UNIVERSIDADE ESTADUAL DE CAMPINAS
FACULDADE DE ODONTOLOGIA DE PIRACICABA

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Correlação entre mudanças na expressão e atividade da enzima anidrase carbônica VI e polimorfismos genéticos neste gene *(CA6)*

Tese de Doutorado apresentada à Faculdade de Odontologia de Piracicaba, da UNICAMP para obtenção do título de Doutor em Biologia Buco-Dental, na área de concentração em Histologia e Embriologia.

Orientador: Prof. Dr. Sergio Roberto Peres Line

Este exemplar corresponde à
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"A ciência não pode prever o que vai acontecer.
Só pode prever a probabilidade de algo acontecer."

César Lattes

RESUMO

As anidrases carbônicas mantém o PH fisiológico catalisando a hidratação do dióxido de carbono na reação $CO_2 + H_2O <=> HCO_3^- + H^+$. A anidrase carbônica VI (AC VI), secretada no meio externo, parece desempenhar um papel importante na homeostase dos tecidos bucais, participando nos processos de gustação, proteção dos tecidos dentais contra a perda de minerais, e possivelmente na formação de cálculos dentários na doença periodontal. Polimorfismos genéticos são variações na sequência do DNA, as quais podem resultar em variação estruturais da proteína. Este estudo teve como objetivo verificar a correlação entre mudanças na expressão e atividade da enzima AC VI e polimorfismos genéticos no seu gene (*CA6*). A população estudada foi constituída de 182 voluntários saudáveis, não fumantes e que não faziam uso de medicamentos, de ambos os sexos, com idade entre 18 e 22 anos. As amostras de saliva total foram obtidas através do método de coleta de saliva estimulada. A concentração de AC VI na saliva foi determinada por Imunoensaio Competitivo (DELFIA® Competitive Time-Resolved Immunofluorometric Assay). Para avaliação da atividade da enzima AC VI todas as amostras foram diluídas para uma concentração de trabalho de 36 ng/ul. A detecção da atividade da AC VI foi feita por um protocolo modificado de Kotwica *et al*, 2006, adaptado para AC VI na saliva. Os géis foram fotografados e as imagens foram quantificadas (área em pixels) pelo *software* Image J, o qual calculou a luminescência e quantificou a atividade da AC VI em valores numéricos**.** Para analisarmos a influência de polimorfismos genéticos na atividade da AC VI humana utilizamos DNA obtido a partir de células epiteliais da mucosa bucal. Amostras de DNA genômico foram genotipadas para os polimorfismos rs2274327 (C/T), rs2274328 (A/C) e rs2274333(A/G) do gene *CA6*, localizado no cromossomo 1 na região 1p36.2 (gene ID: 765), os quais são responsáveis por mudanças do aminoácido na proteína (Tret55Met, Met68Leu e Ser90Gli, respectivamente). A genotipagem foi realizada utilizando a PCR em Tempo Real (ABI Prism 7900HT Applied

Biosystems) com sondas específicas TaqMan (Taqman®SNP Genotyping Assays) de acordo com as instruções do fabricante. Os valores de concentrações e atividade da enzima AC VI obtidos para os diferentes genótipos foram submetidos aos testes Kruskal-Wallis e Dunn**.** Os resultados mostraram que os indivíduos que apresentaram o genótipo TT referente ao rs 2274327 tiveram concentração de enzima AC VI significativamente menor do que indivíduos com genótipos CT ou CC ($p < 0,05\%$). Houve diferença significativa entre os genótipos AG e AA do polimorfismo rs2274333. O fato de não ter havido diferença entre os genótipos GG e AA sugere que a diferença entre os genótipos AG e AA possivelmente ocorreu devido a desequilíbrio de ligação com o polimorfismo rs 2274327. Não foram observadas diferenças significantes entre os genótipos de cada polimorfismo e a atividade da AC VI. Assim, os resultados sugerem que a variação da concentração da AC VI está associada a presença de polimorfismos neste gene.

Palavras-chave: Saliva, enzima, proteína, polimorfismo, anidrase carbônica VI, *CA6* gene

ABSTRACT

The carbonic anhydrases maintain the physiological pH catalyzing the hydration of carbon dioxide in the reaction CO2 + H2O <=> HCO3-+ H +. Carbonic anhydrase VI (CA VI) which is secreted into the external environment appears to play an important role in the homeostasis of oral tissues, participating in the processes of taste, protection of dental tissues against the loss of minerals, and possibly in the formation of dental calculus in periodontal disease. Genetic polymorphisms are variations of DNA sequences occurring in a population which may result in protein structural variants. This study aimed to verify the correlation between changes in expression and activity of human salivary enzyme carbonic anhydrase VI and genetic polymorphisms in this gene (*CA6*). The study population consisted of 182 healthy volunteers, not under medication, of both sexes, aged 18-22 years. Samples of total saliva were obtained by the method of stimulated saliva collection. The concentration of AC VI in saliva was determined by competitive immunoassay (DELFIA® Competitive Time-Resolved Immunofluorometric Assay). In order to evaluate the activity of the enzyme AC VI all samples were diluted to a working concentration of 36 ng/ul. The CA VI activity detection was made by a modified protocol of Kotwica et al 2006**,** adapted to CA VI in saliva in our laboratory. The gels were photographed, and the images were quantified (pixels area) by Image J® software, which calculated the luminescence in the area of the bands and quantified the CA VI activity in numerical value. To analyze the influence of genetic polymorphism in the activity of human AC VI we used DNA obtained from buccal epithelial cells. Samples of genomic DNA were genotyped for polymorphisms rs2274327(C/T), rs2274328 (A/C) e rs2274333 (A/G) in the coding sequences of *CA6* gene, located at chromosome 1, region 1p36.2 (gene ID: 765), which are responsible for the amino acid changes in protein (Tre55Met, Met68Leu and Ser90Gli, respectively). Genotyping was performed by using Taqman®SNP Genotyping Assays and ABI Prism 7900HT

Sequence Detection System (Applied Biosystems, Foster City, CA, USA) according to manufacturer's instructions. The values of concentrations and enzyme activity obtained for the different genotypes were analyzed using the Kruskal-Wallis and Dunn tests. The results showed that the individuals who had the TT genotype of polymorphism rs 2274327 had a significantly lower CA VI concentrations than the individuals with genotypes CT or CT **(***p*< 0,05**%).** There was a significant difference between genotypes AG and AA. The fact that we observed no difference between genotypes GG and AA suggests that the difference between the AG and AA genotypes possibly occurred due to linkage disequilibrium with SNP rs 2274327. There were no significant differences between the genotypes of each polymorphism and the activity of ACVI. Thus, the results suggest that the change in concentration of ACVI is associated with the presence of polymorphisms in this gene.

Key words – Saliva, enzyme, protein, polymorphism, carbonic anhydrase VI*, CA6* gene

SUMÁRIO

1. INTRODUÇÃO

A primeira noção da existência da anidrase carbônica (AC) surgiu no final de 1920, quando uma substância das células vermelhas que catalisavam a hidratação reversível de dióxido de carbono, $CO_2 + H_2O <=> HCO_3^- + H^+$, foi reconhecida em estudos sobre a taxa de escape de dióxido de carbono a partir de sangue hemolisado (Henriques, 1928). Alguns anos mais tarde esta substância foi isolada e purificada parcialmente e verificou-se ser uma enzima, a qual foi denominada anidrase (Meldrum & Roughton, 1933; Edsall, 1968; Carter, 1972). Em 1939, verificou-se ter um peso molecular de cerca de 30 kDa e conter um íon de zinco por molécula (Keilin & Mann, 1939).

As anidrases carbônicas são formadas por um único polipeptídio, e em sua forma nativa contém um íon zinco fortemente ligado, o qual é essencial para a atividade catalítica (Lindskog, 1982). O íon zinco situa-se perto do centro da molécula, na parte inferior de uma cavidade de 15Å de largura e 15Å de profundidade que forma o sítio ativo da enzima. Três resíduos de histidina ligam o íon zinco à estrutura secundária em folha *β* pregueada, sendo o quarto e o quinto sítio ligante do íon provavelmente ocupado por uma molécula de água e um íon hidroxila (Kannan *et al.,* 1977).

As anidrases carbônicas participam em vários processos biológicos envolvidos na manutenção da homeostase do pH, transporte de CO_2 e troca iônica (Tashian, 1989; 1992). Elas também podem agir sobre uma grande variedade de substâncias que sofrem hidratação de aldeídos (Pocker & Meany, 1965; 1967) ou hidrólises de ésteres aromáticos (Schneider & Liefländer, 1963). São encontradas em quase todos os organismos, de algas e bactérias a mamíferos; porém são distintas dependendo de sua localização celular e subcelular, e em funções biológicas (Tashian, 1989; 1992).

As anidrases carbônicas consistem de 5 famílias de gene: *α, β, δ, γ* e *ε* que foram originalmente considerados serem evolutivamente independentes (Hewett-Emmet & Tashian, 1996). As anidrases carbônicas *α* são expressas no reino

animal, plantas, algas verdes, algumas eubactérias e também em alguns vírus (Chegwidden & Carter, 2000; Hewett-Emmett & Tashian, 1996); as anidrases carbônicas *β* têm sido descritas em plantas, eubactérias e algas; as anidrases carbônicas *δ* em algas, as anidrases carbônicas *γ* em Archae, algumas eubactérias e plantas e as anidrases carbônicas *ε* em bactérias. O organismo modelo para estudo de planta, Arabidopsis, tem homólogos de todas as três famílias (Hewett-Emmett & Tashian, 1996).

As anidrases carbônicas *α* estão envolvidas em vários processos biológicos, como regulação do pH, respiração, gliconeogênese, transporte de íons, reabsorção óssea, acidificação renal e formação do líquido cérebro espinhal e ácidos gástricos. Até agora, 13 *α*-isoenzimas ativas foram identificados em mamíferos, as quais têm localizações subcelulares características: cinco no citoplasma (AC I, AC II, AC III, AC VII e AC XIII); cinco associadas à membrana (AC IV, AC IX, AC XII, AC XIV e CA XV), duas no interior da mitocôndria (AC VA e AC VB) e uma forma que é secretada para fora da célula (AC VI) (Hewett-Emmett, 2000; Hewett-Emmett & Tashian, 1996). A maioria dos tecidos do corpo humano contêm pelo menos uma isoenzima anidrase carbônica *α.* Existem também três anidrases carbônicas relacionadas a proteínas (CARPs), que pertencem à família de enzima anidrase carbônica mas não têm a atividade catalítica devido a ausência dos resíduos de histidina na cavidade do sítio ativo (Tashian *et al*., 2000).

A atividade da anidrase carbônica na saliva humana, foi observada pela primeira vez, há aproximadamente 74 anos (Becks & Wainwright, 1939; Rap, 1946), porém estudos sobre seu papel fisiológico na saliva apareceram apenas em 1974, quando Szabó relatou uma média maior no nível de atividade da anidrase carbônica em crianças livres de cárie do que aquelas com cárie ativa.

No final da década de 70 Fernley *et al*., descreveram uma nova anidrase carbônica, expressa na glândula parótida dos ovinos (Fernley *et al.,* 1979). Ao longo dos anos seguintes, a enzima foi primeiro purificada a partir de saliva de rato por Feldstein & Silverman (1984) e mais tarde a partir da saliva humana por

Murakami & Sly (1987) e Kadoya *et al.* (1987), e designada anidrase carbônica VI. Em 1991, Aldred *et al*., clonaram e caracterizaram o DNA complementar que codifica a enzima anidrase carbônica VI humana. Um estudo mostrou que a anidrase carbônica VI também é expressa na glândula mamária e altas concentrações desta enzima podem ser medidas no leite humano. Foi proposto que esta enzima pode ser um fator importante para o crescimento normal e desenvolvimento do trato digestório do lactente (Karhumaa *et al.,* 2001). Outra forma de AC VI foi identificada, a anidrase carbônica tipo b, que pode participar em processos intracelulares induzidos por estresse, incluindo a apoptose (Sok *et al.,* 1999). O gene desta enzima está localizado no braço longo do cromossomo 1 e possui 8 exons e 7 introns (http://genome.ucsc.edu/). Seu peso molecular está entre 39-46kDa (Feldstein & Silverman, 1984; Murakami & Sly, 1987).

A saliva é um líquido claro, levemente ácido de constituição viscosa que é produzida pelas células ductais das glândulas salivares maiores (parótida- 20%, submandibular-65% e sublingual 7%) e menores, além do fluido gengival crevicular. A saliva possui um importante papel na homeostase dos tecidos bucais, participando em várias funções como na lubrificação dos alimentos, digestão, paladar e manutenção da integridade das estruturas dentais. A saliva contém várias proteínas que ajudam no desempenho destas funções. Dentre estas, as anidrases carbônicas desempenham papel importante na homeostase dos tecidos bucais. A importância da anidrase carbônica VI na homeostase é evidenciada pelo seu envolvimento em vários processos patológicos. Sabe-se que o bicarbonato formado na saliva é importante na manutenção do pH esofágico (Helm *et al*., 1984; Sarosiek *et al.,*1996), entretanto a presença da AC VI no muco gástrico contribui para manutenção do pH na superfície das células epiteliais protegendo-as de úlceras gástricas (Parkkila *et al.,1997).*A diminuição da secreção salivar da anidrase carbônica VI tem sido associada à diminuição (disgeusia) e perda (hipogeusia) do paladar e diminuição (disosmia) e perda (hiposmia) do faro (Henkin *et al*., 1999). Concentrações de anidrase carbônica VI na saliva foram associadas com a prevalência de cárie dentária, especialmente em indivíduos com

higiene oral deficiente (Kivela *et al.,* 1999). Evidências experimentais indicam que anidrase carbônica VI salivar penetra na placa e facilita a neutralização dos ácidos presentes nesta região (Feldstein *et al.,* 1984). Embora fosse originalmente previsto que a anidrase carbônica VI regulasse o pH e capacidade tampão salivar (Feldstein *et al*., 1984), alguns estudos indicam que essas variáveis não estão diretamente associadas à concentração de anidrase carbônica na saliva (Parkkila *et al.,*1993; Kivelä *et al.,*1997). Isto possivelmente ocorre devido ao fato de que a expressão desta enzima na saliva é bastante variável entre indivíduos e que outros fatores, como polimorfismos genéticos, ou alterações pós-traducionais possam estar influenciando sua expressão.

A taxa e a eficiência de uma reação bioquímica é determinada pelo nível de expressão e do estado de atividade da enzima (Zeng Y, 2011). Uma enzima pode existir tanto em um estado ativo ou inativo, bem como com altos ou baixos níveis de expressão. Fatores extra e/ou intracelulares podem modular ou regular a expressão das enzimas. Portanto, é possível que a regulação da expressão enzimática possa ser influenciada por outras variáveis como polimorfismos genéticos, visto que polimorfismos, variações na sequência do DNA, podem dar origem a duas ou mais formas alélicas de RNA mensageiros, resultando em variantes estruturais da proteína (Shen *et al.,* 1999).

No presente estudo, investigamos os efeitos dos polimorfismos, rs2274327 (C/T) e rs2274328 (A/C) presentes no exon 2 e do rs2274333(A/G) presente no exon 3 do gene *CA6,* sobre a expressão e a atividade catalítica da enzima anidrase carbônica VI salivar.

2. CAPÍTULO

Essa dissertação está baseada na Resolução CCPG/002/06/UNICAMP que regulamenta o formato alternativo para teses de Mestrado e Doutorado e permite a inserção de artigos científicos de autoria ou co-autoria do candidato.

Por se tratar de pesquisas envolvendo seres humanos, o projeto de pesquisa destes trabalhos foi submetido à apreciação do Comitê de Ética em pesquisa da Faculdade de Odontologia de Piracicaba, tendo sido aprovado (Anexo 1).

Assim sendo, essa tese é composta por um capítulo como descrito a seguir:

Capítulo 1: *"Effect of genetic polymorphisms in CA6 gene on the expression and catalytic activity of human salivary carbonic anhydrase VI".* Artigo submetido à revista *Archives of Oral Biology***.** Carta de envio (Anexo 2).

Effect of genetic polymorphisms in *CA6* gene on the expression and catalytic activity of human salivary carbonic anhydrase VI

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Declaration of interests

The authors declare that there is no potential conflict of interest as none of the authors have any personal or financial relationship that might introduce bias or affect their judgment.

Abstract

Carbonic anhydrase isoenzyme VI (CA VI) plays an important role in the homeostasis of oral tissues participating in the processes of taste, protection of dental tissues against the loss of minerals, and possibly in the formation of dental calculus in periodontal disease. This study aimed to verify the correlation between changes in expression and activity of human salivary carbonic anhydrase VI and genetic polymorphisms in its gene (*CA6*). The study population consisted of 182 healthy volunteers (female and male; aged 18-22). Samples of total saliva were assayed for CA VI concentrations using a specific time-resolved immunofluorometric assay. CA VI catalytic activity was detected by a modified protocol of Kotwica et al 2006[24], adapted to CA VI in saliva. Samples of genomic DNA were genotyped for polymorphisms rs2274327 (C/T), rs2274328 (A/C) and rs2274333 (A/G) by Taqman®SNP Genotyping Assays. The concentration and catalytic activity of the salivary CA VI obtained for the different genotypes were analyzed using the Kruskal-Wallis non-parametric test and the Dunn test. The results showed that individuals with TT genotype (rs2274327) had significantly lower CA VI concentrations than the individuals with genotypes CT or CC ($p < 0,05$**).** There was a significant difference between genotypes AG and AA (rs 2274333). There were no significant differences between the genotypes of each

polymorphism and CA VI activity. Our results suggest that polymorphisms in *CA6* gene are associated with the concentrations of the secreted CA VI enzyme.

Key words – polymorphism; carbonic anhydrase VI; *CA6* gene; gustin; saliva.

Introduction

Carbonic anhydrase (EC 4.2.1.1.; CA) isoenzymes form a family of zinc metalloenzymes, of which the basic physiological function is to catalyze the reversible hydration of carbon dioxide in the reaction $CO_2 + H_2O <=> HCO_3^- + H^+$.[1] They are involved in control of the ion, fluid, and acid-base balance in various tissues.[2,3] Carbonic anhydrase VI (CA VI) is the only secreted form of carbonic anhydrase. This enzyme was first described and characterized in the ovine parotid gland and saliva.[4] In human, CA VI is produced by the serous acinar cells of the parotid and submandibular glands.[1]

To date, sixteen different α-CA isozymes or CA-related proteins (CARP) have been identified in mammals.[5] Some are expressed in almost all tissues, while others are tissue or organ specific.[6] Five are cytosolic isozymes (I, II, III, VII and XIII), five are membrane bound (IV, IX, XII, XIV and XV), two are present in mitochondria (VA and VB), and one is a secretory isozyme (VI). The isozymes of CA enzyme show considerable divergence in DNA sequence, chromosome location and enzymatic properties.[7]

The secreted CA VI is believed to be one of the oldest mammalian CAs in evolutionary terms.[8] CA VI was shown to be expressed in the rat lacrimal glands,[9] lower airways and lung,[10] mouse nasal gland,[11] human lingual serous von Ebner glands,[12] bovine mammary[13] and esophageal glands, as well as in the lining epithelial cells of the large intestine, stomach and esophagus.[14] High levels of CA VI are produced in human mammary glands, and concentrations comparable to salivary CA VI can be detected in milk.[15] The importance of CA VI is evidenced by its involvement in several malfunctions. Low CA VI concentrations in the saliva

were associated with the prevalence of dental caries, especially in individuals with poor oral hygiene.[16] Salivary bicarbonate secretion is also known to be important in the maintenance of esophageal pH homeostasis.[17] Moreover, the presence of CA VI in the gastric mucus contributes to maintaining the pH gradient on the surface epithelial cells, protecting from gastric ulcers.[18] Other possibility is that the expression of the *CA6* gene itself might be altered in certain diseases and cancer, especially those associated with the salivary gland.[8] Thatcher *et al*.[19] suggested that CA VI might be involved in taste bud development and normal taste function because decreased levels of CA VI in human salivary samples were correlated with both loss of taste (hypogeusia) and pathological changes in taste buds.

The rate and efficiency of a biochemical reaction is determined by the expression level and the activity state of the appropriate enzyme.[20] A given enzyme can exist in both active and inactive states as well as high and low expression levels in the cell. Both extra- and intracellular factors can modulate or regulate the enzymes and proteins in both positive and negative ways. Therefore, it is possible that the regulation of CA VI expression in saliva can also be influenced by other variables, such as genetic polymorphisms found in the coding sequences of this enzyme. Single nucleotide polymorphisms (SNPs) can give rise to two or more allelic forms of mRNAs, resulting in protein structural variants.[21]

The purpose of this study was to verify the correlation between the changes in human CA VI expression and activity and genetic polymorphisms in the corresponding *CA6* gene.

Materials and methods

Subject population

This study was carried out on a group of 182 individuals, with the approval of the FOP/UNICAMP Ethics Committee (131/2009), and informed consent was obtained from all subjects who participated in the study. The study participants

were unrelated Brazilians from the Southeastern region of Brazil, male and female, aged 18-22. They were recruited without any exclusion parameters besides being in good health and not under medication.

Concentration of HCA VI

Sample collection

Saliva samples were collected from all volunteers at the same period of the day (1:30-2:00 PM) in order to eliminate circadian differences in CA VI.[22] The subjects chewed PARAFILM® for 5 min to stimulate salivary flow. Saliva from the first 2 min of chewing was swallowed and the rest was collected into 15-mL tubes. After centrifugation (17000 x g) for 2 min, the supernatants were collected and frozen without delay and stored at -20°C until assay.

Labeling of the antigen

Purified human CA VI was labeled with 0.1 mg of Eu-labeling reagent according to slight modifications of the manufacturer's instructions (PerkinElmer, Turku, Finland). The labeling was monitored by sodium docecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and by measuring the fluorescence with a VICTOR3 1420 Multilabel counter (PerkinElmer).

The purified human CA VI was first pretreated by a buffer exchange in a dialysis overnight at 4°C. The enzyme in Tris buffer, pH 7.0, containing 0.4 mol/L NaN_3, 1 mmol/L benzamidine, and 200 mL/L glycerol was applied to a PD-10 column (GE Healthcare) preequilibrated with 25 mL of labeling buffer (9 g/L NaCl, 50 mmol/L $NaHCO_3$, pH 8.5). After discarding the void volume, the 3.5mL enzyme fraction was collected. The actual labeling consisted of the following steps: The pretreated enzyme (0.5 mg in a concentration 2.3 mg/mL) was added to the 0.1 mg of Eu-labeling reagent and incubated for 16 h at room temperature. Free Eu^{3+} was

separated by gel filtration on a PD-10 column equilibrated with 25 mL of Tris buffer, pH 7.75, containing 9 g/L NaCl, 50 mmol/L Tris, and 0.5 g/L NaN_3. Fractions of 1 mL were collected. For long term storage, highly purified, heavy metal-free bovine serum albumin was added at a final concentration of 1.0 g/L to the fraction containing the enzyme peak. The labeled CA VI was stored at 4°C.

Fluoroimmunoassay procedure

DELFIA® anti-rabbit-coated clear plate (PerkinElmer) were washed 6 x 200 µL with Delfia wash solution (PerkinElmer). Anti-human CA VI[23] diluted 1:10 000 in Delfia ® Assay Buffer was applied in the microtitration wells (200 µL/well). After incubation at 22°C for 4 h with continuous gentle shaking, the wells were washed six times with the wash solution. We then added 50 µL of Eu^{3+} -labeled CA VI (diluted appropriately in assay buffer), standards, or saliva samples (50 µL of 1:50-diluted saliva) to the wells, and brought the incubation volume to 200 µL/well with Delfia ® Assay Buffer. The mixture was incubated at 22°C with shaking for 20 h, after which we washed the wells six times with the wash solution and added 200 µL of enhancement solution (PerkinElmer) to each well. After an intense shaking for 5 min the fluorescences were measured with the research fluorometer. Every wash was made using Inteliwasher 3D-IW8 Microplate washer (Biosan, Riga, Latvia). Each saliva sample was assayed in identical triplicate reactions.

Activity of CA VI

After salivary concentration measurement, all samples were brought to the same working concentration of 36 ng/uL in the sample buffer containing 10% SDS, 4 x Tris-HCl (pH 6.8), 30% glycerol and 0.001% bromophenol blue and water. The determination of CA VI activity was performed by a modified protocol of Kotwica [24], adapted to CA VI in saliva in our laboratory. Briefly, the material was stirred for 1 min before being loaded on 12% acrylamide gel. 20 µL sample was placed in each

channel of the gel (samples were run in duplicate), which was run for 2 h at 140 volts at 4°C. After electrophoresis, the gel was washed for 10 min in 10% isopropanol diluted in 100 mmol/L Tris, pH 8.2 followed by one wash of 100 mmol/L Tris, pH 8.2. The gel was incubated in 0.1% bromothymol blue diluted in 100 mmol/L Tris, pH 8.2, for 20 min at 4°C. CA VI activity was observed after immersing the gel in distilled deionized water saturated with CO_2. The gels were photographed, and the images were quantified (pixels area) by Image J ® software [Collins, 2007].

It is important to mention that prior to the commencement of the analysis of enzymatic activity CA VI, Western blotting was performed to assure that the band observed in zymography corresponded to the respective isoenzyme. We used anti-CA VI from Sigma Chemical Company (St. Louis, MO, USA) to detect CA VI protein in Western blotting.

Genotyping Study

DNA extraction

Samples of epithelial oral cells were collected by mouthwash with dextrose 3% and genomic DNA was extracted using 8 mol/L ammonium acetate and 1 mmol/L EDTA according to Aidar & Line.[25] The amount and purity of the DNA was determined by spectrophotometry. The DNA concentration was obtained by readings at 260 nm. The ratio of readings at 260 nm/280 nm was used to estimate the DNA purity.

Genotyping

Samples of genomic DNA were genotyped for 3 SNPs in human *CA6* gene. Genotyping was performed by using Taqman®SNP Genotyping Assays (C___1739308_1, C___1739309_1, and C ___1739329_1 for SNP rs 2274327, rs

2274328 and 2274333 respectively) and ABI Prism 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA) according to manufacturer's instructions. Briefly, 1 µL DNA sample (20 ng/ µL) was mixed with the supplied 2x TaqMan Universal PCR Master Mix and TaqMan Assay Mix to a final volume of 5 µL. Each sample underwent 40 amplifications. Allelic calls were first determined semi-automatically. In brief, the plots of fluorescent intensities per cycle for each reporter fluorophore were visually inspected to choose a baseline level, which was subtracted from each data point. The end-point of each normalized dataset was defined as cycle number 40, as suggested by the manufacturer. End-point fluorescent intensities of each probe were plotted in an allelic discrimination graph (VIC on abscissa, FAM on ordinate), and genomic 'clusters' were defined manually by sectioning the plots into quadrants with horizontal and vertical lines. No discrepancies were detected in the genotyping results of duplicate samples.

CA VI isoforms structure comparison

Polymorphism of *CA6* gene could potentially lead to structural changes in CA VI protein molecule. 3D-structure comparison for the CA VI model was done with the Swiss-Prot Deepview PDB-viewer program (Guex N and Peitsch MC 1997 Swiss-Model and the Swiss-PdbViewer: an environment for comparative protein modeling. Electrophoresis 18, 2714-2723). The model coordinates (3FE4.pdb) were taken from PDB databank. The two isoforms of CA VI contained the following alternative amino acid residues: A) Threonine 55 (rs2274327), Methione 68 (rs2274328), and Serine 90 (rs.2274333); Isoform B) Methione 55 (rs2274327), Leucine 68 (rs2274328), and Glycine 90 (rs.2274333).

Statistical Analyses

All the samples were scored and results were analyzed using the Kruskal - Wallis non parametric test and Dunn test. Relationships yielding P values less than

0.05 were considered to be significant.

Results

Analysis of Genetic Polymorphisms

The results showed that the individuals who had the TT genotype of polymorphism rs 2274327 had a significantly lower CA VI concentrations than the individuals with genotypes CT or CC (p< 0,05%) (Figure 1). There was also a significant difference between genotypes AG and AA. The fact that we observed no difference between genotypes GG and AA suggests that the difference between the AG and AA genotypes possibly occurred due to linkage disequilibrium with SNP rs 2274327. There were no significant differences in CA VI enzymatic activites between the saliva samples obtained from individuals with different *CA6* polymorphisms. Taken together, the results suggest that the change in the concentration of CA VI is associated with the presence of polymorphisms in this gene.

Alterations in the structure of the CA VI isozymes

The modeling of 3D protein structure showed that the substitution of threonine for methionine in position 55 may affect local structure, since it increases the hydrophobicity on the loop area (53-57). This may indicate that the loop orientation (indicated in Fig. 2) is significantly changed, because the methionine residue tries to avoid the hydrophilic environment. Therefore, radical changes in the large β-sheet area of CA VI can occur. Furthermore, original threonine is a hydrogen bond acceptor and donor, whereas methionine is not. In the case of Nr II (Met68→Leu), both the local protein structure and common properties of

sidechains are probably conserved. In Nr III (Ser90→Gly), serine, a small hydrogen bond-forming residue is substituted by even smaller residue.

Discussion

The carbonic anhydrases form a family of zinc metalloenzymes that participate in controlling the ion, fluid and acid base balance in various organs. The only known secreted isoenzyme is CA VI, several milligrams of which are secreted daily into the saliva and pass into the gastrointestinal canal.[26] It is known that despite the careful standardization, salivary CA VI concentrations show a high inter-individual variation, which probably reflects differences in enzyme expression.[27] Notably, CA VI enzyme can probably stay intact long periods of time because it is a very stable enzyme which can withstand the harsh conditions in the alimentary canal, and non-degraded enzyme can be detected even in the gastric juice.[18] Our results showed that CA VI activity is preserved after several frozen and thawing cycles as well as it seems to survive enzymatically active even in denaturing conditions caused by SDS, present in the sample loading buffer, used before polyacrylamide gel electrophoresis. Zymography analysis of CA VI in saliva was shown to be a very sensitive method. The use of zymography allows detection of CA VI activity using only a few microliters of saliva (usually less than 100 ul), and the results can be obtained in a single day.

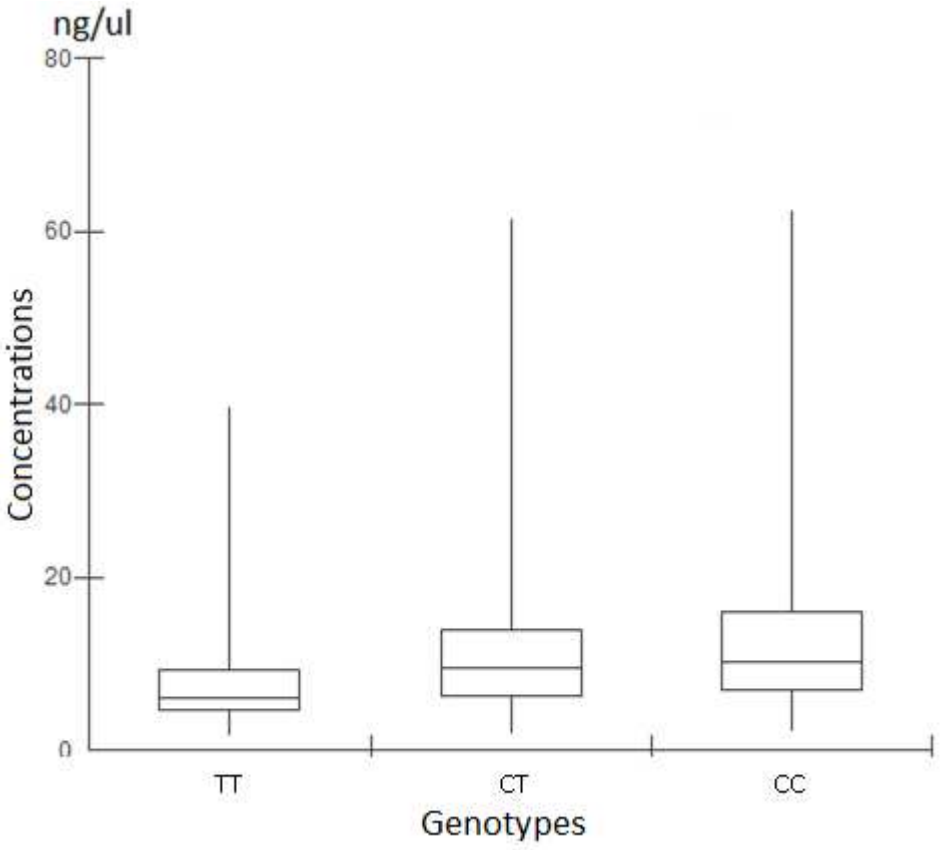
To explore the correlation between changes in expression and activity of human salivary CA VI enzyme and genetic polymorphisms in the corresponding gene, we determined its concentrations and activity in human whole saliva. A highly sensitive and specific time-resolved immunofluorometric assay[25] was used to determine the salivary CA VI concentrations. The CA VI activity detection was made by a modified protocol of Kotwica[24], adapted to salivary CA VI measurement in our laboratory. In the present study, saliva samples were collected at the same time of the day in order to diminish the well-documented circadian variation in CA VI concentrations[22]. The genotypes of 182 healthy saliva donors, regarding 3 SNPs

in *CA6* gene, were assessed by a standard analysis of TaqMan end-point fluorescent data. At standard end-point analysis, clear identification of three tight clusters permitted easy assignment of allele-1, allele-2 and heterozygous genotypes for 3 SNPs. The standard genotype analysis was based on the fluorescent intensities of the two reporter probes near the end of amplification (PCR cycle 40).

The rs2274327 SNP has been previously associated with salivary buffer capacity in children.[28] In agreement with our results the TT genotype was associated with low salivary buffer capacity. Interestingly our results showed that the rs2274327 polymorphic site was associated with the concentrations of CA VI in saliva, but not with enzyme activity. Although we cannot provide a definitive answer at this point, there are some potential explanations. This SNP is predicted to cause changes in protein conformation, however, comparison with sequences of other mammals show that this position is poorly conserved (Human - Thr, mouse - Glu, elephant – Lis, dog - Arg, oposum – Asp). Since aminoacid Thr is the site of o-glycosylation, we tested the potential of this site to be glycosylated using the NetOGlyc 3.1 Server (http://www.cbs.dtu.dk/services/NetOGlyc/[29]) and the GlycoPred server (http://comp.chem.nottingham.ac.uk/glyco/[30]). The NetOGlyc 3.1 Server was ranked the T residue of rs2274327 as the second most likely O-glycosilation site among 32 possible in CA VI, while the GlycoPred server predicted that this site is likely to be glycosylated. O-glycosylation of proteins was shown to be important to protein secretion and possibly protection from proteolysis-mediated inactivation.[31,32] It is also possible that the observed polymorphism could be linked to other, yet unknown, genetic changes in the promoter region of *CA6* gene that could then contribute to the transcription efficacy. Nevertheless, our results pointed out that the protein stability and regulation of CA VI secretion needs to be investigated in detail in the future studies.

Figures

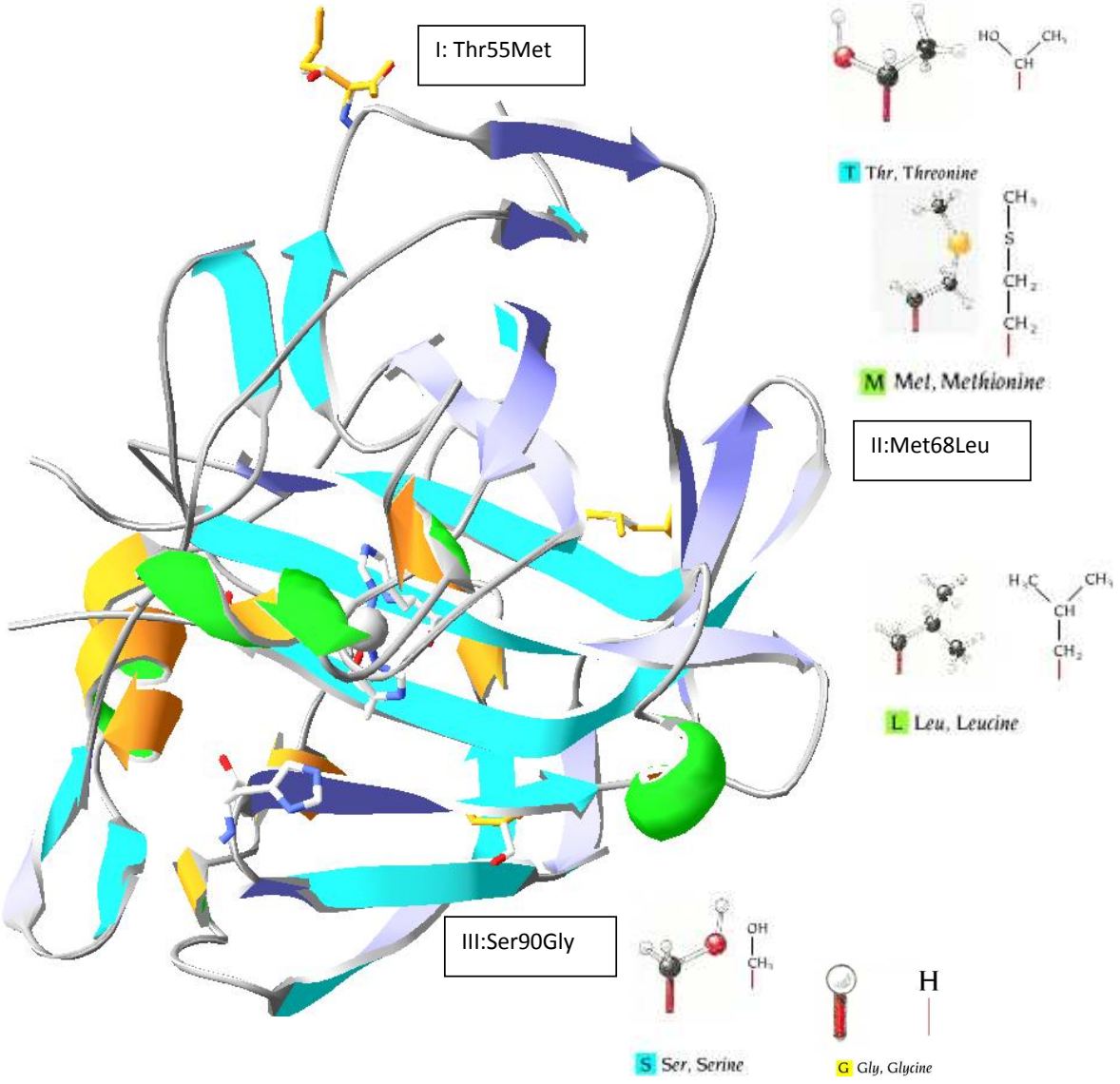
Figure 1: Salivary concentrations of CA VI in the rs2274327 genotypes (median and quartiles). Individuals with genotype TT had smaller concentrations of salivary CA VI than individuals with genotypes CT and CC ($p < 0.05$).



TT significantly lower CA VI concentrations $p < 0.05$

The mean concentrations of CA VI were 12.11 ng/ul (SD±10.27).

Figure 2: Three-dimensional CA VI protein structure - Isoform B



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3. CONCLUSÃO GERAL

Os resultados sugerem que a regulação da expressão da AC VI na saliva pode ser influenciada por variáveis como polimorfismos genéticos encontrados na sequência do código genético do gene (*CA6).*

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5. APÊNDICES

Apêndice 1 Western blot

Western blotting para anidrase carbônica VI

Apêndice 2 Concentrações da enzima Anidrase carbônica VI na saliva

Amostras	[ ]CA VI	Amostras	[ ]CA VI	Amostras	[ ]CA VI	Amostras	[ ]CA VI	Amostras	[ ]CA VI
11	3,445	45	22,337	80	9,563	115	2,203	150	25,32
12	8,327	46	10,553	81	20,722	116	25,159	151	6,304
13	15,873	47	10,394	82	15,601	117	2,34	152	14,489
14	18,915	48	8,534	83	58,639	118	11,448	153	16,103
15	10,162	49	5,886	84	62,209	119	10,028	154	7,175
16	9,013	50	16,917	85	9,336	121	8,439	155	6,436
17	13,062	51	10,41	86	4,617	122	5,238	156	12,042
18	7,039	52	4,43	87	30,442	123	5,567	157	9,711
19	29,691	53	10,267	88	61,364	124	12,128	158	12,916
20	6,628	54	9,619	89	2,364	125	9,797	159	13,225
21	6,661	55	4,841	90	6,048	126	26,512	160	5,475
22	9,698	56	13,771	91	4,705	127	11,573	161	7,256
23	6,122	57	12,148	92	19,441	128	10,374	162	7,721
24	2,287	58	11,902	93	8,002	129	8,11	163	15,7
25	19,847	59	13,646	94	7,403	130	8,736	164	13,152
26	14,544	61	5,349	95	8,474	131	4,109	165	6,108
27	3,259	62	12,988	96	39,062	132	7,568	166	3,615
28	10,158	63	3,761	97	9,438	133	15,392	167	4,053
29	3,467	64	1,794	98	21,493	134	23,939	168	19,982
30	28,543	65	6,726	99	39,598	135	4,138	169	2,735
31	7,348	66	34,777	100	5,388	136	3,237	170	11,652
32	8,574	67	8,897	101	9,963	137	18,601	171	3,507
33	8,255	68	19,574	102	27,681	138	9,845	172	22,527
34	11,689	69	7,486	103	10,712	139	12,809	173	5,044
35	8,91	70	6,282	104	7,642	140	3,403	174	6,534
36	12,843	71	3,748	105	6,141	141	5,355	175	41,094
37	17,701	72	10,486	106	9,41	142	6,148	178	15,226
38	13,724	73	3,391	107	5,397	143	14,01	180	6,131
39	6,252	74	6,028	108	5,217	144	7,602	181	5,46
40	23,889	75	6,293	110	6,611	145	14,121	182	12,659
41	15,808	76	2,414	111	6,702	146	11,195		
42	13,467	77	16,51	112	3,464	147	8,402		
43	7,995	78	8,755	113	14,221	148	5,357		
44	16,713	79	2,007	114	6,838	149	42,086		

Valores das concentrações da enzima AC VI na saliva determinados por Imunoensaio Competitivo (DELFIA® Competitive Time-Resolved Immunofluorometric Assay). Concentrações ([ ]) em ng/ul.

Apêndice 3 Polimorfismos investigados

SNP	rs2274327	rs2274328	rs2274333
Chr	1	1	1
Exon	2	2	3
Chr position	9009406	9009444	9017204
SNP to Chr	+	+	+
SNP to mRNA	+	+	+
Position mRN	188	226	292
Ancestral Allele	C	C	A
Allele	C	A	A
RefSNP Alleles	A/C/G/T	A/C	A/G
Allele change	ACG ⇒ ATG	ATG ⇒ CTG	AGC ⇒ GGC
Function	missense	missense	missense
Protein Position	55	68	90
Residue change	T [Thr] ⇒ M [Met]	M [Met] ⇒ L [Leu]	S [Ser] ⇒ G [Gly]

Apêndice 4 Genotipagem

Amostras	rs2274327	rs2274328	rs2274333	Amostras	rs2274327	rs2274328	rs2274333
1	CC	AA	AA	46	CT	AC	AG
2	CT	AC	AG	47	CC	AA	GG
3	CT	AC	AA	48	CC	AA	AA
4	CC	AA	AG	49	CT	AC	AG
5	TT	CC	AA	50	CT	CC	AA
6	CC	AC	AA	51	CT	AC	AA
7	CT	AC	AA	52	CT	AC	AA
8	CC	AA	GG	53	CC	AA	AG
9	CT	AC	AG	54	CT	AC	AA
10	CT	AC	AA	55	TT	CC	AA
11	CC	AA	AG	56	CC	AA	AG
12	CT	AC	AA	57	CC	AA	AG
13	CT	AC	AG	58	CC	AA	AG
14	CC	AA	GG	59	CC	AA	AG
15	TT	CC	AG	60	-	AA	AA
16	CC	AC	AA	61	CT	AC	AA
17	CT	AC	AG	62	TT	CC	AG
18	TT	CC	AG	63	CT	CC	AA
19	CC	AA	AA	64	TT	CC	AA
20	CC	AA	AA	65	CT	AC	AA
21	CT	AC	AA	66	CC	AA	AG
22	CT	AC	AA	67	CT	CC	AA
23	CC	AC	AG	68	CC	AA	AA
24	CC	AA	AA	69	CC	AA	AG
25	CT	AC	AA	70	CT	AC	AA
26	CC	AA	AG	71	CC	AA	AG
27	TT	CC	AA	72	CT	AC	AG
28	CC	AC	AA	73	CC	AA	AA
29	CT	-	AG	74	CT	CC	AA
30	CC	AC	AG	75	CT	AC	GG
31	CC	AA	AG	76	CC	AA	GG
32	CC	AA	AA	77	CC	AC	GG
33	CC	AC	AA	78	CC	AC	AA
34	CT	AC	AA	79	CT	CC	AA
35	CT	CC	AA	80	CC	AA	AA
36	CT	CC	AA	81	CC	AC	AA
37	CC	AA	GG	82	CC	AA	AG
38	CC	AA	AA	83	CT	AC	AG
39	CT	AC	AG	84	CC	AA	AA
40	CC	AC	GG	85	TT	CC	AA
41	CC	AC	AA	86	TT	CC	AA
42	CT	AC	AA	87	CC	AC	AG
43	TT	CC	AA	88	CT	AC	AG
44	TT	CC	AA	89	CT	AC	AA
45	CT	AC	AA	90	TT	CC	AA

Amostras 1 à 90 - Genotipagem realizada utilizando a PCR em Tempo Real (ABI Prism 7900HT /Applied Biosystems) com sondas específicas TaqMan (Taqman®SNP Genotyping Assays)

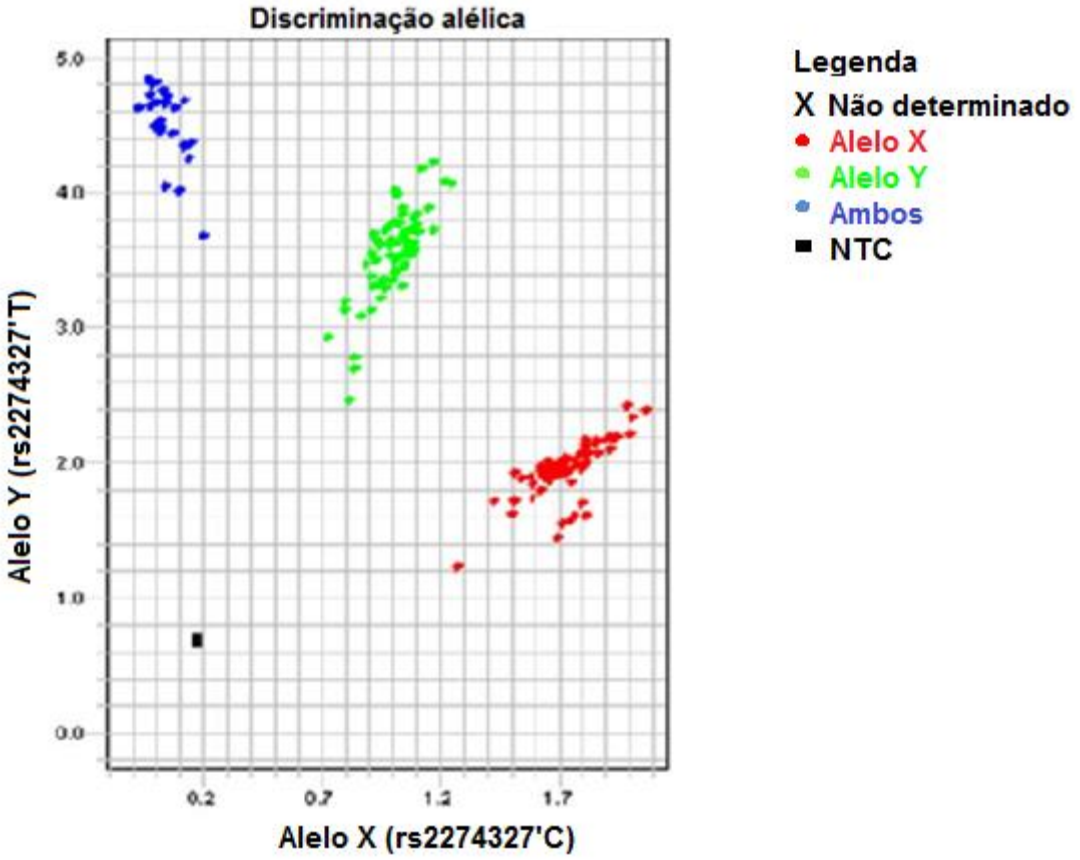
Apêndice 5 Genotipagem

Amostras	rs2274327	rs2274328	rs2274333	Amostras	rs2274327	rs2274328	rs2274333
91	TT	CC	AA	137	CC	AA	AG
92	CT	AC	AG	138	CT	CC	AG
93	CC	AA	AA	139	CC	AC	AA
94	TT	CC	AA	140	CC	AC	AA
95	CT	CC	AG	141	CC	AC	AG
95	CC	AC	AG	142	CT	AC	AA
97	CT	AC	AA	143	CC	AC	AA
98	CC	AA	AG	144	CT	AC	AA
99	TT	CC	AG	145	CT	CC	AA
100	TT	CC	AA	146	CC	AC	AG
101	CC	AC	AG	147	TT	CC	AA
102	CC	AA	GG	148	CT	AC	AA
103	CC	AA	AG	149	CT	CC	AG
104	CC	AC	AG	150	CC	AC	GG
105	CT	AC	AA	151	CT	CC	AG
106	CC	AA	AA	152	CT	AC	AG
107	CC	AA	GG	153	CT	AC	AA
108	TT	CC	AA	154	CT	CC	AG
109	CC	AA	GG	155	CC	AA	AA
110	CC	AA	AG	156	CT	CC	AA
111	CT	AC	AG	157	CT	AC	AA
112	CT	CC	AA	158	CT	AC	AA
113	CC	AC	AG	159	CT	CC	AG
114	CT	AC	AG	160	CT	AC	AG
115	TT	CC	AA	161	CT	AC	AA
116	CT	AC	AG	162	CC	AA	GG
117	CC	AA	GG	163	CT	AC	AA
118	CC	AA	AA	164	CC	AA	AG
119	CT	AC	AA	165	CT	AC	AA
120	TT	CC	AA	166	TT	CC	AA
121	TT	CC	AG	167	TT	CC	AA
122	CT	AC	GG	168	CC	AA	GG
123	TT	CC	AA	169	CC	AC	GG
124	CT	AC	AG	170	CT	CC	AG
125	CC	CC	AA	171	CC	AC	AG
126	CT	CC	AG	172	CC	AA	AG
127	TT	CC	AG	173	CT	AC	AG
128	CT	AC	AA	174	CT	AC	AG
129	CC	AA	AG	175	CT	AC	AG
130	CC	AC	AA	176	CC	AA	AA
131	CC	AC	GG	177	CC	AC	AG
132	CC	AA	AG	178	CT	AC	AG
133	CT	AC	AG	179	CT	AC	AG
134	CT	AC	AA	180	TT	CC	AA
135	CC	AC	AA	181	CC	AA	GG
136	CT	AC	AG	182	TT	CC	AA

Amostras 91 à 182 - Genotipagem realizada utilizando a PCR em Tempo Real (ABI Prism 7900HT /Applied Biosystems) com sondas específicas TaqMan (Taqman®SNP Genotyping Assays)

Apêndice 6 Discriminação alélica

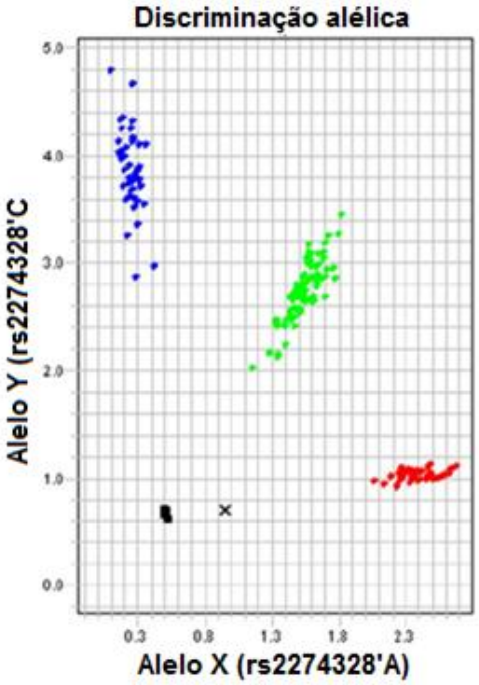
A.



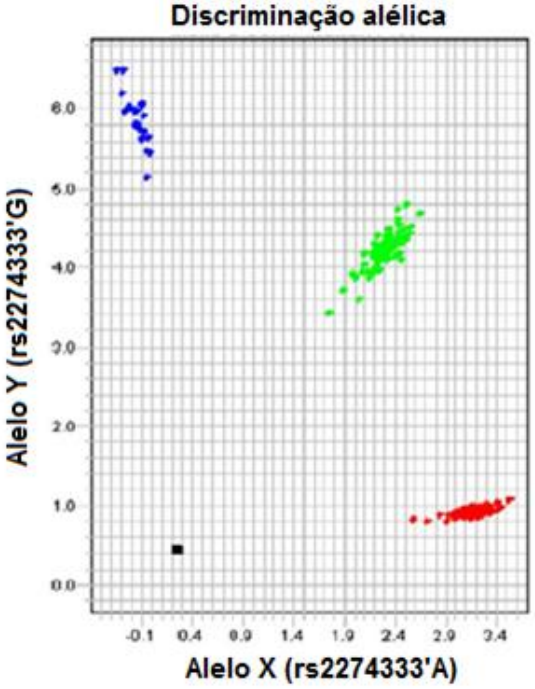
A. SNP rs2274327-182 doadores saudáveis
VIC fluoróforo (eixo-x) - associado com a sonda para o alelo C
FAM fluoróforo (eixo-y) - associado com a sonda para o alelo T

Apêndice 7 Discriminação alélica

B.



C.



B. SNP rs2274328:

VIC fluoróforo (eixo-x) - associado com a sonda para o alelo A

FAM fluoróforo (eixo-y) - associado com a sonda para o alelo C

C. SNP rs2274333:

VIC fluoróforo (eixo-x) - associado com a sonda para o alelo A

FAM fluoróforo (eixo-y)- associado com a sonda para o alelo G

Apêndice 8 Géis de zimografia para atividade da enzima anidrase carbônica VI

Amostras Controle 31 31 32 32 33 33 34 34

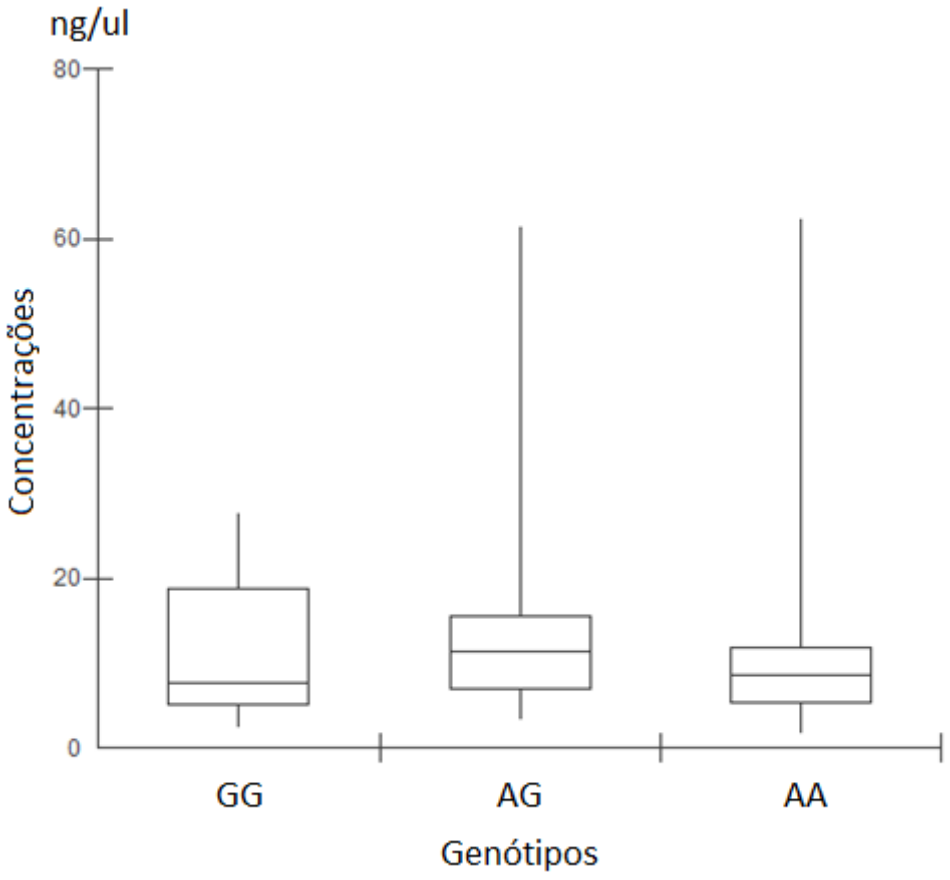
Controle 43 43 52 52 53 53 54 54

Controle 140 140 141 141 142 142 143 143

Controle 112 112 113 113 114 114 115 115

Amostras: Saliva total diluídas para uma concentração de 36ng/ul. Reação da Anidrase Carbônica VI suscitada pela imersão do gel em água saturada com CO_2. A diminuição do pH foi evidenciada pela formação de bandas amarelas. Amostras parciais de 4 géis. Foram corridos um total de 40 géis a fim de que todas as amostras fossem corridas em duplicatas.

Apêndice 9 Concentração salivar da AC VI nos genótipos do rs2274333 (mediana e quartis)



Provável desequilíbrio de ligação

6. ANEXOS

ANEXO 1

COMITÊ DE ÉTICA EM PESQUISA
FACULDADE DE ODONTOLOGIA DE PIRACICABA
UNIVERSIDADE ESTADUAL DE CAMPINAS

CERTIFICADO

O Comitê de Ética em Pesquisa da FOP-UNICAMP certifica que o projeto de pesquisa "**Caracterização genética e bioquímica da atividade da anidrase carbônica salivar IV(CAVI) e sua função na erosão dental por bebidas carbonatadas**", protocolo nº **131/2009**, dos pesquisadores **SERGIO ROBERTO PERES LINE, MARCELO ROCHA MARQUES e MARISI AIDAR**, satisfaz as exigências do Conselho Nacional de Saúde – Ministério da Saúde para as pesquisas em seres humanos e foi aprovado por este comitê em 26/10/2009.

The Ethics Committee in Research of the School of Dentistry of Piracicaba - State University of Campinas, certify that the project "**Biochemical and genetic characterization of the activity of salivary carbonic anhydrase and its participation in dental erosion caused by carbonated beverages**", register number **131/2009**, of **SERGIO ROBERTO PERES LINE, MARCELO ROCHA MARQUES and MARISI AIDAR**, comply with the recommendations of the National Health Council – Ministry of Health of Brazil for research in human subjects and therefore was approved by this committee at 26/10/2009.

Profa. Livia Maria Andaló Tenuta
Secretária
CEP/FOP/UNICAMP

Prof. Jacks Jorge Júnior
Coordenador
CEP/FOP/UNICAMP

Nota: O título do protocolo aparece como fornecido pelos pesquisadores, sem qualquer edição.
Notice: The title of the project appears as provided by the authors, without editing.

ANEXO 2

Mensagem Original ----------

Assunto: Submission Confirmation for Effect of genetic polymorphisms in CA6 gene on the expression and catalytic activity of human salivary carbonic anhydrase VI
De: "Archives of Oral Biology" <AOB@elsevier.com>
Data: Qui, Fevereiro 16, 2012 11:55 am
Para: serglin@fop.unicamp.br

Archives of Oral Biology
Title: Effect of genetic polymorphisms in CA6 gene on the expression and catalytic activity of human salivary carbonic anhydrase VI
Authors: Marisi Aidar, DDS; Marcelo R Marques, DDS, PhD; Ana Paula de Souza, DDS, PhD; Jarkko Valjakka; Nina Mononen; Terho Lehtimäki; Seppo Parkkila; Sergio Roberto Peres Line, PhD, DDS
Article Type: Original Paper

Dear Sergio,

Your submission entitled "Effect of genetic polymorphisms in CA6 gene on the expression and catalytic activity of human salivary carbonic anhydrase VI" has been received by Archives of Oral Biology.

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Kind regards,
(On behalf of the Editors)

Archives of Oral Biology

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