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# Investigation of carbonic anhydrase 6 gene polymorphism rs2274327 in relation to the oral health status and salivary composition in type 2 diabetic patients

Marwa Mrag[a], Haithem Hamdouni[b], Aslem Gouiaa[a], Asma Omezzine[b], Faten Ben Amor[a] and Asma Kassab[a]

[a]University of Monastir, Faculty of Dental Medicine, Oral Health and Oro-Facial Rehabilitation Research Laboratory LR12ES11, Monastir, Tunisia; [b]University of Monastir, Faculty of Pharmacy, LR12SP11, Monastir, Tunisia

**ABSTRACT**

**Objective:** The aim of the present study was to investigate the oral manifestations and salivary composition in type 2 diabetics with periodontitis and to evaluate their association with CA6 gene polymorphism rs2274327.

**Methods:** Oral examination was performed by a single periodontist for 300 type 2 diabetics. Whole unstimulated saliva and blood were collected. The salivary pH, buffer capacity and flow rate were later measured. Immunoglobulin A and electrolytes were assessed using an autoanalyzer. CA6 gene polymorphism rs2274327 was screened by PCR-RFLP assay. The statistical analysis was performed using the SPSS 20.0 version.

**Results:** The salivary pH, buffer capacity and flow rate were significantly lower in the patients carrying TT genotype compared to CC and CT genotype carriers ($p < .05$). Furthermore, the DMFT index, OHI-s, PI, PPD and CAL were significantly higher in the subjects with TT genotype ($p < .05$). Carrying at least one T allele seemed to increase the risk of dental caries (OR = 2.59, $p < .001$), xerostomia (OR = 2.11, $p = .003$) and taste impairment (OR = 1.97, $p < .05$).

**Conclusion:** CA6 gene polymorphism rs2274327 seemed to increase the risk of developing, dental caries, periodontitis, xerostomia and taste impairment in type 2 diabetics.

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Carbonic anhydrase 6; salivary pH; periodontitis; caries; xerostomia

## Introduction

Type 2 diabetes mellitus is a chronic metabolic disease that is prevalent worldwide. This disease is characterised by an insulin resistance-induced hyperglycaemia and it is associated with several problems, including oral complications [1]. Diabetics are often susceptible to xerostomia, dental caries, gingivitis and periodontitis [2]. Several mechanisms, such as basement membrane alteration and autonomic neuropathy [3], have been suggested to participate in the salivary function and the composition impairment reported in diabetic patients [4]. In fact, saliva performs a potential role in the oral microenvironment homeostasis, particularly in dental tissues protection [5]. Imbalanced pH has been suggested to affect the de- and re-mineralisation processes [6]. It has recently been reported that reduced salivary flow rate and pH are associated with high caries scores [7] and periodontitis [8].

Carbonic anhydrase 6 (CA) is the main salivary zinc metalloprotein responsible for salivary pH and buffer capacity regulation. It catalyses reversible hydration of carbon dioxide according to the following reaction: $CO_2 + H_2O \leftrightarrow H_2CO_3 \leftrightarrow H+ + HCO_3-$ [9].

CA6 performs a remarkable role in several physiological processes particularly in oral homeostasis and dental tissue protection through salivary buffer capacity and pH regulation [10]. In fact, experimental evidence states that CA6 is expressed at all the stages of odontogenesis, up to enamel maturation [11]. Enamel quality alteration impairment has been suggested to affect the initial tooth covering protein pellicle, bacteria profile attachment, and therefore participate in tooth tissue destruction under acidic condition [6]. Moreover, the zinc isoenzyme is involved in ion transport and in the secretion process [12]. It has been suggested as an anti-inflammatory, immunomodulating, and mucosa-protecting agent [13]. CA6 is also known as gustin, referring to its involvement in taste perception [14].

The gene encoding this salivary isoenzyme is located at 1p36.2 and is composed of 8 exons and 7 introns [15].

The function of CA6 is likely to be affected by SNPs in the gene coding sequence [8,12]. Therefore, genetic factors may contribute to the development of oral diseases.

A limited number of genetic studies conducted on SNPs in CA6 exon sequences has been performed in relation to dental caries risk [8,15]. Interestingly, the rs2274327 (C/T) variant is thought to be the only SNP related to CA6 dysfunction. In fact, it has been associated with decreased buffer capacity in children [8] and in type 2 diabetic patients [12].

Nevertheless, the CA6 gene polymorphism involvement in oral diseases is still underinvestigated, particularly rs2274327 implication in periodontitis.

CONTACT Marwa Mrag marwa.mrag@yahoo.fr University of Monastir, Faculty of Dental Medicine, Oral Health and Oro-Facial Rehabilitation Research Laboratory LR12ES11, 5019, Monastir, Tunisia

We, therefore, aimed through this work to investigate the oral manifestations and salivary composition in type 2 diabetic patients with periodontitis, and to assess the association between CA6 rs2274327 and the oral aspects in diabetic patients.

## Materials and methods

### Sample size calculation

The following conventional formula [16] was used to determine the convenient sample size based on the prevalence of type 2 diabetes in Tunisia (15.1%) [17] and rs2274327 according to the MAF database (0.27): $n=(z)2\ p\ (1-p)/d2$, with $n$=sample size; $z=1.96$ for a 95% confidence level; p = disease prevalence in a given population; $d$= tolerated margin of error, $d=0.05$). Accordingly, the highest obtained sample size was taken.

### Subjects

The study population involved 300 type 2 diabetic patients with periodontitis recruited from Fattouma Bourguiba and Sahloul hospital. The study was performed at the University Dental Medicine Department over a 15-month period, starting from December 2017 to February 2019. The data were collected via the hospital medical files in accordance with the STROBE recommendations [18]. The study procedures were approved by the Ethics Committee conforming to the Helsinki Declaration (IRB 00008931). A written informed consent was obtained from all the study participants. The exclusion criteria were: completely-edentulous subjects, smokers, alcoholics, pregnant or lactating women, and subjects presenting illnesses that may affect their oral health status.

### Oral health status

Full-mouth periodontal assessments, excluding third molars, were performed on six sites per tooth by a single periodontist using standard William's graduated periodontal probe.

The periodontal examination included the simplified oral hygiene index (OHI-S) of Greene and Vermillion, periodontal probing depth (PPD), clinical attachment loss (CAL), O'Leary Plaque Index (PI), Löe and Silness gingival index (GI), and the percentage of bleeding index on probing (BOP%). Periodontitis severity was graded, according to the 2017 World Workshop on the Classification of Periodontal and Peri-Implant Diseases and Conditions [19], as follows: mild (CAL:1–2 mm and PPD: $\leq$4 mm), moderate (CAL: 3–4 mm and PPD: $\leq$5 mm) and severe periodontitis (CAL $\geq$ 5 mm and PPD: $\geq$6 mm). The caries status was evaluated using the decayed, missing and filled permanent teeth index (DMFT). Xerostomia was diagnosed via the Fox test [20]. Oral candidiasis was diagnosed depending on membranous lesions of the oral mucosa and was confirmed by a laboratory test. Data on halitosis and taste impairment were also recorded via semi structured questionnaire.

### Saliva sampling

Resting whole saliva was collected [21] from the subjects while fasting. The pH was measured using a pH paper (macherey nagel pH-Fix 0–14). The salivary flow rate was calculated and expressed in mL/min [22]. The samples were then subjected to a centrifugation at 5000 rpm for 10 min at 4 °C to reduce salivary debris and viscosity. The buffer capacity was determined according to Ericsson's method: 1.5 ml of 5 mmol/L HCl was mixed with 0.5 ul of saliva and it was vigorously shaken. Then, the mixture was centrifuged for 1 min. After 10 min, the final pH was measured using pH papers [23].

### Biochemical analysis

The salivary assays were performed in the Biochemistry Laboratory at Sahloul hospital, Sousse, Tunisia. Beckman Coulter AU680 (Beckman-Coulter, USA) autoanalyzer was used for the estimation of chloride, potassium (potentiometric method) and calcium (Arsenazo method). The IMMAGE Immunochemistry System (Beckman Coulter, USA) was used for Total IgA levels assessment by the nephelometry method according to Beckman manufacturers.

### rs2274327 genotyping

Four ml of venous blood was drawn into EDTA tube for DNA extraction according to the salting out method [24]. The PCR-RFLP assay was applied to identify Carbonic Anhydrase 6 gene polymorphism rs2274327 (C>T position 8949347) using forward primer 5′-GACCTGCTTCTGCTTTCTGG-3′ and reverse primer 5′-ACACGTGTAACCTGCCTTCC-3′. The reaction was carried out in a final volume of 50 µl with 0.2 mM of each primer, 0.1 mM dNTP,100 ng of genomic DNA, and 2 units of Taq DNA polymerase. The PCR process included initial denaturation at 95 °C for 5 min, followed by 32 cycles of denaturation at 95 °C for 30 s, annealing for 30 s at 62 °C and synthesis for 45 s at 72 °C. The final extension was carried out at 72 °C for 7 min. The PCR product (387 bp) was digested by 5 units of BseGI restriction enzyme for 2 h 30 min at 55 °C, and it was visualised on a 2% agarose gel. The RFLP products were classified as C/C (387 bp), C/T (387, 226 and 161 bp) and T/T (226 and 161 bp). All the subjects were successfully genotyped.

### Statistical analysis

Data analysis was performed using the SPSS 20.0 version. When the quantitative parameters distribution was Gaussian, they were evaluated by the two-way analysis of variance and they were compared by Student's t-test. They were expressed as mean ± standard deviation. Otherwise, they were represented as median [min-max] and were compared by non-parametric *U* test of Mann–Whitney and Kruskal–Wallis test. The Tamhane's T2 *post-hoc* Test was conducted to determine which genotypes pairwises comparisons (CC vs. CT, CC vs. TT and CT vs. TT) differed significantly. The

qualitative variables were compared using Pearson-chi square test and they were expressed as *n* (%).

The studied SNP was tested for Hardy–Weinberg equilibrium in cases. The genotypic association was investigated using Chi squared analysis. The dominant model was used for risk analysis. The Association between the studied polymorphism and oral diseases was reported as odds ratio (OR) and 95% confidence intervals (CI).

## Results

The clinical features of the study population are presented in Table 1. The study included 300 type 2 diabetics with periodontitis. The periodontal examination revealed that 54% of them were diagnosed with severe periodontitis. The allele frequencies of rs2274327 polymorphism were in Hardy–Weinberg equilibrium ($p$=.207). The CC genotype of the rs2274327C>T was found in 97 (32.3%) subjects, the CT genotype in 142 (47.3%) and the TT genotype in 61 (20.4%) subjects. The C and T allele frequencies were 0.56 and 0.44, respectively.

CA6 gene rs2274327 polymorphism was statistically analysed in relation to the salivary physico-chemical parameters (Table 2). Interestingly, the patients carrying TT genotype showed the lowest values of salivary pH and flow rate compared to the patients with CC genotypes ($p < .05$). Furthermore, TT genotype carriers had statistically significant lower value of buffer capacity than CT and CC genotypes ($p < .05$). The total IgA, calcium, potassium and chloride rates did not differ significantly between the genotypes.

Comparison of the clinical parameters according to the genotypes (Table 3) revealed that the patients carrying TT genotype were characterised by higher scores of PI, PPD and CAL compared to the patients with CC genotypes ($p < .05$). Whereas, CC genotype carriers had lower scores of DMFT index and OHI-s compared to the patients with CT and TT genotypes ($p < .05$).

Moreover, xerostomia and taste impairment were significantly more frequent in patients with TT genotype compared to other genotypes ($p < .05$). However, GI, BOP, halitosis and oral candidiasis did not differ significantly according to genotypes ($p$>.05). Moreover, the dominant model was used to calculate the risk of developing oral complications such as dental caries, xerostomia, taste impairment, halitosis, and oral candidosis (Table 3). Carrying at least one T allele seemed to significantly multiply the risk of developing dental caries and xerostomia by over two folds. Moreover, T allele seemed to increase the risk of taste impairment by 1.97 fold ($p$=.006). However, no association was found between this allele and halitosis or oral candidosis.

**Table 1.** Characteristics of the study participants.

| Parameters | Type 2 diabetics with periodontitis ($n = 300$) |
|---|---|
| Gender *n* (%) | |
| Male | 114 (47.5) |
| Female | 126 (51.5) |
| Age (mean ± SD) years | 62.05 ± 11.3 |
| BMI (mean ± SD) (kg.m$^{-2}$) | 28.69 ± 4.68 |
| Diabetes duration (years) | 11 [6.5–28] |
| Diabetes treatment *n* (%) | 280 (93.3) |
| Diabetic complications *n* (%) | |
| Microangiopathy | 161 (53.7) |
| Macroangiopathy | 23 (7.7) |
| Tooth brushing frequency/day Med [min-max] | 2 [0–3] |
| Mouthwash *n* (%) | 72 (46.2) |
| Adjuvant means *n* (%) | 38 (41.3) |
| DMFT | 10 [4–24] |
| Periodontitis *n* (%) | |
| Mild | 50 (16.7) |
| Moderate | 88 (29.3) |
| Severe | 162 (54) |

SD: standard deviation; med: median; min: minimum; max: maximum.

## Discussion

CA6 is involved in several physiological processes, including buffer capacity maintenance and pH regulation that are important for dental tissue protection.

The aim of the present study was to investigate whether the rs2274327C>T polymorphism of CA6 is associated with the oral health status and the salivary factors in diabetic patients with periodontitis.

In the present work, diabetic subjects carrying TT genotype were characterised by more altered salivary flow rate, pH value and buffer capacity than those with CT and particularly CC genotype.

These findings are in accordance with the evidence from the literature reporting a decreased buffer capacity in type 2 diabetic patients [12], as well as in healthy children carrying the TT genotype of the CA6 rs2274327 [8]. However,

**Table 2.** Comparison of salivary parameters according to CA6 rs2274327C>T genotypes.

| Parameters/Genotypes | Type 2 diabetics with periodontitis ($n = 300$) | | | |
|---|---|---|---|---|
| | CC | CT | TT | *p* Value |
| Salivary pH (mean ± SD) | 6.95 ± 0.77 | 6.84 ± 0.83 | 6.59 ± 0.72[b] | .021 |
| Buffer capacity (mean ± SD) | 4.28 ± 0.11 | 4 ± 0.19[b] | 3.52 ± 0.11[b,c] | <.001 |
| Salivary flow rate (mean ± SD) (mL/min) | 0.52 ± 0.11 | 0.49 ± 0.13 | 0.45 ± 0.12[b] | .005 |
| Total IgA med [min; max] (g/L) | 0.19 [0.07–2.59] | 0.20 [0.07–2.59] | 0.25 [0.07–2.59] | .320[a] |
| Potassium (mean ± SD) (mmol/L) | 29.94 ± 10.45 | 29.69 ± 16.15 | 29.3 ± 9.73 | .957 |
| Calcium (mean ± SD) (mmol/L) | 1.52 ± 0.66 | 1.5 ± 0.68 | 1.56 ± 0.71 | .832 |
| Chloride (mean ± SD) (mmol/L) | 27.77 ± 12.2 | 29.19 ± 14.82 | 30.42 ± 14.93 | .499 |

SD: standard deviation; med: median; min: minimum: max: maximum.
[a]*p* Value calculated by Kruskal–Wallis test.
[b]$p$<.05 compared to CC genotype (The Tamhane's T2 *post-hoc* analysis).
[c]$p$<.05 compared to CT genotype (The Tamhane's T2 *post-hoc* analysis).

**Table 3.** Comparison of oral health status according to CA6 rs2274327 C > T genotypes.

| | Type 2 diabetics with periodontitis ($n = 300$) | | | |
|---|---|---|---|---|
| Parameters/Genotypes | CC | CT | TT | *p* Value |
| OHI-S (mean ± SD) | 2.21 ± 0.53[c] | 2.47 ± 0.41 | 2.57 ± 0.47[b] | <.001 |
| PI (mean ± SD) | 62.45 ± 21.99 | 68.52 ± 22.94 | 70.50 ± 21.98[b] | .018 |
| GI ( mean ± SD) | 2.05 ± 0.52 | 2.09 ± 0.57 | 2.11 ± 0.58 | .801 |
| BOP ( mean ± SD) | 71.17 ± 21.49 | 68.76 ± 21.52 | 65.06 ± 21.16 | .220 |
| PPD (mean ± SD) | 6.74 ± 1.32 | 7.07 ± 1.23 | 7.23 ± 1.25[b] | .040 |
| CAL (mean ± SD) | 4.74 ± 1.29 | 5.05 ± 1.24 | 5.25 ± 1.28[b] | .037 |
| DMFT index med [min;max] | 8 [1–21][c] | 10 [2–23] | 10 [4–24][b] | .036[a] |
| | | | | OR[d]=2.59 [1.57–4.28], *p*<.001 |
| Xerostomia *n* (%) | | | | |
| Yes | 41 (23.56) | 80 (45.98) | 53 (30.46) | <.001 |
| No | 56 (44.44) | 62 (49.21) | 8 (6.35) | OR[d]=2.11 [1.28–3.48], *p*=.003 |
| Taste impairment *n* (%) | | | | |
| Yes | 33 (22.3) | 73 (49.32) | 42 (28.38) | .003 |
| No | 64 (42.1) | 69 (45.4) | 19 (12.5) | OR[d]=1.97 [1.21–3.23], *p*=.006 |
| Halitosis *n* (%) | | | | |
| Yes | 40 (30.3) | 69 (52.3) | 23 (17.4) | .287 |
| No | 57 (33.9) | 73 (43.5) | 38 (22.6) | OR[d]=1.18 [0.72–1.92], *p*=.505 |
| Oral Candidiasis *n* (%) | | | | |
| Yes | 20 (28.2) | 41 (57.7) | 10 (14.1) | .110 |
| No | 77 (33.6) | 101 (44.1) | 51 (22.3) | OR[d]=1.29 [0.71–2.31], *p*=.391 |

DMFT: decayed-missing-filled permanent-teeth; med: median; min: minimum; max: maximum; OHI-S: simplified oral hygiene index; SD: standard deviation; PI: plaque index; GI: gingival index; BI: bleeding index; PPD: periodontal probing depth; CAL: clinical attachment loss.
[a] *p* value calculated by Kruskal–Wallis test.
[b] p<0.05 compared to CC genotype (The Tamhane's T2 Post-Hoc analysis).
[c] *p*<.05 compared to CT genotype (The Tamhane's T2 *post-hoc* analysis).
[d] CA6 rs2274327 risk allele was calculated according to the dominant model (TT+CT vs. CC) and presented as: OR, 95%IC, p.

investigating the same SNP, Sengul et al. reported no association between the salivary physicochemical parameters (pH and flow rate) and the genotypes [25].

The CA6 rs2274327 polymorphism (Chr1: g. 9,009,406C>T; c.164C>T) results in a missense substitution of a polar amino acid (Threonine) to a non-polar amino acid (Methionine) in position 55 of the enzyme. This position is present in a O-glycosylation site important to protein maturation. In fact, the rs2274327 was suggested to affect protein conformation, and eventually its expression level, which is probably reflected by a decreased CA6 concentration reported in subjects carrying TT genotype [26]. Moreover, recent research has suggested that impairment of the salivary pH or the buffer capacity strongly reflects CA6 concentration alteration [15,25], which in its turn has been associated with increased caries risk [9].

In the present study, a significant increase of the DMFT index, which is the dental caries status indicator, was noted in the patients with TT genotype. Moreover, the risk calculation showed that carrying at least one T allele seemed to increase the dental caries susceptibility in diabetic patients by 2.59 fold. Recent studies [6,15] are consistent with the CA6 SNP-caries association and they have reported that genetic variation in the CA6 gene promotes cariogenic species colonisation and therefore affects the oral microbiota ecology, which enhances the dental caries risk [6]. Moreover, rs17032907 SNP in the CA6 intronic region is reported to be associated with the caries risk in an adult Chinese population [27]. In fact, CA6 has the ability to diffuse into the dental plaque where it neutralises acidity by removing hydrogen ions [28], and therefore reduces enamel demineralisation [29]. Further findings have indicated that CA6 protects from dental demineralisation through the neutralisation of the lactic and acetic acids produced by the bacterial plaque [28]. It consequently prohibits cariogenic pathogens colonisation [30].

In the present work, high OHI-s, presenting the dental calculus-debris index and PI index were associated with rs2274327 TT genotype which was in turn associated with a reduced CA6 buffer capacity. In fact, the buffer capacity alteration is suggested to be involved in dental plaque and calculus reticulation [31]. This phenomenon is considered as a potential activator for periodontal disease aggravation, found in 54% of our diabetic subjects. Furthermore, diabetics carrying TT genotype were characterised by high PPD and CAL periodontal scores which may be enhanced by dental plaque and calculus accumulation due to a decreased salivary pH, buffer capacity and flow rate.

Interestingly, we noted a significant association between the genotypic and allelic CA6 rs2274327 polymorphism and xerostomia. Indeed, carrying the homozygous or heterozygous T allele seemed to predispose the diabetic subjects to xerostomia by 2.11 folds. As CA6 is known to be involved in saliva secretion [12], this association could be explained by an impairment of secretion, subsequent to rs2274327 CA6 polymorphism.

Moreover, diabetic subjects carrying at least one T allele were more likely to develop taste impairment by 1.97 fold ($p$=.006). It was suggested that CA6 genetic variation affects the zinc binding site of gustin (CA6) and therefore influences the gustin taste function [32]. Similar to our results, alteration of CA6 secretion is associated with different taste dysfunctions, including dysosmia, dysgeusia, hyposmia and hypogeusia [8].

However, our study presents some limitations. An evaluation of additional CA6 polymorphisms could have increased the strength of the work.

In summary, to the best of the authors' knowledge, this is the first study investigating the rs2274327 SNP of CA6 gene in relation to the oral conditions and the salivary parameters among a Tunisian population. In diabetics, TT genotype was simultaneously associated with the decrease of salivary physicochemical parameters (pH, buffer capacity and flow rate) and the increase of periodontal scores (PPD, CAL, PI, OHI-s) as well as the DMFT index, compared to CC or CT genotypes. Furthermore, carrying at least one T allele seemed to multiply the risk of dental caries, xerostomia and taste impairment.

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## Disclosure statement

No potential conflict of interest was reported by the author(s).

## ORCID

Haithem Hamdouni http://orcid.org/0000-0001-5746-8075

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