Research Article
Title
Association between Carbonic Anhydrase VI *(CA VI)* gene copy number and dental caries experience

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Short title: Association between *CA VI* gene and caries

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Abstract

The current study examined the association between the carbonic anhydrase VI (*CA VI)* copy number variations (CNVs) and dental caries experience in adults. In total, 202 of 35-72 years old subjects participating in the Lithuanian National Oral Health Survey (LNOHS) agreed to provide saliva samples, thus their data were included in the current study. Information about sociodemographic, environmental, and behavioural determinants was acquired via the self-administered World Health Organisation (WHO) questionnaire. Fluoride levels in the drinking water were recorded based on information provided by water suppliers. Dental caries experience was recorded by one calibrated examiner using the WHO criteria for recording caries on smooth (including proximal, buccal, and oral) or occlusal surfaces. Caries experience was measured as the total number of decayed (D_3), missing (M), filled (F) surfaces (D_3MFS). DNA was extracted from saliva samples to examine *CA VI* CNVs using the QX200 droplet digital PCR system. Negative binomial regression and Poisson regression analyses were employed for data analyses. Based on multivariable regression analyses, higher copy number of *CA VI* were associated with higher caries experience on smooth surfaces (IRR 1.04, 95% CI 1.005 – 1.08) and occlusal surfaces (IRR 1.02, 95% CI 1.003 – 1.04). Positive associations between higher copy number of *CA VI* and higher caries experience on smooth and occlusal surfaces were found, suggesting that the *CA VI* coding gene may be associated with caries development. Future studies are needed to validate our results and to examine the underlying mechanisms of such associations.

Introduction

Dental caries is a non-communicable multifactorial oral condition determined by a combination of sociodemographic, environmental, behavioural, and genetic factors [World Health Organization, 2010]. Several studies reported that a substantial proportion of the variation in dental caries may be due to genetics [Vieira, 2021]. This could explain, at least in part, why some subjects are more likely than others to be impacted by caries when exposed similarly to social, environmental, and behavioural risk factors [Gustafsson et al., 1954; Yildiz et al., 2016]. Possibly, the composition of saliva, being one of the host factors may modify the dynamic process of dental caries. Previous studies demonstrated that some salivary proteins could be used as biomarkers to identify individuals with higher caries risk [Tulunoglu et al., 2006; Roa et al., 2008]. The carbonic anhydrase VI (CA VI) isoenzyme was found to have a role in dynamics of dental caries [Kimoto et al., 2006; Esberg et al., 2019]. The CA VI is the only secretory isoenzyme in the mammalian carbonic anhydrase family produced by the serous acinar cells of the parotid and submandibular glands and this isoenzyme is one of the major protein constituents of the human parotid saliva [Fernley et al., 1995]. The CA VI supports the maintenance of salivary pH by contributing to the total saliva's buffering capacity through the bicarbonate system ($CO_2 + H_2O \leftrightarrow HCO_3^- + H^+$) which is activated after exposure to fermentable carbohydrates. The CA VI isoenzyme is encoded by *CA VI* gene which is located at chromosome 1 p36.22-p36.33 [Sutherland et al., 1989; Jiang and Gupta, 1999].

Inconsistent results regarding the association between CA VI salivary concentration and dental caries were reported [Kivela et al., 1999; Ozturk et al., 2008; Picco et al., 2017; Picco et al., 2019]. However, more consistent findings were reported concerning the association between the CA VI activity and dental caries [Borghi et al., 2017; Picco et al., 2017; Picco et al., 2019; de Sousa et al., 2021; de-Sousa et al., 2021]. CA VI concentration and its activity in saliva can be altered by genetic factors, such as single nucleotide polymorphism (SNP) in the gene coding

sequences [Tashian, 1989]. There are indications that *CA VI* gene SNP and haploblocks of *CA VI* may be associated with *Streptococcus mutans* colonization [Esberg et al., 2019]. However, inconsistent results were reported regarding the relationships among SNPs, and multisite haploblocks in the *CA VI* gene, and CA VI concentration and its activity in saliva [Peres et al., 2010; Koc Ozturk et al., 2012; Aidar et al., 2013; Li et al., 2015; Yildiz et al., 2016].

Copy number variations (CNVs) is a common feature of the human genome that plays an important role in evolution, contributes to diversity of population, development of certain diseases, as well as influences host microbiome interactions [Zhang et al., 2009; Greenblum et al., 2015; Hu et al., 2018; Poole et al., 2019]. In this regard, the association between dental caries and the CNVs of *AMY1* gene coding of the salivary protein α-Amylase was suggested [Stangvaltaite-Mouhat et al., 2021a]. To the best of our knowledge, the role *CA VI* CNV plays in caries has not been described before. Considering the role CA VI may play in the dynamics of caries process, the current study tested the association between *CA VI* CNVs and dental caries.

Material and methods

Study design and participants

This cross-sectional gene-focused study used data collected during the Lithuanian National Oral Health Survey (LNOHS). In this survey, a random, stratified sample of individuals aged 35-74 years from 15 geographic locations (the 5 biggest cities and 10 randomly selected peri-urban/rural areas, one from each of the 10 Lithuanian counties) were invited to participate. More details about the LNOHS design and its sampling framework can be found elsewhere [Stangvaltaite-Mouhat et al., 2020; Stangvaltaite-Mouhat et al., 2021b; Stankeviciene et al., 2021]. Of all, 202 participants (14%) of the LNOHS sample agreed to provide their saliva

samples for subsequent genetic analysis, and these subjects were generally healthy, not related and had regular access to dental care.

Sociodemographic, environmental and behavioural determinants

Additional information about sociodemographic and behavioural determinants was acquired via the WHO self-administered "Oral health questionnaire for adults" [World Health Organization, 2013]. Sociodemographic determinants included sex, education (total number of years) and age (in full years). Information about the fluoride levels in the drinking water (environmental determinant) was acquired from 15 geographic locations based on information provided by the water suppliers. Subsequently, this variable was dichotomised with a cut-off point of 1 ppm. Behavioural determinants included consumption of sugar-containing foods, frequency of tooth brushing and the time of the last dental visit. Frequency of sugar-containing foods or drinks consumption was calculated based on responses to 8 questions, each question presented a different sugar-containing food or drink group, for each item responses were structured as follows: '1' rarely/never, '2' several times a month, '3' once a week, '4' several times a week, '5' every day, '6' several times a day. This variable was further dichotomized into a several times a week or less (less than daily) versus every day or several times a day consumption (daily). Frequency of tooth brushing was coded as twice a day or more versus once a day or less, and the last dental visit being within the last 12 months versus more than 12 months ago.

Assessments of dental caries experience

Dental caries experience (on smooth or occlusal surfaces) was assessed at a total of 28 teeth by one calibrated examiner (IS) and recorded as a total number of decayed, missing, filled surfaces (D_3MFS) following the WHO criteria [World Health Organization, 2013]. The level of intra-

examiner agreement based on duplicate recordings on 15 random selected subjects was considered satisfactory as indicated by high intra-class correlation coefficients; 1.00 for D_3, 0.99 for M, and 1.00 for F components. The D_3MFS score was computed separately for smooth surfaces (proximal, buccal and oral surfaces) and occlusal surfaces [Stangvaltaite-Mouhat et al., 2021a].

Measurement of the CA VI copy number (CN)

Collection of saliva samples and DNA isolation

Unstimulated saliva samples were collected for 5 min following the oral examination using 50mL tubes (Falcon®, UK), and saliva was stored in -20°C freezers for further analyses [Stangvaltaite-Mouhat et al., 2021a]. For the DNA extraction, a total of 600µL from each saliva sample was placed in a 2-mL tube before being centrifuged for 5 min at 20,000*g* to pellet cells. The supernatant was then discarded, and the pellet captured. The QIAamp DNA Mini Kit (Qiagen, Heidelberg, Germany) and QIAcube (Qiagen, Hilden, Germany) automated system with a preprogramed protocol was used to extract DNA according to the manufacturer's instructions with slight modifications. The extracted DNA was eluted in 100µL in 10 mM Tris buffer. The quality and yield of the extracted genomic DNA were analysed by agarose gel electrophoresis before determining the DNA concentration with the Qubit 3.0 fluorometer (Life Technologies, CA, USA) following the manufacturer's instructions.

Droplet Digital PCR and Analysis

The extracted DNA was used to examine *CA VI* CNVs using the QX200 Droplet Digital PCR (ddPCR) system (Bio-Rad, Pleasanton, CA, USA). Each ddPCR reaction consisted of 10µL of Supermix for probes with no dUTP (Bio-Rad), 1µL of human *CA VI* assay (Hs06589443_cn, catalog number: 4400291), 1µL of human *AP3B1* assay (dH- saCP1000001: 900nM primers

and 250nM HEX probe; Bio-Rad), 0.5µL AluI restriction enzyme (10 U/µL), 1µL of DNA (containing on average 40ng of DNA), and molecular biology-grade water to make a total reaction mix of 20µL. The ddPCR reaction mix was used to generate droplets using the droplet generator (Bio-Rad). A total of 40µL of reaction mix was transferred into a 96-well plate and then sealed with pierceable foil using a PX1 PCR sealer (Bio-Rad) before being incubated for 30 min at 37°C for enzymatic digestion. The plate was then transferred to a deep-well C1000 Touch^{TM} thermocycler (Bio- Rad) for amplification as follows: Initialization step at 94°C for 5 min, denaturation 94°C for 1 min (×39), annealing 58°C for 30 sec (×39), elongation step at 72°C for 30 sec (×39), final elongation at 72°C for 5 min and held at 4°C indefinitely. After PCR amplification, a droplet reader (Bio-Rad) was used to detect fluorescence, i.e., FAM or HEX, and to assign droplet status as positive or negative. The nuclease-free water served as a negative control and two samples with 11and 6 CNVs as positive controls were included in the experiments. The ddPCR results were analysed using QuantaSoft^{TM} Analysis Pro software v1.0. The software was used to determine the *CA VI* CNVs for each study subject based on the ratio between *CA VI* signals detected in the QX200 system and those detected for the *AP3B1* reference gene (2 diploid copies) to calculate *CA VI* CNVs.

Statistical analyses

Statistical analyses were performed employing the Statistical Package for the Social Sciences software version 28.0, (IBM, Armonk, NY, USA). Univariable and multivariable negative binomial regression analyses tested the associations between caries experience on smooth-surfaces and levels of *CA VI* CN. Univariable and multivariable Poisson regression analyses were employed to test the association between occlusal-surface caries experience and levels of *CA VI* CN. In multivariable analyses, these associations were adjusted for sociodemographic characteristics (sex, education, age), environmental factor (fluoride level in drinking water) and

behavioural factors (frequency of sugar-containing foods or drinks consumption, frequency of tooth brushing and last dental visit). The level of significance for all tests was set at $p < 0.050$. The size of associations was presented as incidence rate ratios with their 95% confidence intervals.

Results

The mean (standard deviation (SD)) age of study subjects was 40.1 (5.4) years and 60% of the study subjects (n=119) were females. The *CA VI* CN varied between 1 and 15 (median 4; mean 4.9, SD 2.9) (Supplementary Figure 1). On smooth surfaces the mean (SD) of D_3MFS score was 27.5 (20.9) and on occlusal surfaces it was 10.1 (3.6) (Table 1). *CA VI* CN distribution by smooth-surface and occlusal-surface caries experience is presented in supplementary Figure 2-3. Based on multivariable regression analyses, one *CA VI* CN increment was associated with 4% higher smooth-surface caries experience (IRR 1.04; 95% CI 1.005 – 1.08) and 2% higher occlusal-surface caries experience (IRR 1.02; 95% CI 1.00 – 1.04) (Table 2).

Higher education (IRR 0.97, 95% CI 0.94 – 0.99) and higher fluoride levels in the drinking water (IRR 0.7, 95% CI 0.5 – 0.9) were associated independently with lower smooth-surface caries experience, while older age (IRR 1.04, 95% CI 1.02 – 1.07) and last dental visit within the last 12 months (IRR 1.4, 95% CI 1.1 – 1.8) were associated independently with higher smooth-surface caries experience.

For occlusal-surface caries experience, female sex associated with higher caries experience (IRR 1.2, 95% CI 1.1 – 1.4), while higher levels of fluoride in the drinking water associated with 20% lower occlusal-surface caries experience (IRR 0.8, 95% CI 0.7 – 0.9) (Table 2).

Discussion

This is the first study to examine the association between *CA VI* CNVs and dental caries experience, which demonstrated that participants having higher *CA VI* CN had higher experience of both; smooth-surface and occlusal-surface caries. In addition, our study was the first investigation to focus on CNVs of *CA VI* rather than on its concentration, activity, and polymorphism [Tashian, 1989; Peres et al., 2010; Koc Ozturk et al., 2012; Aidar et al., 2013; Li et al., 2015; Yildiz et al., 2016]. Previous studies reported that CA VI protein levels related to several outcomes such as dental caries, periodontal conditions, cancer of the salivary glands and taste processes, but the mechanisms behind these relationships are still not fully understood [Kivela et al., 1999; Patrikainen et al., 2014; Arabacı et al., 2015; Picco et al., 2017; Picco et al., 2019]. Inconsistent results were provided on association between dental caries and CA VI concentration and activity [Borghi et al., 2017; Picco et al., 2017; Picco et al., 2019; de Sousa et al., 2021; de-Sousa et al., 2021]. It has been suggested that CA VI expression in the saliva (e.g., either its concentration or activity) can be modified by a single nucleotide polymorphism (SNP) in the gene coding sequences, but the clinical implications of such *CA VI* SNP-related findings have been inconclusive [Peres et al., 2010; Koc Ozturk et al., 2012; Aidar et al., 2013; Li et al., 2015; Yildiz et al., 2016]. It was suggested that CNVs can influence the gene expression through complex mechanisms [Gamazon and Stranger, 2015]. A tighter coupling between gene CNVs at the transcript level than between CNVs and protein level changes was reported [Geiger et al., 2010]. The *CA VI* CNVs may influence the *CA VI* expression in the saliva, a hypothesis that is warranted to be tested. Unfortunately, it was not feasible in the present study to measure the CA VI protein concentration and its activity, as saliva samples were collected at different times of the day, meaning that the effect of the circadian periodicity known to influence the salivary CA VI concentration was not controlled for [Parkkila et al., 1995].

There are preliminary indications that some genetic factors may impact susceptibility of dental surfaces differently, while others are common genes for smooth-surface and occlusal-surface caries [Shaffer et al., 2012; Stangvaltaite-Mouhat et al., 2021a]. Therefore, the dental caries experience total scores in the current study were calculated separately for smooth and occlusal surfaces. Moreover, in the current study there was no restriction on age, given levels of *CA VI* CNVs do not change over time [Wang et al., 2008].

Other well-known determinants than genetic determinants have been associated with dental caries, this in accordance to our study findings demonstrating that sociodemographic, environmental, and behavioural determinants were independently associated with dental caries. Therefore, future studies should focus on different samples. For example, children population, where the effect of genetic determinants on dental caries may be more pronounced compared to sociodemographic, environmental, and behavioural determinants. Populations with cavities-free individuals could also be a choice as this would allow a comparison with those who had developed dental caries.

Our findings may contribute towards identification of a genetic biomarker for a high caries risk, consequently genetic saliva assessment can be an integral part of a more personalized clinical approach to prevent dental caries. However, the contribution of CNVs to human gene expression, especially in complex diseases, is still unknown [Shao et al., 2019]. For example, deletion of one copy can result in defects that are linked to gene function. Duplication involving a dosage-sensitive gene can be the cause of some other diseases [Zhang et al., 2009]. Therefore, additional genetic studies focusing on the inherent mechanisms, particularly performed in different populations are needed to validate our findings.

To conclude, the present study demonstrated a positive association between *CA VI* CNVs and higher smooth-surface and occlusal-surface caries experience, suggesting that *CA VI* coding gene may be common for caries development on both smooth and occlusal surfaces. The current

work is a first step towards a more personalized approach to prevent dental caries. However, future studies are needed to validate our results and to examine the underlying mechanisms of such associations.

Statement of Ethics: This study was performed in compliance with Good Clinical Practice and the Declaration of Helsinki. Approvals were obtained from the Lithuanian Bioethical Committee (reference number 158200-17-920-426), the Personal Data Protection Authority (reference number 2R-4077), and the Regional Committee of Medical and Health Research Ethics in Northern Norway (reference number 2017/805). Participation was voluntarily and based on a signed written informed consent form.

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Data Availability Statement: All data generated or analysed during this study are included in this article. Further enquiries can be directed to the corresponding author.

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All authors approved the final version of the manuscript and agreed to be personally accountable for their own contributions and to ensure that questions related to the accuracy or integrity of any part of the work, even parts in which the author was not personally involved, are appropriately investigated, resolved, and the resolution documented in the literature.

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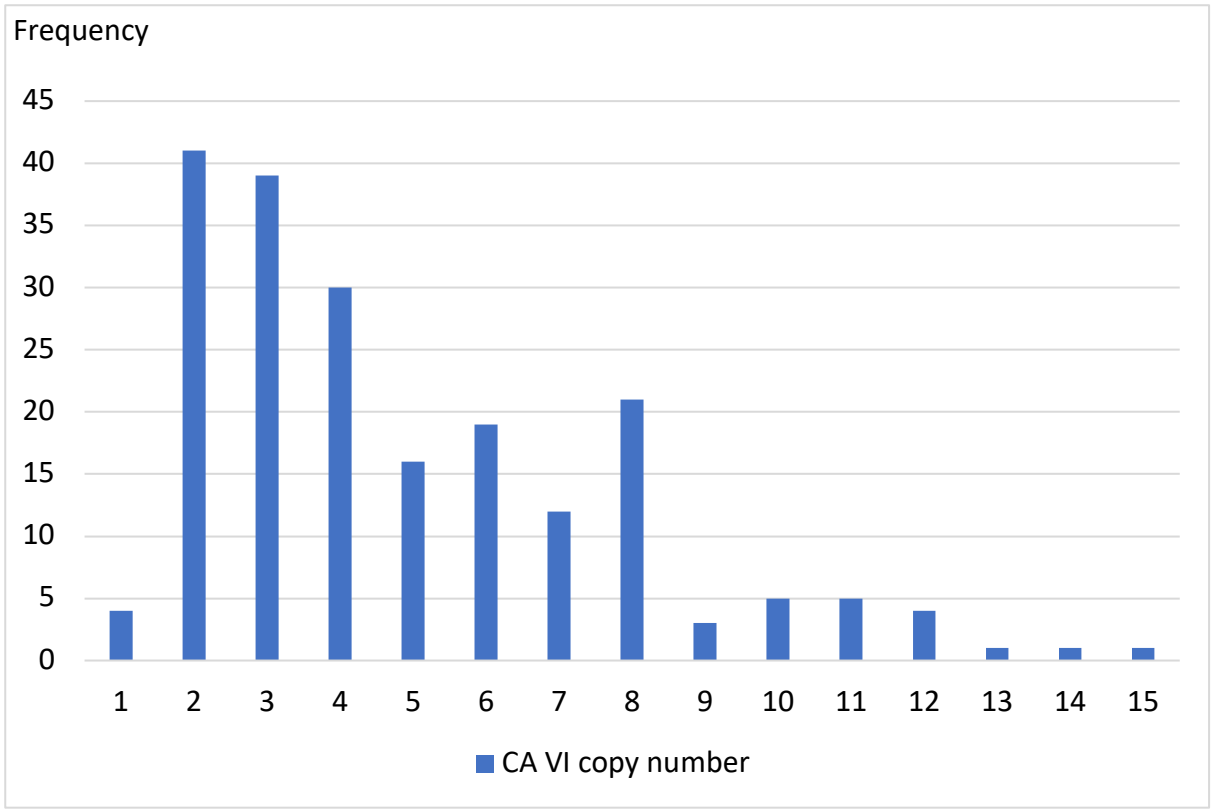
Table 1. *CA VI* copy number (CN), smooth- and occlusal-surface dental caries experience (decayed, missing, filled surfaces (D_3MFS) score), sociodemographic, environmental, and behavioural determinants among participants with recorded *CA VI* copy number (CN).

Variables	**N (%)**
***CA VI* copy number (predictor)**	N=202
Mean (SD)	4.9 (2.9)
Median (IQR)	4 (4)
Dental caries experience (outcomes)	
Smooth-surface D_3MFS	N=202
Mean (SD)	27.5 (20.9)
Median (IQR)	22.5 (22)
Occlusal-surface D_3MFS	N=202
Mean (SD)	10.1 (3.6)
Median (IQR)	10.0 (6)
Sociodemographic determinants	
Sex	N=202
Male	83 (41)
Female	119 (59)
Education	N=202
Mean (SD)	16.3 (3.8)
Age	N=202
Mean (SD)	40.1 (5.4)
Environmental determinant	
Fluoride level in drinking water	N=202
ppm≤1.0	181 (90)
ppm>1.0	21 (10)
Behavioural determinants	
Frequency of sugar-containing foods or drinks consumption	N=167
Less than daily	67 (40)
Daily	100 (60)
Frequency of tooth brushing	N=199
Twice a day or more	101 (51)
Once a day or less	98 (49)
Last dental visit	N=201
More than 12 months ago	61 (30)
Within the last 12 months	140 (70)

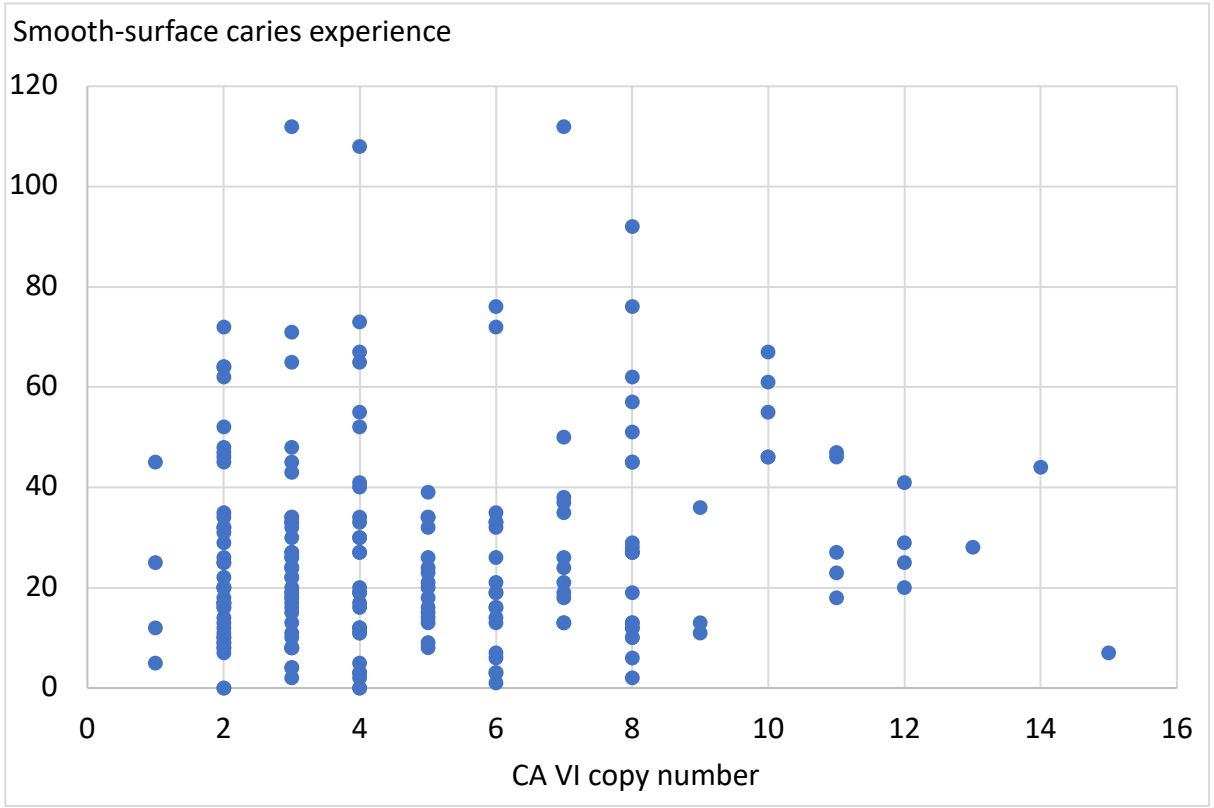
Table 2. Incidence rate ratios (IRR) with their 95% confidence intervals (95% CIs) for the association between smooth-surface or occlusal-surface dental caries experience (decayed, missing, filled surfaces (D_3MFS) score) and *CA VI* copy number according to univariable and multivariable negative binomial regression and Poisson regression analyses, respectively.

Determinants		Smooth-surface caries experience*		Occlusal-surface caries experience**	
		Crude IRR (95% CI) p-value	Adjusted IRR (95% CI) N=167 p-value	Crude IRR (95% CI) p-value	Adjusted IRR (95% CI) N=167 p-value
CA VI copy number#		1.03 (0.9 – 1.1) 0.105	1.04 (1.005 – 1.08) 0.027	1.02 (1.0 – 1. 03) 0.02	1.02 (1.003 – 1.04) 0.021
Sex	Males Females	1 1.2 (1.0 – 1.5) 0.049	1 1.3 (0.9 – 1.6) 0.054	1 1.2 (1.1 – 1.3) <0.001	1 1.2 (1.1 – 1.4) <0.001
Education#	(in years)	0.9 (0.9 – 1.0) 0.050	0.97 (0.94 – 0.99) 0.043	1.0 (0.9 – 1.0) 0.744	0.9 (0.9 – 1.0) 0.309
Age#	(in years)	2.0 (1.3; 2.5) <0.001	1.04 (1.02 – 1.07) <0.001	1.01 (1.003 – 1.02) 0.004	1.0 (0.9 – 1.0) 0.069
Fluoride level in drinking water	ppm≤1.0 ppm>1.0	1 0.8 (0.6 – 1.1) 0.202	1 0.7 (0.5 – 0.9) 0.032	1 0.9 (0.7 – 1.01) 0.062	1 0.8 (0.7 – 0.9) 0.024
Frequency of sugar-containing foods or drinks consumption	Less than daily Daily	1 1.1 (0.9 – 1.4) 0.479	1 1.0 (0.8 – 1.2) 0.663	1 0.9 (0.8 – 1.1) 0.270	1 0.9 (0.9 – 1.0) 0.255
Frequency of tooth brushing	Twice a day or more Once a day or less	1 0.9 (0.7 – 1.1) 0.226	1 0.9 (0.7 – 1.1) 0.285	1 0.9 (0.8 – 0.9) 0.007	1 0.9 (0.8 – 1.0) 0.188
Last dental visit	More than 12 months ago Within the last 12 months	1 1.1 (0.9 – 1.4) <0.001	1 1.4 (1.1 – 1.8) 0.009	1 1.1 (0.9 – 1.2) 0.059	1 1.1 (0.9 – 1.2) 0.387

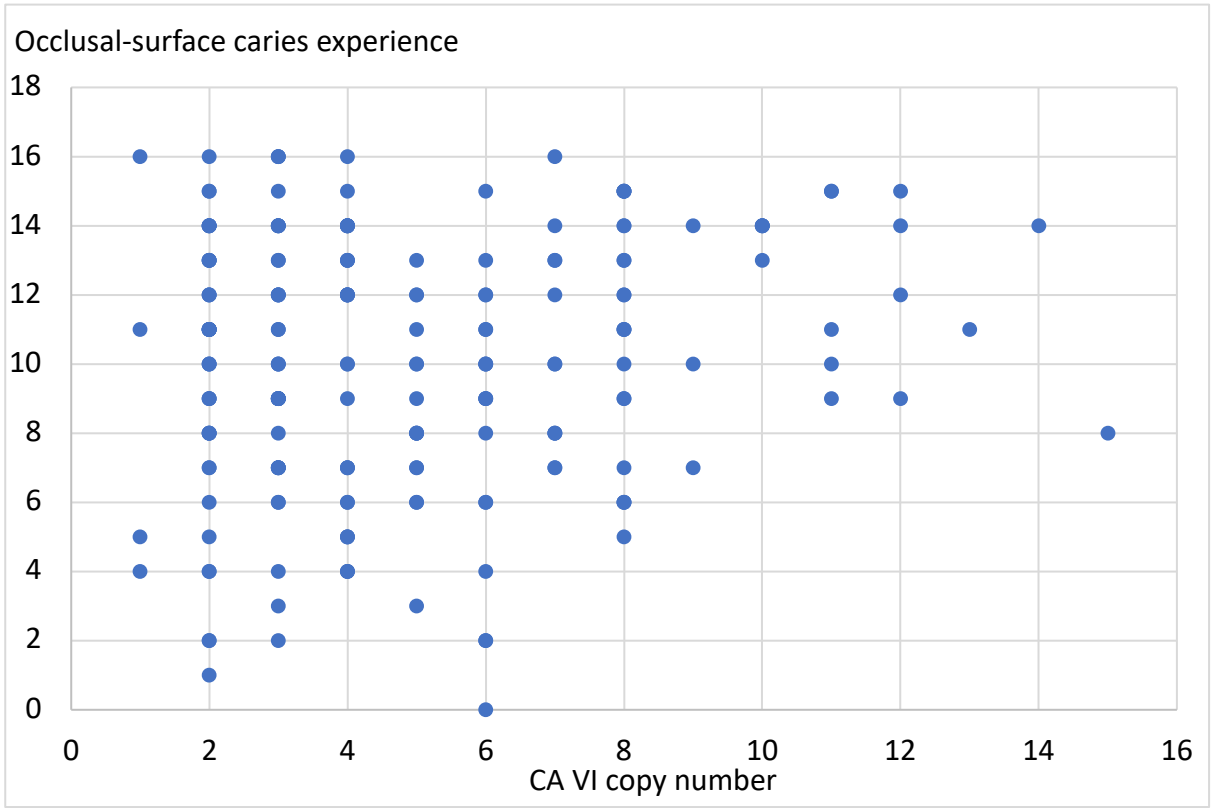
*Negative binomial regression analysis; ** Poisson regression analysis; #Continuous scale variable.



Supplementary Figure 1. Frequency distribution of *CA VI* copy number in 202 adult participants.



Supplementary Figure 2. *CA VI* copy number distribution by smooth-surface dental caries experience (decayed, missing, filled surfaces (D3MFS) score) among 202 adult participants.



Supplementary Figure 3. *CA VI* copy number distribution by occlusal-surface dental caries experience (decayed, missing, filled surfaces (D3MFS) score) among 202 adult participants.