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MOLECULAR STUDIES IN THE HUMAN
SALIVARY PROTEIN CARBONIC ANHYDRASE VI

(BIO/10)

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The work presented in this thesis is the subject of the following scientific publications:

- Padiglia A, Zonza A, **Atzori E**, Chillotti C, Calò C, Tepper BJ, Barbarossa IT. (2010) “Sensitivity to 6-n-propylthiouracil is associated with Gustin (carbonic anhydrase VI) gene polymorphism, salivary zinc, and body mass index in humans”. *Am J Clin Nutr* 92:539–545
- Iole Tomassini Barbarossa, **Elena Atzori**, Andrea Zonza and Alessandra Padiglia. (2011) “A Rapid Screening Method for the Identification of a Single-Nucleotide Polymorphism in the Carbonic Anhydrase VI Gene in Studies of Sensitivity to the Bitter Taste of 6-n-Propylthiouracil”. *Genetic testing and molecular biomarkers* Volume 15, Number 10, Pp.721–724
- Melania Melis, **Elena Atzori**, Stefano Cabras, Andrea Zonza, Carla Calò, Patrizia Muroi, Mariella Nieddu, Alessandra Padiglia, Valeria Sogos, Beverly J Tepper, Iole Tomassini Barbarossa (2013) “The gustin (CA6) gene polymorphism, rs2274333 (A/G), as a mechanistic link between PROP tasting and fungiform taste papilla density and maintenance”. *Plos One*; Vol. 8; Issue 9; e74151

Abstract

The genetic ability to feel the bitter taste of thioureas, such as PROP, varies greatly among individuals influencing the choice of food and body composition.

Sensitive and non-sensitive individuals were defined respectively as “ *Taster* ” and “ *No Tasters* ”.

The term “ *Super Tasters* ” is used to distinguish individuals who perceive PROP as most bitter to those defined as “ *Medium Tasters* ” who perceive the bitter taste moderately. The sensitivity to PROP is associated with the haplotypes (PAV and AVI) receptor gene TAS2R38, and may be associated with polymorphisms of the gene gustina (CA6). The gustina is a zinc dependent enzyme present in human saliva implicated in the development of taste buds.

The aim of this work was to analyze the association between sensitivity to PROP , the polymorphism rs2274333 (A / G) gene gustina, zinc and salivary polymorphisms of TAS2R38 and BMI .

In 75 volunteers aged between 21 and 28 years were determined by BMI and Zn²⁺ + salivate. The sensitivity to PROP was determined by evaluation of the intensity of the sensation evoked by suprathreshold solutions and determining the threshold of perception. Molecular analysis of the gene and gustina receptor gene TAS2R38 were performed by means of PCR, PCR-RFLP and sequencing of fragments obtained .

The average values of the concentration of zinc salivary and BMI were higher in individuals defined as “ *No Tasters* ” than those determined in the “ *Super Tasters* ”.

The low taste sensitivity to PROP of “ *No Tasters* ” was strongly associated with the G allele of the gene polymorphism of gustina and the variant of the TAS2R38 AVI, while the high sensitivity of the “ *Super Tasters* ” is strongly associated allele A gene gustina all'aplotipo PAV and the TAS2R38. Moreover, while the A allele of the gene of gustina is found to be more important for the perception of low concentrations of PROP, the variant of the TAS2R38 PAV is most important result for the evaluation of the intensity of the sensation evoked by high concentrations of PROP.

These data show that the sensitivity to PROP is inversely related to BMI and Zinc salivary and directly associated with the gene dimorphism gustina is assumed that might influence the function of the protein. In addition, these new findings explain

how the combination of gene gustina and TAS2R38 genotype may modulate the phenotype of sensitivity to PROP providing an additional tool for the evaluation of human eating behavior and nutritional status.

Keywords: Sensitivity to PROP, Carbonic anhydrase, gustina (CA6), *TAS2R38*.

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Introduction

The goal of my PhD work was to undertake a study of the molecular to load a portion of the CA6 gene coding for the protein salivary Carbonic Anhydrase (CA VI) and analyze the association between sensitivity to PROP, the polymorphism rs2274333 (A / G) and polymorphisms of the TAS2R38.

This goal represents an important step that is part of the molecular a research project whose purpose and correlate the presence of any changes in the gene CA6, with differences in taste sensitivity to the bitter taste, and especially for the thiourea 6-n propylthiouracil (PROP).

In fact, the interest in the study of this polymorphism is born during an investigation aimed at analyzing the gene coding for the protein isoform salivary carbonic anhydrase VI in individuals with differences in taste sensitivity to 6-n propylthiouracil thiourea (PROP).

The scientific world in the field of physiology and biochemistry of taste, for some time now, in fact, considering the possibility that individual differences in sensitivity to the bitter taste, typical of thioureas, in addition to being genetically inherited can be correlated with differences in some salivary proteins involved in taste function . Furthermore, it has been demonstrated by some authors that just the taste sensitivity to the bitter taste, which varies widely between individuals , is able to significantly influence food preferences and the achievement of the feeling of satiety [48] .

A few years ago it was revealed that among the rose of proteins considered to play an important role in taste function , involving the protein CA VI Zn²⁺ dependent, [35] which expressed predominantly in the parotid gland, is about 3% of the salivary proteome, playing an important role in maintaining the pH of the saliva [28,47] .

Recent studies have also highlighted another important role of the protein CA VI was, in fact, demonstrated that the protein acts as a trophic factor for the growth and development of circumvallate taste buds , through its action on stem cells [23].

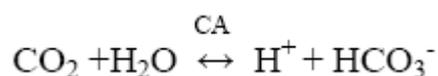
CA6 The gene consists of 8 exonic sequences, coding for a protein of molecular weight 35 kDa approximately. In the 2nd, 3rd and 4th exon of the gene, are located codons encoding for amino acid residues that enter in the formation of the catalytic site, in correspondence of which is present one zinc ion coordinated with three histidines (H 111, H 113, H 138) and a water molecule to form a tetrahedral complex [34].

In the present work, the molecular investigation was centered in the study of 2nd and 3rd exon in the virtue of the vital role that serve after the path that leads to the expression of the gene, and for the correct folding for both the biological function of the protein.

Therefore, an important objective of the study of the molecular 2nd and 3rd exon was to highlight the presence of any polymorphisms can generate, after the expression of the gene, protein products with differences in level of the primary structure and secondary education.

Carbonic anhydrase: isoenzymes and classification.

Carbonic anhydrase (CA) is an enzyme that catalyzes the reversible hydration of CO₂.



The enzyme has been identified for the first time in 1933 , in red blood cells of cattle. Since then, it has been found in abundance in all tissues of mammals, and also in plants, algae and bacteria.

Of this enzyme are known three distinct classes (alpha, beta and gamma). The members of the different classes while not presenting a high sequence homology, perform the same function and require zinc ion in their active site. The carbonic anhydrase in mammals belong to the class alpha enzymes of plants belonging to the beta class, while the enzymes of the methanogenic bacteria that live on the bottom of the marshes, belong to the class gamma.

It appears evident that these classes of enzymes have evolved independently , preserving coding for amino acid residues of the catalytic sites structurally related . Regarding mammals , were characterized 15 different isoforms localized in different tissues [46]. These isoforms are further divided into 4 groups:

- 1) CA cytosolic , including isoforms CA I, CA II , CA III , CA VII and CA XIII
- 2) CA mitochondrial isoform including CA V
- 3) CA secretion , including the isoform CA VI
- 4) CA membrane , including the isoforms CA IV , CA IX , CA XII, CA XIV and CA XV

They were also described three other isoforms CA VIII, CA X and CA XI, whose function has not yet been clarified.

In all isoforms is present, as a cofactor, one Zn^{2+} ion coordinated with three histidines and with a water molecule to form a tetrahedral complex.

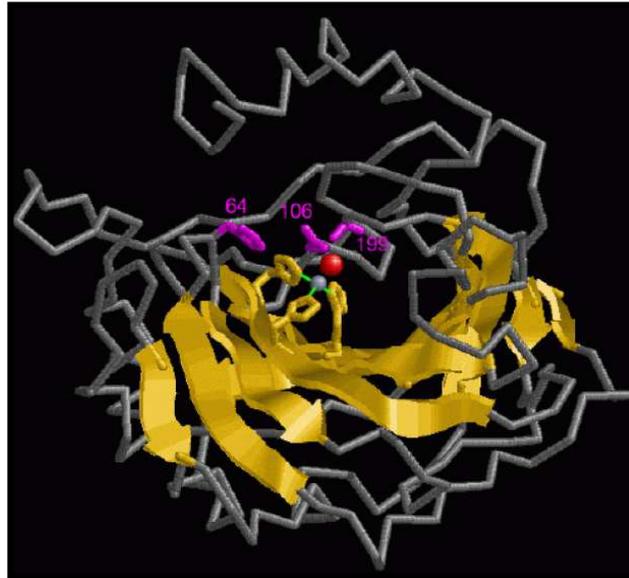


Fig. 1. Tertiary structure of bovine carbonic anhydrase

Receptor TAS2R38

The thioureas are chemical compounds that contain the Working Party was $\text{NC} = \text{S}$ which is responsible for its bitter taste [10-20]. The chemical thiocyanate group is also present in glucosinati and goitrine [48], substances commonly found in cruciferous vegetables and other plants belonging to the family Brassicaceae. [14] A greater rejection towards the Brassicaceae would be a mechanism of advantage for the selection of those individuals with higher sensitivity for bitterness .

The bitter taste is mediated by G-protein coupled receptors belonging to the subfamily of TAS2R [7,41] cell membrane buds. These receptors are seven trans-membrane domain alpha-helix and a conserved amino acid residue. The man owns ~ 25 TAS2R receptors for bitter encoded by genes located on chromosomes Cluster of 5p , 7q , 12p [44] and so far , we have identified approximately 550 molecules able to bind to these receptors [50].

However, this number represents only a small fraction of the thousands of bitter-tasting compounds of plant origin that exist in nature. Because the number of compounds greatly exceeds the number of receptors, it seems likely that individual receptors respond to more than one type of chemical compound flavor bitter [8]. It well known that there're receptors that respond to a limited number of compounds, whereas others respond to a wide range of chemical substances [33]. The TAS2R8 is an example of a highly selective receptor for which are known only 3 ligands with narrow structural similarities. The receptors TAS2R10, -14 and - 46 receptors are highly promiscuous. In fact, when expressed on cells, were able to respond to 50% of bitter compounds tested. The receptor TAS2R38 that binds to the chemical group C = S of the thioureas, such as feniltiocarbamide (PTC) and 6 -n- propiltiouracile (PROP) [26], is considered a receptor modestly restrictive and has been shown capable of responding also stimuli lacking the C = S group [33].

Individual differences in the perception of the bitter taste are inherited genetically [12] and reflect the rich diversity of allelic receptors TAS2Rs .

The ability to feel the bitter taste of thioureas is associated with haplotypes that geneTAS2R38 derived from three individual polymorphisms resulting in three amino acid substitutions (Pro49Ala, Ala262Val, and Val296Ile) [26] (Fig.6).

Experimental Section

As previously mentioned, the CA VI protein plays in the oral cavity an important dual role:

- 1) maintaining the pH of saliva.
- 2) stimulation of the development and growth of circumvallate taste buds through its action on stem cells.

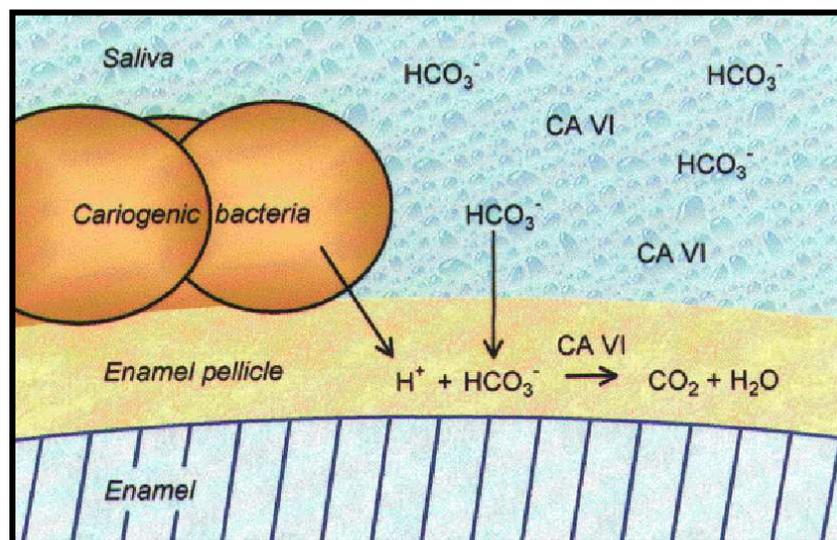


Fig. 2. Reaction catalyzed by CA VI in saliva.

In this work, the more that aspect aimed at the assessment of the enzymatic activity of CA VI in saliva, I have dealt with taking into account the molecular aspects related to gene CA6, in order to evaluate whether individual differences in taste sensitivity, widely found in individuals selected for this research, could be related with differences at the genetic level. The CA VI is usually highlighted in the saliva with immunohistochemical methods, which however, do not provide any information on possible structural abnormalities in the protein load [36].

The study of the gene may help to understand , albeit in an indirect way , which will be the primary, secondary and sometimes tertiary protein, and then get the important data that can be the starting point for cross-sectional studies of biochemical, physiological and cytomorphological.

Therefore, I started to study the molecular sequences from the two exons 2 and 3 articulating the research through the development of the following experimental steps :

Selection of a sample of 75 individuals volunteers and their classification according to the taste perception, through the use of solutions of different concentrations PROP.

These 2 experimental phases were conducted at the laboratories of the Section of General Physiology, Department of Experimental Biology. The selected subjects were classified, according to their taste perception [2], as follows:

- *Tasters*: individuals are able to perceive the bitter taste of PROP.
- *No Taster*: subjects insensitive to thiourea.

Individuals classified as Tasters were further divided into:

- *Super Tasters*: have a very high sensitivity to thiourea.
- *Medium Tasters*: have a moderate sensitivity to thiourea.

The subjects had a normal body mass index (BMI) ranging from 18.6 to 25.3 kg/m² and showed no changes in body weight greater than 5 kg in the last 3 months.

No one followed a prescribed diet or taking medications that may interfere with the perception of taste. The subjects did not have food allergies.

Spectrophotometric determination of zinc salivary through the use of a cationic porphyrin.

In human saliva, zinc is mainly linked to the carbonic anhydrase type VI (CAVI). We then proceeded to determine the concentration of free zinc salivate, as cofactor protein CAVI.

The method consists nell'utilizzo cationic porphyrin 5,10,15,20-Tetrakis (4-trimethylammoniophenyl) porphyrin tetra (p-toluensulfonate)-(ttmapp) and the compound 7-Iodo-8-hydrossiquinoline-5-sulfonic acid (Ferron), which acts as a catalyst for the incorporation of zinc into the porphyrin ring. The samples, prepared according to the following protocol, are analyzed spectrophotometrically at λ of 425 nm.

Preparation of saliva sample.

Immediately after collection, the saliva sample is centrifuged at a speed of 12000 rpm for 10 minutes (Eppendorf centrifuge, model 5417C, Eppendorf, Hamburg, Germany), and the supernatant is removed. The supernatant obtained from the centrifuged samples, was then removed with Pasteur pipettes, transferred to a sterile tube. The samples are prepared in accordance with the following table:

	H ₂ O	Saliva	TFTA	Buffer Acetato
Blank Reagent TFTA	150µl	-	600µl	-
TEST SALIVA	-	150µl	600µl	-
Blank SALIVA	-	150µl	-	600µl

All samples were mixed very well and incubated for 60' at room temperature. After zeroing the spectrophotometer against H₂O, the samples are to be read at λ 425 nm.

Calculation of the concentration of Zn

$$\begin{array}{c}
 \text{Concentration of Zn in saliva } (\mu\text{mol/l}) \\
 \downarrow \\
 \frac{\left[A (\text{test saliva}) - A (\text{BK saliva}) \right] - A (\text{reagent BK})}{A (\text{Standard Zn}) - A (\text{reagent BK})} \times 30.6 \times \text{dilution}
 \end{array}$$

The absorbance of each sample was measured at 425 nm and the total concentration of Zn²⁺ was expressed in micrograms per deciliter.

Extraction of genomic DNA from saliva samples

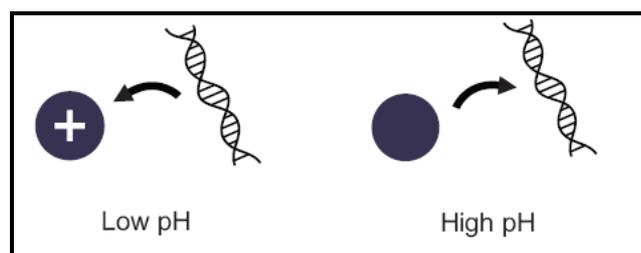
The saliva (1 ml) was taken from 75 subjects previously classified as *Super Taster* (27 individuals), *Medium Tasters* (28 individuals) and *No Taster* (20 individuals).

Specifically:

- 1) *Super Tasters* have a very high sensitivity to thiourea.
- 2) *Medium Tasters* have a moderate sensitivity to thiourea.
- 3) *No Taster* insensitive to thiourea.

The DNA and was purified from saliva samples (1 ml), using an extraction method that employs the use of a solution of magnetic beads, called Charge Switch.

The magnetic beads used in conditions of moderately acid pH (pH <6.5), have a positive charge capable of binding the DNA phosphodiester bridges negatively charged, thereby allowing to separate, efficiently and in a few steps, the nucleic acid from intracellular proteins and other macromolecules. Subsequently bringing the pH of the solution at basic pH values (pH \approx 8.5), the beads lose their positive charge in solution, releasing the DNA.



Once the extraction of DNA, we have determined spectrophotometrically its purity and its concentration.

To be able to proceed to the next stage of amplification by PCR is necessary to evaluate the amount of DNA present in each sample after the extraction.

In this regard, the spectrophotometer that is used, recording the ultraviolet light absorbed by the sample, provides us with a precise quantification. In this type of analysis takes into account two wavelengths: 260 and 280 nm.

The purity of the various samples of nucleic acid was estimated, and then with the “ ratio ” (260nm/OD OD 280nm), all samples showed a good degree of purity, having values between 1.8-2.0.

Analysis by PCR (*Polymerase Chain Reaction*), of the nucleotide sequences of the exon 2nd and 3rd of the CA6 gene and of the three polymorphisms *TAS2R38* gene

In our work, the purified DNA was subjected to PCR reactions in the presence of pairs of primers specific for DNA regions delimiting the nucleotide sequences of the second and third exon in which are located the residues histidine ligands and Zinc ion polymorphisms of the *TAS2R38*.

The PCR reactions were carried out using 10-20 ng of purified genomic DNA from saliva, according to the method described previously.

The samples were amplified in a final reaction volume of 25 μ l, containing 10 mM Tris-HCl, pH 8.5, formamide, 50 mM KCl, 200 μ M of each dNTP, 2 units' of Taq polymerase, and a suitable concentration of each forward and reverse primer, specific for the target sequences of respectively 2nd and 3rd exon of the CA6 gene. Using the following primer pairs:

➤ For - GGCCTCCTTCTGGGGGACCTGCTTCTGC

Rev - CAGCTCTGAGGCCGTGCTCTGTGTCTCT

specific for the 2nd exon

➤ For - GTGGGAGGTGAGCAGAGAAG

Rev - GCAGTGAGCTGAGATTGTGC

specific for the 3rd exon

We obtained amplification products respectively of 458 bp and 225 bp (base pair, bp) respectively.

The PCR experiments were conducted using an amplification program of 35 cycles, with an annealing temperature 62° C for 2nd exon and 50° C for 3rd exon. The PCR products were then separated by electrophoresis on polyacrylamide gel at 6%, or 2% agarose gel, and visualized by staining with DNA intercalators (ethidium bromide and SYBR Green) and with the silver nitrate .

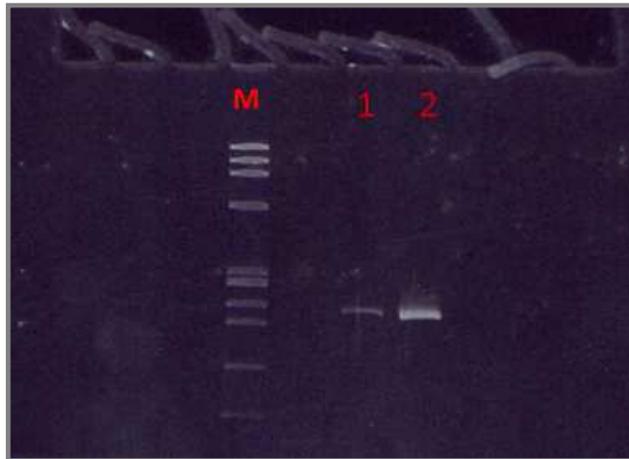


Fig. 3. Electrophoresis on polyacrylamide gel at 6%. The marker (M) DNA fragments used is the $\lambda\phi$ X 174. Fragment of 225 bp relative to the 3rd exon obtained with 10 ng (1) and 20 ng (2) DNA.

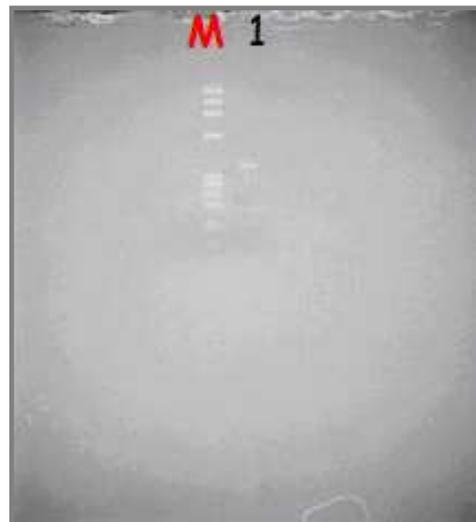


Fig. 4. Electrophoresis on agarose gel at 2%. The marker (M) DNA fragments used is the $\lambda\phi$ X 174. Fragment of 458 bp (1) relative to the second exon obtained with 15 ng DNA.

Were also studied three single nucleotide polymorphisms (SNPs) of the TAS2R38 locus.

The receptor gene TAS2R38, localized on the long arm of chromosome 7, is constituted by a single exon in which there are three SNPs in position 145 (C/G), 785 (C/T) and 886 (G/A).

The three SNPs are not synonyms and therefore responsible of three amino acid substitutions in the protein at the following locations:

P49A (rs713598 Prolina/Alanina)

A262V (rs1726866) (Alanina/Valina)

V296I (rs10246939) (Valina/Isoleucina)

samples were amplified in a final reaction volume of 25 µl, containing 10 mM Tris-HCl, pH 8,5, formamide, 50 mM KCl, 200 µM each dNTP, 2 unit of Taq polimerase, and a suitable concentration of each forward and reverse primer, specific for the target sequences.

Using the following pair of primers for the polymorphism at position 145:

➤ For-CC TTCGTTTCTTGGTGAATTTTGGGATGTAGTGAAGAGGCGG

➤ Rev- AGGTGGCTTGGTTTGCAATCATC

Obtaining an amplification product of 221 bp (base pair, bp).

Regarding the polymorphisms at positions 785 and 886 were identified from a single PCR reaction using the following primer pair:

2 For-TCGTGACCCCAGCCTGGAGG

2 Rev-GCACAGTGTCCGGGAATCTGCC

defining a fragment of 298 bp (base pair).

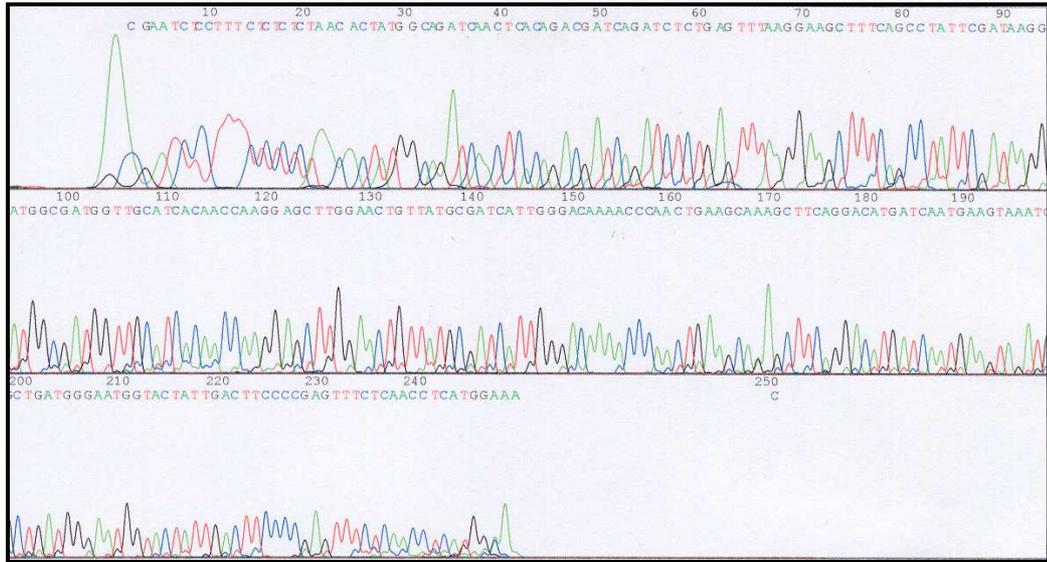
The amplification protocol consisted of 35 cycles, with an annealing temperature of 60° C.

The PCR products were then separated by electrophoresis on polyacrylamide gel at 6%, or 2% agarose gel, and visualized by staining with DNA intercalators (ethidium bromide and SYBR Green) and with the silver nitrate .

Sequencing of the products obtained and sequence analysis.

The DNA fragments obtained in the course of the PCR experiments, were sequenced using the technique of BigDye Terminator Kit (Applied Biosystems). The products of the sequencing reactions were separated using the ABI Prism automatic sequencer (Applied Biosystems).

The general principle underlying this technique is the use of di- deoxynucleotides which are marked with a different color dye (fluorescein, NBD, tetramethylrhodamine and Texas Red). Indeed, the replication of the fragments is stopped by the incorporation of a specific di- deoxynucleotide that is marked with the same color, for example blue for C, red for T, for the orange and green G for A. The four mixtures reaction are then mixed and loaded on a sequencing gel . The electrophoresis is made to go to continuous and the color of each band is read in the moment in which it passes in the beam of a photometer at laser placed at the base of the gel. The raw data read from the sequencer are constituted by four series of partially resolved fluorescence peaks , one for each of the 4 bases. Each peak corresponds to the signal emitted from a DNA fragment which ends with a ddNTP labeled with the fluorochrome. An algorithm of signal processing separates the individual peaks , proceeds to the shift of the 4 series of peaks relative to one another and attaches a base in each peak , as shown in the electropherogram below.



The nucleotide sequence obtained was analyzed using bioinformatic programs portal ExPASy.

Sequence analysis and experimental results related to the phases described.

With regard to the analysis of sequences related to the 2nd exon, molecular analysis has not revealed deletions or insertions in exon sequence of load studied. It was instead observed a difference in level of nucleotide 164 of the coding sequence (corresponding to amino acid 55th of the primary structure of the protein) in which is located a single nucleotide polymorphism (C/T), as shown in the following figure:

```
gcctgtggggccagagacagtgcctatcaacctacagaggaCgaaggtgcggtacaac 180
A C G G Q R Q S P I N L Q R T K V R Y N 60
ccctcctgaaggggctcaatatgacaggctatgagaccaggcaggggagttcccatg 240
P S L K G L N M T G Y E T Q A G E F P M 120
```

```
gcctgtggggccagagacagtgcctatcaacctacagaggaTgaaggtgcggtacaac 180
A C G G Q R Q S P I N L Q R M K V R Y N 60
ccctcctgaaggggctcaatatgacaggctatgagaccaggcaggggagttcccatg 240
P S L K G L N M T G Y E T Q A G E F P M 120
```

The single nucleotide polymorphism or SNP (Single Nucleotide Polymorphism) is a variation in the level of a nucleic acid sequence that occurs between individuals of the same species, characterized by a difference in load of a single nucleotide. For example, if the sequences are identified in two subjects, as in the case of our sequence exon, and GGACGAA GGATGAA, this is a SNP that distinguishes the two alleles C and T. SNPs can occur within a coding sequence of a gene, as in the case of the gene CA6, or within an intronic region or in an intergenic region. SNPs within a gene, does not necessarily alter the amino acid sequence encoded, since the genetic code is degenerate. A SNP that generates in all its forms, the same peptide is said synonymous (synonymous), otherwise it is said non-synonymous (non-synonymous).

In light of these considerations, we can say that the SNP to load the allelic form (C/T), is non-synonymous, as it generates the appearance of an amino acid substitution in the protein, at codon 55 (I \rightarrow M).

Regarding the examination of the sequences of the third exon, molecular analysis has not revealed deletions or insertions; was observed instead a difference at the nucleotide level in position 269 of the coding gene, in which is located a single nucleotide polymorphism borne allele rs2274333 (A/G).

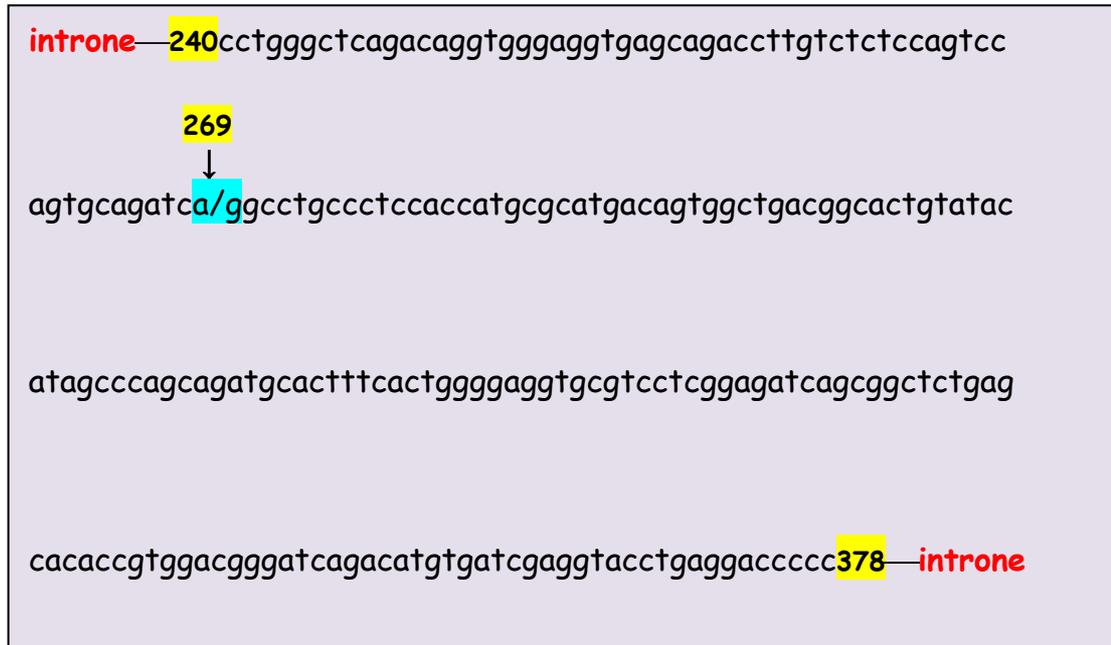


Fig.5 Nucleotide sequence of the third exon of the CA6 gene. In blue is shown the polymorphism of allele rs2274333 (A/G).

The SNP allele borne rs2274333 (A/G), also non-synonymous, because it creates the appearance of an amino acid substitution at codon 90 (S → G) in the protein.

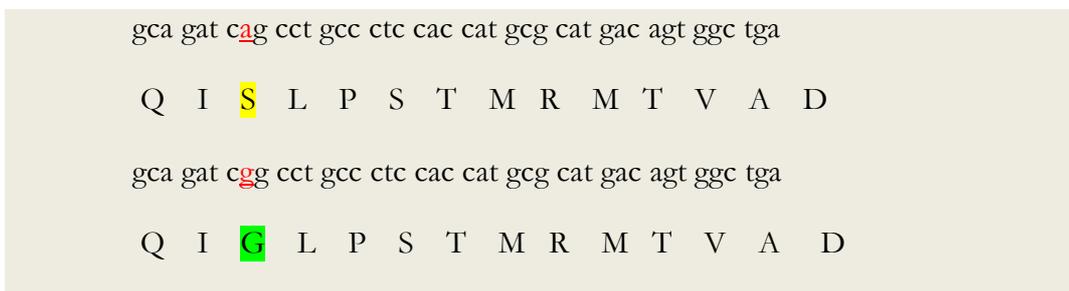


Fig.6a. Nucleotide sequence of the third exon of the CA6 gene.

10	20	30	40	50	60
MRALVLLLSL	FLLGGQAQHV	SDWTYSEGL	DEAHWPQHYP	ACGGQRQSPI	NLQRTKVRYN
70	80	90	100	110	120
PSLKGLNMTG	YETQAGEFPM	VNNGHTVQIS/G	LPSTMRMTVA	DGTVYIAQQM	HFHWGGASSE
130	140	150	160	170	180
ISGSEHTVDG	IRHVIEI <u>HIV</u>	HYSKYKSYD	IAQDAPDGLA	VLAAFVEVKN	YPENTYYSNF
190	200	210	220	230	240
ISHLANIKYP	GQRTTLTGLD	VQDMLPRNLQ	HYITYHGSLT	TPPCTENVHW	FVLADEVKLS
250	260	270	280	290	300
RTQVWKLNS	LLDHRNKTIH	NDYRRTQPLN	HRVVESNFPN	QEYTLGSEFQ	FYLHKIEEIL
308					
DYLRRALN					

Fig.6b. CA6 human gene. In the figure is highlighted in bold the amino acid sequence encoded by third exon. The histidine zinc ligands are indicated by underlining.

TECHNICAL PCR -RFLP

Identification of SNPs borne by allele rs2274333 (A/G).

A useful method to identify SNPs is the evaluation of the length polymorphisms of restriction fragments, or RFLP (Restriction Fragment Length Polymorphisms).

If an allele contains a recognition site for a restriction enzyme and another does not , digestion of the two alleles will generate two fragments of different size . In the case of the allele rs2274333 (A/G), the exon portion characterized by the sequence AGCCT is easily distinguishable from the sequence GGCCT, since replacement of the A in G at position 269 of the gene object of our study, in addition to determining the change in the codon CAG coding for a serine, in CGG coding for a glycine, also creates a recognition site for the restriction enzyme Hae III (Haemophilus aegyptius III). Indeed , Hae III is able to cut DNA at specific recognition sequence of making a clean cut , as shows the position of the arrow.

↓

5'-GGCC-3'

3'-CCGG-5'

All PCR fragments, given the presence in 3rd exon of a specific restriction site, were analyzed at the molecular level by associating to the study of the sequences, the identification of the recognition site for the restriction enzyme Hae III. Then, once the amplified fragment of interest, to verify the presence of the polymorphism rs2274333 (A/G), the amplification products were then subjected to enzymatic digestion, incubating the reaction mixture at 37° C for 120 minutes. The digested product was subsequently subjected to electrophoretic migration on polyacrylamide gels. From cutting endonuclease, in the case in which there is the restriction site of the enzyme, you create two fragments of 50 bp (sequence highlighted in green) and 165 bp respectively (sequence highlighted in fuchsia).

intron

```
intron—240cctgggctcagacaggtgggaggtgagcagacctgtctctcagtc  
agtcagatcgg↓cctgccctccaccatgcgcatgacagtggtgacggcactgtatac  
atagcccagcagatgacttcactgggaggtgcgtcctcggagatcagcggctctgag  
cacaccgtggacgggatcagacatgtgatcgaggtacctgaggaccccc378—intron
```

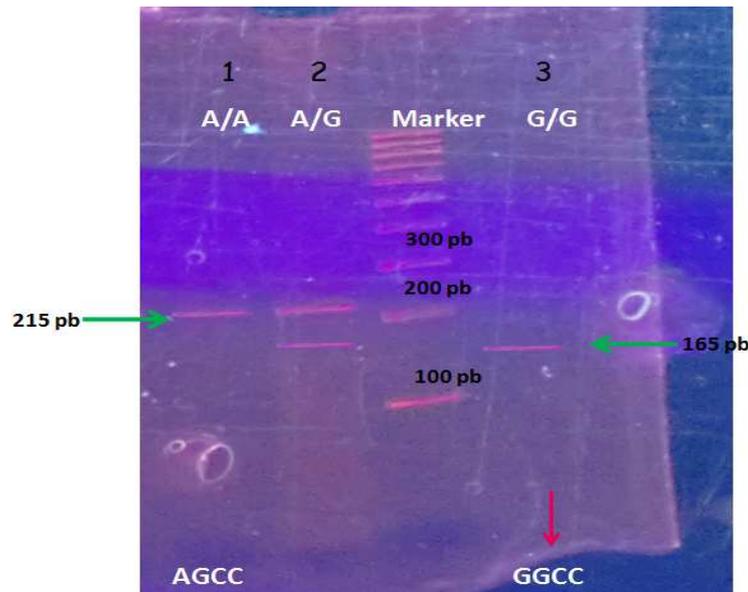


Fig. 7. The figure shows the results of the enzymatic digestion of three of the 75 samples analyzed.

Observing the electrophoretic profiles of the enzymatic digestion shows that:

- 1) It's a condition of homozygosity for the A allele (genotype A/A), does not occur when the cut by restriction enzyme. Therefore, individuals in the A/A is this sequence AGCC.
- 2) It's a condition of heterozygosity for alleles AG (genotype A/G). The PCR product was only partially digested, as indicated by the two electrophoretic bands obtained, respectively, 215 bp and 165 bp, indicating the presence of two sequences GGCC and AGCC. The digestion product of 50 bp is not visible as it was electrophoretically eluted from the gel.
- 3) It's a condition of homozygosity for the G allele (genotype G / G). The PCR product was completely digested in fact showing an electrophoretic band of 165 bp. Even in this case, the digestion product of 50 bp is not visible electrophoretically.

Designing of primers for training artificial restriction sites upstream and downstream of SNPs.

In the case of the three alleles rs713598 G /C, rs1726866 C/T and rs10246939 A/G, the analysis of the nucleotide sequences, upstream and downstream of SNPs, did not show the presence of restriction sites for endonucleases no. Therefore, we have developed a strategy to create, at each SNP, restriction sites for a particular enzyme. Specifically, each SNP has been incorporated within a PCR fragment obtained with primers capable of promoting a site-directed mutagenesis in the DNA. The primers used to introduce the amplified fragments, upstream or downstream of the SNP, appropriate nucleotide substitutions that would generate a palindromic region intercepted by an endonuclease. Ultimately, the SNP will be one of the nucleotides present in a restriction site created artificially.

Polymorphism rs713598 G / C

For the identification of this polymorphism, we used a pair of primers able to amplify a fragment of 203 bp, from nucleotide 98 to nucleotide 300. In the figure below, nucleotides highlighted in yellow with a capital letter, mark the boundaries of the fragment amplificato. Le nucleotide sequences on which were drawn the sense and antisense primers are shown in bold, while the polymorphic nucleotides are shown in red. The nucleotide highlighted in blue, is the target of nucleotide substitution in the PCR fragment. Polimorfismo rs713598 G/C.

1F mut →

61 atttcagtcctggagtttgagtgagggtttctgacca**A**tgcttcgttttcttggtgaat
121 tttgggatgtagtgaagaggc**a**gc/gcactgagcaacagtgattgtgtgctgctgtctc
181 agcatcagccggcttttctgcatggactgctgttctgagtgctatccagcttaccac
241 ttccagaagttgagtgaaccactgaaccacagct**acc**aagccatcatcatgctatggat**G**
← 1R

The sense primer (1F mut) used for amplification of the fragment is the following:

5'-Atgccttcgttttcttggtgaattttgggatgtagtgaagaggc**G**g-3'

The primer was designed by introducing, in correspondence of the penultimate nucleotide a **G** in place of **a** (as shown above in the sequence). This mismatch is fundamental for the PCR experiments, because the nucleotide **a** in the sequence of the gene TAS2R38, is replaced by a **G** in each of the amplification products. This creates the first **G** of the recognition sequence **G**CC of HaeIII, thus allowing the cut of the sequence only when present in the fragment and the **C** allele, present in the *Taster*.

atttcagtcctggagtttgagtgagggtttctgacca**A**tgcttcgttttcttggtgaat
tttgggatgtagtgaagaggc**G**gccaactgagcaacagtgattgtgtgctgctgtgt
ctcagcatcagccggcttttctgcatggactgctgttctgagtgctatccagcttaccact
ttccagaagttgagtgaaccactgaaccacagct**acc**aagccatcatcatgctatggat**G**

Ultimately, with the changes introduced in the fragment, you can recognize the allele form C by allele form G of the SNP rs713598 G allele G / C, because in the presence of endonuclease Hae III PCR fragment is digested into two fragments of 47 and 156bp respectively.

```

AtgccttcgttttcttggtgaatttttgggatgtagtgaagaggcGg 47 bp
                ccactgagcaacagtgattgtgtgctgctgtgtctc
agcatcagccggcttttctctgcatggactgctgttctctgagtgctatccagcttaccac
ttccagaagttgagtgaaccactgaaccacagctaccaagccatcatcatgctatggatc 156 bp
  
```

Polymorphisms rs1726866 C/T e rs10246939 A/G

For the identification of these two polymorphisms we designed a single pair of primers able to amplify a fragment of 193 bp (from nucleotide 737 to nucleotide 941), containing both polymorphisms within its sequence. Nucleotides highlighted in yellow with a capital letter, as shown in the figure below, mark the boundaries of the amplified fragment. The sequences on which the primers were designed sense and antisense are in bold, while the polymorphic nucleotides are shown in red. The two nucleotides highlighted in blue represent the nucleotides that will be replaced artificially in the PCR fragment.

1F mut →

721 gcccacattaagccctc**A**agtccttctctcctttttctgcttctttgtgatatcatcc

781 **tg**gc**/tt**gccttcatctctgtgccctactgattctgtggcgcgacaaaataggggtgatg

841 gtttgtgtgggataatggcagcttgcctctgggcatgcagccg/**at**ct**gatctcaggc**

901 **aatgccaagttgaggagagctgtgatgacca**T****tctgctctgggctcagagcagcctgaag

← **2R mut**

The sense primers (*2F mut*) and antisense (*2R mut*) used in the amplification reaction are the following:

5'-aagtctctgtctccttttctgctctttgtgatatcatcctgAg-3'(senso)

5'-atggcatcacagctctcctcaactggcattgcctgagatcagT-3' (antisenso)

The sense primer was designed introducing in correspondence of the penultimate nucleotide an A instead of a t (as shown in the sequence above). This mismatch, as previously seen, is crucial for the PCR experiments, because the nucleotide t in the sequence of the gene TAS2R38, is replaced by an A in each of the amplification products. This creates the first A of the recognition sequence AGCT of AluI, thus allowing the cut of the sequence only when the fragment is the allele C.

721 gcccacattaagcctcAagtctctgtctccttttctgctctttgtgatatcatcc

781 tgAgctgccttcattctgtgccctactgattctgtggcgcgacaaaataggggtgatg

841 gtttgtgtgggataatggcagcttgcctctgggcatgcagccgtActgatctcaggc

901 aatgccaagttgaggagagctgtgatgaccaTctgctctgggctcagagcagcctgaag

The reverse primer, PCR product by introducing a T instead of a C in position 888, leading to the formation of a restriction site GTAC, specific for the enzyme RsaI, in which the G allele, is the first nucleotide of the sequence palindromic recognized.

Therefore, subjecting the PCR fragment to enzymatic digestion with AluI, you get two restriction fragments of 46 and 148 bp respectively, only if the SNP rs1726866 shows the C allele, characteristic of the *Super Taster*.

Aagtcctctgtctccttttctgcttctttgtgatcatcctg**Ag** 46 bp

ctgccttcactctgtgccctactgattctgtggcgcgacaaaataggggtgatggtttgtgtgggataatggcagcttgcctctgggc
atgcagcc**gtAct**gatctcaggcaatccaagttgaggagagctgtgatgacca**T** 148 bp

If the same fragment is subjected to digestion with the enzyme *RsaI*, the fragment is cut in correspondence of the sequence **GTAC**, giving a fragment of 149 and one of 45 bp, only if the SNP rs10246939 presents the **G** allele (characteristic of the *Super Taster*).

Aagtcctctgtctccttttctgcttctttgtgatcatcctg**Ag**ctgccttcactctgtgccctactgattctgtggcgcgacaaa
ataggggtgatggtttgtgtgggataatggcagcttgcctctgggcatgcagcc**gt** 149 bp

Actgatctcaggcaatccaagttgaggagagctgtgatgacca**T** 45 bp

Each purified DNA sample was subjected to different PCR reactions, and in particular:

- 1) in PCR reactions aimed to direct sequencing in the presence of the pair of specific primers **1F** and **1R**, for the isolation of the DNA region containing the SNP **C/G**, and primers **2F** and **2R** for the isolation of the region containing the SNPs **C/T** and **G/A**.
- 2) in PCR reactions aimed at the analysis of restriction in the presence of the pair of the primers **1F mut** and **1R**, for the isolation of the DNA region containing the SNP **C/G**, and mut primers **2Fmut** and **2R** for the isolation of the region containing the SNP **C/T** and **G/A**.

The PCR experiments were conducted using an amplification program of 35 cycles, with an annealing temperature of 50° C for the fragment *1F/1R* and 62° C for the fragment *2F/2R*.

Using pairs of primers *1F mut/1R* specific for the analysis of 1st SNP (C/G) and *2F mut/2R mut* specific for the analysis of the 2nd and 3rd SNP (C/T and G/A), we obtained amplification products of 203 and 194 bp respectively.

The PCR experiments were conducted using an amplification program of 35 cycles, with an annealing temperature of 63° C for the fragment *1Fmut/1R* and 68° C for the fragment *2Fmut/2Rmut*.

Figure 8 below shows the electrophoretic profile of one of the 60 samples analyzed.

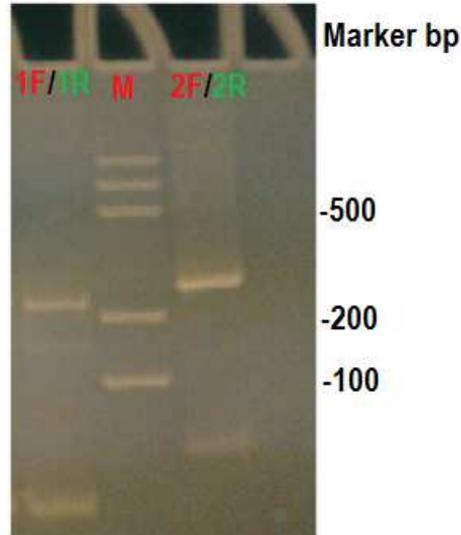


Fig.7

Correlation between phenotype and genotype

Molecular analysis conducted on 75 individuals, classified in *Super Tasters*, *Medium Tasters* and *No Tasters* depending excessive, moderate or absent taste sensitivity to the bitter taste, showed a significant correlation between the difference in taste perception and individual polymorphism rs2274333 (A / G) present in 3rd exon.

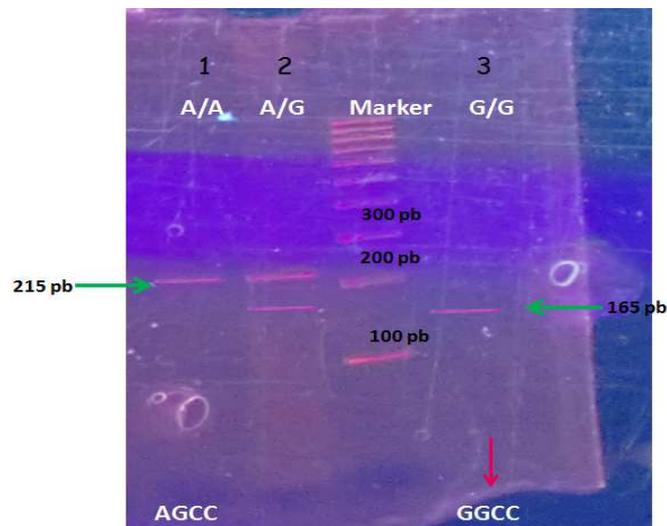


Fig. 9 . The figure shows the results for three of the 75 samples subjected to digestion.

Using statistical methods, and in particular the method of Fisher, is showed that the genotype A/A and the A allele is much more frequent in individuals *Super tasters*, while the genotype G/G and the G allele is much more frequent in subjects *No tasters*.

Specifically, for each group of subjects analyzed, and 'result as follows:

A) *Super Tasters*: 20 of 27 subjects analyzed well have the genotype A/A, only 7 are heterozygous A/G and none on the other hand shows the genotype G/G.

B) *No Tasters*: 11 of 20 of those surveyed have the genotype G/G, 4 are heterozygous A/G and 5 have the genotype A/A. It thus appears that the 92.59% of the super tasters leads the A allele while 65.00% of non-tasters leads the G allele at this position.

C) *Medium Tasters*: of 28 samples, 14 are homozygous A/A, 10 are heterozygous A/G and 4 homozygous G/G, in these individuals the A allele is much more frequent (67.86%) compared with the G allele whose frequency is less than half (32.14%).

However, with respect polymorphism C/T detected in the 2nd exon, statistical methods have not revealed a significant correlation between genotype and phenotype. The condition of homozygosity for the allele C or T, and heterozygous (C/T), does not seem to be closely associated with a *No taster* or *Super taster status*.

The 60 samples subjected to restriction analysis regarding the locus TAS2R38, showed genotypic profiles and the frequency of haplotypes, reported in the following Table 1.

Tabella 1

	<i>Super Taster</i>		<i>Medium Taster</i>		<i>Non Taster</i>	
	n=20	%	n=20%		n=20	%
Genotipo						
PAV/PAV	9	45.00	21	0.00	–	0
AVI/AVI	–	0	1	5.00	19	95.00
PAV/AVI	11	55.00	17	85.00	1	5.00
Aplotipo						
PAV	29	72.50	21	52.50	1	2.50
AVI	11	27.50	19	47.50	39	97.50

To confirm the results of the restriction analysis, PCR products related to fragments 1F/1R (1st SNP) and 2F/2R (2nd and 3rd SNP), were purified from primers and dNTPs present in the reaction mixtures, and then sent to an external sequencing service. The nucleotide sequences obtained, analyzed using bioinformatics programs portal ExPASy, have fully confirmed, for all 60 subjects, the results of restriction analysis.

The statistical methods (Fisher's method) showed significant correlation with (p-value <0.0001) and by PCR-RFLP than with direct sequencing, the PAV haplotype is widely distributed in the general PROP taster, while the AVI haplotype among individuals *No taster*. As shown in Table 1, the large proportion of heterozygous PAV/AVI in *Super taster* phenotype suggests that gene haplotypes TasR38 only

justified in part the sensitivity to the bitter taste of PROP, as the TAS2R38 gene haplotypes differ only two phenotypes for sensitivity to PROP: the *Tasters* and *No tasters*. Molecular analysis of the polymorphism rs2274333 (A/G) gene CA6, previously conducted in the laboratory on the same 60 subjects, have shown that the A allele and the AA genotype is more prevalent in *Super taster* phenotype, while the GG genotype and the G allele in *No taster* (table 2).

Tabella 2

	Fenotipo (n=60)		
	Super taste (n= 20)	Medium taster (n= 20)	Nontaster (n= 20)
Genotipo rs2274333 (A/G)			
AA	17	10	5
AG	3	7	4
GG	0	3	11
Frequenza allele A	92,5%	67,5%	35%

In light of the results obtained, we have put in correlation with polymorphisms of the gene TAS2R38 with the polymorphisms of CA6 gene in the 3 groups of subjects (Table 3).

Tabella 3

Genotipo	Super Taster		Medium Taster		Non Taster	
	n	%	n	%	n	%
TasR38 CA6						
PAV/PAV-AA	8	40.00	1	5.00	—	0
PAV/PAV-AG	1	5.00	—	0	—	0
PAV/PAV-GG	—	0	1	5.00	—	0
AVI/AVI-AA	—	0	1	5.00	6	30.00
AVI/AVI-AG	—	0	—	0	1	5.00
AVI/AVI-GG	—	0	—	0	12	60.00
PAV/AVI-AA	9	45.00	9	45.00	—	0
PAV/AVI-AG	2	10.00	7	35.00	—	0
PAV/AVI-GG	—	0	1	5.00	1	5.00

As can be seen from the results shown in Table 3, the high percentage of subjects PAV/AVI can justify a *Super taster* phenotype, when the heterozygote is in association with the A allele of the rs2274333 polymorphism. Ultimately, the high sensitivity to PROP in *Super tasters* seems determined by the association (calculated using Markov Chain method) of the haplotype PAV of TasR38 gene with at least one A allele of the CA6 gene.

Prediction of the tertiary structure

Studies of secondary structure prediction , we have associated studies of prediction of the tertiary structure , using the technique dell' *homology modeling*. The *homology modeling* is one of the best methods currently used for the prediction of the three-dimensional structure (3D) of those proteins for which you know only the primary sequence .

The purpose dell'homology modeling is therefore to build a 3D model for a specific protein of unknown structure (target sequence) based on the sequence similarity with protein (template) whose three-dimensional structures are instead already been resolved by means of common techniques crystallographic (X - ray crystallography or NMR) .

To be able to build a realistic model and it is requires that the target protein and the protein mold must have a percentage of similarity of primary sequence sufficiently high (25-30% or more), in this case it is sure of the fact that the two or more proteins are considered homologues, that derive from a common ancestral progenitor . The theoretical structures of the polymorphic forms of the CA VI were constructed using the homology modeling program ESyPred3D portal ExPASy (Lambert et al., 2002), and using the mold as a protein 3D structure of carbonic anhydrase II (Lloyd et al. 2005). The 3D structure of CA II (PDB code 3CAJ) and was chosen as a template for two main reasons: (1) the alignment of the primary structure of CA II and CA VI vs of about 40%, (2) the domain which localized the catalytic site in which are present the three histidine residues binders zinc ion that is extremely conserved (homology of about 50). Using the crystal structure of human CA II , we then reconstructed in silico 3D structures of the 4 polymorphic variants **Threonine/Serine, Threonine/Glycine, Methionine/Serine,**

Methionine/Glycine , resulting from the association of alleles C/T of 2nd and exon A/G 3rd exon.

The diagram below illustrates the hypothetical structures of polymorphic variants **Methionine/Methionine** and **Serine/Glycine**.

It not shows the structures of polymorphic variants with the threonine residue (**Threonine/Serine**, **Threonine/Glycine**), because this substitution associated with amino acid substitutions in position 90, three-dimensional structures from place to absolutely comparable to those in which the present methionine. The presence of the serine or glycine residue of upstream of the catalytic site, leads instead to structural modifications apparent to loading of the protein domain in which are located the three histidines binders zinc ion (H 111 , H 113 , H 138) , as it can be observed by analyzing the figures 10 (b).

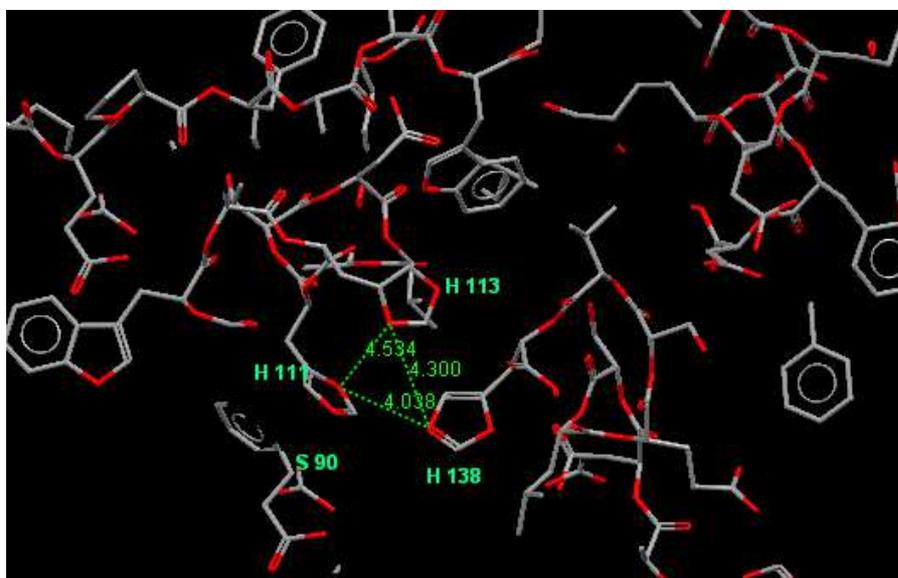


Fig. 11 a. 3D structure of the catalytic site of CA VI, polymorphic variant Methionine/Serina. In the figure shows the distances between the histidine residues H 111, H 113, H 138, and the residue S90.

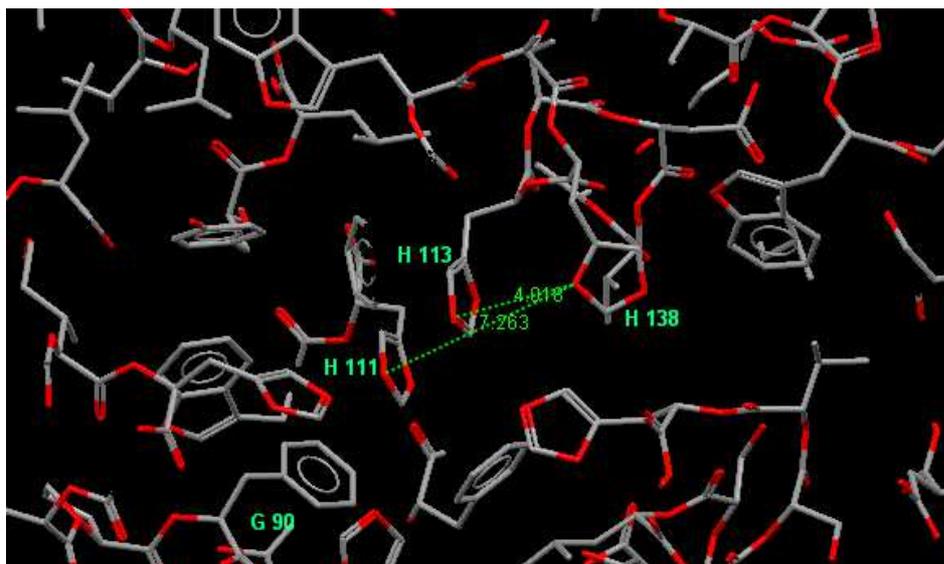


Fig. 11 b. 3D structure of the catalytic site of CA VI, polymorphic variant methionine / glycine. In the figure shows the distances between the histidine residues H 111, H 113, H 138, and the residue G90.

As can be seen by analyzing the structures *in silico* 3D, when in the primary structure is present the serine in position 90, the three histidine residues are located at a distance is 4 Å about, assuming an arrangement comparable to that described for the CA II, and in general suitable for binding with the zinc ion, placed at a distance of 2 Å about from the histidine residues. The analysis of the active site of CA II through the crystal structure, showing the presence of a hydrophobic cavity in which zinc occupies the central part, being placed at a distance of 2 Å from each histidine residue. In the cavity the distance between the histidine residue and 'therefore of 4 Å about.

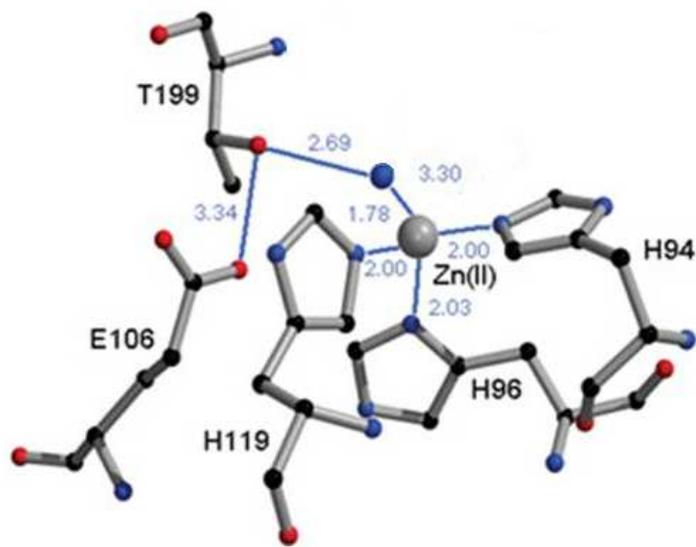


Fig. 12. 3D structure of CA II (PDB 3CAJ). In the figure is indicated the arrangement of zinc in the catalytic site and its distance from the histidine residue

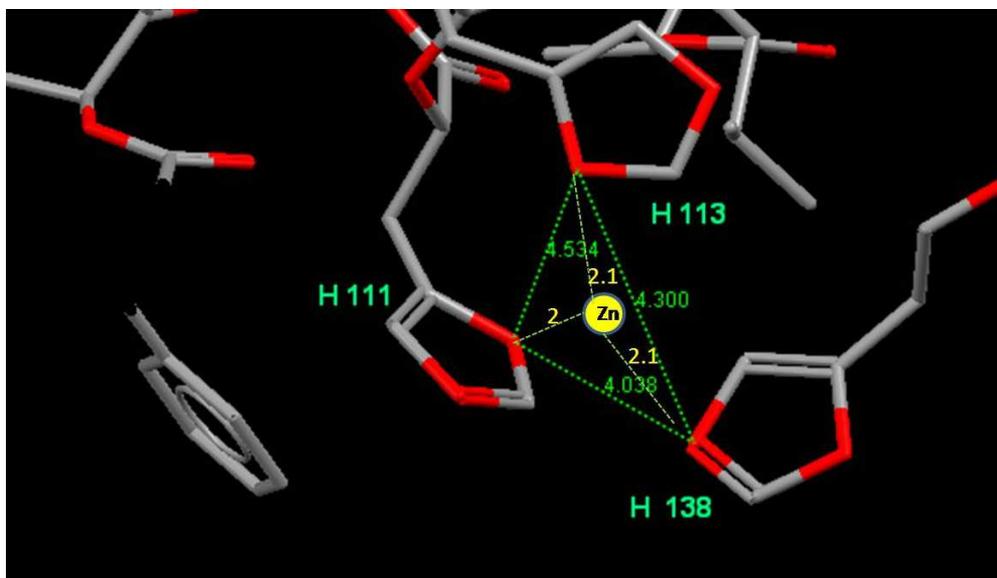


Fig. 13. 3D structure of CA VI polymorphic variant Methionine / Serina. In the figure is indicated the possible arrangement of zinc in the catalytic site and its distance from the histidine residue.

The substitution S → G in position 90, would lead to a removal of the H 111 from the H 138, producing a corresponding conformation of the protein domain deformed when compared with the CA II isoform taken as a model, however different conformation with the CA VI in which is present theresidue S 90.

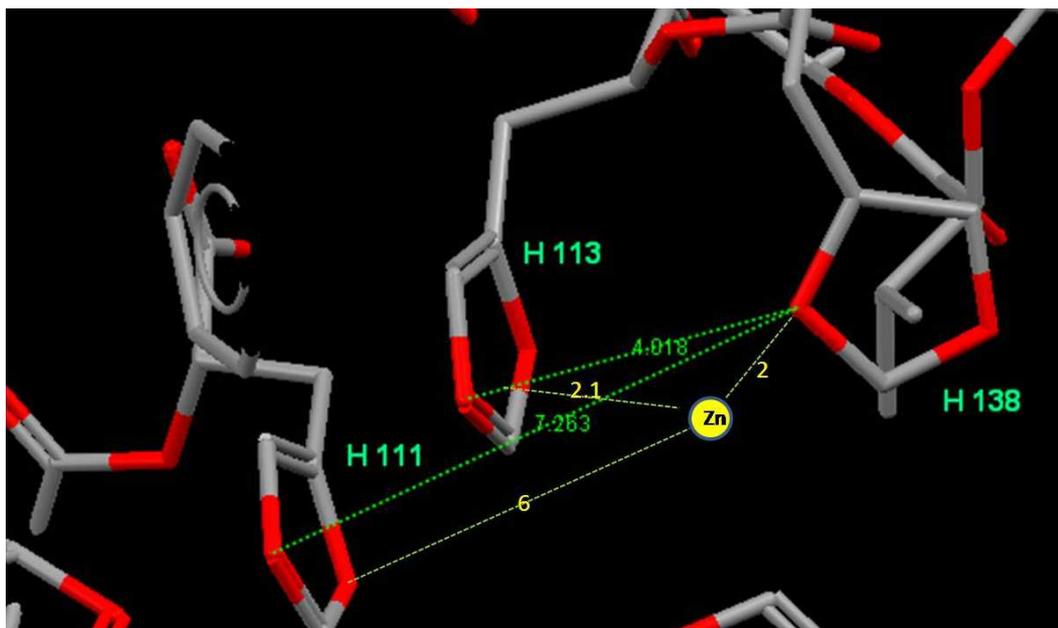


Fig. 14. 3D structure of CA VI polymorphic variant Methionine/Glycine. In the figure is indicated the possible arrangement of zinc in the catalytic site and its distance from the histidine residue

Protein Purification CA VI

Therefore, in light of the results previously obtained, and the research work was directed towards an experimental strategy whose purpose was to isolate the protein CA VI from the saliva of two *Super taster* homozygous for serine (SS) , and saliva of two *No tasters* homozygous for glycine (GG) to assess the potential activity of the stem cell line of taste .

The purification of the protein from the saliva of two *Super taster* homozygous SS and two *No tasters* homozygous GG, it was conducted through the use of the techniques of affinity chromatography.

Saliva samples (250 mL) collected within vials polyethylene, were centrifuged at 16000×g for 30 minutes, in order to eliminate the cells of the oral mucosa by salivary secretion. After centrifugation, the supernatant was mixed with a buffer solution containing benzamidine 0.2 M, Tris- SO₄ 0.1 M and 0.2 M Na₂SO₄, pH 7 (ratio supernatant/buffer 1:3). The use of this solution is important to promote the inhibition of salivary protease. The mixture thus obtained was then used for the chromatographic experiments.

Affinity chromatography by the use of the inhibitor

The fixed phase of affinity chromatography was prepared as reported in the literature (Khalifah et al., 1977), by binding to the resin (carboxy methyl Bio-Gel, Bio-Rad, Richmond, CA), the p-amino metilbensulfonamide, that functions as a competitive inhibitor of CA VI. The fractions containing the purified CA VI, have been collected on the basis of spectrophotometric absorbance values at 280 nm.

The concentration of the purified protein was quantized by using the method of Bradford (Bradford, 1976) and its purity assessed by SDS-PAGE.

Bradford's method

It is a colorimeter method used to assess the protein concentration. This method is based on the observation that an acidic solution of Coomassie Brilliant Blue G-250 undergoes a shift in the absorbance maximum from 465 nm to 595 nm when the dye binds the protein material. Specifically, the dye forms non-covalent complexes with proteins, thanks to electrostatic interactions that are established with amino and carboxyl groups, and van der Waals interactions. This method involves the use of a calibration line, obtained using a standard solution of bovine serum albumin (BSA, bovine serum albumin) at a known concentration.

The concentration of protein in the sample is calculated by measuring the absorbance at 595 nm, and using the calibration line.

Standard Protein

As a standard protein, for the preparation of the standard curve, it was used with the BSA concentrations of 2, 4, 8, 16, 20 $\mu\text{g/mL}$.

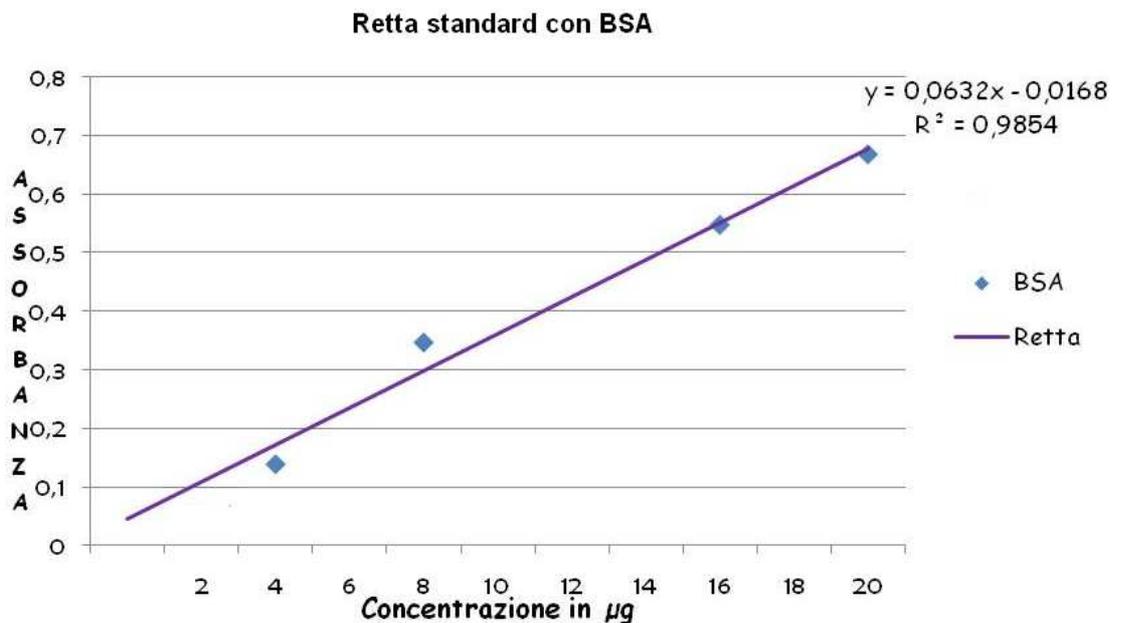
Each incubated was prepared in duplicate according to the scheme shown in the table

	2 $\mu\text{g/mL}$	4 $\mu\text{g/mL}$	8 $\mu\text{g/mL}$	16 $\mu\text{g/mL}$	20 $\mu\text{g/mL}$	Bianco
BSA	40 μL	40 μL	40 μL	40 μL	40 μL	-
Coomassie	2 mL	2 mL	2 mL	2 mL	2 mL	2 mL
H ₂ O	-	-	-	-	-	40 μL

The samples were left to incubate at room temperature for 5', and readings were made spectrophotometrically at λ of 595 nm.

For the purposes of the construction of the standard curve, the absorption values obtained for the different concentrations of BSA, was subtracted from the value of absorption of the white, and the data obtained have been reported in a graph absorbance vs concentration. The calibration line was obtained from a linear regression with the least squares method.

The correlation coefficient, $r = 0.985$, reported a strong mutual correspondence between values of absorbance vs concentration ($A = \epsilon c l$).

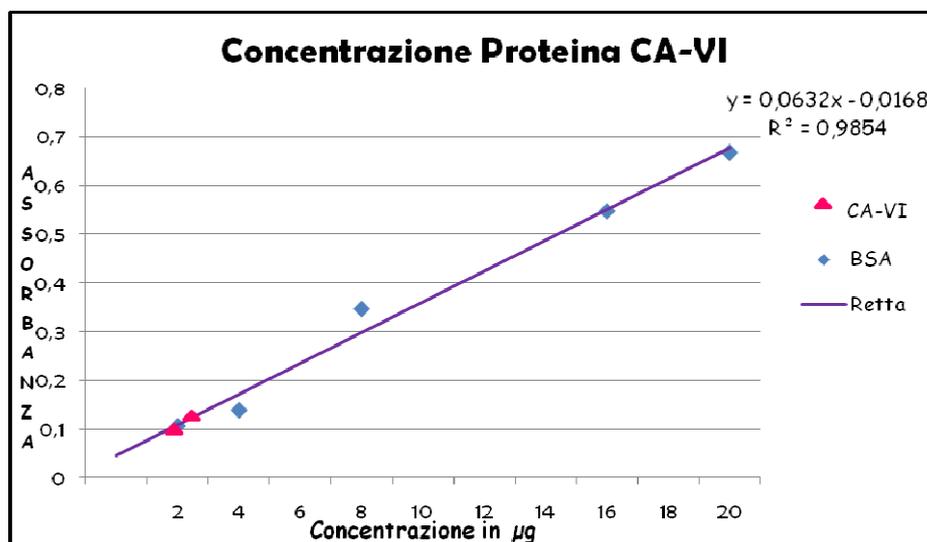


Determination of the concentration of CA VI

Immediately before to performing the test, the purified protein was centrifuged at 12,000 rpm for 20 minutes. Even in this case, for a more rigorous experimental, the samples were prepared in duplicate, according to the diagram in the figure.

	H ₂ O	CA VI	Coomassie
Bianco	40μL	-	2 mL
TEST	-	40μL	2 mL

All samples were then mixed well, and incubated for 5 minutes at room temperature. After the incubation time, the samples were read spectrophotometrically at λ of 595 nm. After subtracting the absorption of the white from the respective test [(A test - A Blank)], the concentration of the samples was determined by standard linear regression.



From 250 mL of saliva, with a total protein content of 60mg, were purified 1.5 mg of CA VI.

The degree of purity of the protein

The degree of purity of the protein isolated from the saliva samples and was assessed by electrophoresis in denaturing SDS-PAGE, which allows the separation of proteins according to their molecular mass, according to the Laemmli method (Laemmli UK, 1970).

The separating gel (running gel) was prepared in gradient of acrylamide 7-12% and 8-16% (w/v) (acrylamide/bisacrylamide = 30/0.8%), while the gel pre-stroke (stacking gel) is 4.5% acrylamide (w/v).

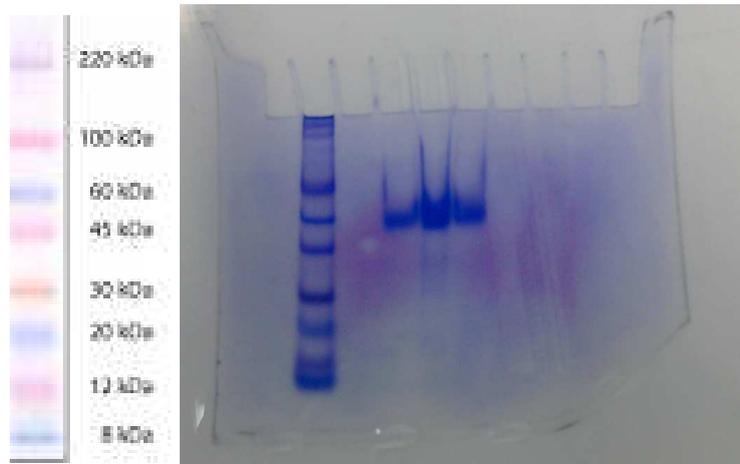
The buffer of the separating gel contains Tris-HCl 0.375 M at pH 8.8, while the buffer of the gel pre-stroke contains Tris-HCl 0.125 M at pH 6.8. The samples were solubilized in Tris-HCl 62.5 mM pH 6.8, SDS 2% (w/v), and β -mercaptoethanol 5%, 100° C for 5 minutes.

At the end were weighted with concentrated glycerol (80%) containing bromophenol blue. The buffer used for the electrophoretic run contains: Tris-HCl 0.025 M, glycine 0.192 M and SDS 0.1% (w/v) at pH 8.3.

The gels obtained by the electrophoresis were fixed in a solution of methanol: water: acetic acid = 5:5:1 (v/ v/v), and then were stained for 20 minutes with 0.25% of Coomassie Brilliant Blue R-250 in methanol: water: acetic acid. The protein bands are evident after discoloration of the gel in a solution containing 10% acetic acid and 5% methanol.

After leaving the gel in destaining solution over night, we detected a single protein band of approximately 42 kDa PM.

M 1 2 3



The lane M and is relative to the marker of molecular weights; as standards of molecular weights it was used Chemichrome Western Control consists of a mixture of proteins with a molecular weight range between 8 and 220 kDa. The lanes 1, 2 and 3, are related to the protein samples with different concentrations of protein (50, 75, 100 ng).

The purification of salivary protein CA VI is the achievement of an important goal experimental allow us to check if indeed the polymorphism rs2274333 (A / G) of the encoding gene is capable to influencing the activity of protein as trophic factor cell.

CONCLUSIONS

For the first time we have shown that the taste sensitivity to PROP is inversely related to the concentration of the zinc salivary and directly associated with the polymorphism rs2274333 (A/G) in 3rd exon of the gene CA6, which seems to be linked with the protein's ability to bind zinc.

A comparative analysis of the rs2274333 polymorphism allowed us to combine the highest sensitivity to PROP *Super tasters* of the AA genotype and allele A, the lowest sensitivity to PROP *No tasters* of the GG genotype and G allele, and the moderate sensitivity to PROP of *Medium tasters* in the presence of at least one allele A.

Our results suggest that the polymorphism rs2274333 (A/G) influence the binding of zinc to CA6. The activity of gustina depends on the presence of zinc at the active site of the protein [118,119], and it has been demonstrated that its full functional activity is crucial for the function gustatory [116,117,120,121].

Although speculative, we can speculate that the small difference in level of the primary structure related to the presence of the SNP rs2274333 (A / G) present in 3rd exon CA VI, may determine profound differences at level of protein folding, due to the presence of a small package β localized between amino acids 96-99, only when in position 90 there's the glycine.

The 3D structure obtained by homology modeling experiments, has highlighted the possible structural abnormalities at the expense of protein domain that contains the histidine residue, which could make it difficult to bond with the zinc ion.

We've hypothesized that the amino acid residue Ser in position 90, seen in “*Super tasters*”, would lead to optimal geometry of the active site of the protein that would favor binding to zinc. In contrast, the presence of Gly, observed in “*No tasters*”, may

make it difficult to bond the zinc to the catalytic site of the protein thus affecting its enzymatic activity.

Then, the high concentration of zinc in the saliva of “*No tasters*” could be correlated with a non-optimal geometry of the active site which makes it difficult the bond of the zinc to histidines 111 and 113.

In the first phase of this work the aim was to determine how the TAS2R38 haplotypes and genotypes of the polymorphism rs2274333 (A/G) gene gustina can co-operate in the modulation of PROP phenotype.

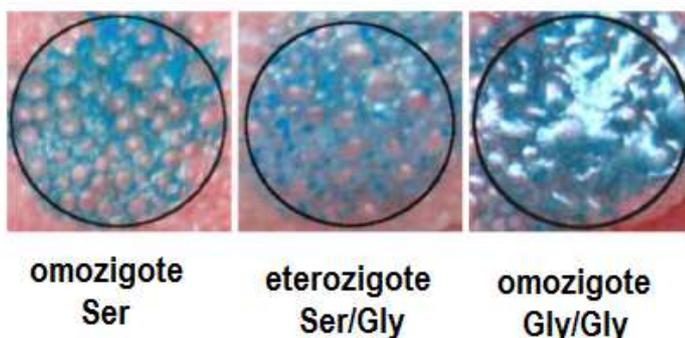
The analysis of the genotype distribution and allele frequencies of the TAS2R38 gene and gustin according to PROP status showed that the locus TAS2R38 only distinguishes the “*Tasters*” from “*No tasters*”, while the gene locus of gustina differentiates all three PROP taster groups. Furthermore, the results of this analysis with those of the analysis of the distribution of the combinations of the genotypes of the two loci showed that, while the locus TAS2R38 genetically characterizes individuals “*No tasters*”, the locus of the gene of gustina characterizes the “*Super tasters*”.

In fact, the lowest sensitivity to PROP in “*No tasters*” is strongly associated with non sensitive variant of the TAS2R38 AVI (95% of the “*No tasters*” brings the AVI haplotype and 90% are homozygous AVI) in those with at least 1 G allele gene gustina. Secondly, the higher sensitivity to PROP in “*Super tasters*” is strongly associated with the A allele and the AA genotype (93% of super tasters leads the A allele and 85% are homozygous A).

The purification of the protein salivary CA VI is the achievement of an important milestone experimental as recently has allowed to verify that the polymorphism rs2274333 (A / G, S → G) gene CA6 is able to influence the activity of the

protein as a factor trophic cell. In fact, in a recent work done in collaboration with the Section of Physiology and Cytomorphology of the Department of Biomedical Sciences (University of Cagliari) showed that in vitro treatment of fetal cells from goats of the taste line (ZZ-R-127) with isoform protein Ser90, is able to further promote cell proliferation compared to that observed after treatment of the same cell line with the isoform protein Gly90.

The same work has revealed that individuals homozygous for the polymorphism protein S90, have a greater number of taste buds on the tongue surface for cm², compared to subjects homozygous for the Gly90 isoform protein.



The largest number of taste buds, closely correlated with the presence of a greater number of taste receptors, could explain the greater sensitivity gustatory for the bitter taste of PROP of subjects homozygous for protein polymorphism Ser90 and then to the A allele of the SNP rs2274333, compared to subjects homozygous for the G allele, and then with the protein polymorphism Gly90.

In conclusion, our results summarizing previous work showing that the TAS2R38 alone can not explain the high sensitivity of the gustatory *Super tasters*, extend our knowledge by identifying the polymorphism rs2274333 (A/G) of gustin gene a new

candidate who, by modulating the functional activity of the protein as a trophic factor that promotes the growth and development of taste buds, may explain the high skill of *Super tasters* to discriminate a wide range of oral stimuli bitter and not bitter , individual differences in chemosensory perception, preferences food (including those in respect of fats) and BMI differences that are reported here as well as in other studies [2-6 , 13, 17,18 , 25, 32 , 38, 49] .

Ethics Statement

All subjects were verbally informed about the procedure and the purpose of the study. They reviewed and signed an informed consent form. The study was in compliance with the ethical principles established by the recent revision of the Declaration of Helsinki and the procedures were approved by the Committee of the Hospital of the University of Cagliari (Protocol No. 451/09 dated 15/10/2009 and verbal amendment Ranked # 8 of 29/11/2010).

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I

A Rapid Screening Method for the Identification of a Single-Nucleotide Polymorphism in the Carbonic Anhydrase VI Gene in Studies of Sensitivity to the Bitter Taste of 6-n-Propylthiouracil

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The ability to perceive the bitter taste of 6-n-propylthiouracil (PROP) is a variable phenotype that has been associated with body mass index (in kg/m²) and linked to food choice and satiety. PROP-sensitive and -nonsensitive individuals are defined as tasters and nontasters, respectively. Sensitivity to PROP is a heritable trait based on the *TAS2R38* gene on chromosome 7q34. In a recent study we demonstrated an association between PROP sensitivity and the single-nucleotide polymorphism (SNP) rs2274333 (+292A/G) within a coding sequence of the gustin/carbonic anhydrase VI gene. The purpose of this study was to develop a rapid and inexpensive screening method for identification of the rs2274333 SNP in individuals with varying sensitivity to PROP. Our results show that the methodology employed allows distinguishing A/G alleles perfectly, with a simple DNA digestion of a polymerase chain reaction fragment covering the SNP site of interest. So, the polymerase chain reaction followed by restriction fragment length polymorphism assay described in this article can be used as an alternative to sequencing in bitter taster status research, and could be employed as a survey tool in nutrigenomic studies.

Introduction

SENSITIVITY TO THE bitter taste of thiourea, in the forms of phenylthiocarbamide and 6-n-propylthiouracil (PROP), varies widely among individuals. The ability to perceive the bitter taste of PROP is the more common phenotype, and has been associated with body mass index, food choice, and satiety (Tepper, 2008). The individual variability to taste thioureas is a heritable trait, depending on alleles of the *TAS2R38* gene, which expresses a receptor that binds the N-C=S group, allowing perception of bitter taste in response to thiourea (Fox, 1932; Harris and Kalmus, 1949; Guo and Reed, 2001). Kim *et al.* (2003) cloned the *TAS2R38* gene (SWISS-PROT accession number P59533), a member of the bitter taste receptor family, and identified three nonsynonymous coding single-nucleotide polymorphisms (SNPs) (Pro49Ala, Ala262Val, and Val296Ile) giving rise to two common molecular haplotypes of this receptor (PAV and AVI, depending on the amino acid present at each SNP). The human haplotype PAV is associated with tasting, whereas the other common form, AVI, is associated with nontasting.

Recently, Padiglia *et al.* (2010) introduced another factor to the genetic basis of bitter tasting by demonstrating an asso-

ciation between the PROP phenotype and a SNP within the carbonic anhydrase VI (CA6) gene (SWISS-PROT accession number P23280), which is assumed to be a taste buds trophic factor (Henkin *et al.*, 1988). The CA6 SNP is an A-to-G transition at position 292 (+292A/G) of exon 3, resulting in substitution of the Ser residue with Gly at position 90 of the mature protein. The G allele of +292A/G, which has been associated with conformational changes of the protein, could establish a mechanistic link between PROP nontaster status and reduced taste bud density and functionality (Padiglia *et al.*, 2010). Recently, a study demonstrated that insensitivity to PROP was associated with increased adiposity in women, and might be linked to increased selection of dietary fats (Tepper *et al.*, 2010). Earlier investigations suggested that nontasters may not experience the bitter taste of nicotine in cigarettes, leading to an increased risk to health due to prolonged smoking (Duffy *et al.*, 2004). Some authors have also shown that PROP bitterness is associated with oral sensations from alcohol. Individuals who taste PROP as most bitter may experience more negative oral sensations from alcoholic beverages (bitterness and irritation) and fewer positive sensations (sweetness), and therefore experience a sensory hindrance of overconsumption (Guinard *et al.*, 1996; Intranuovo

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and Powers, 1998; Duffy *et al.*, 2004). Given the implications of PROP tasting status on nutritional conditions and other health parameters, the goal of this study was to find a rapid and inexpensive method to detect the CA6 +292A/G SNP, to employ instead of already used DNA sequencing. This molecular approach was possible since the A-to-G substitution not only changes the codon CAG (serine) to a nonsynonymous codon CGG (glycine), but also creates the nucleotide sequence GGCC recognized by the *Hae*III restriction enzyme.

Materials and Methods

Subjects

A total of 60 healthy nonsmokers, already genotyped for the rs2274333 SNP by direct sequencing (Padiglia *et al.*, 2010), were recruited for this research. Their mean age was 25 years, ranging from 20 to 29 years. All had measurable thresholds for common chemosensory stimuli and were free of medications that might affect taste or odor perception. The 60 participants were classified for PROP bitter taste status as supertasters, medium tasters, or nontasters by standard procedure (Rankin *et al.*, 2004; Padiglia *et al.*, 2010). Each group had an equal supertasters:medium tasters:nontasters ratio (20:20:20). The participants were verbally informed about the procedure and the aim of the work. Each subject reviewed and signed an informed consent form at the beginning of the protocol. The study was approved by the Ethics Committee of the University Hospital of Cagliari, Italy.

Preparation of template DNA and polymerase chain reaction conditions

Genomic DNA was extracted from saliva samples using the Invitrogen Charge Switch Forensic DNA Purification kit (Invitrogen) according to the manufacturer's protocol. The concentration was estimated by measurements of OD260. Purified DNA samples were stored at or below -20°C until use. A 245 bp fragment surrounding the SNP was amplified with primers 5'-GACCCCTCTGTGTTACCTA-3', and 5'-TATGGGGTTCAAAGGAAAGA-3' (Fig. 1). The polymerase

chain reaction (PCR) was carried out in a total volume of 25 μL and contained 250 ng DNA, 10 pmol of each primer, 1.5 mM MgCl_2 , 100 mM Tris-HCl, pH 8.3, 50 mM KCl, 200 μM of dNTP mix, and 1.5 U of Hot Master Taq Eppendorf. Reactions were performed in a Personal Eppendorf Mastercycler (Eppendorf). Amplification conditions consisted of initial denaturation at 95°C for 5 m, followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 54°C for 30 s, and extension at 72°C for 30 s. A final extension was conducted at 72°C for 5 m. Negative controls (water instead of human DNA) were run with every PCR, and standard precautions were taken to avoid contamination.

Restriction fragment length polymorphism analysis for genotyping

A 3 μL aliquot of the PCR was mixed with a 17 μL solution containing 2 μL $10\times$ NE Buffer (50 mM NaCl, 10 mM Tris-HCl, 10 mM MgCl_2 , and 1 mM dithiothreitol, pH 7.9), 0.2 μL *Hae*III (10,000 U/mL) (Sigma-Aldrich), and 14.8 μL sterile deionized H_2O , and then incubated at 37°C for 2 h. The digest was mixed with 5 μL of loading buffer and electrophoresed on a 10% vertical polyacrylamide gel. Silver nitrate and ethidium bromide staining were carried out according to Herring *et al.* (1982) and Sambrook *et al.* (1989), respectively. An *Hae*III digest of pUC18 DNA was used as an MW marker (Sigma-Aldrich). All PCRs and digestions were conducted in triplicate for each DNA sample. To verify the accuracy of the PCR-restriction fragment length polymorphism (RFLP) method, all PCR samples were sequenced with an ABI Prism automated sequencer. Translation of nucleotide sequences was performed using ExPASy translate routine software (<http://ca.exPASy.org/>).

Allelic frequencies were determined and statistical analysis was performed using the chi-square (χ^2) test.

Results

Sixty volunteer subjects classified by their PROP taster status were genotyped for CA6 +292A/G SNP by PCR-RFLP

FIG. 1. Nucleotide and deduced amino acid sequences of carbonic anhydrase VI exon 3. The nucleotide sequence is numbered in the 5' to 3' direction. Exon 3 sequence is in capitals and the corresponding amino acid sequence is under the coding sequence. The intronic flanking sequences is in lowercase. Arrows above underlined sequences indicate sense (\rightarrow) and antisense primers (\leftarrow) derived from the intronic flanking sequences and used to amplify the genomic region covering the single-nucleotide polymorphism (SNP) of interest (A/G), shown in bold. *Hae*III recognition sequence is boxed and the cleavage site is indicated by arrow (\downarrow).

ctgtaggcctgacccctctgtgttcacctaactctgctctcagccccacctgtctctccatg	
CAG ATC A/GGC CTG CCC TCC ACC ATG CGC ATG ACA GTG GCT GAC GGC	330
Q I S/G L P S T M R M T V A D G	102
ACT GTA TAC ATA GCC CAG CAG ATG CAC TTT CAC TGG GGA GGT GCG TCC	388
T V Y I A Q Q M H F H W G G A S	118
TCG GAG ATC AGC GGC TCT GAG CAC ACC GTG GAC GGG ATC AGA CAT GTG	436
S E I S G S E H T V D G I R H V	134
ATC GAG gtacctgaggacccccactgtgtcctctttcctttgaaccccatagtcacttttc	442
I E	136

assay. Since this SNP changes the codon from CAG to CGG generating the nucleotide sequence GGCC recognized by *Hae*III, it was possible to employ the fast and inexpensive proposed method. As previously described, fragments of 245 bp covering the SNP of interest, were first amplified in triplicate for each DNA sample, and subsequently digested with *Hae*III. Since the restriction enzyme recognizes only sequences with the G allele, the resulting digested fragments revealing the genotype of each individual were analyzed on polyacrylamide gel with the untreated PCR products. Presence of the G allele of the +292A/G SNP results in digestion of the 245 bp PCR product into two fragments of 185 and 60 bp. Presence of the A allele removes the *Hae*III digestion site. A heterozygous individual would therefore have three fragments of 245, 185, and 60 bp (Fig. 2). The results of DNA electrophoretic profiles after digestion were compared with electropherogram data by direct sequencing of the same PCR samples, obtaining a full correspondence (100%) between the two methods.

As expected, we observed that 17 out of 20 supertasters analyzed showed undigested PCR products, meaning the homozygous presence of the A allele. Three out of 20 supertasters showed an electrophoretic profile consisting of an undigested fragment of 245 bp and two smaller fragments of

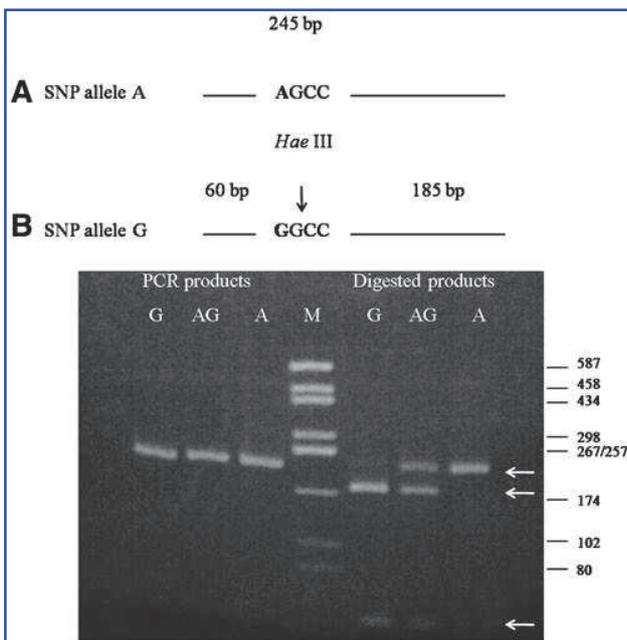


FIG. 2. Assay design (A) and example of the results (B) obtained using polymerase chain reaction (PCR)–restriction fragment length polymorphism method. A 245 bp fragment of carbonic anhydrase VI gene covering the SNP site of interest was first amplified and subsequently digested with *Hae*III restriction enzyme that recognizes only the G allele. The resulting fragments revealing the genotype of each individual were analyzed on polyacrylamide gel in parallel with the untreated PCR products. M, size marker; G, A/G, and G correspond to the SNP alleles of each genotype. The arrows indicate the DNA fragments (245, 180, and 60 bp) obtained after *Hae*III restriction enzyme digestion. The gel was stained with ethidium bromide.

TABLE 1. POLYMORPHISM +292A/G DEDUCED BY POLYMERASE CHAIN REACTION–RESTRICTION FRAGMENT LENGTH POLYMORPHISM ANALYSIS AND ITS CORRELATION WITH 6-N-PROPYLTHIOURACIL PHENOTYPE

	Phenotype (n=60)		
	Super taster (n=20)	Medium taster (n=20)	Nontaster (n=20)
Genotype (RFLP products)			
AA (245 bp product)	17	10	5
AG (245/185/60 bp products)	3	7	4
GG (185/60 bp products)	0	3	11
Alleles A frequencies (undigested PCR products)	92.5%	67.5%	35%

PCR, polymerase chain reaction; RFLP, restriction fragment length polymorphism.

185 and 60 bp product of enzymatic digestion, confirming the presence of both AG alleles. Nobody from this group had the GG genotype (Table 1). Eleven out of 20 nontasters showed an electrophoretic profile consisting of two digested fragments of 185 and 60 bp, corresponding to the homozygous presence of the G allele, 4 were heterozygous AG and 5 had the AA genotype, exhibiting partially and nondigested PCR fragments, respectively. Thus, 92.5% of the supertasters carried the A allele, whereas 65.00% of nontasters carried the G allele at this location. In medium tasters the undigested products linked to presence of allele A were more frequent (67.5%) than fully digested products related to the presence of allele G (Table 1).

Conclusion

Until recently, *TAS2R38* was considered the only gene involved in bitter taste variation in humans, but new data suggest that *CA6* gene polymorphisms may also contribute to variation in PROP taste sensitivity and impact food perception and preference (Padiglia *et al.*, 2010). Using a PCR-RFLP technique, we established a simple, low-cost, and efficient method that can be used to determine the *CA6* +292A/G SNP genotype, in bitter taste studies. In fact, the results obtained, also confirmed by direct sequencing data, showed the three genotypes (AA, GG, and AG) expected in the population studied for SNP rs2274333. In summary, our study indicates that RFLP assay based on digesting *CA6* amplicons with a single endonuclease *Hae*III may represent an easy specific marker to employ for *CA6* +292A/G genotypization of those individuals with differences in taste perception. Moreover, this easy experimental strategy may help to strengthen the genetic basis of food preference, which is of great importance to nutrigenomics and public health.

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Disclosure Statement

No competing financial interests exist.

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II

The Gustin (CA6) Gene Polymorphism, rs2274333 (A/G), as a Mechanistic Link between PROP Tasting and Fungiform Taste Papilla Density and Maintenance

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Abstract

Taste sensitivity to PROP varies greatly among individuals and is associated with polymorphisms in the bitter receptor gene *TAS2R38*, and with differences in fungiform papilla density on the anterior tongue surface. Recently we showed that the PROP non-taster phenotype is strongly associated with the G variant of polymorphism rs2274333 (A/G) of the gene that controls the salivary trophic factor, gustin. The aims of this study were 1) to investigate the role of gustin gene polymorphism rs2274333 (A/G), in PROP sensitivity and fungiform papilla density and morphology, and 2) to investigate the effect of this gustin gene polymorphism on cell proliferation and metabolic activity. Sixty-four subjects were genotyped for both genes by PCR techniques, their PROP sensitivity was assessed by scaling and threshold methods, and their fungiform papilla density, diameter and morphology were determined. *In vitro* experiments examined cell proliferation and metabolic activity, following treatment with saliva of individuals with and without the gustin gene mutation, and with isolated protein, in the two iso-forms. Gustin and *TAS2R38* genotypes were associated with PROP threshold ($p=0.0001$ and $p=0.0042$), but bitterness intensity was mostly determined by *TAS2R38* genotypes ($p<0.000001$). Fungiform papillae densities were associated with both genotypes ($p<0.014$) (with a stronger effect for gustin; $p=0.0006$), but papilla morphology was a function of gustin alone ($p<0.0012$). Treatment of isolated cells with saliva from individuals with the AA form of gustin or direct application of the active iso-form of gustin protein increased cell proliferation and metabolic activity ($p<0.0135$). These novel findings suggest that the rs2274333 polymorphism of the gustin gene affects PROP sensitivity by acting on fungiform papilla development and maintenance, and could provide the first mechanistic explanation for why PROP super-tasters are more responsive to a broad range of oral stimuli.

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Introduction

Individual variability in sensitivity to the bitter taste of phenylthiocarbamide was first recognized by Fox more than eight decades ago [1]. Since that time, steady progress has been made in elucidating the psychophysical features [2–5], population genetics [6,7] and molecular basis of this trait [8,9]. PTC/PROP tasting has also gained considerable attention as an oral marker for food preferences and eating habits that ultimately impacts nutritional status and health [10]. This role is based on data showing that the PROP phenotype associates with variation in perception and preference for fat [11–13], energy intake and body weight [14,15], selection of fruits and

vegetables [16–18], plasma antioxidant status [19] and the risk of colon cancer [20–22]. This involvement remains controversial since some studies have failed to show the expected associations between PTC/PROP status and health outcomes [23–25]. These controversies could also be explained by confounding factors (such as cognitive control of eating behavior or the endocannabinoid system) that may play a prominent role in determining these associations [26,27].

The bitterness of PTC /PROP is due to the presence of the N–C=S group within these molecules. The human gene that expresses receptors that bind this chemical group is known as *TAS2R38*. Individuals can be divided into three taster groups (non-taster, medium taster and super-taster) based on

behavioral testing assessing their PTC/PROP sensitivity. The percentage of non-taster individuals greatly varies among populations: from less than 7% to more than 40% [28]. There are two classes of screening methods: threshold determinations and suprathreshold measures that address stimulus detection and responsiveness at higher concentrations, respectively [2,10,13,14,29–36].

Allelic diversity in the *TAS2R38* bitter receptor gene is primarily responsible for PROP tasting [8,9]. Three polymorphic sites in the *TAS2R38* sequence, result in amino acid substitutions at positions Pro49Ala, Ala262Val, and Val296Ile, giving rise to two common haplotypes: PAV, the dominant (taster) variant and AVI, the recessive (non-taster) one. PROP-taster individuals possess the PAV/PAV or PAV/AVI diplotype, whereas non-tasters are homozygous for the recessive haplotype (AVI/AVI). Rare haplotypes (AAV, AAI, PVI, and PAI) have also been observed [6]. *In vitro* experiments [9] and receptor modelling [37,38] suggest that the PAV variant defines the active binding site of the receptor.

TAS2R38 is reported to account for majority (50–85%) of the variation in the phenotype [8,9], but a variety of observations suggest that other genes [39,40] may also be involved. On the other hand, a recent genome-wide association study revealed that only loci within the *TAS2R38* gene were associated with the perception of PROP [5]. This latter finding is consistent with the idea that the *TAS2R38* receptor is specific for thiourea substances, and is not activated by bitter compounds lacking the thiourea group [41,42]. Nevertheless, recent data suggest that salivary proteins may complement the direct effects of DNA sequence variation in *TAS2R38* on PROP tasting, further refining bitterness perception. Specifically, Cabras et al. [43] showed that PROP super-tasting was associated with higher salivary levels of Ps-1 and II-2 peptides belonging to the basic proline-rich protein (bPRP) family of peptides, and that oral supplementation with Ps-1 peptide enhanced the bitterness of PROP [44]. These data are consistent with the role of bPRPs as modifiers of taste and astringent molecules [45–47].

Our laboratory has also been studying the role of the zinc dependent salivary protein, gustin (also known as carbonic anhydrase VI (CA6)), in PROP tasting [48,49]. Gustin/CA6 is a 42 kDa protein secreted by the parotid, submandibular and von Ebner glands [50–52]. Gustin is considered a trophic factor that promotes growth and development of taste buds since disruptions in this protein are known to decrease taste function [53]. Padiglia et al. [48] showed that the rs2274333 (A/G) polymorphism of the gustin gene results in an amino acid substitution at position Ser90Gly in the peptide, leading to a structural modification of the gustin active site, reduced zinc binding, and the accumulation of zinc ions in saliva. This gustin polymorphism is also strongly associated with PROP tasting [48] such that PROP super-tasters more frequently carried the AA genotype of gustin and expressed the native form of the protein, whereas PROP non-tasters more frequently carried the GG genotype and expressed the less functional form [49]. PROP super-tasters have a greater density of fungiform taste papillae on the anterior surface of the tongue [2,34,54–56]. Considering gustin's role in taste bud development and the close association between the rs2274333 polymorphism of

gustin and PROP tasting, it is plausible that the relationship between papillae density and PROP status is mediated by gustin. To date, no studies have examined the effects of gustin on taste papilla morphology and physiology, particularly with respect to PROP taster status.

The objectives of this study were to investigate the effect of gustin gene polymorphism rs2274333 (A/G) and *TAS2R38* polymorphisms on PROP sensitivity and fungiform papillae density and morphology in a genetically homogeneous cohort. In addition, *in vitro* experiments, examined 1) the effect of treatment with saliva collected from individuals with genotype AA and GG of polymorphism rs2274333 on cell development and metabolic activity, and 2) the effect of treatment with isolated gustin, in the two iso-forms resulting from this polymorphism, on cell metabolic activity.

Materials and Methods

Ethical statement

All subjects was verbally informed about the procedure and the aim of the study. They reviewed and signed an informed consent form. The study was conformed to the standards set by the latest revision of Declaration of Helsinki and the procedures have been approved by the Ethical Committee of the University Hospital of Cagliari, Italy.

Subjects

Sixty-three non-smoking Caucasian healthy, young subjects (22 males, 42 females, age 25 ± 3 y) from Sardinia, Italy were recruited at the local University. They had a normal body mass index (BMI) ranging from 18.6 to 25.3 kg/m² and showed no variation of body weight larger than 5 kg over the previous 3 months. None were following a prescribed diet or taking medications that might interfere with taste perception. Subjects neither had food allergies, nor scored high on eating behaviour scales (assessed by the Three-Factor Eating Questionnaire [57]). Thresholds for the 4 basic tastes (sweet, sour, salty, bitter) were evaluated in all subjects in order to rule out any gustatory impairment.

PROP taste sensitivity assessments

The PROP phenotype of each subject was assessed by both threshold and suprathreshold measures. PROP (Sigma-Aldrich, Milan, Italy) thresholds were determined using a variation of the ascending-concentration, 3-alternative forced-choice (3-AFC) procedure [58]. PROP solutions in spring water ranged from 0.00001 to 32 mM in quarter-log steps.

Taste intensity ratings for a single suprathreshold PROP (3.2 mM) solution [49] were collected using the Labeled Magnitude Scale (LMS) [59] in which subjects placed a mark on the scale corresponding to his/her perception of the stimulus. The LMS scale gave subjects the freedom to rate the PROP bitterness relatively to the “strongest imaginable” oral stimulus they had ever experienced in their life.

For both methods, the solutions were prepared the day before each session and stored in the refrigerator until 1 h

before testing. The stimuli were presented at room temperature as 10 ml samples.

Molecular analysis

Subjects were genotyped for polymorphism rs2274333 (A/G) of the gustin (CA6) gene that consists of a substitution of amino acid Ser90Gly. They were also genotyped for three single nucleotide polymorphisms (SNPs) at base pairs 145 (C/G), 785 (C/T), and 886 (G/A) of the *TAS2R38* locus (through the manuscript the name of the gene is identified in italics, while its corresponding encoded protein by plain text). The *TAS2R38* SNPs give rise to 3 non-synonymous coding exchanges: proline to alanine at residue 49; alanine to valine at residue 262; and valine to isoleucine at residue 296. These substitutions result in two major haplotypes (PAV and AVI) and three rare (AAI, PVI and AAV). The DNA was extracted from saliva samples using the Invitrogen Charge Switch Forensic DNA Purification kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Purified DNA concentration was estimated by measurements of OD260. PCR techniques were employed to amplify the gustin gene region including rs2274333 polymorphism, and the two short region of the *TAS2R38* gene including the three polymorphisms of interest.

To genotype gustin gene polymorphism rs2274333, a fragment of 253 bp was amplified with forward 5'TGACCCCTCTGTGTTACCT3' and reverse 5'GTGACTATGGGGTTCAAAGG3' primers. The reaction mixtures (25 μ L) contained 250 ng DNA, 10 pmol of each primer, 1.5 mM MgCl₂, 100 mM Tris-HCl at pH 8.3, 50 mM KCl, 200 μ M of dNTP mix, and 1.5 units of Hot Master Taq Eppendorf. Thermal cycles of amplification were carried out in a Personal Eppendorf Master cyler (Eppendorf, Germany). The amplification protocol included an initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 54°C for 30 s, and then extension at 72°C for 30 s. A final extension was carried out at 72°C for 5 min. Amplified samples were digested with HaeIII enzyme at 37°C for 4 hours. The digested fragments were electrophoresed on 2% agarose gel and stained with ethidium bromide.

To determine *TAS2R38* haplotypes, PCR amplification followed by restriction analysis using *HaeIII* for SNP detection at the 145 nucleotide position, and direct sequencing (using forward and reverse primers) for SNPs identification at the 785 and 886 nucleotide position. The following primer set was used to amplify a fragment of 221 bp including the first of three SNPs : F5'-CCTTCGTTTTCTGGTGAATTTTTGGGATGTAGTGAAGAGGCGG-3' R 5'-AGGTTGGCTTGGTTTGAATCATC-3'. The forward primer binds within the *TAS2R38* gene, from nucleotides 101–144. There is a single mismatch at position 143, where the primer has a G (underlined in bold) and the gene has an A. This mismatch is crucial to the PCR experiment, because the A nucleotide in the *TAS2R38* gene sequence, is replaced by a G in each of the amplified products. This creates the first G of the *HaeIII* recognition sequence GGCC, allowing the amplified taster allele to be cut. The amplified non taster allele reads GGGC and is not cut. The PCR reaction mixtures (25 μ L) contained 250 ng DNA, 10 pmol

of each primer, 1.5 mM MgCl₂, 100 mM Tris-HCl at pH 8.3, 50 mM KCl, 200 μ M of dNTP mix, and 1.5 units of Hot Master Taq Eppendorf. Thermal cycles of amplification were carried out in a Personal Eppendorf Master cyler (Eppendorf, Germany). The amplification protocol consisted of initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 64°C for 45 s, and then extension at 72°C for 45 s. For the analysis of the polymorphism G/C at position 143, a 3 μ l aliquot of PCR products was mixed with a 17 μ l solution containing 2 μ l 10 \times NE Buffer (50mM NaCl, 10mM Tris-HCl, 10mM MgCl₂, 1mM dithiothreitol, pH 7.9), 0.2 μ l HaeIII (10 000 U ml⁻¹; Sigma-Aldrich, St Louis, MO), and 14.8 μ l sterile deionized H₂O. The solution was incubated at 37°C for 4 h. The digest was mixed with 5 ml of loading buffer and electrophoresed on a 10% vertical polyacrylamide gel. The DNA bands were evidenced by ethidium bromide staining. The PCR 100 bp Low Ladder DNA was used as Mr markers (Sigma-Aldrich). Polymorphisms at the 785 and 886 nucleotide position were identified by a single PCR reaction using the sense primer 5'-TCGTGACCCAGCCTGGAGG-3' and the antisense primer 5'-GCACAGTGTCCGGGAATCTGCC-3' delimiting a 298 bp fragment. The PCR reaction mixtures (25 μ L) contained 250 ng DNA, 10 pmol of each primer, 1.5 mM MgCl₂, 100 mM Tris-HCl at pH 8.3, 50 mM KCl, 200 μ M of dNTP mix, and 1.5 units of Hot Master Taq Eppendorf. Thermal cycles of amplification were carried out in a Personal Eppendorf Master cyler (Eppendorf, Germany). The amplification protocol consisted of initial denaturation at 95°C for 5 min, followed by 30 cycles of denaturation at 95°C for 30 s, annealing at 60°C for 30 s, and then extension at 72°C for 30 s. PCR products were sequenced with an ABI Prism automated sequencer. Nucleotide and deduced amino acid sequence analyses were performed with the OMIGA version 2.0 software (Oxford Molecular, Madison, WI).

Fungiform papillae identification and measurements

The method to identify fungiform papillae was similar to that developed by Shahbake et al. [56] and is briefly described as follows. The tip of the anterior tongue surface was dried with a filter paper and stained by placing (for 3 s) a piece of filter paper (circle 6 mm in diameter) that contained a blue food dye (E133, Modacor Italiana, Italy) at the left side of the midline. Photographic images of the stained area were taken using a Canon EOS D400 (10 megapixels) camera with lens EFS 55-250 mm. Three to ten photographs were taken of each subject, and the best image was analyzed. The digital images were downloaded to a computer and were analyzed using a "zoom" option in the Adobe Photoshop 7.0 program. The fungiform papillae were identified from the digital images by their mushroom-shape, they were readily distinguished from filiform papillae by their very light staining with the food dye compared to the latter papillae which stained dark [60].

The number of papillae in the stained area was counted for each subject, and the density in (1 cm²) was calculated. The diameter of each papilla was measured in 4 dimensions (at 0, 45, 90 and 135°) and the standard deviation (SD) was calculated. This procedure was repeated for all papillae in a counting area. A fungiform papilla was considered distorted

when the SD was ≥ 0.088 . This value corresponded to 2 SDs. The grand mean of diameters, the mean of SDs, and the percentage of distorted fungiform papillae were determined for each subject. Papillae were separately evaluated by three trained observers who were blind to the PROP status of the subjects. The final measurements were based on the consensus assessment of all observers.

Experimental procedure

Subject testing was carried out in three visits on different days separated by a 1-month period. Subjects were requested to abstain from eating, drinking and using oral care products or chewing gums for at least 8 h prior to testing. They had to be in the test room 15 min before the beginning of the session (9.00 AM) in order to adapt to the constant environmental conditions (23-24°C; 40-50% relative humidity). In the first visit, a 3 ml sample of whole saliva was collected from each subject, into an acid-washed polypropylene test tube by means a soft plastic aspirator. Samples were stored at -80°C until molecular analyses were completed as described above. After 15 min, subjects rinsed their mouth with distilled water, then the tongue was dried and stained as described above, and photographs of the tip of the tongue were recorded.

Taste assessments were carried out in the 2nd and 3rd visits. In women, visits were scheduled around the sixth day of the menstrual cycle to avoid taste sensitivity changes due to the estrogen phase [61]. In the second visit, after rinsing the mouth with spring water, subjects were instructed to swish the entire contents of one cup (10 mL of PROP 3.2 mM) in their mouth for 10 s and then to spit it out. After tasting, the subjects evaluated bitterness intensity of the solution using the LMS. PROP thresholds were determined for each subject at the third visit. All rinsed their mouth with spring water before the experimental session. They were presented with 3 cups positioned in a random order, one with a given PROP concentration and two containing spring water. They were instructed to swish the entire contents of one cup in their mouth for 5 s and then to spit it out. Before moving onto the next cup, they rinsed their mouth with spring water. After tasting all 3 samples, they were asked to choose which one was different from the other two samples. The detection threshold was designated as the lowest concentration at which the subject correctly identified the target stimulus on three consecutive trials. The inter-stimulus interval as well as inter-trial interval was set at 60 s.

In vitro experiments

Two cell-based experiments were conducted. The first experiment tested the effects of treatment with saliva collected from individuals with genotype AA and GG of polymorphism *rs2274333* on cell proliferation and metabolic activity. The second one tested the effects of treatment with the two gustin iso-forms isolated from saliva of donors homozygous for AA and GG, on cell metabolic activity.

Cell cultures. A fetal goat tongue-derived epithelial cell line (ZZ-R 127) supplied by the Collection of Cell Lines in Veterinary Medicine of the Friedrich Loeffler Institute was used [62]. Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Gibco, USA) plus 10% (v/v) fetal calf serum

(FCS, Gibco) at 37°C in a humidified atmosphere of 5% CO₂. Cells were plated in 24-well plates at a density of 8×10^4 cells/well. After 24 h, cells in DMEM plus 10% FCS were treated for 72 h with 10% saliva from donors (or gustin iso-forms) depending on the experimental conditions.

Effects of saliva on growth and metabolic activity. For the first experiment, saliva was collected from a total of 24 subjects; 12 subjects with genotype AA at the gustin locus (*TAS2R38* genotypes were as follows: 8 heterozygous and 4 PAV homozygous) and 12 subjects with genotype GG at the gustin locus (*TAS2R38* genotypes were: 6 AVI homozygous, 4 heterozygous and 2 PAV homozygous). Saliva was collected on the same day as the *in vitro* experiments, and centrifuged at 12,000 RPM for 10 minutes. The supernatant was filtered with a sterile 0.22- μ m pore filter, and then added to the cell cultures, as described below. Gustin protein was still present in filtered supernatants, as demonstrated by immunoblot experiments (data not shown). Three experimental treatments were used: (1) saliva from subjects with genotype AA; (2) saliva from subjects with genotype GG; and (3) control (DMEM plus 10% FCS alone). Saliva from each subject was assayed separately. After 72 h treatment, cells were trypsinized and counted with a hemocytometer under inverted microscope.

Cell metabolic activity was determined by the resazurin system (Tox-8 assay kit, Sigma, USA) in which metabolically active cells convert resazurin into a fluorescent dye, resorufin, by the intracellular reduction enzymes. This assay represents a simple, accurate and reproducible tool for measuring the metabolic activity of living cells [63]. After 72h treatment with saliva, resazurin dye solution was added to cells in an amount equal to 10% of the culture medium volume (100 μ l/well) and cells were cultured for a further 4 h. Fluorescence of converted dye was measured using a fluorescent microplate reader (VICTOR X Multilabel Plate Readers, PerkinElmer) at a wavelength of 590 nm using an excitation wavelength of 560 nm.

Mean values of cell number and fluorescence emission after treatments with saliva of subjects with genotype AA (n=12) and genotype GG (n=12) were calculated and are presented graphically.

Effects of gustin iso-forms on metabolic activity. In the second experiment, cells were treated with isolated gustin in the two iso-forms resulting from the polymorphism *rs2274333* (A/G). Saliva was collected from one super-taster donor homozygous for the AA form of gustin (*rs2274333*) and from one non-taster donor homozygous for the GG form (both heterozygous for *TAS2R38*), and used to purify the two iso-forms of carbonic anhydrase VI. The preparation of saliva samples and all purification steps were conducted using the method of Murakami and Sly [64]. The same experimental procedure was used for the purification of each iso-form. Volunteers expectorated in a frozen bottle containing 2 ml of 0.2 M benzamidine (Sigma-Aldrich, St. Louis, MO) in 0.1 M Tris-SO₄, and 0.2 M sodium sulfate, at pH 8.7. Saliva samples were collected after lunch, because food intake enhances the secretion of saliva from the parotid glands which are the primary site for gustin protein production [65]. Samples of whole saliva were collected from each subject, after stimulation

with citric acid. This produced large amounts (~40 mL) per collection. The collection procedure was repeated in different days until a pooled sample of 250 ml of saliva for each genotype was obtained. Samples were stored at -80° then thawed and centrifuged (16,000 x g, 15 min) to remove foreign material. The supernatant was diluted to 1 liter with 0.1 M Tris-SO₄, and sodium sulfate 0.2 M at pH 8.7.

The purification of carbonic anhydrase VI was carried out through the use of affinity chromatography, preparing the column matrix as reported by Khalifah et al. [66]. Specifically, carboxy methyl Bio-Gel A (Bio-Rad Laboratories, Richmond, CA) was linked to the sulfonamide inhibitor p-aminomethylbenzenesulfonamide (Gallade Chemical; Newark, CA). EDAC [1-(3-dimethylamionpropyl)-3-ethyl carbodiimide hydrochloride] obtained from Sigma-Aldrich (St. Louis, MO), was used to activate the column matrix carboxyl groups. The purified fractions containing the carbonic anhydrase VI were collected based on spectrophotometric absorbance values at 280 nm. Then, as reported by Murakami and Sly [64], fractions containing the protein were applied to a diethylaminoethyl - sephacel (Sigma-Aldrich, St. Louis, MO) ion-exchange column. The concentration of purified protein was quantified by the method of Lowry et al. [67] using bovine serum albumin as a standard, and its purity was determined by SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis). SDS-PAGE (12% acrylamide) was performed according to Laemmli [68]. Sigma, Marker product code C 4236 (Sigma-Aldrich, St. Louis, MO) with range 8-210 kDa was employed as a standard in electrophoresis. The gel was stained with Coomassie Brilliant Blue R-250 (Sigma-Aldrich, St. Louis, MO), using the typical Coomassie staining procedure [69]. The yield of the purification was approximately 1 mg of protein starting from 250 ml of whole saliva.

The mean concentration of gustin in human saliva is about 5 ± 0.2 µg/ml [70]. Since gustin binds an ion of Zn with a stoichiometry of 1:1 [50], we used a protein concentration of 8 µg/ml corresponding to 0.2 nmoles, and 0.2 nmoles of added Zn. Four experimental treatments were used: (1) gustin Ser90 + Zn; (2) gustinGly90 + Zn; (3) control (DMEM plus 10% FCS alone); and (4) control + Zn. The Tox8 assay (previously described) was used to obtain fluorescence emissions using the same procedures as the saliva experiment. Since we were able to obtain a large amount of isolated protein, each treatment was repeated 33 times (to maximize the reliability of the assay) and the mean values of the replicates are presented graphically.

Statistical analyses

Hardy Weinberg equilibrium for the *TAS2R38* gene and polymorphism 2274333 (A/G) of the gustin gene was verified through the Markov Chain test (Genepop software version 4.0; <http://kimura.univ-montp2fr/~rousset/Genepop.htm>). Linkage disequilibrium (LD) between the two loci was verified by the Markov Chain algorithm (Genepop software version 4.0.5.3; <http://kimura.univ-montp2fr/~rousset/Genepop.htm>). We stratified our sample based on *TAS2R38* and gustin genotypes, and tested both the additive and dominant models for the PAV

Table 1. Number of occurrences of each combination of the *TAS2R38* and gustin gene genotypes in a genetically homogeneous cohort.

Genotype	Subjects (n)
AVI/AVI - GG	5
AVI/AVI - AG	6
AVI/AVI - AA	9
PAV/AVI - GG	1
PAV/AVI - AG	12
PAV/AVI - AA	20
PAV/PAV - GG	2
PAV/PAV - AG	2
PAV/PAV - AA	6

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and A variants, respectively, with the Chi square test to show the two genes are independent.

Main effects ANOVA was used to examine the effects of the *TAS2R38* gene and polymorphisms 2274333 (A/G) of the gustin gene on PROP threshold, bitterness intensity rating (PROP 3.2 mM), and fungiform papilla density and diameter. Main effects ANOVA was used to assess the first-order (non-interactive) effects of multiple categorical independent variables.

One-way ANOVA was used to compare the SD of diameter of fungiform papillae and the percentage of distorted fungiform papillae across gustin gene genotypes, and the effect of treatments on cell metabolic activity. Post-hoc comparisons were conducted with the Newman-Keuls test.

Stepwise, multiple linear regression was used to predict PROP phenotype (threshold and bitterness intensity rating), fungiform papilla density and morphology using gustin and *TAS2R38* genotypes, gender and age as predictor variables. The relative contribution of each significant variable and semipartial correlations (*sr*) for each variable are reported in the tables. Cell growth (expressed as percentage of control values) was compared between cells treated with saliva from individuals with genotype AA and GG of the gustin gene using the Student's *t*-test. Statistical analyses were conducted using STATISTICA for WINDOWS (version 7.0; StatSoft Inc, Tulsa, OK, USA). *p*-values <0.05 were considered significant.

Results

The Markov Chain test showed that the population meets the Hardy Weinberg equilibrium both for *TAS2R38* and gustin gene ($p=0.6154$ and $p=0.1174$, respectively). The distribution of the *TAS2R38* and gustin gene genotype associations is shown in Table 1. Markov Chain algorithm showed that the two loci were not in linkage disequilibrium ($p=0.1782$). Chi square test showed that carriers of the taster form of *TAS2R38* were not more likely to have the functional variant of the gustin gene in either the additive ($\chi^2=6.5$; $p=0.17$) or the dominant model ($\chi^2=2.54$; $p=0.11$).

PROP Thresholds and Bitterness Intensity

Molecular analysis for polymorphism *rs2274333* (A/G) of the *gustin* (CA6) gene allowed us to identify the genotype of sixty-three subjects: 35 were homozygous AA, 20 were heterozygous and 8 were homozygous GG. The analysis at the three SNPs of the *TAS2R38* locus identified 10 subjects who were PAV homozygous, 33 were heterozygous and 20 were AVI homozygous.

PROP threshold values and bitterness intensity ratings (PROP 3.2 mM) of individuals with genotypes AA, AG and GG of the *gustin* gene and with genotypes PAV/PAV, PAV/AVI and AVI/AVI of *TAS2R38* are shown in Figure 1A and B. Main effects ANOVA revealed a strong association between PROP threshold and the *gustin* gene polymorphism ($F_{[2,58]} = 10.502$; $p=0.00013$). Post-hoc comparisons showed that thresholds were statistically higher in individuals with genotype GG of the *gustin* gene than in the other genotypes ($p \leq 0.000119$; Newman-Keuls test), but not different between AA and AG individuals ($p > 0.05$). Although thresholds were variable in those with the GG genotype, thresholds were more than 10-fold higher in these individuals than in the other groups. Main effects ANOVA also showed an association between PROP threshold and *TAS2R38* genotypes ($F_{[2,58]} = 6.0189$; $p=0.0042$). Thresholds of individuals with the AVI/AVI genotype were higher than those of individuals with genotypes PAV/PAV and PAV/AVI ($p \leq 0.00158$; Newman-Keuls test), that did not differ from each other ($p > 0.05$).

PROP bitterness intensity ratings (3.2 mM) were strongly associated with *TAS2R38* genotypes ($F_{[2,58]} = 32.468$; $p < 0.000001$) and less so with the *gustin* gene polymorphism ($F_{[2,58]} = 3.4330$; $p=0.038$). *TAS2R38* bitterness ratings of PAV/PAV individuals were statistically higher than those of heterozygous individuals ($p \leq 0.0173$; Newman-Keuls test) who in turn gave higher intensity ratings to PROP than individuals with the AVI/AVI genotype ($p=0.00011$; Newman-Keuls test). In the case of *gustin*, post hoc comparisons showed that PROP bitterness was statistically higher in individuals with genotype AA than in those with the other genotypes ($p \leq 0.0471$; Newman-Keuls test), but not different between GG and AG individuals ($p > 0.05$).

Papillae Density and Morphology

Figure 2 shows the mean densities (\pm SEM) of fungiform papillae on the anterior part of the tongue of individuals with genotypes AA, AG and GG of the *gustin* gene (upper graph) and of individuals with genotypes PAV/PAV, PAV/AVI and AVI/AVI of *TAS2R38* (lower graph). Also shown are representative images of the tongue tip stained area where measures were taken. ANOVA calculations showed that fungiform papillae density on the anterior part of the tongue was strongly associated with the *gustin* gene ($F_{[2,58]} = 8.5270$; $p=0.00057$) and less so with *TAS2R38* polymorphisms ($F_{[2,58]} = 4.6147$; $p=0.0138$). In the case of *gustin*, fungiform papillae density values were lower in individuals with the GG genotype than in those with genotypes AG and AA ($p \leq 0.0379$; Newman-Keuls test). Papillae density was not different between AA and AG individuals ($p > 0.05$). In the case of *TAS2R38* genotypes, post hoc comparison showed that individuals with the

PAV/PAV genotype had a higher fungiform papillae density than those with PAV/AVI and AVI/AVI genotypes ($p \leq 0.0094$; Newman-Keuls test); the density values of the latter two groups were not different from each other ($p > 0.05$).

ANOVA revealed that mean fungiform papilla diameter was associated with the *gustin* gene polymorphism ($F_{[2,58]} = 7.5920$; $p=0.00118$), but not with *TAS2R38* genotypes ($F_{[2,58]} = 0.7191$; $p=0.491$). Post-hoc comparisons showed that mean papilla diameter determined in those with genotypes AA and AG were lower than those of homozygous GG individuals ($p \leq 0.00053$; Newman-Keuls test) (Figure 3).

ANOVA was also used to examine relationships between fungiform papilla morphology and *gustin* and *TAS2R38* genotypes. However, only associations between these features and *gustin* were statistically significant. In fact, both the SD of papilla diameter (Figure 4A) and the percentage of distorted papillae (Figure 4B) depended on *gustin* genotype ($F_{[2,60]} = 11.765$; $p=0.00005$ and $F_{[2,60]} = 9.787$; $p=0.00021$, respectively). Post-hoc comparisons showed that individuals with the GG genotype had papillae with greater variation in shape (higher SDs in papilla diameter) as well as a higher percentage of distorted papillae than the other genotypes ($p \leq 0.00019$ and $p \leq 0.00017$; Newman-Keuls test). No differences were found between AA and AG individuals ($p > 0.05$).

Multiple Regression Modeling

Multiple linear regression was used to assess the relative contributions of *gustin* and *TAS2R38* polymorphisms to PROP tasting and papillae density and morphology (Tables 2 and 3). Accordingly, *gustin* genotypes, *TAS2R38* genotypes and age were significant predictors of PROP threshold, with each factor contributing 17.72%, 11.18% and 5.45%, respectively, to the model. The overall model predicted 31.01% of the variance in threshold sensitivity. In the case of PROP bitterness intensity, *TAS2R38* and *gustin* genotypes were the only significant contributors in the model, predicting 55.16% of the variance in PROP bitterness intensity. However, *TAS2R38* genotype was a much stronger predictor in this model (49.75% variance) than was *gustin* genotype (6.18% variance).

Gustin genotypes and age were the only significant contributors to fungiform papillae density with the overall model explaining 30.90% of the variance. Finally, *gustin* genotype was the only significant contributor to fungiform papillae diameter, SD of papilla diameter and percentage distortion. However, the predictive power of these models were relatively low, explaining 13.2-16.11% of the variance in these measures.

In vitro experiments

The effect of *gustin* gene polymorphism *rs2274333* (A/G) from the *in vitro* experiments is shown in Figure 5. The number of cells, expressed as a percentage of control, treated with the saliva of subjects with genotype AA ($n=12$) was higher than the number of cells treated with saliva of subjects with genotype GG ($n=12$) ($p=0.0135$; Student's *t* test) (Figure 5A). ANOVA showed that the fluorescence emission at a wavelength of 590 nm, as a function of cell metabolic activity, depended on treatments performed with the saliva of subjects with different genotypes for the polymorphism in the *gustin* gene ($F_{[2,33]} =$

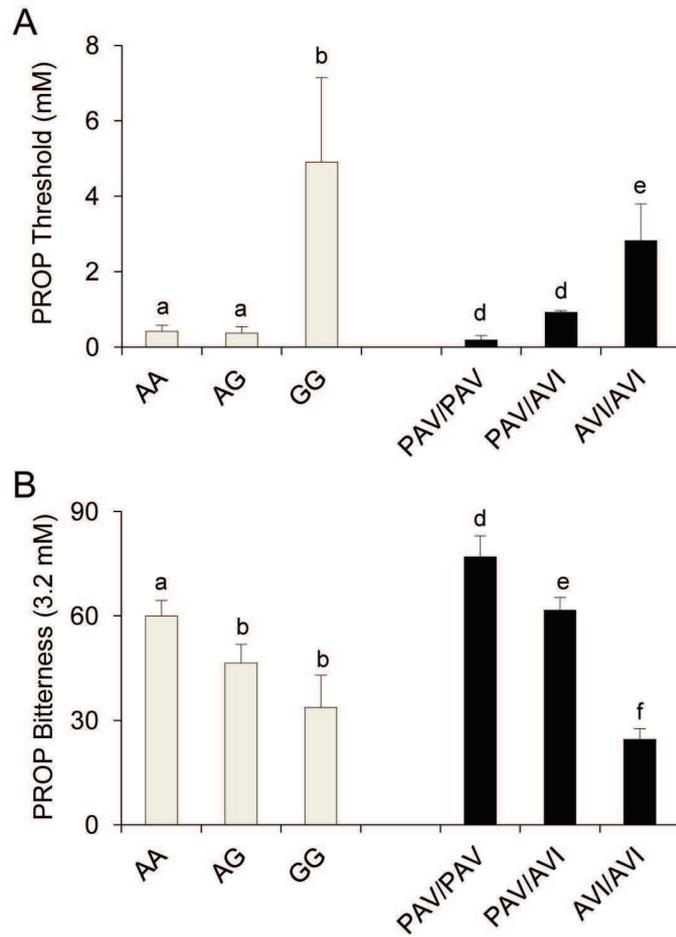


Fig. 1

Figure 1. Relationship between PROP phenotype and gustin gene and *TAS2R38* polymorphisms. PROP threshold (A) and bitterness intensity ratings (3.2 mM) (B) of individuals with genotypes AA, AG and GG of gustin (CA6) polymorphism *rs2274333* (A/G), and of individuals with genotypes PAV/PAV, PAV/AVI and AVI/AVI of *TAS2R38*. All values are mean (\pm SEM). $n=63$. Different letters indicate significant difference ($p \leq 0.0471$; Newman-Keuls test subsequent to main effects ANOVA).

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16.628; $p=0.00001$) (Figure 5B). Post hoc comparisons showed a higher emission of fluorescence from cells treated with saliva of subjects with genotype AA than that obtained from cells treated with saliva of genotypes GG ($p=0.000137$; Newman-Keuls test) or control ($p=0.000229$; Newman-Keuls test). No

differences were found between treatment with saliva of genotypes GG and control ($p > 0.05$).

ANOVA also showed that the fluorescence emission depended on treatments performed with the two iso-forms of gustin (gustin Ser90 or gustin Gly90) ($F_{[3,128]} = 10.463$; $P <$

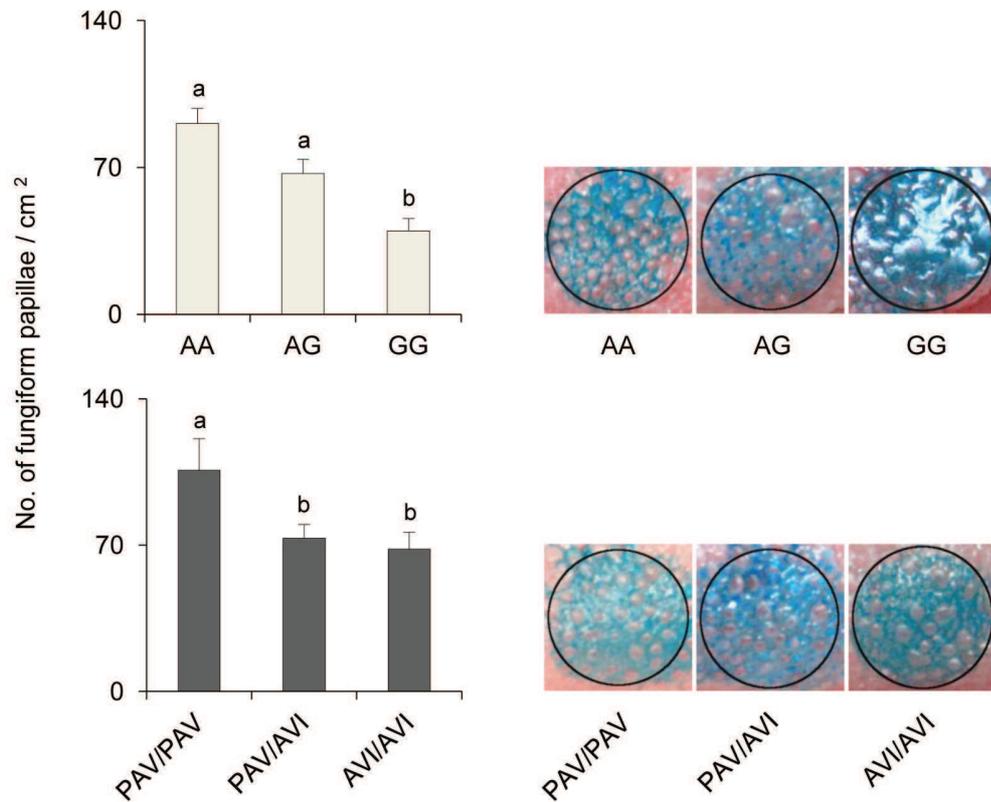


Fig. 2

Figure 2. Relationship between density of fungiform papillae and gustin gene and *TAS2R38* polymorphisms. Mean values \pm SEM of density of fungiform papillae (No. /cm²) on the anterior part of the tongue of individuals with genotypes AA, AG and GG of gustin (CA6) polymorphism rs2274333 (A/G) (upper graph) and of individuals with genotypes PAV/PAV, PAV/AVI and AVI/AVI of *TAS2R38* (lower graph). n=63. Different letters indicate significant difference ($p \leq 0.0379$; Newman-Keuls test subsequent to main effects ANOVA). Examples of the 6-mm-diameter stained area of the tongue tip where measures were taken are shown to the right of the graphs.

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0.00001) (Figure 5C). Pairwise comparisons showed that cells treated with gustin 90Ser + Zn emitted a higher fluorescence

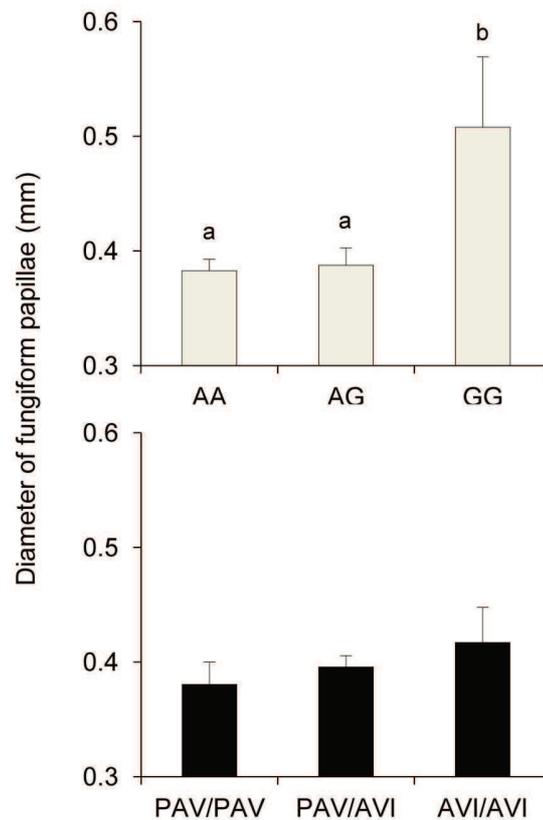


Fig. 3

Figure 3. Relationship between fungiform papillae diameter and gustin gene and *TAS2R38* polymorphisms. Mean values \pm SEM of the diameter of fungiform papillae of individuals with genotypes AA, AG and GG of gustin (CA6) polymorphism rs2274333 (A/G) (upper graph) and of individuals with genotypes PAV/PAV, PAV/AVI and AVI/AVI of *TAS2R38* (lower graph). $n=63$. Different letters indicate significant difference ($p \leq 0.00053$; Newman-Keuls test subsequent to main effects ANOVA).

doi: 10.1371/journal.pone.0074151.g003

Table 2. Stepwise forward multiple regression models for PROP phenotype (threshold and bitterness intensity).

PROP phenotype	Variable	Overall model		Parameter estimate		Each step (R^2)
		(adj R^2)	(p)	(sr)	(p)	
Threshold	Gustin	0.3101	<0.001	-0.38	<0.001	0.1772
	<i>TAS2R38</i>			-0.31	0.005	0.2890
	Age			0.23	0.031	0.3435
Bitterness intensity	<i>TAS2R38</i>	0.5516	<0.001	0.65	<0.001	0.4975
	Gustin			0.26	0.004	0.5593

Independent variables for both models included: Gustin genotypes, *TAS2R38* genotypes, age and gender. Only the significant variables are indicated. Adj, adjusted; sr , semipartial correlation.

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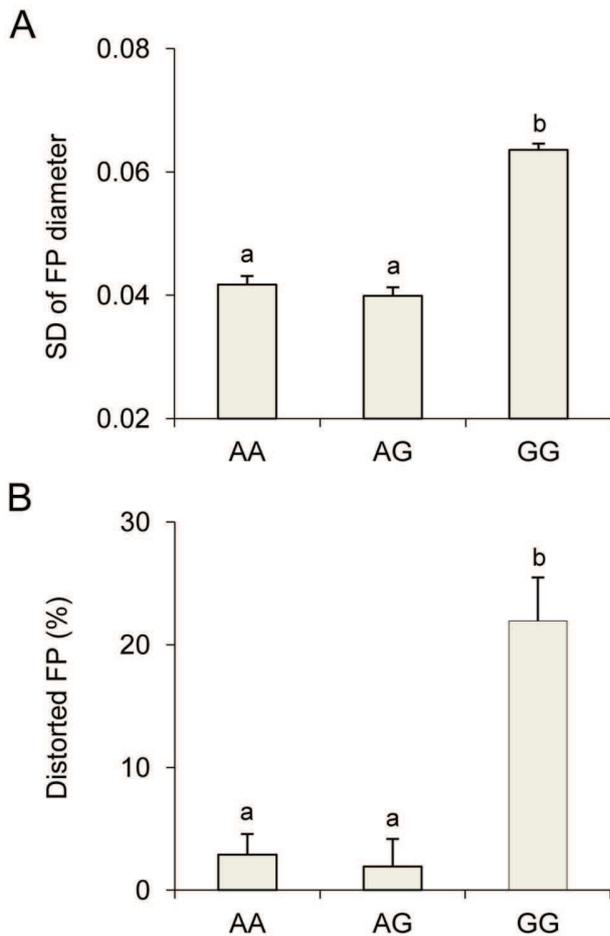


Fig. 4

Figure 4. Relationship between fungiform papillae distortion and gustin gene polymorphism. Standard deviation (SD) of diameter of fungiform papillae (A) and percentage of distorted fungiform papillae (B) in individuals with genotypes AA, AG and GG of gustin (CA6) polymorphism rs2274333 (A/G). All values are mean (\pm SEM). $n=63$. Different letters indicate significant difference ($p \leq 0.00019$; Newman-Keuls test subsequent to one-way ANOVA).

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than those treated with gustin 90Gly + Zn or with control + Zn or control ($P \leq 0.0067$; Newman-Keuls test). No differences were found between these last three treatments $P > 0.05$.

Discussion

One aim of the present study was to determine the effects of *TAS2R38* genotypes and the rs2274333(A/G) polymorphism in the gustin gene on PROP tasting, fungiform papillae density and morphology. Results showed that PROP thresholds and bitterness intensity ratings were associated with *TAS2R38* and gustin gene genotypes, as reported previously [49]. Importantly, those who were homozygous GG for the gustin SNP had thresholds that were more than 10-fold higher than those who carried either the AA or AG forms suggesting that gustin has a fundamental role in the ability to taste PROP at low concentration. Both gustin and *TAS2R38* genotypes were associated with fungiform papillae density with a stronger effect for gustin than for *TAS2R38*. However, only gustin was associated with morphological changes in fungiform papillae such as larger size, greater variation in shape and more distortions.

Regression modelling permitted us to assess the relative contributions of gustin and *TAS2R38* genotypes to these same outcomes. Both genes contributed to threshold acuity, however, *TAS2R38* polymorphisms made a much greater contribution to PROP bitterness intensity than did gustin. These data confirm the findings of Calò et al. [49] showing a much stronger effect of *TAS2R38* genotypes on suprathreshold intensity than threshold sensitivity. The reasons for these differential effects are unclear, but we can speculate that at low stimulus concentrations, that are further diluted in the oral cavity, both papillae features (as determined by gustin) and the presence of the functional, PAV form of the *TAS2R38* receptor are critical for tasting PROP. At higher concentrations, when there is a higher probability that the stimulus molecules arrive at the receptor site, the number of functional (PAV) receptors may be more important for enhancing peripheral nerve signalling than the number of taste cells that are present. This explanation may be overly simplistic as it fails to account for a number of factors that affect taste function such as smoking, damage to taste nerves [71,72] and variability in *TAS2R38* receptor expression. These factors need to be considered in future studies to obtain a more complete picture of the physiological mechanisms contributing to PROP tasting.

Our data showed that *TAS2R38* genotypes were associated with papillae number, and PAV homozygous individuals had a higher papillae number with respect to other genotypes. However, in the regression analysis, that looks at multiple variables at the same time, the *TAS2R38* genotypes were not significant predictors of papillae number or their other morphological features. It is important to note however, that gustin genotypes predicted only a small percentage of the variance in papillae size, and shape, suggesting that other factors define these morphological characteristics. We did not investigate brain-derived neurotrophic factor (BDNF) which has also been implicated in papillae development and maintenance [73–75], and this also needs to be pursued in future investigations.

Numerous studies have report greater papillae densities in PROP super-tasters compared to those who perceive PROP as less intense [2,34,54–56,76]. In agreement with these

Table 3. Stepwise forward multiple regression models for fungiform papilla density and morphology (diameter of papillae, SD of diameter and percentage of distorted papillae).

	Variable	Overall model		Parameter estimate		Each step
		(adj R ²)	(p)	(sr)	(p)	(R ²)
Density of papillae	Gustin	0.3090	<0.001	0.43	<0.001	0.1952
	Age			-0.31	0.004	0.3060
Diameter of papillae	Gustin	0.1218	0.007	-0.36	0.004	0.1320
SD of diameter	Gustin	0.1342	0.005	-0.34	0.005	0.1358
% of distorted papillae	Gustin	0.1538	0.002	-0.38	0.002	0.1611

Independent variables for all models included: Gustin genotypes, *TAS2R38* genotypes, age and gender. Only the significant variables are indicated. Adj, adjusted; sr, semipartial correlation.

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studies we found that homozygous individuals for the sensitive allele (PAV) of *TAS2R38*, who perceived the highest PROP bitterness, had higher papillae densities compared to those who perceived PROP as less intense. Our results complement these earlier observations by also showing that a single A allele in the gustin gene was sufficient to increase papillae density. In addition, we studied for the first time, the relationship between papillae distortion, which seems to be a measure of functionality [53], and genotypes for the two loci. We found that a single A allele in the gustin gene produced small papillae with a regular morphology; these effects were not found for *TAS2R38* genotypes.

Hayes et al. [35] reported no association between *TAS2R38* genotypes and papillae densities. In our previous work [49] we found that *TAS2R38* and the gustin gene had independent effects in modulating PROP phenotype in an ethnically homogeneous population where the majority of PAV homozygotes also carried the AA (functional) form of the gustin *rs24743333* polymorphism. In contrast, a majority (55%) of AVI homozygotes carried the GG (less functional) form. In the present study, fewer AVI homozygotes (25%) carried the GG form. Nevertheless, the presence of the AA form of gustin was more common in those with at least one PAV allele for *TAS2R38*. Thus, it is plausible that the higher papillae densities we observed in PAV homozygotes (although the sample size for this group was low) may better reflect the actions of gustin rather than *TAS2R38* genotypes. Future studies will have to confirm this finding. Our results should not lead to the conclusion that *TAS2R38* genotypes predict gustin genotypes. The two loci are independent (not in linkage disequilibrium) and, in fact, reside on different chromosomes. Why these two discrete loci appear to have functional overlap in defining PROP tasting and papillae density and morphology is presently unknown. The answer to this question cannot be resolved here and will come from more comprehensive genetic studies.

Up to now, only few populations have been tested for variants in the gustin gene, but the allele frequencies in these populations are not known. Variations in the frequency of gustin A and G alleles across populations could produce discrepant findings across studies, and could explain why a genome wide phenotype-genotype association study of PROP threshold failed to detect a relationship with variants in the gustin gene [5]. Both confounding and heterogeneity of

populations are common contributors to the problem of non replication in genetic studies of complex traits [77]. On the other hand, the study of ethnically homogeneous populations can be expected to reduce noise in genetic association studies by diminishing ancestral diversity [77–79]. The genetic homogeneity of the population we studied might have allowed us to observe the effect of the gustin gene as growth factor of taste buds. We also found that in regression analysis, *TAS2R38* accounted for less variance in the threshold response to PROP than in previous studies [8,35,80]. This finding could also reflect underlying differences in population characteristics.

For more than 40 years, gustin has been described as a trophic factor responsible for the growth and maintenance of taste buds [50]. This role was based on observations of patients with taste loss who exhibited pathological changes in taste buds accompanied by low salivary gustin and zinc levels. Administration of zinc to a subset of these patients improved taste function, increased salivary gustin and normalized taste bud morphology [53]. However, direct evidence that gustin increases cell growth has been lacking. Our *in vivo* studies showed that treatment of cells with saliva from individuals with the AA genotype of gustin resulted in increased cell proliferation and metabolic activity, whereas similar treatment with saliva from individuals with the GG genotype did not. Furthermore, direct treatment of cells with the active iso-form of the protein (gustin90Ser) increased cellular metabolic activity, while treatment with the inactive iso-form (gustin 90 Gly) failed to do so. These novel findings confirm, for the first time, a role for gustin in cell proliferation and maintenance.

In conclusion, our findings in an genetically homogeneous cohort suggest that the gustin (CA6) gene polymorphism, *rs2274333* (A/G), affects PROP tasting by acting on the density and maintenance of fungiform papillae, and that between the two protein iso-forms that result from this polymorphism, gustin 90Ser exhibits full functional activity, compared to the gustin 90Gly iso-form. In addition, the results of this work, if confirmed in different populations, will provide a mechanistic explanation of why PROP super-taster individuals have a higher density of fungiform papillae than PROP non-tasters, and why they show greater oral responsiveness to a wide range of stimuli that are not mediated via the *TAS2R38* bitter taste receptor.

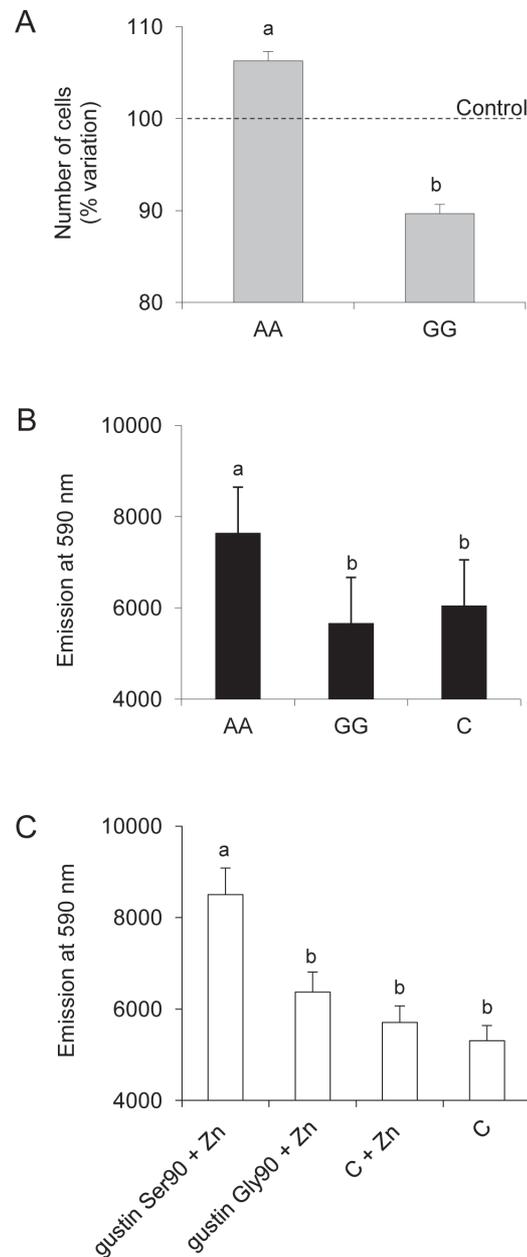


Fig. 5

Figure 5. Effect of gustin gene polymorphism rs2274333 (A/G) in vitro experiments. A, Number of cells, expressed as percentage of control, after treatments with saliva of subjects with genotype AA (n=12) or with saliva of subjects with genotype GG (n=12); different letters indicate significant difference ($p=0.0135$; Student's *t* test). B, Fluorescence emission at a wavelength of 590 nm obtained from cells treated for 72 h with saliva of subjects with genotype AA, genotype GG and control; n=12; different letters indicate significant differences ($p\leq 0.00023$; Newman-Keuls test subsequent to one-way ANOVA). C, Fluorescence emission at a wavelength of 590 nm obtained from cells treated for 72 h with the two iso-forms of isolated gustin (gustin Ser90 or gustin Gly90) + Zn, control + Zn, or control; n=33; different letters indicate significant differences ($p\leq 0.00067$; Newman-Keuls test subsequent to one-way ANOVA).

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