Elevated Dipeptides and Agrochemicals in the Saliva of Type 2 Diabetes Mellitus Patients: A Dual Origin Metabolomic Insights

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ABSTRACT

Introduction and aims: Type 2 diabetes mellitus (T2DM) is a prevalent metabolic disorder influenced by internal metabolic disruptions and external exposures, known as exposomes, which increase disease risk. Identifying salivary metabolites is a promising method to detect biomarkers for both endogenous and environmental factors. This study utilised a dual approach to profile salivary endogenous metabolites and exposomes, aiming to provide a comprehensive understanding of T2DM by integrating biological and environmental factors, thereby improving biomarker discovery and risk prediction.

Methods: Salivary metabolites were analysed via ultraperformance liquid chromatography coupled with Q-Exactive mass spectrometry in samples from women with T2DM (n = 39) and healthy controls (n = 40). The groups were matched for age, sex, periodontitis, dyslipidaemia, and hypertension. The identified metabolites were mapped to the Human Metabolome Database and the Blood Exposome List using U.S. Environmental Protection Agency resources.

Results: Principal component analysis revealed distinct clusters for endogenous metabolites and exposomes, leading to separate analyses. In the endogenous metabolite category, 64.5% of the metabolites significantly differing between DM and non-DM groups were dipeptides (false discovery rate <0.05, variable importance for the projection >2). Among the dipeptides, Gln-Trp and Phe-Asn were identified as the top predictors of T2DM, with an area under the curve of 0.87, while His-Phe, His-Tyr, Met-Tyr, and Leu-Gln had area under the curve of 0.85. In the exposome category, univariate regression revealed significant associations between synthetic dipeptides and agrochemical exposomes and fasting plasma glucose levels, with daminozide exhibiting the greatest effect size.

Conclusion: Leveraging saliva's noninvasive collection, these findings underscore the diagnostic potential of salivary dipeptides and emphasise the importance of addressing exposomes in T2DM management.

Clinical relevance: By integrating endogenous and exposome profiling, this study offers a novel approach for identifying metabolic and environmental risk factors, advancing biomarker discovery and risk prediction to improve early diagnosis and personalised management of T2DM.

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Introduction

The number of people with type 2 diabetes mellitus (T2DM) is anticipated to increase by 25% by 2030 and 51% by 2045 from the current level of just under half a billion.[1] To ensure early detection of diabetes and reduce complications, it is essential to implement practical, reliable, and cost-effective screening measures. While traditional glycaemic markers, such as glucose and HbA1c,[2] and new glycaemic markers, such as glycated albumin,[3] fructosamine,[4] and 1,5-anhydroglucitol,[5] have proven effective at reflecting glycaemic status, they all require blood samples. Blood sampling is invasive, necessitating the expertise of a phlebotomist, and since diabetes requires regular monitoring, repeated blood draws can cause significant patient discomfort. Saliva, on the other hand, offers a promising noninvasive and cost-effective alternative for assessing and monitoring the physiological status of healthy individuals and T2DM-affected individuals.[6]

Recent advances in metabolomics, particularly using mass spectrometry (MS)-based untargeted approaches, have revealed that, like blood, saliva is also a rich source of low-molecular-weight metabolites, comprising carbohydrates, lipids, amino acids, vitamins, organic acids, and thiols, which can serve as important indicators of health and disease.[7,8] Through a multi-platform approach complemented by computer-aided literature mining, 853 nonredundant salivary metabolites were identified and annotated.[9] This emerging information on the salivary metabolome has helped identify distinct metabolomic fingerprints in the saliva of patients with colorectal cancer,[10] breast cancer,[11] schizophrenia,[12] periodontal disease,[8,13] or burning mouth syndrome.[14] Since T2DM is a metabolic disorder, a metabolomics approach is particularly promising for biomarker discovery because it reveals the molecular pathways involved in its development and progression.[15] However, profiling of the salivary metabolome in people with T2DM has not yet reached its full potential for biomarker discovery, primarily because the existing studies explored the association of T2DM with other coexisting conditions, such as periodontitis or cardiometabolic traits [16,17] or failed to discriminate between type 1 and type 2 diabetes,[18] limiting our understanding of T2DM-specific salivary metabolites.

Over the last two decades, transformative research has emphasised that environmental factors, known as exposomes, play a more significant role in the risk of chronic diseases, including T2DM, than genetic or endogenous factors.[19] Exposomes encompass all the exogenous environmental exposures a person encounters throughout their lifetime, such as chemical, pollutant, and lifestyle factors, which interact with their biological system to influence health outcomes.[20] Researchers are now using a balanced strategy to investigate both endogenous factors and exposomes to determine the causes of chronic diseases.[21] Saliva contains valuable molecular information that can be explored through exposome-wide association studies to discover exposure–risk factors for chronic diseases.[22] Based on the literature and open-source saliva–metabolome database, Bessonneau et al[22] categorised the exposomes in saliva according to their origins, including food, drugs, pollutants, microbes and metals, in addition to metabolites originating from the host's endogenous sources. These were mapped to human metabolic diseases, central nervous system diseases, and neoplasms, indicating that the salivary metabolome captures a biologically meaningful fraction of exposomes associated with human diseases. Despite this progress, no studies have examined environmental salivary metabolites influencing T2DM.

Endogenous metabolites can serve as diagnostic markers reflecting the physiological and pathological state of the host. In contrast, exposome metabolites provide insights into external risk factors that may contribute to disease development or progression. To this end, we conducted an exploratory salivary metabolomics study to identify signature metabolites linked to T2DM, uniquely integrating endogenous and exposome metabolites to capture intrinsic biological changes and extrinsic environmental factors. This dual approach, combined with a matched and adjusted case-control group for periodontitis, cardiometabolic (dyslipidaemia and hypertension), and anthropometric factors (age and sex), addresses gaps in current research and enhances the precision of biomarker discovery for T2DM.

Methods

Study population

All subjects in this cross-sectional study were recruited from the Diabetes and Metabolic Centre at Singapore General Hospital between October 2021 and December 2022. The study included 79 women, comprising 39 diagnosed with T2DM (DM group) and 40 free of T2DM (non-DM group). T2DM is defined based on the recommendations of the American Diabetes Association: fasting plasma glucose (FPG) ≥126 mg/dL (7.0 mmol/L), 2-hour postplasma ≥200 mg/dL (11.1 mmol/L) during an oral glucose tolerance test, or HbA1c ≥6.5% (48 mmol/mol).[23] The controls were matched to the patients for age (±3 years), as these factors influence metabolic profiles[24,25] and are thus potential confounders in the association between metabolites and diabetes risk. Information on demographics, anthropometric measurements, and medical history was obtained from the hospital's medical records. Body mass index (BMI) is defined as an individual's body weight divided by the square of his or her height (kg/m^2). All individuals were enrolled voluntarily and provided written informed consent. The study was approved by the SingHealth Centralized Institutional Review Board (CIRB Ref No. 2020/2698).

Oral examination and saliva collection

A single examiner carried out the oral examination (PB). Periodontitis was defined as an interdental clinical attachment level detectable at ≥2 nonadjacent teeth or by the presence of a buccal or lingual/palatal clinical attachment level ≥3 mm with pocket depth >3 mm detectable at ≥2 teeth.[26] Bleeding on probing was assessed by gently inserting a periodontal probe into the gingival sulcus at six sites per tooth, and the presence of bleeding was recorded within 30 seconds. For saliva sample collection, subjects were asked to refrain from eating, drinking, or performing oral hygiene procedures for at least 30 to 60 minutes before collection. Participants

subsequently rinsed their mouths with water for 1 minute to remove any remaining food particles. Afterwards, unstimulated saliva was collected using the spit technique according to published protocols.[27]

Untargeted liquid chromatography coupled with MS (LC–MS)-based metabolomics

The separation and detection of metabolites were performed on a Waters UPLC I-Class Plus (Waters Corporation) coupled with a Q Exactive high-resolution tandem mass spectrometer (UPLC-Q Exactive MS) (Thermo Fisher Scientific). For chromatographic separation, 5 μL of extracted saliva was injected into a Waters Acquity UPLC BEH C18 column (1.7 μm, 2.1 mm $\times$ 100 mm; Waters Corporation), and the column temperature was maintained at 45°C. A pooled QC sample was initially injected five times to ensure system equilibrium, and then, it was injected every 10 samples during saliva sample detection to further monitor system stability (Figure S1). The mobile phase consisted of 0.1% formic acid (A) and acetonitrile (B) in positive mode and 10 mM ammonium formate (A) and acetonitrile (B) in negative mode. The gradient conditions were as follows: 0 to 1 minute, 2% B; 1 to 9 minutes, 2% to 98% B; 9 to 12 minutes, 98% B; 12 to 12.1 minutes, 98% B to 2% B; and 12.1 to 15 minutes, 2% B, with a flow rate of 0.35 mL/min. The normalised collision energy was set to 20, 40, and 60 eV. The ESI parameters were as follows: sheath gas flow rate, 40 $\times$; auxiliary gas flow rate, 10 $\times$; spray voltage, 3.80 kV; and negative-ion mode, 3.20 kV; capillary temperature, 320°C; and auxiliary gas heater temperature, 350°C. MS data were acquired using Q Exactive (Thermo Fisher Scientific) in full-scan range mode at 70 to 1050 m/z with a mass resolution of 70,000. The automatic gain control target for MS data acquisition was set to 3e6 with a maximum ion injection time of 100 ms. Sample preparation, instrument parameters, and data processing were performed at the Beijing Genome Institute (BGI LTD).

Metabolite ion peak extraction and metabolite identification

The generated raw data were processed using Compound Discoverer 3.2 (Thermo Fisher Scientific) for ion peak extraction, peak alignment, and peak integration. In brief, the raw files were aligned using an adaptive curve setting with a parent ion mass deviation of <5 ppm, a fragment ion mass deviation of <10 ppm, and a retention time deviation of <0.2 minutes. Metabolites identified in the processed raw data with mass spectral peaks were searched against bmdb (BGI metabolome database), the ChemSpider online database, the mzCloud spectral library, the Human Metabolome Database (HMDB), and PubChem. The data matrix, containing information such as metabolite peak area and metabolite identification results, was acquired into MetaX[28] for data preprocessing. The data were normalised by applying probabilistic quotient normalisation methods to improve the comparability between samples,[29] and quality control-based robust loess signal correction was used to correct batch effects.[30] Metabolites with a coefficient of variation greater than 30% in the QC samples were removed (Figure S2).

Statistical analysis

The demographic and clinical data are presented as the mean $\pm$ SD for continuous data and as the frequency (percentage) for categorical variables. T tests and chi-square tests were used to compare differences between two groups for categorical and continuous data, respectively.

The metabolites selected for analysis were filtered using HMDB,[31] and the exposomes were identified using the Blood Exposome List from U.S. Environmental Protection Agency (EPA) resources.[32] Principal component analysis (PCA) was also conducted to compare exposomes and endogenous metabolites, and a PERMANOVA was performed to assess these differences statistically. The analyses were performed in R using the 'vegan' and 'factoextra' packages. Metabolomic data analysis was performed using MetaboAnalyst 6.0 separately for endogenous metabolites and exposomes. Initial data filtering was conducted with an interquartile range (IQR) threshold of 40% to remove low-variance metabolites. The filtered data was normalised by log transformation (base 10) and Pareto scaling (mean-centred and divided by the square root of the standard deviation of each variable). Multivariate analysis was performed using orthogonal partial least squares discriminant analysis (OPLS-DA) to distinguish and visualise the discriminatory metabolites between the DM and non-DM groups. The OPLS-DA model was validated using permutation testing with 100 permutations to assess the significance of the model's predictive ability, and the empirical P value for Q2 was less than 0.05 for both the endogenous metabolite and exposome datasets, indicating that the model has a statistically significant predictive ability. The variable importance for the projection (VIP) score from the OPLS-DA model was used to measure each metabolite's impact strength and explanatory power in discriminating DM and non-DM group samples. For univariate analysis, we used an equal variance and false discovery rate correction with a significance threshold set at $q < 0.05$ to account for multiple tests and identify significantly altered metabolites. The criteria for differentially abundant metabolite screening were defined as a false discovery rate <0.05 and a VIP > 2.

Receiver operating characteristic (ROC) curve analysis was performed on endogenous metabolites ranked by VIP score using MetaboAnalyst's Explorer module, including age and BMI. ROC curves were generated using linear support vector machine models, and Monte Carlo cross-validation with balanced subsampling was employed to ensure robust performance evaluation. The area under the curve (AUC) was computed using bootstrapping with 95% confidence intervals. For the exposome metabolites, univariate regression analysis was conducted using the 'stats' package in R, with FPG as the dependent variable and adjusting for age and BMI.

Results

Clinical measurements

Table 1 shows the clinical characteristics and measurements of the DM and non-DM groups. The DM group had

Table 1 – Demographic and clinical characteristics of individuals with type 2 diabetes mellitus (DM) and without (non-DM).

	DM (n = 39)	Non-DM (n = 40)	P value
Age in years	50.4 ± 11.8	54.6 ± 11.3	.10
BMI in kg/m^2	26.8 ± 4.1	23.2 ± 5.2	**<.01**
Smoking			
Current smoker (N/%)	2 (5.1%)	0	.16
Nonsmoker (N/%)	37 (94.8%)	40 (100%)	
FPG in mmol/L	9.3 ± 3.1	5.3 ± 0.6	**<.0001**
HbA1c (N (mean ± SD))	38 (8.3 ± 1.5)	19 (5.4 ± 0.3)	**<.0001**
Insulin therapy (N/%)	21 (53.8%)	NA	-
Oral antidiabetic drugs (N/%)	36 (92.3%)	NA	-
History of hypertension (N/%)	15 (38.4%)	9 (22.5%)	.09
History of dyslipidaemia (N/%)	15 (38.4%)	10 (25.0%)	.14
Periodontal parameters			
Percentage of sites affected	14.4 ± 17.7	9.0 ± 13.5	.13
PD > 3 mm	4.0 ± 0.1	4.0 ± 0.1	.48
PD > 5 mm	6.8 ± 0	0	-
CAL > 3 mm	4.4 ± 0.5	4.3 ± 0.3	.47
CAL > 5 mm	6.1 ± 0.2	6.1 ± 0.3	.71
Bleeding on probing	39.3 ± 18.4	30.9 ± 17.7	.05
Mean plaque score	51.0 ± 16.2	47.5 ± 15.4	.30

BMI, body mass index; CAL, clinical attachment level; FPG, fasting plasma glucose; HbA1c, glycated haemoglobin; PD, pocket depth.
The data are expressed as the mean ± SD unless otherwise indicated. Continuous variables were compared with t tests, and categorical variables were compared with chi-square tests. Statistical significance was defined as a P value < .05 and is indicated in bold.

significantly greater FPG and HbA1c levels than the non-DM group (P < .001). The BMI of participants with T2DM was significantly greater than that of participants without diabetes (P < .01). Periodontal parameters and cardiometabolic factors like dyslipidaemia and hypertension showed no statistically significant differences between the groups.

Salivary metabolome profile

Salivary metabolomics data were acquired using UPLC-Q Exactive MS. After removing mass ions with a relative standard deviation >30%, 3216 metabolites were identified in both positive and negative ion modes. These metabolites were mapped to the HMDB, which identified 2020 metabolites. Among these, 1005 metabolites were identified as exposomes based on the Blood Exposome List from the U.S. EPA resources. Since the study did not focus on the effects of therapeutic agents on T2DM, 337 metabolites identified exclusively as drugs were excluded. Consequently, 1683 metabolites were selected for further analysis; these included 758 metabolites that were categorised as 'Exposomes', and the remaining 925 metabolites were categorised as 'Endogenous Metabolites'. Exposomes refer to the metabolites derived from external exposures, while endogenous metabolites originate within the body as physiological metabolic byproducts. Details of the selected metabolites in the two categories are provided in the Supplementary File (Table S1).

PCA was conducted to determine whether there were distinctions between endogenous and exposome metabolites. A significant difference was noted between the two categories, with the first principal component (PC1) accounting for 84.5% of the variance (R^2 = 0.008, P = .002) (Figure 1). Due to the different origins and biological relevance of the endogenous and exposome metabolites, the two categories were analysed independently using MetaboAnalyst to decipher the differences in metabolic profiles between the DM and non-DM groups.

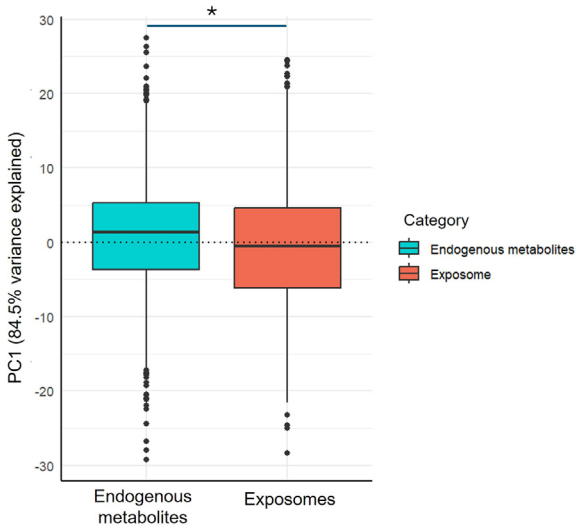

Fig. 1 – A box plot depicting the distribution of principal component 1 (PC1) scores by category. The plot illustrates the variability of PC1 across different groups, with each box representing the interquartile range (IQR) of the PC1 score and the line within the box indicating the median value. The dotted grey line at zero marks the baseline reference point for PC1. The upper and lower whiskers extend to the maximum and minimum values within 1.5 times the IQR from the quartiles, respectively. Data points outside this range are shown as outliers. *Represents a P value <.01.

Characterisation of endogenous metabolites

Data filtering of the endogenous metabolites using an IQR threshold of 40% eliminated 370 low-variance metabolites. By setting an IQR threshold of 40%, we ensured that we retained metabolites that exhibited sufficient variability to detect significant associations while filtering out those that were less likely to be informative. The remaining 555 metabolites were subsequently normalised via log transformation and Pareto scaling. The OPLS-DA score scatter plot demonstrated separation between the DM and non-DM groups, with an overlap for the filtered endogenous metabolites (Figure 2A). Of the 555 metabolites analysed, 48 significantly differed between the DM and non-DM groups, meeting the screening criteria of VIP > 2 and $q < 0.05$ (Table S2). Among these, 32 (66.6%) were dipeptides. Notably, 14 out of the 15 metabolites identified by VIP scores were dipeptides, underscoring their potential as key discriminators between the two groups (Figure 2B, Table 2). In addition to dipeptides, the melatonin radical, a metabolite of the melatonin hormone, was also significantly increased in DM patients.

Given the significant contribution of dipeptides in distinguishing between patients with and without diabetes, as indicated by their high VIP scores, we focused subsequent analysis exclusively on these endogenous dipeptides. Endogenous metabolites are intrinsic to physiological processes and are ideal biomarkers for identifying disease states. Hence, ROC curve analysis was used to evaluate the diagnostic performance of the selected dipeptides, as it quantitatively measures their ability to distinguish between the DM and non-DM groups, validating their potential as biomarkers. This model also included age and BMI for each dipeptide because they are well-established risk factors for T2DM. A multivariate ROC model was generated using a linear support vector machine for the 14 dipeptides to assess their performance across all possible thresholds via Monte Carlo cross-validation. Notably, the top predictive performances were observed for the dipeptides Gln-trp and Phe-Asn, with an AUC of 0.87, followed by His-phe, His-tyr, Met-tyr, and Leu-gln, with an AUC of 0.85 (Table 3).

Characterisation of exposomes

Data filtering of the exposomes using an IQR threshold of 40% eliminated 303 low-variance metabolites. The remaining 454 metabolites were subsequently normalised via log transformation and Pareto scaling. The exposome category analysis revealed moderate separation between salivary metabolites from the DM and non-DM groups on the OPLS-DA score scatter plot (Figure 3A). A total of 16 metabolites significantly differed between the groups, meeting the stringent screening criteria of VIP > 2 and $q < 0.05$ (Table S3). Interestingly, among the 15 metabolites identified by VIP scores, nine were dipeptides, and five were agrochemicals (Figure 3B, Table 4). L-coprine was significantly lower in DM participants.

Exposomes are external exposures that may influence health outcomes. Unlike endogenous metabolites, for which we utilised ROC analysis to identify biomarkers, we applied regression analysis to exposomes. This approach allows us to investigate the potential of these factors as risk factors for T2DM by modelling and quantifying the associations between specific exposures and health outcomes. Using the top 15 metabolites identified by VIP scores in exposome categories as independent variables, univariate linear regression

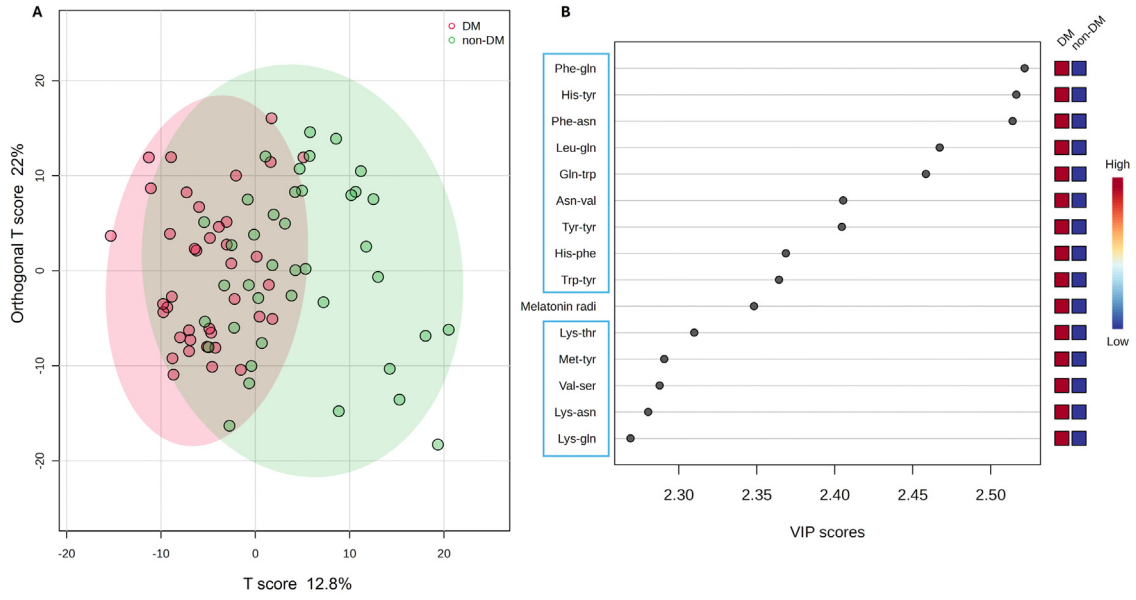

Fig. 2 – Salivary profile of metabolites in the Endogenous metabolite category. (A) Orthogonal partial least squares discriminant analysis (OPLS-DA). A score plot illustrating the separation of salivary endogenous metabolites between individuals with type 2 diabetes mellitus (DM) and those without (non-DM). Each data point represents a saliva sample, with colours indicating the respective group. (B) A variable importance in projection (VIP) score plot displaying the discriminant power of individual metabolites for distinguishing between the DM and non-DM groups. The metabolites marked within the blue boxes are dipeptides.

Table 2 – Comparison of salivary metabolites between individuals with type 2 diabetes mellitus (DM) and those without (non-DM) diabetes mellitus in the endogenous metabolite category.

HMDB ID	FDB ID	Metabolite	VIP	Fold change (DM/non-DM)	FDR
28744	111800	Asn-val	2.401	1.901	0.002
28808	111854	Gln-trp	2.456	2.205	<0.001
28892	111921	His-phe	2.366	1.682	0.002
28897	111926	His-tyr	2.512	2.097	0.001
28927	111954	Leu-gln	2.465	2.016	0.001
28946	111969	Lys-asn	2.279	1.913	0.001
28949	111972	Lys-gln	2.269	1.809	0.001
28961	111984	Lys-thr	2.310	1.999	0.001
60070	-	Melatonin radical	2.349	1.994	0.002
28985	112005	Met-tyr	2.291	1.782	0.002
28990	112010	Phe-asn	2.509	2.397	<0.001
28993	112013	Phe-gln	2.519	2.229	<0.001
29095	112099	Trp-tyr	2.358	1.650	0.004
29117	112120	Tyr-tyr	2.407	1.921	0.001
29136	112137	Val-ser	2.287	1.461	0.019

FDB, food database; FDR, false discovery rate; HMDB, human metabolome database; VIP, variable importance for the projection.

analysis was performed with FPG as the dependent variable after adjusting for age and BMI. All the exposomes except Arg-pro and L-coprine were significantly associated with the FPG level (Table 5). This included eight dipeptides and five agrochemicals. Among them, daminozide had the greatest effect size, with a coefficient = 0.20 and 95% CI [0.06, 0.34], $P = .006$.

Discussion

The present study investigated the salivary metabolomic profiles of individuals with and without T2DM using an MS-based untargeted metabolomics approach. Our study represents a novel approach by categorising the salivary metabolome into endogenous and exposome-derived metabolites, accounting for periodontitis, cardiometabolic, and anthropometric factors. This novel annotation identified endogenous salivary dipeptides as potential biomarkers for T2DM disease detection and synthetic dipeptides and agrochemical exposomes as potential risk factors for T2DM after adjusting for age and BMI.

Table 3 – A multivariate receiver operating characteristic (ROC) curve model including age and BMI for type 2 diabetes mellitus prediction.

	Multivariate ROC	
Metabolite	AUC	95% CI
Gln-trp	0.87	0.76; 0.97
Phe-asn	0.87	0.77; 0.97
Met-tyr	0.85	0.73; 0.96
His-phe	0.85	0.75; 0.95
His-tyr	0.85	0.74; 0.96
Leu-gln	0.85	0.75; 0.95
Phe-gln	0.84	0.72; 0.94
Tyr-tyr	0.84	0.73; 0.95
Lys-asn	0.83	0.73; 0.95
Lys-gln	0.83	0.71; 0.95
Lys-thr	0.83	0.73; 0.95
Trp-tyr	0.83	0.71; 0.94
Asn-val	0.83	0.71; 0.94
Val-ser	0.81	0.68; 0.93

95% CI, 95% confidence interval; AUC, area under the ROC curve.

Endogenous metabolites originate from diet or drug intake and are utilised in various physiological and metabolic pathways, becoming part of the body's internal biochemistry. In contrast, exposomes are synthetic chemicals derived from the diet and drugs in the form of food additives, pesticides, or medical-grade plastics that can disrupt normal physiological processes, contributing to disease development or affecting overall health. We employed an untargeted metabolomics approach to profile salivary metabolites and annotated endogenous metabolites using the HMDB and exposomes, utilising resources from the U.S. EPA. PCA demonstrated a significant difference between endogenous metabolites and exposomes, indicating that distinct metabolic signatures were associated with endogenous and exogenous factors. As the levels of endogenous metabolites are expected to be higher than those of the exposomes, it is more effective to investigate metabolites from the two categories distinctly.[33] Hence, considering the different origins and biological relevance of endogenous and exposome metabolites, we performed independent analyses to decipher their role in discriminating between the DM and non-DM groups.

The analysis of endogenous metabolites revealed numerous dipeptides as key discriminators in T2DM patients. Among the statistically significant metabolites, 66.6% were dipeptides, and 14 of the 15 metabolites with the highest VIP scores were dipeptides. Dipeptides are organic compounds containing a sequence of exactly two amino acids joined by a peptide bond.[31] While few dipeptides are known to have physiological or cell-signalling effects, most of these proteins serve as incomplete breakdown products of protein digestion or protein catabolism. There are three potential causes for the elevated levels of dipeptides in the saliva of patients with diabetes. Firstly, diabetes is often associated with altered

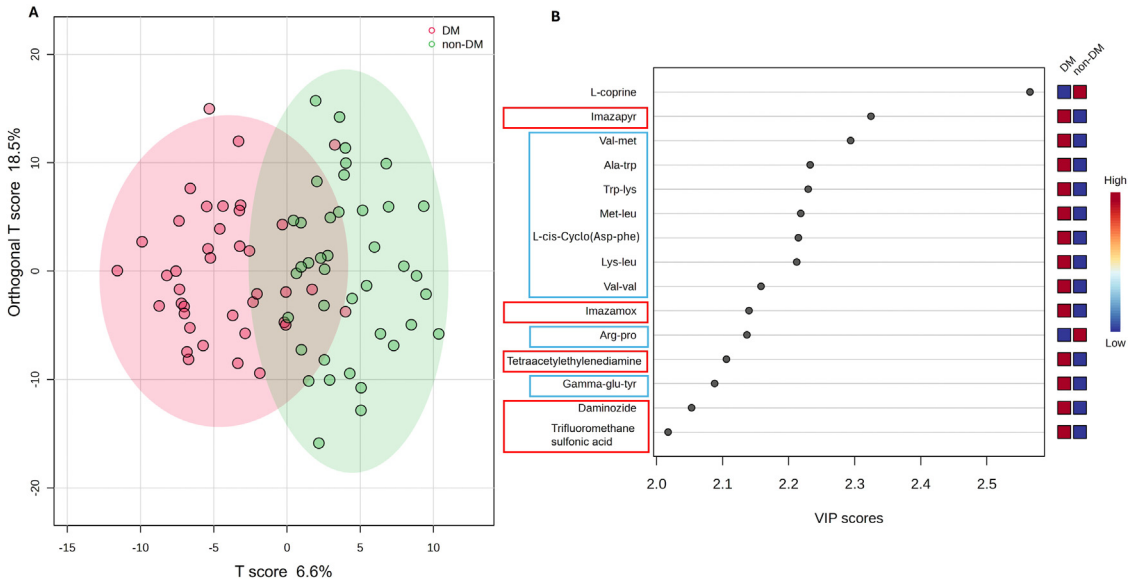

Fig. 3 – Salivary profile of metabolites in the exposome category. (A) Orthogonal partial least squares discriminant analysis (OPLS-DA). A score plot illustrating the separation of salivary exposomes between individuals with type 2 diabetes mellitus (DM) and those without (non-DM). Each data point represents a saliva sample, with colours indicating the respective group. (B) A variable importance in projection (VIP) score plot displaying the discriminant power of individual metabolites for distinguishing between the DM and non-DM groups. The metabolites marked within the blue boxes are dipeptides, and those within the red boxes are agrochemicals.

protein metabolism, which leads to increased protein breakdown.[34] This process may produce dipeptides and amino acids in the body, which are subsequently excreted in saliva. The entry of proteins and dipeptides into whole saliva via salivary glands or gingival crevices is facilitated by the enhanced basement membrane permeability associated with microvascular defects in diabetes.[35,36] Second, the overrepresentation of dipeptides in the DM group could be produced by the enhanced peptidase and hydrolase activities of the salivary gland, which can break down the protein-rich saliva typical of individuals with diabetes.[37,38] A previous study using non-obese diabetic mouse models showed that saliva and salivary glands exhibit high proteolytic enzyme activity, resulting in abnormally processed protein constituents.[39] Moreover, patients with T2DM have a higher total protein content in their saliva than healthy individuals, providing abundant substrates for dipeptide formation.[40,41] Finally, a high abundance of dipeptides in saliva could be the product of oral

Table 4 – Comparison of salivary metabolites between individuals with type 2 diabetes mellitus (DM) and those without (non-DM) diabetes mellitus in the exposome category.

HMDB ID	DTXSID	Metabolite	VIP	Fold change (DM/non-DM)	FDR
34266	974419	L-coprine	2.57	0.43	0.002
253405	8034665	Imazapyr	2.32	2.70	0.001
259742	70303398	Val-met	2.29	2.39	0.001
29088	10199717	Trp-lys	2.23	2.27	0.001
13209	20874543	Ala-trp	2.23	1.89	0.001
28977	60392372	Met-leu	2.22	2.32	0.001
31360	4044244	L-cis-cyclo(Asp-phe)	2.21	2.23	0.001
28955	70426798	Lys-leu	2.21	1.98	0.001
29140	40959967	Val-val	2.16	1.96	0.002
28717	70178886	Arg-pro	2.14	0.48	0.024
253404	3034664	Imazamox	2.14	2.91	0.002
11741	995841	Gamma-glu-tyr	2.09	2.05	0.001
40573	5040752	Tetraacetylethylenediamine	2.11	2.00	0.002
250838	9020370	Daminozide	2.05	1.40	0.015
36577	2044397	Trifluoromethanesulfonic acid	2.02	2.50	0.002

DTXSID, distributed structure-searchable toxicity (DSSTox) substance identifier; FDR, false discovery rate; HMDB, human metabolome database; VIP, variable importance for the projection.

Table 5 – Univariate linear regression analysis for salivary exposomes after adjusting for age and BMI.

Exposome	Coefficient (95% CI)	*P* value
Cyclo (Asp-Phe)	0.13 (0.04; 0.21)	.003
gamma-Glu-tyr	0.10 (0.04; 0.15)	.001
Met-Leu	0.06 (0.02; 0.09)	.004
Val-met	0.06 (0.02; 0.11)	.004
Trp-lys	0.10 (0.04; 0.16)	.002
Ala-Trp	0.10 (0.05; 0.16)	.001
Arg-pro	−0.03 (−0.09; 0.03)	.350
Lys-leu	0.11 (0.04; 0.18)	.002
Val-val	0.08 (0.02; 0.14)	.011
L-coprine	−0.07 (−0.16; 0.01)	.080
Daminozide	0.20 (0.06; 0.34)	.006
Imazamox	0.08 (0.03; 0.14)	.002
Imazapyr	0.11 (0.05; 0.17)	.001
Tetraacetyl ethylenediamine	0.09 (0.01; 0.16)	.020
Trifluoromethanesulfonic acid	0.05 (0.01; 0.10)	.016

95% CI, 95% confidence interval.
Significance was set at a *P* value <.05.

bacterial metabolism. Periodontal bacteria possess dipeptidyl peptidases (DPPs) and exopeptidases in their periplasmic space, which release various dipeptides from the N-terminus of polypeptides.[42] However, since the DM and non-DM groups had comparable levels of periodontitis, this explanation is less likely.

The dipeptides identified in our study have not been previously reported or discussed in the literature. Consequently, the source and pathophysiological mechanisms underlying their association with diabetes remain largely unknown. However, these findings can be attributed to the altered protein metabolism associated with diabetes, according to the available evidence. A cross-sectional study revealed that muscle histidine-containing dipeptides increased with progressive glucose intolerance.[43] Since T2DM is linked to a greater proportion of fast glycolytic type 2 muscle fibres, which store abundant histidine-containing dipeptides, this shift towards faster fibre types in diabetic muscle likely contributes to elevated dipeptide levels.[44] In diabetic rats, the increased levels of the collagen-derived dipeptides Ala-Pro and Pro-Pro were attributed to heightened prolidase activity, leading to augmented collagen breakdown and dipeptide release.[45,46] T2DM patients also exhibit elevated circulating DPP-4 levels, which cleave incretin hormones such as glucagon-like peptide-1 and glucose-dependent insulinotropic polypeptide, exacerbating hyperglycemia.[47] Despite its relatively specific substrate preference, DPP-4 has been shown to cleave various protein substrates to form dipeptides in pharmacological, in vitro, and animal studies.[48] These findings suggest elevated dipeptide levels in diabetes patients are likely linked to increased protein content and proteolytic activity. The high predictive power of some of the dipeptides identified in this study emphasises the significance of dipeptides as biomarkers and highlights the need for further investigation into their potential roles and mechanisms in T2DM development.

The analysis of metabolites in the exposome category revealed more distinct clusters on the OPLS-DA plot than did the analysis of endogenous metabolites, suggesting that the exposomes of individuals with and without diabetes are more differentiated than their endogenous metabolomes are. A comparison between the DM and non-DM groups revealed that dipeptides, agrochemicals, and mushroom-derived L-coprine were the primary discriminators of T2DM, as these compounds had the highest VIP scores. Regression analysis showed that all exposomes, except Arg-pro and L-coprine, were significantly associated with FPG levels. Although dipeptides were also found in the endogenous metabolites category, the dipeptides identified as exposomes are not naturally occurring and may have potential health implications. The dipeptide cyclo(Asp-Phe) was positively associated with FPG, with an estimated coefficient of 0.13 in the regression model. It is a metabolite of the dipeptide sweetener aspartame, which decomposes to cyclo-Asp-Phe when beverages are exposed to elevated temperature, pH, and moisture extremes.[49] Although cyclo(Asp-Phe) was detected in diabetic saliva with notable significance, its presence is rather expected in participants with diabetes and is not inherently pathological. Gamma-Glu-Tyr and Trp-Lys, with regression coefficients of 0.1, have been identified as novel DPP-IV inhibitors, suggesting their potential use as alternative treatments for T2DM.[50,51] However, these metabolites are listed in the Comparative Toxicogenomic Database and are considered chemicals of emerging concern.[52,53] Other dipeptides, such as Met-Leu and Lys-Leu, have been detected but not quantified in animal meat, except for Val-Met.[54] Its presence in humans is predicted to result from likely exposure to far-field pesticides and residential chemicals.[54]

Numerous epidemiological studies have linked human pesticide exposure to the prevalence of insulin resistance-related metabolic diseases, including T2DM.[55] For example, daminozide, which had the highest regression coefficient among all exposomes analysed, is a plant growth regulator and is a known inhibitor of KDM2A (lysine demethylase 2A), which is a negative gluconeogenesis regulator.[56] Imazapyr and imazamox are imidazolinone herbicides. Imazamox-based herbicide formulations reduce the size of β-islet cells and increase serum glucose and calcium levels.[57] Tetraacetylethylenediamine, a bleach activator and antimicrobial pesticide used in food-contact paper, dairy processing equipment, and food processing equipment,[58] is produced by acetylation of ethylenediamine, which is reported to exacerbate diabetes in partially depancreatised rats via activation of the anterior pituitary-adrenal cortex axis.[59] Trifluoromethanesulfonic acid, a perfluorinated compound, is used in the pharmaceutical, agrochemical and fine chemical industries.[60] Exposure to low concentrations of trifluoromethanesulfonic can disturb liver lipid metabolism, possibly by altering the gut microbiota, suggesting health risks.[61] Although the regression coefficient for exposome-related risk factors for T2DM was relatively low, this does not diminish their potential impact on disease development. Exposomes typically enter the body in small quantities. However, continuous daily exposure to these endocrine-disrupting chemicals, even at concentrations below the established tolerance threshold for individual substances in the human body, can significantly elevate the risk of hormonal and metabolic disorders, including diabetes.[62] Our findings suggest that inadvertent and persistent exposure to exposomes plays a critical role in diabetes

management, as it can disrupt metabolic pathways and impair insulin sensitivity, thereby increasing the risk of diabetes development. These factors may hinder dietary control and therapeutic efforts by interfering with the body's metabolic processes and responses to interventions. Consequently, a comprehensive diabetes management strategy should incorporate measures to reduce dietary and environmental exposures to mitigate disease risk effectively.

While saliva collection after a 30 to 60-minute fasting period minimised the likelihood of food contamination, supporting the endogenous origin of the dipeptides, a key limitation of this study is the lack of dietary information, which could have offered further insights into altered protein metabolism and the dipeptides' endogenous nature. Although the global incidence of T2DM is greater in men than in women, we selected only women for this study because they face a more significant burden of risk factors at the time of their T2DM diagnosis, mainly due to obesity.[63] Women's lives are marked by substantial hormonal fluctuations and body changes driven by reproductive factors, which differentiate their metabolic profiles from those of men.[64] Exposomes also differ between women and men due to variations in exposure patterns and biological factors resulting from differences in lifestyle behaviours. Women may encounter distinct physicochemical exposures through personal care products, household cleaners, and dietary and environmental pollutants, leading to different health outcomes between sexes.[65] Given these sex-specific differences in risk factors, comorbidities, complications, and exposures, focusing on women in this study allows us to more effectively identify relevant salivary biomarkers associated with T2DM in this population.

In this study, we accounted for endogenous metabolites, exposomes, and systemic and anthropometric attributes, capturing a broader spectrum of risk factors for T2DM. Firstly, detecting a significantly elevated number of dipeptides in the saliva of patients with T2DM, irrespective of their endogenous or exogenous origin, suggests a strong association between dipeptide formation and T2DM. Secondly, even low-level exposure to agrochemicals through dietary intake could affect diabetes status, highlighting the need for additional research and regulatory scrutiny.

Author contributions

P. Balan and J. Seneviratne designed the study. W. Lim, Q Chen, and YM. Bee and P. Balan collected the samples and clinical data. BGI, Hong Kong, conducted the metabolomic experiments. P. Balan, F. Leite, and J. Seneviratne analysed the data. P. Balan wrote the manuscript. P. Balan, W. Lim, and YM. Bee, F. Leite, and J. Seneviratne contributed to conceptualisation, supervision, management, manuscript review, and editing. All authors read and approved the final manuscript.

Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this article.

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Supplementary materials

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