in any saliva samples,

1   while hypoxanthine was elevated in saliva (1.35-fold). However, GMP (1.57-fold) was

2   increased to a greater extent than guanine (1.22-fold). Therefore, the regulation of these

3   metabolites in saliva is more complicated than that in blood, and cannot be explained by

4   the activity of HGPRT alone.

5   Several molecules in the glycolysis pathway, including lactate (1.73-fold) and

6   pyruvate (1.23-fold), were elevated in saliva from smokers, although not significantly.

7   These results are consistent with those in the study by Takeda et al. (Takeda et al., 2009).

8   Previous studies have also reported reduced salivary enzyme activities, including lactic

9   dehydrogenase (LDH) and amylase (Nagler et al., 2000). These findings imply that the

10   pyruvate/lactate ratio is elevated; however, our observation was inconsistent with this

11   notion. By contrast, chronic cigarette smoking increased LDH activity in blood

12   (Padmavathi et al., 2009). Therefore, altered LDH activity might provide only a small

13   contribution to the elevated lactate level; instead, the secretion of lactate from the

14   salivary gland might have a greater influence.

15   Saliva from individuals who regularly consumed alcohol showed no significant

16   differences in PC1 and PC2 (Table 1, Supplementary Fig. S1d, Supplementary Table

17   S10) Thus, alcohol consumption did not seem to chronically affect salivary metabolites.

18   Many medications and nutritional supplements are known to decrease or increase

17

1 the rate of saliva secretion (Lukkari et al., 1997, Ryo et al., 2011). However, we found

2 no significant differences in the concentrations of the identified metabolites in subjects

3 who used medications (P = 0.987) or nutritional supplements (P = 0.3138)

4 (Supplementary Fig. S2), nor were PC1 and PC2 significantly different (Table 1,

5 Supplementary Figs. S1e, S1f). Only two metabolites were decreased in saliva from

6 subjects who used medications (n =5, P < 0.05); however, the q-values were relatively

7 large (> 0.95) (Supplementary Table S11). These results should be validated in a larger

8 cohort because all of these subjects used different types of medications. Saliva from

9 subjects who used nutritional supplements, including vitamin drops (n = 4) and black

10 vinegar (n = 1), showed elevated glutarate concentrations (fold-change = 7.1), a

11 common component of nutritional supplements (Kleber et al., 1979), as well as

12 histamine (fold-change = 1.4) and 1-methylhistamine (fold-change = 2.4)

13 (Supplementary Table S12). The salivary histamine concentration was reported to be a

14 marker for periodontal disease in patients with diabetes (Venza et al., 2006). However,

15 no subjects with periodontal disease were included in our study; therefore, the changes

16 in the histamine pathway might be due to a factor that was not evaluated here.

17

18 **3.6. Oral conditions**

1   In terms of oral cavity conditions, we examined the effects of abnormalities of the oral

2   cavity and tooth alignment, the number of dental caries, and TMD. PCA showed no

3   significant differences in the PC1 or PC2 values in these cases (Table 1, Supplementary

4   Tables S13–S16, Supplementary Fig. S1g–S1j)

5   Several studies have shown metabolic abnormalities in subjects with dental caries.

6   For example, the concentrations of arginine and lysine in stimulated parotid saliva were

7   higher in subjects without caries than in those with caries (Van Wuyckhuyse et al.,

8   1995). Similarly, central carbon metabolism was reported to be abnormal in

9   supragingival plaque and several oral bacterial species have been detected in subjects

10   with dental caries (Takahashi et al., 2010). Therefore, we can expect abnormalities in

11   salivary metabolomic profiles in patients with dental caries. Indeed, we found that

12   arginine (fold-change = 1.1, P = 0.27) and lysine (fold-change = 1.57, P = 0.16) saliva

13   concentrations were slightly elevated, although not significantly. Several metabolomics

14   studies have investigated the changes in metabolites in subjects with periodontal

15   diseases. For example, it was reported that the concentrations of metabolites in the

16   purine degradation pathway were increased in gingival fluid in subjects with periodontal

17   disease (Barnes et al., 2009); however, these metabolites, including hypoxanthine

18   (fold-change = 0.97, P = 0.39), were unchanged in whole saliva in our study. Similarly,

1   it was previously reported that saliva phenylalanine and valine concentrations were

2   increased and pyruvate concentrations were decreased in subjects with periodontal

3   disease (Aimetti et al., 2011); however, these changes were not observed in our study.

4   Our observations suggest that these metabolite changes are specific to periodontal

5   diseases, rather than dental caries.

6   In the subject with TMD, the q-values for alanine and thymine were smaller than

7   those for metabolites associated with other oral conditions (Supplementary Table S16).

8   The P-value for PC2 in TMD ($P = 0.1075$) was much smaller than those for the PC

9   values of other oral conditions, although these values were not statistically significant.

10   These results indicate that TMD has a greater effect on saliva profiles compared with

11   the other oral cavity parameters evaluated in this study. The effects of TMD on salivary

12   metabolites were expected because patients with TMD have abnormal urinary amino

13   acid profiles (McGregor et al., 2003) and elevated salivary cortisol levels (Da Silva

14   Andrade et al., 2008). The increased oxidized glutathione concentration suggests the

15   presence of severe oxidative stress in these patients. In fact, estrogen activates

16   inflammation by inducing the proliferation and differentiation of macrophages in

17   temporomandibular joint cellular elements (Galal et al., 2008). Only the increases in

18   alanine concentrations (fold-change = 1.33, $P = 0.0067$) were consistently correlated

1   with the concentrations of metabolites secreted from activated macrophages. By

2   contrast, there were no increases in other metabolites that are abundantly secreted from

3   macrophages in the saliva from subjects with TMD (Sugimoto et al., 2012c), which

4   indicates that these changes are mainly derived from other mechanisms. Interestingly,

5   salivary D-alanine was not derived from bacteria or food; instead, the submandibular

6   gland and epithelial cells are the main sources of this metabolite, without transferring

7   D-alanine from blood (Nagata et al., 2006). This suggests that D-alanine more clearly

8   reflects the metabolic status of host cells in the oral cavity than other metabolites, which

9   are secreted by multiple sources. However, our CE-MS method could not separate L-

10   and D-alanine. Therefore, further studies are needed to identify the sources of these

11   metabolites in TMD to understand the underlying mechanisms.

12

13   **3.7. Other factors and limitations**

14   Aging may also affect salivary metabolomic profiles. For example, it was reported that

15   salivary glycine and lysine concentrations increased with aging, independent of sex

16   (Tanaka et al., 2010). Although the effects of age were not assessed in our study, we

17   found no significant changes in these metabolites, nor were they associated with the

18   factors that were evaluated in this study, supporting their use as markers for aging. To

1   generalize the results of the current study, further analyses are needed in a larger cohort

2   of subjects/samples, particularly for the factors with a relatively small number of

3   samples in this study e.g., collection method, the use of medications or nutritional

4   supplements, and especially oral cavity disorders that were analyzed as a single type

5   while different disorder may have different effects on salivary metabolite profiles.

6   Another limitation is that we analyzed the effects of individual parameters on the

7   salivary metabolomic profile. For example, in the analyses of sex and collection time,

8   the age distribution was not significantly different in parametric tests ($P = 0.20$ for sex

9   and $P = 0.30$ for collection time; Student's t-test) but it was significantly different in

10   non-parametric tests ($P = 0.02$ for sex and $P = 0.03$ for collection time; Mann–Whitney

11   test). Thus, age might influence the effects of these parameters on metabolite profiles. In

12   addition, the profiles of weakly charged metabolites that cannot be detected by CE-MS

13   should be analyzed simultaneously (Alvarez-Sanchez et al., 2012).

14   The parameters used in this study were assessed by questionnaire and several

15   parameters were self-diagnosed, such as TMD. Therefore, these parameters might be

16   inaccurate and future analyses should involve confirmation of the disease/condition by

17   physicians or dentists to reach a more definitive conclusion. It will also be necessary to

18   determine correlations between the saliva and blood concentrations of each metabolite

1 (Shiiki et al., 2011) to identify the source of each molecule. To provide comprehensive

2 datasets, we are currently developing a web-based database that incorporates all of the

3 metabolomic concentrations and the relevant parameters that might help to understand

4 the associations between specific phenotypes and metabolomic profiles (Sugimoto et al.,

5 2012a).

6

4. Concluding remarks

8 In conclusion, our study revealed that salivary metabolite profiles were associated with

9 many physiological and environmental factors. Saliva collection method, sex, BMI, and

10 smoking had the greatest effects on metabolite profiles. Saliva collection time also

11 affected a greater number of salivary metabolites compared with other factors. However,

12 age might contribute to the differences in sex and collection time. Based on these

13 findings, the interpretation of salivary metabolomic profiles should take into account

14 these factors and the corresponding changes in specific metabolites when using saliva to

15 diagnose specific diseases.

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7 **Disclosure:** The authors have no conflicts of interest to declare.

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11

1   **Figure Legends**

2

3   **Fig. 1.** Study design. To determine the effects of collection time, for example, saliva

4   samples were collected in the morning ($n = 86$) and in the afternoon ($n = 18$). Saliva

5   samples collected in the morning were also used to determine differences between

6   smokers ($n = 14$) and non-smokers ($n = 70$). Because data were missing for some

7   subjects, the total number of subjects included in the analysis of smoking status ($n = 84$)

8   was less than the total number of saliva samples ($n = 86$). [a,b]Saliva samples were

9   collected in the afternoon from different subjects ($n = 51$). [c]Saliva samples were

10   collected using both methods in each subject ($n = 18$).

11

12   **Fig. 2.** Principal component (PC) score plots for sex (**a**) (circles, females; triangles,

13   males), BMI (**b**) (circles, < 18.73 kg/m^2; triangles, $18.73–21.00$ kg/m^2; squares, > 21.00

14   kg/m^2), breakfast (**c**) (circles, not eaten; triangles, eaten), and smoking (**d**) (circles,

15   non-smokers; triangles, smokers). The accumulated contribution ratios (%) for PC1 and

16   PC2 were 36.5 and 53.7 in (**a**), 25.3 and 40.6 in (**b**), 23.6 and 38.5 in (**c**), and 24.0 and

17   39.1 in (**d**), respectively. The other plots are shown in Supplementary Information Fig.

18   2.

1

Fig. 3. Principal components (PC) analysis of the metabolic profiles for saliva samples

collected using salivettes and the passive drool method. Parallel coordinate plots of PC

scores (**a**), loading plots (**b**), and concentrations of representative metabolites (**c**). The

symbols S and P represent saliva samples collected using salivettes and the passive

drool method, respectively. The accumulated contribution ratios for PC1 and PC2 were

46.2% and 60.2%, respectively.

8

Figure 1

Figure 2

a Sex

b BMI

c Breakfast

d Smoking
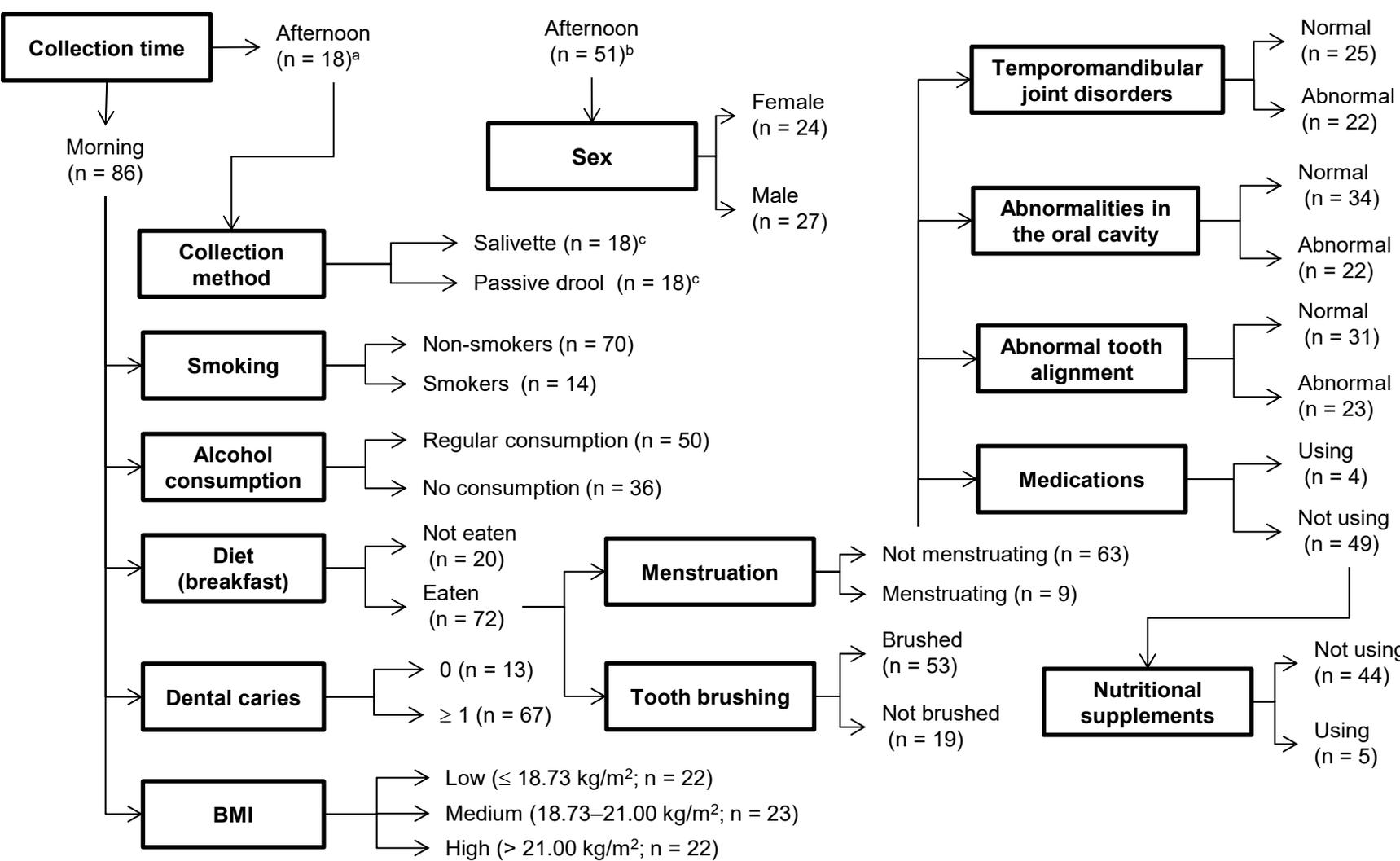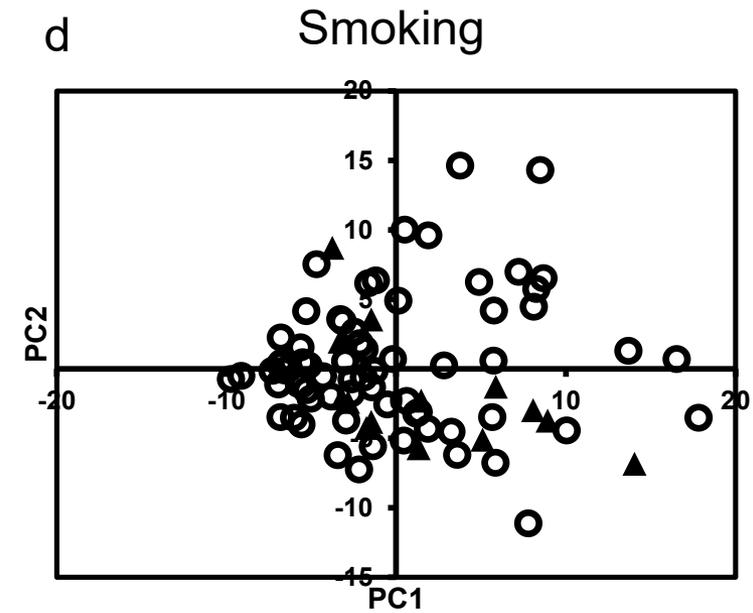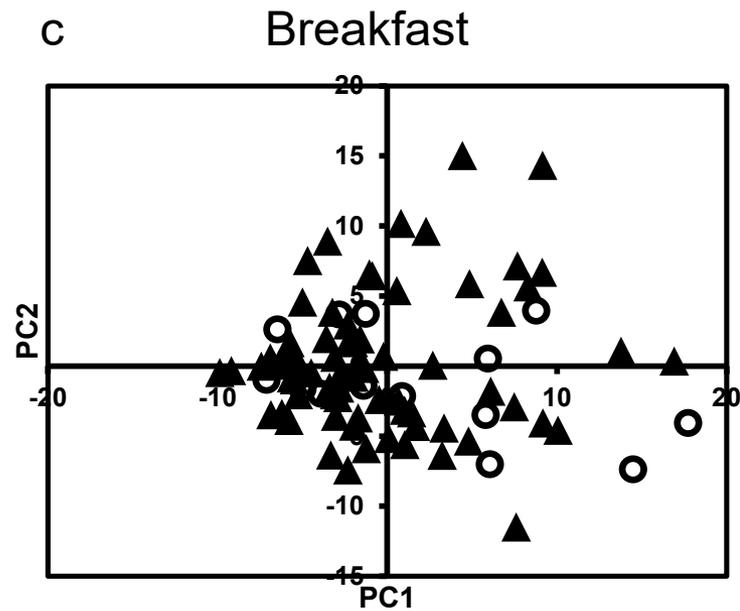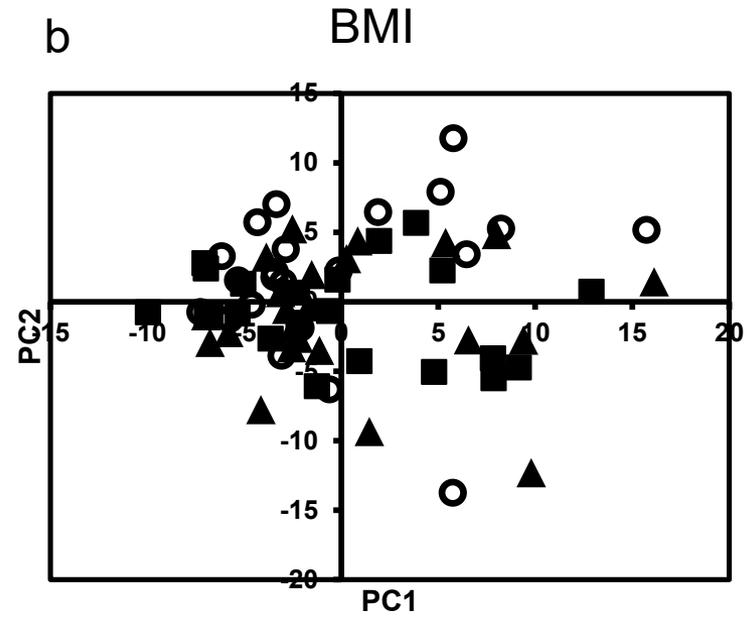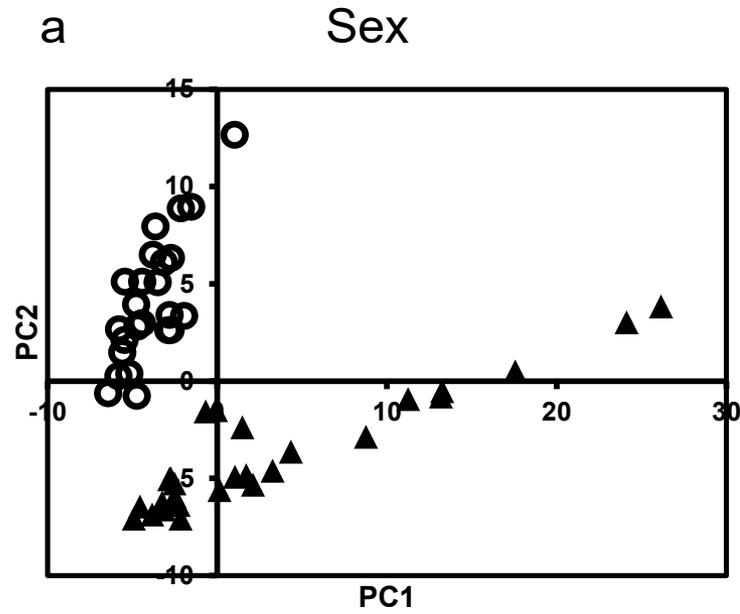
Figure 3
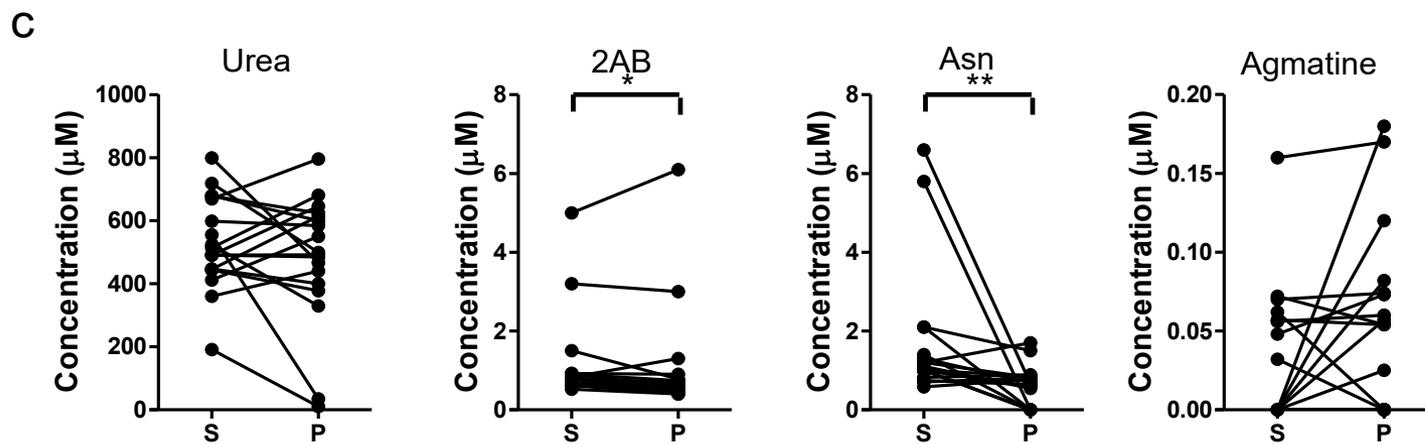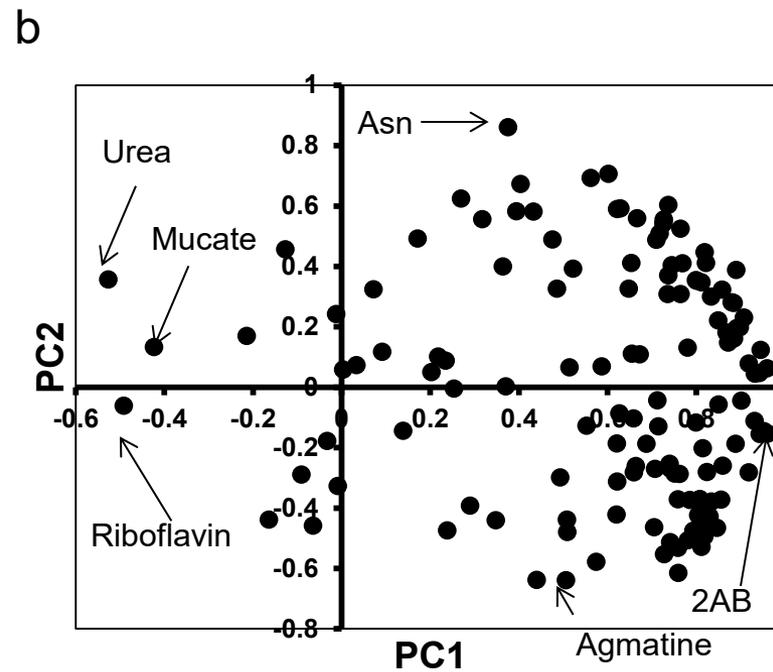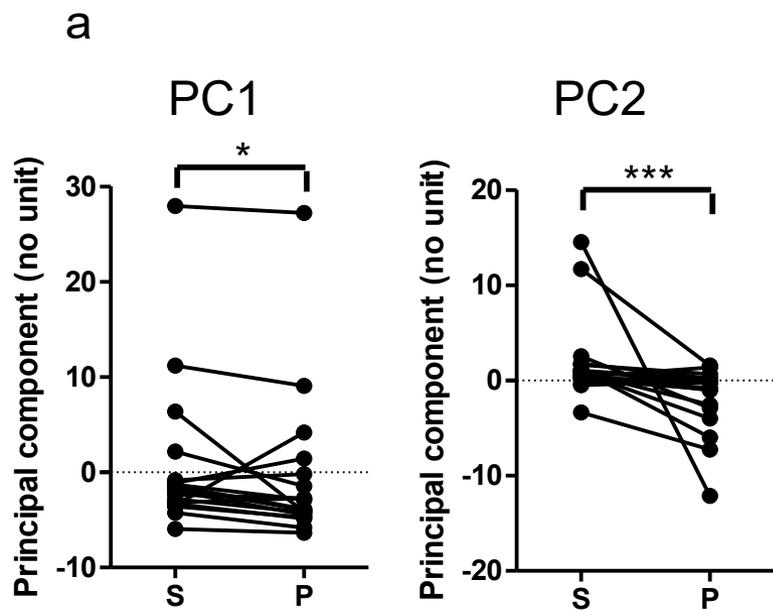
Supplementary Information

Physiological and environmental parameters associated with mass spectrometry-based salivary metabolomic profiles

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Material and Methods

Instrumental parameters for capillary electrophoresis-time-of-flight-mass spectrometry (CE-TOFMS)

The instrumentation and measurement conditions used for CE-TOFMS are described elsewhere (Soga et al., 2006; Soga et al., 2009). Briefly, cation analysis was performed using an Agilent CE capillary electrophoresis system, an Agilent G6220A LC/MSD TOF system, an Agilent 1100 series isocratic HPLC pump, a G1603A Agilent CE-MS adapter kit, and a G1607A Agilent CE-ESI-MS sprayer kit (Agilent Technologies, Waldbronn, Germany). Anion analysis was performed using an Agilent CE capillary electrophoresis system, an Agilent G6210A LC/MSD TOF system, an Agilent 1200 series isocratic HPLC pump, a G1603A Agilent CE-MS adapter kit, and a G1607A Agilent CE-electrospray ionization (ESI) source-MS sprayer kit (Agilent Technologies). For the cation and anion analyses, the CE-MS adapter kit includes a capillary cassette that facilitates thermostating of the capillary. The CE-ESIMS sprayer kit simplifies coupling of the CE system with the MS system, and is equipped with an electrospray source. For system control and data acquisition, we used G2201AA Agilent ChemStation software for CE and Agilent MassHunter software for TOF-MS. The original Agilent SST316Ti stainless steel ESI needle was replaced with a passivated SST316Ti stainless steel and platinum needle (passivated with 1% formic acid and 20% isopropanol aqueous solution at 80°C for 30 min) for anion analysis.

For cationic metabolite analysis using CE-TOFMS (Soga et al., 2006), sample separation was performed in fused silica capillaries (50 μm i.d. × 100 cm total length) filled with 1 mol/L formic acid as the reference electrolyte. Sample solutions were injected at 50 mbar for 3 s and a voltage of 30 kV was applied. The capillary temperature was maintained at 20°C and the temperature of the sample tray was kept below 5°C. The sheath liquid, composed of methanol/water (50% v/v) and 0.1 μmol/L hexakis (2,2-difluoroethoxy) phosphazene (Hexakis), was delivered at 10 μL/min. ESI-TOF-MS was conducted in the positive ion mode. The capillary voltage was set at 4 kV and the flow rate of nitrogen gas (heater temperature = 300°C) was set at 10 psig. In TOF-MS, the fragmentor, skimmer and OCT RF voltages were 75, 50 and 125 V, respectively. Automatic recalibration of each acquired spectrum was performed using reference standards ($[^{13}C$ isotopic ion of protonated methanol dimer $(2MeOH + H)]^+$, m/z 66.063199) and ($[$protonated Hexakis $(M + H)]^+$, m/z 622.028963). Mass spectra were acquired at the rate of 1.5 cycles/s over a m/z range of 50–1,000.

For anionic metabolite analysis using CE-TOFMS (Soga et al., 2009), a commercially available COSMO(+) capillary (50 μm i.d. × 110 cm, Nacalai Tesque, Kyoto, Japan), chemically coated with a cationic polymer, was used for separation. Ammonium acetate solution (50 mmol/L; pH 8.5) was used as the electrolyte for separation. Before the first use, the new capillary was flushed successively with the running electrolyte (pH 8.5), 50 mmol/L acetic acid (pH 3.4), and then the electrolyte again for 10 min each. Before each injection, the capillary was equilibrated for 2 min by flushing with 50 mM acetic acid

(pH 3.4) and then flushed for 5 min with the running electrolyte. A sample solution (30 nL) was injected at 50 mbar for 30 s, and a voltage of –30 kV was applied. The capillary temperature was thermostated to 20°C and the sample tray was cooled below 5°C. An Agilent 1100 series pump equipped with a 1:100 splitter was used to deliver 10 μL/min of 5 mM ammonium acetate in 50% (v/v) methanol/water, containing 0.1 μM Hexakis, to the CE interface. Here, it was used as a sheath liquid surrounding the CE capillary to provide a stable electrical connection between the tip of the capillary and the grounded electrospray needle. ESI-TOF-MS was conducted in the negative ionization mode at a capillary voltage of 3,500 V. For TOF-MS, the fragmentor, skimmer and Oct RF voltages were set at 100, 50 and 200 V, respectively. The flow rate of the drying nitrogen gas (heater temperature = 300°C) was maintained at 10 L/min. Automatic recalibration of each acquired spectrum was performed using reference standards ([^{13}C isotopic ion of deprotonated acetic acid dimer (2 CH_3COOH - H)]$^-$, m/z 120.03841), and [Hexakis-deprotonated acetic acid (CH_3COOH - H)]$^-$, m/z 680.035541). Exact mass data were acquired at a rate of 1.5 spectra/s over a m/z range of 50–1,000.

Figure legends

Supplementary Figure S1

Principal component (PC) score plots for collection time (**a**) (circles, morning; triangles, afternoon), menstruation (**b**) (circles, not menstruating; triangles, menstruating), tooth brushing (**c**) (circles, brushed; triangles, not brushed), alcohol consumption (**d**) (circles, no consumption; triangles, regular consumption), use of medications (**e**) (circles, no use of medications; triangles, use of medications), use of nutritional supplements (**f**) (circles, no use of supplements; triangles, use of supplements), oral cavity disorders (**g**) (circles, normal; triangles, abnormal), tooth alignment disorders (**h**) (circles, normal; triangles, abnormal), carious teeth (**i**) (circles, no carious teeth; triangles, presence of carious teeth), and (**j**) TMD (circles, normal structure; triangles, with TMD). The accumulated contribution ratios (%) for PC1 and PC2 were 32.0 and 44.7 in (**a**), 23.9 and 41.4 in (**b**), 24.0 and 41.1 in (**c**), 19.8 and 33.0 in (**d**), 22.6 and 38.1 in (**e**), 22.5 and 38.4 in (**f**), 21.5 and 37.9 in (**g**), 21.9 and 38.0 in (**h**), 22.2 and 37.8 in (**i**), and 25.3 and 40.96 in (**j**), respectively.

Supplementary Figure S2

Concentrations of the identified salivary metabolites in subjects who did/did not use medications (**a**) or nutritional supplements (**b**).

Reference

Soga T, Baran R, Suematsu M, Ueno Y, Ikeda S, Sakurakawa T et al. (2006). Differential metabolomics reveals ophthalmic acid as an oxidative stress biomarker indicating hepatic glutathione consumption. *J Biol Chem* 281(24):16768-16776.

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Supplementary Figure S1

a Collection time

b Menstruation

c Tooth brushing

d Alcohol consumption

e   Use of medications

f   Use of nutritional supplements

g   Oral cavity disorders

h   Tooth alignment disorders
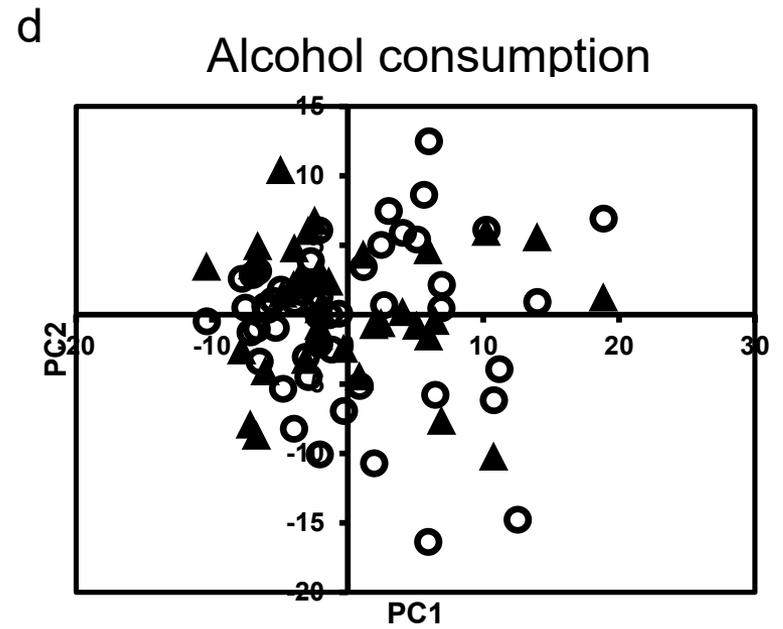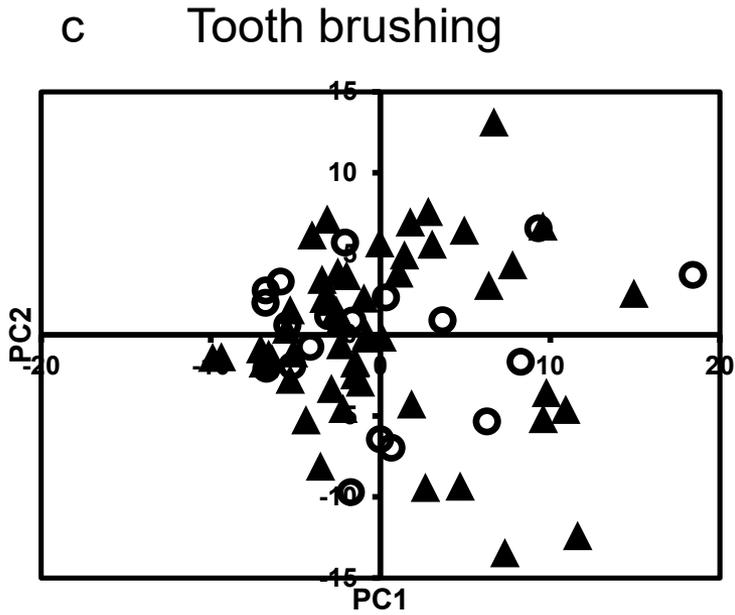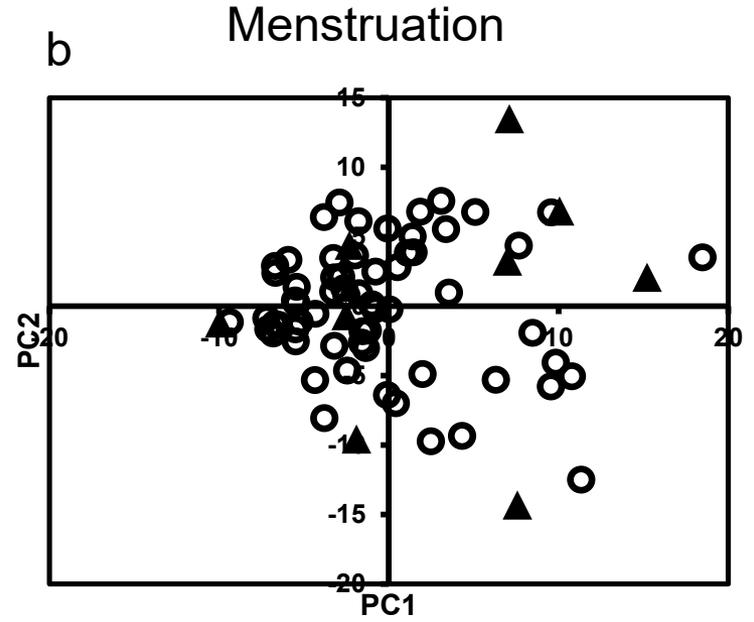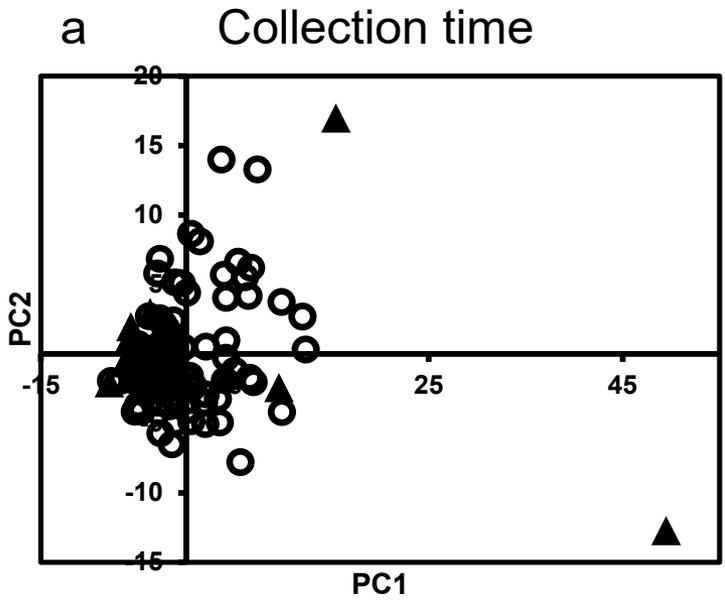
Supplementary Figure S1 (Continued)

i   Carious teeth

j   Suspected TMD

Supplementary Figure S2

a   Use of medications

b   Use of nutritional supplements
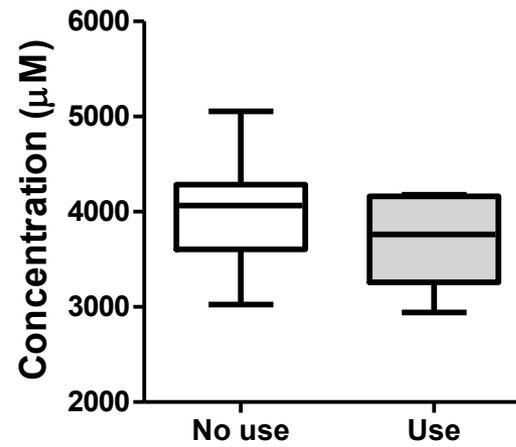
Table S1 Effects of collection method

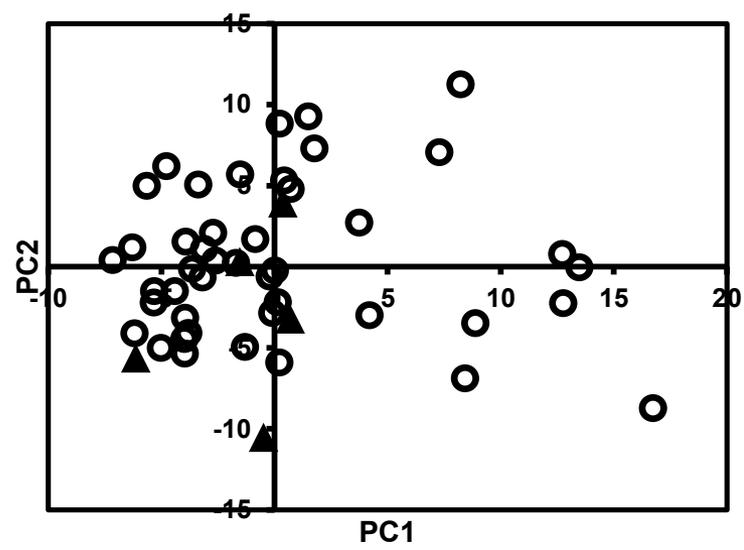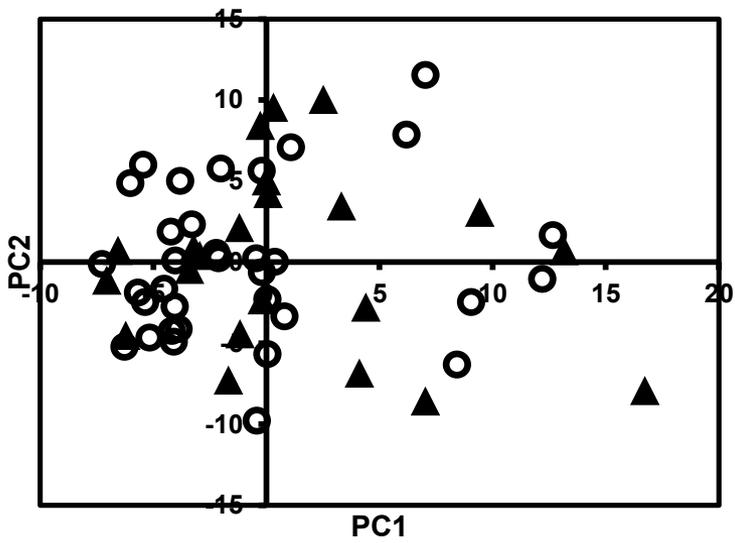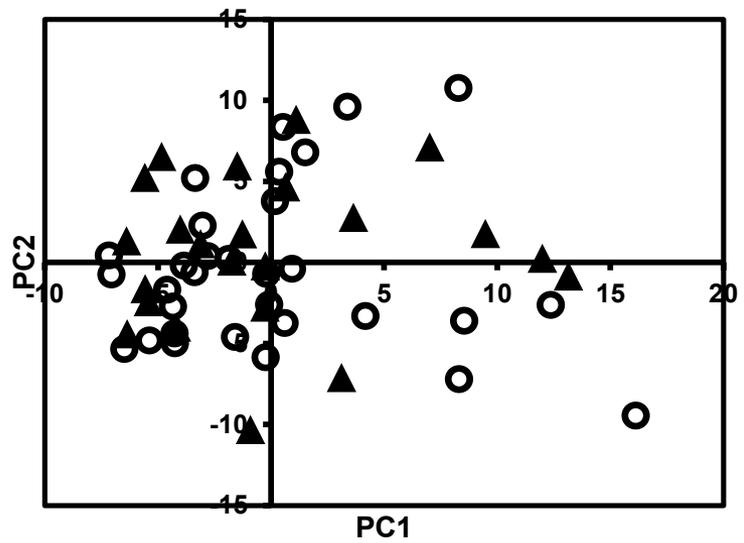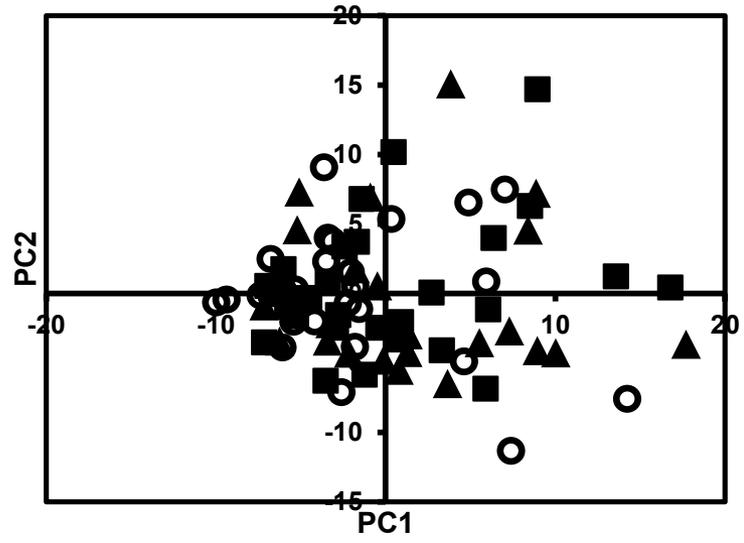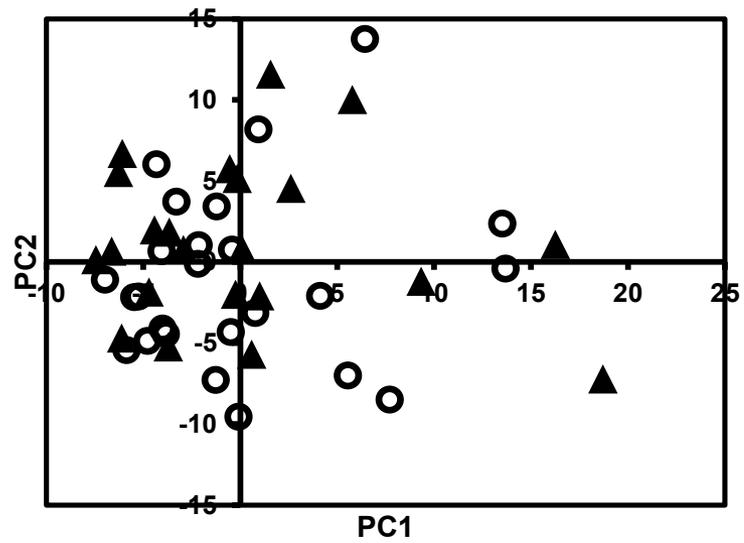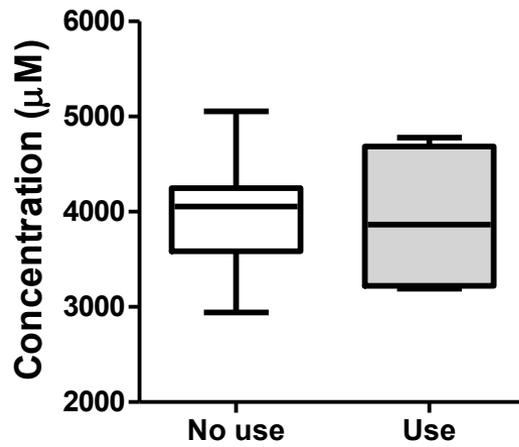| ChEBI	Name	Salivettes (n = 18)		Possive drool method (n = 18)		FC	P-value	q-value
		Mean	SD	Mean	SD			
17858	Glutathione (oxidized)	0.350	1.05	0.0156	0.0359	0.0446	1.09×10^{-3}	3.37×10^{-3}
16761	ADP	0.317	0.491	0.0257	0.0618	0.0809	7.21×10^{-4}	3.34×10^{-3}
48131	Azelate	2.06	1.85	0.221	0.218	0.108	2.13×10^{-4}	1.66×10^{-3}
15702	Terephthalate	3.95	1.89	0.546	0.381	0.138	2.14×10^{-4}	1.66×10^{-3}
30817	Urocanate	6.13	16.3	1.04	2.19	0.170	2.51×10^{-3}	4.54×10^{-3}
16010	5-Oxoproline	15.0	32.5	3.82	5.16	0.255	3.20×10^{-4}	1.85×10^{-3}
16027	AMP	1.19	2.66	0.321	0.506	0.270	9.19×10^{-4}	3.37×10^{-3}
16668	Hypotaurine	0.842	1.41	0.240	0.461	0.285	0.0376	0.0311
16345	Cysteine sulfinate	0.546	0.508	0.163	0.345	0.298	2.53×10^{-3}	4.54×10^{-3}
17822	Ser	28.1	60.9	8.91	9.17	0.317	6.48×10^{-3}	8.22×10^{-3}
17196	Asn	1.70	1.69	0.580	0.511	0.341	2.64×10^{-3}	4.54×10^{-3}
17345	GMP	0.262	0.501	0.0898	0.212	0.342	0.0167	0.0175
17750	Betaine	8.51	19.4	3.17	2.68	0.373	5.64×10^{-3}	7.67×10^{-3}
16870	Glycerophosphorylcholine	2.53	4.35	1.05	0.248	0.415	8.37×10^{-4}	3.37×10^{-3}
16857	Thr	7.78	13.5	3.50	4.89	0.450	2.27×10^{-3}	4.54×10^{-3}
6650	Malate	4.15	6.21	1.87	1.06	0.452	1.08×10^{-3}	3.37×10^{-3}
30887	Isocitrate	0.616	0.351	0.294	0.267	0.478	2.09×10^{-3}	4.54×10^{-3}
17821	Thymine	5.91	11.2	2.89	4.53	0.490	0.0455	0.0345
32805	cis-Aconitate	0.358	0.198	0.176	0.107	0.491	2.49×10^{-4}	1.66×10^{-3}
18257	Ornithine	28.9	33.1	14.2	11.1	0.493	2.45×10^{-3}	4.54×10^{-3}
17061	Gln	22.8	44.0	11.3	14.5	0.495	1.26×10^{-3}	3.44×10^{-3}
28358	Lactate	5.11×10^2	6.12×10^2	2.62×10^2	2.80×10^2	0.514	3.85×10^{-4}	1.98×10^{-3}
30915	2-Oxoglutarate	2.65	1.07	1.53	1.18	0.578	5.27×10^{-3}	7.39×10^{-3}
27570	His	9.93	9.50	6.02	4.39	0.606	4.04×10^{-3}	5.92×10^{-3}
16977	Ala	30.5	39.6	18.8	20.4	0.617	2.65×10^{-3}	4.54×10^{-3}
15960	o-Acetylcarnitine	1.53	1.23	0.955	0.442	0.622	1.08×10^{-3}	3.37×10^{-3}
32816	Pyruvate	68.6	69.1	43.3	65.1	0.631	6.58×10^{-3}	8.22×10^{-3}
18132	Phosphorylcholine	5.42	3.54	3.46	1.59	0.639	1.26×10^{-3}	3.44×10^{-3}
28631	3-Phenylpropionate	3.54	2.71	2.34	2.49	0.661	0.0294	0.0257
11851	2-Oxoisopentanoate	0.656	0.399	0.434	0.382	0.662	0.0418	0.0333
17553	Ethanolamine phosphate	76.2	64.8	50.9	34.1	0.668	1.99×10^{-3}	4.54×10^{-3}
15428	Gly	27.7	25.9	18.6	16.4	0.672	0.0120	0.0129
17295	Phe	8.24	6.51	5.55	4.30	0.674	0.0115	0.0127
16919	Creatine	33.9	45.9	23.2	29.4	0.684	7.63×10^{-5}	1.66×10^{-3}
3424	Carnitine	0.924	0.960	0.656	0.734	0.710	1.91×10^{-4}	1.66×10^{-3}
30769	Citrate	16.9	9.95	12.2	7.87	0.720	7.69×10^{-3}	9.36×10^{-3}
30794	Malonate	0.504	0.177	0.369	0.217	0.731	0.0209	0.0206
18186	Tyr	15.1	10.3	11.1	7.65	0.732	0.0245	0.0230
27596	3-Methylhistidine	0.134	0.110	0.0982	0.128	0.735	0.0463	0.0345
48430	4-Methyl-2-oxopentanoate	1.24	0.728	0.944	0.442	0.758	4.09×10^{-3}	5.92×10^{-3}
27911	N_8-Acetylspermidine	0.178	0.156	0.136	0.190	0.767	0.0179	0.0184
16953	N-Acetylaspartate	1.08	0.586	0.827	0.521	0.769	1.51×10^{-3}	3.88×10^{-3}
7307	1-Methyl-2-pyrrolidinone	26.8	8.32	20.8	4.73	0.775	4.04×10^{-3}	5.92×10^{-3}
17084	2-Hydroxyglutarate	1.08	0.629	0.881	1.13	0.813	0.0346	0.0291
15978	Glycerophosphate	6.33	3.24	5.30	2.20	0.837	0.0385	0.0312
37023	a-Aminoadipate	0.952	1.49	0.804	1.44	0.844	0.0184	0.0185
16958	β-Ala	1.14	1.34	1.02	1.25	0.893	0.0244	0.0230
28340	2AB	1.14	1.14	1.07	1.39	0.932	0.0450	0.0345
15901	Dihydrouracil	2.00×10^3	2.23×10^2	2.24×10^3	3.80×10^2	1.12	8.96×10^{-3}	0.0104
15676	Allantoin	23.9	3.67	27.5	5.12	1.15	3.77×10^{-3}	5.92×10^{-3}
16750	Guanosine	0.227	0.617	0.262	0.285	1.15	0.0278	0.0252
17363	Ru5P	0.818	1.58	0.992	0.935	1.21	0.0331	0.0284
17015	Riboflavin	0.578	0.157	0.710	0.180	1.23	3.75×10^{-3}	5.92×10^{-3}
16335	Adenosine	0.242	0.133	0.306	0.156	1.27	0.0294	0.0257
30746	Benzoate	13.9	3.52	19.5	8.70	1.40	0.0249	0.0230
15888	4-Acetylbutyrate	0.366	0.242	0.523	0.224	1.43	8.40×10^{-3}	9.96×10^{-3}

506227	*N*-Acetylglucosamine	2.40	2.35	4.41	5.98	1.84	5.96×10^{-3}	7.88×10^{-3}
17797	R5P	0.264	0.406	0.608	0.811	2.30	0.0455	0.0345
15746	Spermine	0.0962	0.262	0.242	0.565	2.52	1.95×10^{-3}	4.54×10^{-3}
18127	Cadaverine	2.74	5.58	7.40	22.4	2.70	0.0106	0.0119
16610	Spermidine	0.487	1.02	1.38	2.59	2.82	2.51×10^{-4}	1.66×10^{-3}
17562	Cytidine	0.0683	0.0749	0.237	0.184	3.47	2.51×10^{-4}	1.66×10^{-3}

Table S2 Effects of collection time (morning vs afternoon)

ChEBI	Name	Morning (n = 86)		Afternoon (n = 18)		FC	*P*-value	*q*-value
		Mean	SD	Mean	SD			
-	Syringate	1.59	0.118	0.00	0.00	0.00	1.21×10^{-11}	7.10×10^{-10}
64277	7,8-Dihydrobiopterin	10.8	14.7	1.22	3.60	0.113	2.36×10^{-4}	1.73×10^{-3}
17869	6-Hydroxyhexanoate	0.315	0.250	0.0489	0.142	0.155	7.69×10^{-5}	1.05×10^{-3}
16831	3-Hydroxy-3-methylglutarate	0.0917	0.0851	0.0201	0.0639	0.219	1.22×10^{-4}	1.08×10^{-3}
17797	R5P	0.720	0.402	0.264	0.406	0.367	3.27×10^{-5}	6.39×10^{-4}
15946	Fluctose 6-phosphate	0.403	0.286	0.173	0.176	0.429	1.07×10^{-3}	4.83×10^{-3}
48928	6-Phosphogluconate	0.253	0.218	0.117	0.228	0.461	1.78×10^{-3}	7.05×10^{-3}
30915	2-Oxoglutarate	5.27	3.61	2.65	1.07	0.502	1.29×10^{-4}	1.08×10^{-3}
1178	2-Isopropylmalate	0.218	0.138	0.114	0.0493	0.522	8.96×10^{-5}	1.05×10^{-3}
17562	Cytidine	0.123	0.0900	0.0683	0.0749	0.555	0.0461	0.0696
30887	Isocitrate	0.988	0.531	0.616	0.351	0.623	7.80×10^{-3}	0.0240
17533	*N*-Acetylglutamate	0.0700	0.0525	0.0448	0.0564	0.639	0.0433	0.0696
16576	Ala-Ala	0.184	0.138	0.119	0.176	0.649	0.0289	0.0576
16682	N-Acetyl-β-alanine	0.214	0.154	0.151	0.335	0.704	0.0110	0.0269
16695	UMP	0.708	1.98	0.508	1.22	0.718	0.0404	0.0696
18021	PEP	0.419	0.270	0.312	0.332	0.744	7.14×10^{-3}	0.0232
17015	Riboflavin	0.754	0.222	0.578	0.157	0.767	1.81×10^{-3}	7.05×10^{-3}
6650	Malate	5.22	3.49	4.15	6.21	0.794	6.05×10^{-4}	3.55×10^{-3}
17672	Carbamoylphosphate	38.4	8.24	32.1	7.53	0.836	0.0106	0.0269
16446	*N*-Acetylglucosamine 1-phosphate	0.326	0.327	0.282	0.623	0.868	9.64×10^{-3}	0.0269
15901	Dihydrouracil	2.21×10^{3}	3.53×10^{2}	2.00×10^{3}	2.23×10^{2}	0.908	4.58×10^{-3}	0.0168
15966	Glu	14.9	7.60	14.5	19.8	0.968	0.0163	0.0364
18012	Fumarate	0.798	0.396	0.778	1.07	0.975	0.0103	0.0269
17363	Ru5P	0.830	0.542	0.818	1.58	0.985	0.0300	0.0576
17858	Glutathione (oxidized)	0.349	0.436	0.350	1.05	1.00	0.0437	0.0696
15741	Succinate	15.0	15.5	15.6	27.7	1.03	0.0314	0.0576
17794	3PG	1.54	1.04	1.66	2.97	1.08	0.0139	0.0325
15784	*N*-Acetylglucosamine 6-phosphate	0.173	0.163	0.192	0.574	1.11	0.0174	0.0364
17497	Glycolate	9.67	3.35	10.9	2.45	1.12	0.0173	0.0364
28837	Octanoate	0.629	0.249	0.738	0.193	1.17	7.05×10^{-3}	0.0232
15721	S7P	0.299	0.236	0.360	1.20	1.20	1.06×10^{-3}	4.83×10^{-3}
18406	5-Aminoimidazole-4-carboxamide ribo	0.441	0.133	0.548	0.153	1.24	3.00×10^{-4}	1.95×10^{-3}
4170	G6P	1.73	1.28	2.23	5.59	1.29	9.55×10^{-3}	0.0269
15676	Allantoin	18.1	5.09	23.9	3.67	1.32	1.15×10^{-6}	3.37×10^{-5}
-	Cysteine-glutathione disulphide	0.234	0.267	0.362	1.18	1.54	0.0315	0.0576
17460	Lipoamide	0.278	0.208	0.439	0.254	1.58	1.06×10^{-3}	4.83×10^{-3}
18257	Ornithine	16.7	14.6	28.9	33.1	1.73	0.0368	0.0653
16345	Cysteine sulfinate	0.316	0.424	0.546	0.508	1.73	0.0463	0.0696
32980	3-(4-Hydroxyphenyl)propionate	1.59	2.05	3.11	5.21	1.96	0.0447	0.0696

Table S3 Effects of sex (female vs male)

ChEBI	Name	Female (n = 24)		Male (n = 27)		FC	*P*-value	*q*-value
		Mean	SD	Mean	SD			
16870	Glycerophosphorylcholine	1.84	0.652	0.00	0.00	0.00	3.66×10^{-11}	1.79×10^{-10}
18086	Indole-3-acetaldehyde	7.24	1.16	0.00	0.00	0.00	3.68×10^{-11}	1.79×10^{-10}
18406	5-Aminoimidazole-4-carboxamide ribo	0.504	0.126	0.00	0.00	0.00	3.69×10^{-11}	1.79×10^{-10}
17015	Riboflavin	0.880	0.375	0.00	0.00	0.00	3.69×10^{-11}	1.79×10^{-10}
17084	2-Hydroxyglutarate	1.44	1.33	0.00	0.00	0.00	3.71×10^{-11}	1.79×10^{-10}
28179	Benzamide	24.3	12.3	0.00	0.00	0.00	3.71×10^{-11}	1.79×10^{-10}
7971	5-Methoxy-3-indoleacetate	1.09	2.13	0.00	0.00	0.00	3.71×10^{-11}	1.79×10^{-10}
-	Mucate	0.266	0.160	0.00	0.00	0.00	2.17×10^{-9}	4.88×10^{-9}
17858	Glutathione (oxidized)	0.207	0.264	0.00	0.00	0.00	8.35×10^{-8}	1.13×10^{-7}

ID	Compound							
-	Cysteine-glutathione disulphide	0.227	0.209	0.00	0.00	0.00	8.35×10^{-8}	1.13×10^{-7}
16761	ADP	0.298	0.316	0.00	0.00	0.00	8.35×10^{-8}	1.13×10^{-7}
17859	Glutarate	0.501	1.36	0.00	0.00	0.00	6.06×10^{-6}	5.53×10^{-6}
16668	Hypotaurine	0.614	0.660	0.00	0.00	0.00	1.61×10^{-5}	1.23×10^{-5}
16796	Melatonin	0.191	0.201	0.00	0.00	0.00	4.15×10^{-5}	2.86×10^{-5}
30831	2-Oxobutyrate	4.06	8.39	0.0412	0.214	0.0101	4.64×10^{-9}	9.20×10^{-9}
15702	Terephthalate	3.72	1.60	0.0785	0.0591	0.0211	1.02×10^{-9}	2.86×10^{-9}
48131	Azelate	1.73	1.02	0.0648	0.127	0.0374	2.44×10^{-10}	3.24×10^{-10}
27596	3-Methylhistidine	0.126	0.139	9.50×10^{-3}	0.0494	0.0757	1.10×10^{-6}	1.24×10^{-6}
17748	Thymidine	2.52	0.579	0.221	0.800	0.0877	8.95×10^{-9}	1.68×10^{-8}
15746	Spermine	0.0665	0.0665	7.33×10^{-3}	0.0381	0.110	6.43×10^{-5}	4.25×10^{-5}
16345	Cysteine sulfinate	0.627	0.484	0.0732	0.284	0.117	2.55×10^{-5}	1.81×10^{-5}
16027	AMP	1.04	1.08	0.124	0.284	0.120	1.40×10^{-6}	1.52×10^{-6}
17553	Ethanolamine phosphate	66.2	20.4	14.8	17.4	0.223	1.72×10^{-8}	2.64×10^{-8}
32816	Pyruvate	62.5	47.1	16.0	18.3	0.256	6.60×10^{-7}	7.67×10^{-7}
7307	1-Methyl-2-pyrrolidinone	25.0	7.93	6.51	5.41	0.260	1.40×10^{-8}	2.37×10^{-8}
16919	Creatine	30.7	14.6	8.32	3.30	0.271	1.46×10^{-9}	3.52×10^{-9}
27911	N_8-Acetylspermidine	0.150	0.0787	0.0437	0.129	0.292	4.41×10^{-7}	5.72×10^{-7}
28837	Octanoate	0.741	0.162	0.218	0.635	0.294	5.31×10^{-7}	6.64×10^{-7}
15901	Dihydrouracil	2.35×10^3	6.54×10^2	7.00×10^2	1.99×10^2	0.297	1.30×10^{-9}	3.37×10^{-9}
15676	Allantoin	24.0	4.41	8.69	3.25	0.362	2.31×10^{-9}	4.88×10^{-9}
17672	Carbamoylphosphate	33.4	10.6	12.4	14.8	0.372	8.69×10^{-6}	7.52×10^{-6}
37023	α-Aminoadipate	0.821	0.408	0.336	0.475	0.409	5.94×10^{-4}	3.06×10^{-4}
15956	Biotin	0.296	0.150	0.123	0.141	0.416	1.99×10^{-4}	1.20×10^{-4}
28358	Lactate	2.31×10^2	1.12×10^2	96.4	68.3	0.417	1.89×10^{-6}	1.95×10^{-6}
18132	Phosphorylcholine	5.21	2.21	2.21	2.22	0.423	1.81×10^{-5}	1.33×10^{-5}
16953	N-Acetylaspartate	1.54	1.13	0.658	0.331	0.427	4.95×10^{-4}	2.69×10^{-4}
30915	2-Oxoglutarate	3.78	2.33	1.64	3.47	0.435	2.59×10^{-4}	1.53×10^{-4}
29069	Phthalate	0.0841	0.0878	0.0397	0.0623	0.472	0.0495	0.0174
18021	PEP	0.463	0.275	0.219	0.312	0.472	6.00×10^{-4}	3.06×10^{-4}
32805	cis-Aconitate	0.363	0.169	0.172	0.179	0.473	2.54×10^{-3}	1.19×10^{-3}
18095	Hydroxyproline	0.873	0.537	0.432	0.431	0.495	1.42×10^{-3}	6.92×10^{-4}
15960	o-Acetylcarnitine	1.68	0.780	0.843	0.441	0.501	1.81×10^{-5}	1.33×10^{-5}
11851	2-Oxoisopentanoate	0.679	0.369	0.345	0.858	0.508	1.19×10^{-4}	7.43×10^{-5}
16446	N-Acetylglucosamine 1-phosphate	0.251	0.167	0.135	0.363	0.539	3.01×10^{-4}	1.72×10^{-4}
15946	Fluctose 6-phosphate	0.370	0.197	0.208	0.352	0.560	6.53×10^{-3}	2.74×10^{-3}
4324	3-Hydroxybutyrate	4.71	7.21	2.64	1.83	0.561	0.0452	0.0162
16695	UMP	0.510	0.518	0.286	0.458	0.561	0.0190	7.29×10^{-3}
16108	DHAP	3.68	1.39	2.08	1.62	0.566	8.92×10^{-4}	4.43×10^{-4}
18295	Histamine	0.374	0.527	0.216	0.492	0.577	0.0166	6.50×10^{-3}
17361	CMP	0.0965	0.0870	0.0573	0.176	0.594	5.19×10^{-3}	2.30×10^{-3}
30769	Citrate	18.1	10.3	10.9	8.35	0.604	2.30×10^{-3}	1.09×10^{-3}
15891	Taurine	57.8	24.3	40.1	24.5	0.694	6.58×10^{-3}	2.74×10^{-3}
17794	3PG	1.69	0.706	1.26	1.21	0.746	5.51×10^{-3}	2.41×10^{-3}
17460	Lipoamide	0.549	0.674	0.424	0.941	0.772	0.0130	5.27×10^{-3}
16411	Indole-3-acetate	1.08	1.40	0.859	2.16	0.797	6.14×10^{-3}	2.64×10^{-3}
17497	Glycolate	10.7	1.79	8.64	3.09	0.806	5.69×10^{-4}	3.00×10^{-4}
17533	N-Acetylglutamate	0.0723	0.0367	0.0695	0.137	0.961	9.48×10^{-3}	3.90×10^{-3}
16737	Creatinine	2.84	0.880	3.93	1.43	1.38	2.92×10^{-3}	1.32×10^{-3}
16958	β-Ala	1.01	0.448	1.48	0.851	1.46	0.0139	5.59×10^{-3}
1178	2-Isopropylmalate	0.159	0.0746	0.247	0.161	1.56	0.0222	8.43×10^{-3}
16235	Guanine	0.769	0.404	1.51	1.37	1.96	0.0495	0.0174
30794	Malonate	0.396	0.159	0.865	0.291	2.18	1.64×10^{-8}	2.63×10^{-8}
-	5-Oxoproline	3.84	2.81	8.70	7.55	2.26	0.0169	6.56×10^{-3}
15354	Choline	3.92	2.23	8.94	5.57	2.28	4.63×10^{-4}	2.56×10^{-4}
18019	Lys	19.2	12.8	45.1	45.0	2.35	0.0300	0.0112
17012	N-Acetylneuraminate	15.9	11.2	41.1	34.6	2.58	2.69×10^{-3}	1.23×10^{-3}
16708	Adenine	0.448	0.309	1.19	1.25	2.65	6.18×10^{-3}	2.64×10^{-3}
15741	Succinate	9.03	5.22	24.3	20.4	2.69	2.61×10^{-3}	1.21×10^{-3}
17053	Asp	6.54	2.35	18.2	14.6	2.78	9.20×10^{-6}	7.57×10^{-6}

ChEBI	Name	Mean	SD	Mean	SD	FC	P-value	q-value
17431	Agmatine	0.0578	0.0466	0.170	0.210	2.95	0.0444	0.0161
18101	4-Hydroxyphenylacetate	2.70	4.48	8.18	11.0	3.03	0.0334	0.0124
16610	Spermidine	0.402	0.320	1.27	1.70	3.15	0.0414	0.0152
15966	Glu	13.5	8.75	43.4	55.1	3.20	7.29×10^{-4}	3.67×10^{-4}
16467	Arg	9.00	2.75	28.9	20.1	3.21	6.75×10^{-6}	6.00×10^{-6}
48928	6-Phosphogluconate	0.171	0.231	0.557	0.537	3.27	5.59×10^{-4}	3.00×10^{-4}
17368	Hypoxanthine	1.55	0.973	5.83	5.65	3.77	1.32×10^{-4}	8.09×10^{-5}
15428	Gly	23.2	11.6	92.0	83.7	3.96	2.57×10^{-5}	1.81×10^{-5}
15727	Carnosine	0.115	0.290	0.469	0.434	4.07	2.69×10^{-6}	2.67×10^{-6}
17822	Ser	7.77	4.10	31.7	24.5	4.08	1.48×10^{-5}	1.16×10^{-5}
17768	*N*-Acetylputrescine	1.04	0.694	5.06	7.64	4.87	0.0145	5.75×10^{-3}
16349	Citrulline	4.61	2.82	26.2	33.1	5.68	1.79×10^{-3}	8.61×10^{-4}
15603	Leu	2.79	1.21	16.0	21.5	5.72	9.68×10^{-5}	6.16×10^{-5}
30817	Urocanate	0.942	1.54	5.49	5.14	5.82	1.25×10^{-5}	1.00×10^{-5}
17363	Ru5P	0.825	0.406	5.09	4.91	6.17	5.96×10^{-7}	7.18×10^{-7}
17203	Pro	18.8	13.5	1.18×10^2	1.25×10^2	6.29	3.49×10^{-4}	1.96×10^{-4}
506227	*N*-Acetylglucosamine	3.14	2.38	21.4	18.6	6.82	9.19×10^{-6}	7.57×10^{-6}
17562	Cytidine	0.0787	0.0807	1.05	0.828	13.4	1.19×10^{-8}	2.11×10^{-8}
17191	Ile	0.0890	0.330	6.60	9.47	74.1	9.99×10^{-10}	2.86×10^{-9}
16643	Met	0.0354	0.0873	2.88	5.55	81.4	2.81×10^{-4}	1.63×10^{-4}
-	Gly-Leu	2.96×10^{-3}	0.0145	0.918	1.35	3.10×10^2	6.90×10^{-5}	4.48×10^{-5}
-	Syringate	0.00	0.00	1.71	0.0913	N.A.	5.49×10^{-11}	2.74×10^{-10}
39708	8-Anilino-1-naphthalene sulfonate	0.00	0.00	0.330	0.182	N.A.	1.11×10^{-10}	4.17×10^{-10}
35697	trans-Cinnamate	0.00	0.00	0.869	1.14	N.A.	1.91×10^{-6}	1.95×10^{-6}
47977	Glucosamine	0.00	0.00	3.02	4.13	N.A.	4.70×10^{-6}	4.40×10^{-6}
15940	Nicotinate	0.00	0.00	2.10	2.46	N.A.	4.70×10^{-6}	4.40×10^{-6}
-	Isopropanolamine	0.00	0.00	0.324	0.413	N.A.	5.82×10^{-5}	3.93×10^{-5}

Table S4 Effect of BMI (< 18.73 kg/m^2 vs ≥ 18.73 kg/m^2)

ChEBI	Name	BMI < 18.73 kg/m^2 (n = 22)		BMI ≥ 18.73 kg/m^2 (n = 45)		FC	*P*-value	*q*-value
		Mean	SD	Mean	SD			
18295	Histamine	0.114	0.160	0.255	0.360	0.448	0.0170	0.215
32980	3-(4-Hydroxyphenyl)propionate	1.03	1.26	2.02	2.48	0.509	0.0296	0.263
18127	Cadaverine	1.32	1.41	2.50	2.62	0.526	0.0190	0.220
28631	3-Phenylpropionate	2.40	3.87	4.07	3.99	0.591	4.78×10^{-3}	0.215
17148	Putrescine	12.7	13.0	20.6	14.7	0.617	0.0237	0.235
6128	2-Hydroxy-4-methylpentanoate	0.425	0.279	0.688	0.530	0.618	9.81×10^{-3}	0.215
15887	5-Aminovalerate	74.2	1.05×10^2	1.19×10^2	93.3	0.623	8.68×10^{-3}	0.215
18257	Ornithine	12.5	10.8	18.3	14.2	0.680	0.0439	0.287
1941	γ-Butyrobetaine	0.754	0.692	1.10	0.713	0.687	9.04×10^{-3}	0.215
32978	3-Phenyllactate	0.427	0.354	0.596	0.337	0.717	0.0135	0.215
30772	Butanoate	13.3	12.8	18.3	13.0	0.725	0.0336	0.275
15611	Sarcosine	2.53	1.74	3.48	2.23	0.726	0.0453	0.287
545959	Homovanillate	1.43	1.13	1.94	1.15	0.738	0.0170	0.215
30776	Hexanoate	6.93	4.92	8.47	4.43	0.818	0.0385	0.276
16199	Urea	4.95×10^2	1.73×10^2	4.76×10^2	1.98×10^2	1.04	0.0303	0.263
6650	Malate	6.35	3.56	4.53	2.22	1.40	0.0398	0.276
18012	Fumarate	0.997	0.492	0.707	0.303	1.41	0.0163	0.215
16870	Glycerophosphorylcholine	1.98	1.45	1.38	0.531	1.44	0.0146	0.215
17196	Asn	1.74	0.972	1.20	0.809	1.46	0.0219	0.234
15946	Fluctose 6-phosphate	0.551	0.364	0.368	0.249	1.50	0.0382	0.276
16027	AMP	1.51	1.44	0.793	0.705	1.91	0.0484	0.293
16761	ADP	0.387	0.462	0.169	0.234	2.30	0.0158	0.215

Table S5 Effect of BMI (< 21.00 kg/m^2 vs ≥ 21.00 kg/m^2)

ChEBI	Name	BMI < 21.00 kg/m^2 (n = 45)		BMI ≥ 21.00 kg/m^2 (n = 22)		FC	*P*-value	*q*-value
		Mean	SD	Mean	SD			
30831	2-Oxobutyrate	4.12	4.41	2.62	4.13	0.635	0.0449	0.816
4324	3-Hydroxybutyrate	5.00	4.25	3.22	2.77	0.686	9.42×10^{-3}	0.816

ChEBI	Name	Mean	SD	Mean	SD	FC	P-value	q-value
11851	2-Oxoisopentanoate	1.06	0.436	0.752	0.287	0.788	0.0293	0.816
15888	4-Acetylbutyrate	0.417	0.137	0.349	0.124	0.875	0.0459	0.816

Table S6 Effects of menstruation

ChEBI	Name	Not menstruating (n = 63)		Menstruating (n = 9)		FC	*P*-value	*q*-value
		Mean	SD	Mean	SD			
17460	Lipoamide	0.281	0.203	0.153	0.182	0.545	0.0342	0.504
11955	4-Amino-3-hydroxybutyrate	0.0410	0.110	0.205	0.259	5.00	3.36×10^{-3}	0.314

Table S7 Effects of eating breakfast

ChEBI	Name	Not eaten (n = 72)		Eaten (n = 20)		FC	*P*-value	*q*-value
		Mean	SD	Mean	SD			
30817	Urocanate	7.25	12.7	1.23	1.70	0.170	5.64×10^{-5}	7.94×10^{-3}
-	5-Oxoproline	18.2	20.9	5.65	4.71	0.310	6.21×10^{-4}	0.0291
17822	Ser	32.0	41.4	10.0	6.21	0.314	1.15×10^{-3}	0.0403
64277	7,8-Dihydrobiopterin	23.6	28.7	8.25	8.34	0.349	9.09×10^{-3}	0.153
28358	Lactate	6.20×10^{2}	6.25×10^{2}	2.27×10^{2}	1.32×10^{2}	0.366	3.22×10^{-4}	0.0226
-	Methionine sulfoxide	0.110	0.126	0.0444	0.0842	0.405	0.0275	0.322
16643	Met	0.418	0.420	0.176	0.152	0.420	0.0126	0.177
30833	Adipate	0.171	0.159	0.0813	0.111	0.476	0.0339	0.341
16857	Thr	7.98	7.32	3.96	2.50	0.496	0.0218	0.279
17196	Asn	2.17	1.41	1.24	0.768	0.574	9.79×10^{-3}	0.153
15603	Leu	6.61	4.84	3.80	2.10	0.575	9.71×10^{-3}	0.153
48131	Azelate	2.74	1.71	1.69	0.876	0.616	7.75×10^{-3}	0.153
15702	Terephthalate	5.01	1.19	3.94	1.61	0.786	3.36×10^{-3}	0.0947
32980	3-(4-Hydroxyphenyl)propionate	1.32	2.81	1.64	1.89	1.24	0.0383	0.360
-	Mucate	0.172	0.160	0.312	0.202	1.81	0.0303	0.328

Table S8 Effects of tooth brushing

ChEBI	Name	Not brushed (n = 19)		Brushed (n = 53)		FC	*P*-value	*q*-value
		Mean	SD	Mean	SD			
30883	Adipate	0.124	0.115	0.0658	0.107	0.529	0.0410	0.978
15888	4-Acetylbutyrate	0.446	0.157	0.357	0.167	0.801	0.0265	0.978
18132	Phosphorylcholine	4.44	2.30	5.24	1.97	1.18	0.0455	0.978
17562	Cytidine	0.0886	0.0760	0.125	0.0812	1.41	0.0469	0.978

Table S9 Effects of smoking

ChEBI	Name	Non-smoker (n = 70)		Smoker (n = 14)		FC	*P*-value	*q*-value
		Mean	SD	Mean	SD			
32362	Heptanoate	0.218	0.144	0.111	0.139	0.509	0.0221	0.299
16695	UMP	0.676	2.15	0.893	1.08	1.32	0.0364	0.299
17368	Hypoxanthine	1.57	1.14	2.12	1.17	1.35	0.0476	0.299
16682	*N*-Acetyl-β-alanine	0.199	0.152	0.299	0.141	1.51	0.0424	0.299
4170	G6P	1.60	1.19	2.42	1.55	1.52	0.0476	0.299
17345	GMP	0.199	0.245	0.312	0.229	1.57	0.0406	0.299
16610	Spermidine	0.331	0.265	0.552	0.329	1.67	0.0129	0.257
16027	AMP	0.912	0.994	1.60	1.12	1.76	0.0267	0.299
17821	Thymine	1.81	2.51	3.51	3.10	1.94	0.0340	0.299
28931	3'-AMP	0.137	0.204	0.269	0.162	1.96	5.23×10^{-3}	0.257
16761	ADP	0.207	0.324	0.433	0.363	2.10	0.0124	0.257
16264	UDP-*N*-acetylglucosamine	0.110	0.199	0.272	0.271	2.47	9.02×10^{-3}	0.257
30817	Urocanate	1.49	1.98	5.78	13.1	3.87	0.0279	0.299
17688	Nicotine	2.92×10^{-3}	0.0177	0.402	0.287	1.38×10^{2}	1.34×10^{-14}	1.34×10^{-12}

Table S10 Effects of alcohol consumption

ChEBI	Name	No consumption (n = 36)		Regular consumption (n = 50)		FC	*P*-value	*q*-value
		Mean	SD	Mean	SD			
17431	Agmatine	0.0403	0.0425	0.0232	0.0382	0.576	0.0490	0.983

ChEBI	Name					FC	P-value	q-value
506227	N-Acetylglucosamine	3.78	3.93	2.23	1.83	0.590	0.0198	0.983
17012	N-Acetylneuraminate	17.1	12.1	12.5	10.8	0.733	0.0239	0.983
-	Syringate	1.57	0.123	1.62	0.105	1.03	0.0246	0.983

Table S11 Effects of the use of medications

ChEBI	Name	Not used (n = 49)		Used (n = 4)		FC	P-value	q-value
		Mean	SD	Mean	SD			
32383	6-Hydroxyhexanoate	0.358	0.404	0.0718	0.144	0.201	0.0267	0.958
29069	Phthalate	0.174	0.499	0.146	5.66×10^{-3}	0.840	0.0249	0.958

The medications included (1) vitamin drops (containing ascorbic acid and pantothenic acid), (2) theodur, (3) theophylline and olopatadine, (4) mecobalamin, and (5) furosemide.

Table S12 Effects of the use of nutritional supplements

ChEBI	Name	Not used (n = 44)		Used (n = 5)		FC	P-value	q-value
		Mean	SD	Mean	SD			
15946	Fluctose 6-phosphate	0.413	0.225	0.210	0.164	0.509	0.0268	0.738
15966	Glu	14.5	6.75	8.91	3.90	0.615	0.0345	0.738
16108	DHAP	3.00	1.57	1.87	0.809	0.623	0.0420	0.738
28340	2AB	0.986	0.285	0.637	0.120	0.646	8.84×10^{-3}	0.415
18295	Histamine	0.179	0.368	0.253	0.176	1.41	0.0244	0.738
32362	Heptanoate	0.189	0.155	0.338	0.0993	1.79	0.0376	0.738
7548	1-Methylhistamine	0.0125	0.0348	0.0301	0.0193	2.41	8.64×10^{-3}	0.415
17859	Glutarate	0.0770	0.0431	0.546	0.712	7.09	1.67×10^{-3}	0.234

The saliva donors took vitamin drops ($n = 3$), black vinegar ($n = 1$), and flavin adenine dinucleotide sodium and pyridoxal phosphate hydrate ($n = 1$).

Table S13 Effects of oral cavity disorders

ChEBI	Name	Normal (n = 25)		Abnormal (n = 22)		FC	P-value	q-value
		Mean	SD	Mean	SD			
7548	1-Methylhistamine	0.0117	0.0359	0.0203	0.0254	1.74	0.0154	0.822
16414	Val	3.63	1.66	4.93	2.31	1.36	0.0344	0.822
17394	N-γ-Ethylglutamine	0.0499	0.108	0.110	0.132	2.19	0.0390	0.822

Table S14 Effects of tooth alignment disorders

ChEBI	Name	Normal (n = 31)		Abnormal (n = 23)		FC	P-value	q-value
		Mean	SD	Mean	SD			
17562	Cytidine	0.132	0.0808	0.0795	0.0643	0.613	0.0186	0.968
15676	Allantoin	18.9	4.50	17.1	5.00	0.926	0.0470	0.968
16750	Guanosine	0.101	0.0647	0.0609	0.0661	1.07	0.0260	0.968
4324	3-Hydroxybutyrate	3.88	4.26	5.04	3.02	1.19	0.0117	0.968

Table S15 Effects of dental caries

ChEBI	Name	None (n = 13)		With carious teesh (n = 67)		FC	P-value	q-value
		Mean	SD	Mean	SD			
16010	5-Oxoproline	0.618	0.758	0.481	0.853	0.848	0.0289	0.880
17968	Butanoate	12.5	8.39	20.5	16.5	1.64	4.84×10^{-3}	0.778
17858	Glutathione (oxidized)	0.249	0.397	0.441	0.403	1.77	0.0199	0.880
30854	Indole-3-acetate	0.151	0.478	0.571	0.992	3.80	0.0203	0.880
28179	Benzamide	0.151	8.15	24.7	12.7	1.64×10^{2}	0.0299	0.880

Table S16 Effects of TMD

ChEBI	Name	Normal (n = 25)		Abnormal (n = 22)		FC	P-value	q-value
		Mean	SD	Mean	SD			
6650	Malate	3.79	1.80	5.36	2.50	1.23	0.0251	0.767
16977	Ala	17.0	9.27	23.0	8.42	1.33	6.77×10^{-3}	0.599
28358	Lactate	1.59×10^{2}	71.4	2.50×10^{2}	1.42×10^{2}	1.35	0.0146	0.767
17858	Glutathione (oxidized)	0.216	0.250	0.434	0.405	1.53	0.0401	0.767
17821	Thymine	0.655	1.44	2.61	2.39	1.68	1.66×10^{-3}	0.294