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Title: The gustin (CA6) gene polymorphism, rs2274333 (A/G), is associated with fungiform papilla density, whereas PROP bitterness is mostly due to TAS2R38 in an ethnically-mixed population

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Abstract: PROP responsiveness is associated with TAS2R38 haplotypes and fungiform papilla density. Recently, we showed that a polymorphism in the gene coding for the salivary trophic factor, gustin (CA6), affects PROP sensitivity by acting on cell growth and fungiform papillae maintenance, in a genetically homogeneous cohort. Since population homogeneity can lead to over estimation of gene effects, the primary aim of the present work was to confirm gustin's role in PROP bitterness intensity and fungiform papillae density in a genetically diverse population. Eighty subjects were genotyped for both genes by PCR techniques. PROP responsiveness was assessed by filter paper method and fungiform papilla density was determined in each subject. As expected, PROP bitterness ratings were lower in individuals with the AVI/AVI diplotype of TAS2R38 than in individuals with PAV/PAV and PAV/AVI diplotype. However, no differences in PROP bitterness among genotypes of the gustin gene, and no differences in the density of fungiform papillae related to TAS2R38 diplotype were found. In contrast, the density of fungiform papillae decreased as the number of minor (G) alleles at the gustin locus increased. In addition, the distribution of TAS2R38 genotypes within each gustin genotype group showed that the occurrence of recessive alleles at both loci was infrequent in the present sample compared to other populations. These findings confirm that papillae density is associated with gustin gene polymorphism, rs2274333 (A/G), in an ancestrally heterogeneous population, and suggest that variations in the frequency of allele combinations for these two genes could provide a salient explanation for discrepant findings for gustin gene effects across populations.

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We wish to submit the enclosed manuscript: "**The gustin (CA6) gene polymorphism, rs2274333 (A/G), is associated with fungiform papilla density, whereas PROP bitterness is mostly due to *TAS2R38* in an ethnically-mixed population**" to be considered for publication in Physiology & Behavior. The choice of the Journal was dictated by the topic of our study which is closely related to the aim and scope of Physiology & Behavior.

Our work provides additional insights on the role of the gustin (CA6) gene polymorphism, rs2274333 (A/G), in PROP sensitivity and fungiform papilla density by analyzing an ethnically-mixed sample, and identify confounding factors which may explain discrepant findings for gustin gene effects across populations.

Kind regards,

Beverly J Tepper, PhD
Professor

Highlights of the manuscript: "**The gustin (CA6) gene polymorphism, rs2274333 (A/G), is associated with fungiform papilla density, whereas PROP bitterness is mostly due to TAS2R38 in an ethnically-mixed population**"

- *TAS2R38* accounted for 58 % of the phenotypic variance in PROP bitterness intensity
- Gustin locus predicted 16 % of the variance in fungiform papilla density
- The GG genotype in gustin gene was associated with the lowest papilla density
- Occurrence of recessive alleles at both loci was infrequent in the tested sample

The gustin (CA6) gene polymorphism, rs2274333 (A/G), is associated with fungiform papilla density, whereas PROP bitterness is mostly due to *TAS2R38* in an ethnically-mixed population

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1. Introduction

The large individual variability in the ability to detect the bitter taste of 6-n-propylthiouracil (PROP) has been well studied since the early 20th century, and is used as genetic marker for individual differences in food preferences and eating habits [1-3]. PROP tasting is a common genetic trait that is present in all population groups across the globe where it has been studied [4]. Individuals can be divided into non-tasters, medium-tasters and super-tasters based on their ability to taste PROP. The distribution of PROP groups among populations varies markedly and depends on race and ethnicity [5]. In Caucasians of North American and European ancestry, the estimated frequency of PROP groups is approximately 30% non-tasters, 45% medium-tasters and 25% super-tasters [5]. PROP tasters (and particularly super-tasters) are more sensitive to the bitterness of PROP as well as other bitter substances found in foods [6-9]. They are also more sensitive to sweetness [10-12], sourness [13], the creaminess of dairy products [14-16], the heat of chili pepper [17-19], and the bitterness and astringency of alcohol [19, 20]. These heightened sensitivities are generally associated with decreased preferences for foods that illustrate these sensory qualities [8, 15, 21-25]. Those who are taste blind to PROP (i.e., non-tasters) show the opposite responses, they are less sensitive to the aforementioned sensory qualities and more likely to prefer foods with these sensory attributes than tasters [26]. Other reports show no association between PROP taster status and these variables [27, 28].

In the last decades, the genetic basis for differences in the ability to taste PROP has been mostly identified with allelic diversity in the *TAS2R38* gene [29, 30]. This gene codes for the TAS2R38 receptor, which specifically binds the bitter thiourea moiety present in compounds, such as PROP and its chemical relative phenylthiocarbamide (PTC) [30]. The allelic diversity of the *TAS2R38* locus is due to three single-nucleotide

polymorphisms (SNPs) resulting in three amino acid substitutions (Pro49Ala, Ala262Val, and Val296Ile) [29, 30] that give rise to two common haplotypes: PAV, the dominant taster variant, and AVI, the non-taster recessive one. Rare haplotypes (AAV, AAI, PVI, and PAI) have also been observed but are limited to specific populations [31]. Non-tasters are homozygous for the AVI haplotype, whereas the degree of perceived PROP bitterness varies in individuals with the taster haplotype (PAV), with some classified as super-tasters, and others defined only as tasters according to psychophysical scaling methods [32, 33]. *TAS2R38* genotypes do not completely explain the oro-sensory differences across PROP phenotypes, especially between medium and super-tasters [34]. These data imply that other factors may be involved in defining PROP phenotypes [5, 30, 34, 35]. Principal among these factors is the density of fungiform papillae [33, 36-40] and the composition of saliva [41-44].

Recently, Melis et al [36] showed that polymorphism *rs2274333* (A/G) in the gene encoding gustin (also known as carbonic anhydrase VI (CA6)),was strongly associated with PROP taste sensitivity and papillae density. Gustin is a 42 kDa protein secreted by the parotid, submandibular and von Ebner glands [45-48] that has previously been described as a trophic factor for growth and development of taste buds [49]. The polymorphism, *rs2274333* (A/G), of the gustin gene results in the amino acid substitution at position Ser90Gly in the protein sequence. According to Melis et al. [36] PROP super-tasters more frequently were homozygous for the A allele of the gustin gene and expressed the more active enzyme iso-form, whereas non-tasters more frequently carried the GG genotype and expressed the less functional form. Individuals with the GG genotype also had a lower density of fungiform papillae and exhibited more gross morphological changes in fungiform papillae, than did subjects with the more functional allele, suggesting an association of the gustin gene with growth and

maintenance of taste papillae. Moreover, the authors showed, in *in vitro* experiments, that isolated cells thrived better when exposed to saliva from AA subjects or the corresponding active iso-form (Ser90) of protein. Together, these data provide physical and mechanistic evidence for a robust association between variation in the gustin gene and the formation and function of fungiform papillae.

The study of Melis et al. [36] was conducted in a relatively homogenous cohort residing on the island of Sardinia. However, two recent studies in ethnically-mixed populations in Brazil [50] and the U.S. [51] failed to find associations between PROP tasting and gustin genotypes. The aim of this study was to confirm the role of the gustin gene polymorphism in PROP sensitivity and fungiform papilla density in an ethnically-mixed sample, and to identify confounding factors which may explain discrepant findings for gustin gene effects across populations.

2. Material and methods

2. 1. Subjects

A total of 91 healthy, adults (18-35 years of age) were recruited from the Rutgers community through email distribution lists and notices placed around campus. Subjects completed the 3-Factor Eating Questionnaire by Stunkard & Messick [52] for assessment of general eating attitudes. The suitability for the study of each volunteer were evaluated by a screening questionnaire with demographic and health information. Exclusion criteria include major diseases (diabetes, kidney disease, etc.), pregnancy or lactation, food allergies, and the use of medications that interfere with taste or smell and with saliva flow (e.g., steroids, antihistamines, certain antidepressants). The study was approved by the Rutgers University Institutional Review Board. All subjects signed an

informed consent to participate in the study and received financial compensation for their participation.

2. 2. Density of fungiform papillae

Fungiform papillae density was measured according to Melis et al. 2013 [36] and is briefly described below. Subjects rinsed their mouth with spring water prior to commencing a measurement session. During the session, each subject sat on a chair, with the elbows on a table, supporting the head with the hands to minimize movement. The anterior left side of the tip of the tongue surface was dried by lightly blotting the area with filter paper. The area was stained by placing a filter paper circle (6 mm in diameter) on the tongue for 3 sec which had been previously soaked in blue food coloring (McCormick & Co. Sparks, MD). Multiple photographs were taken of each subject using a Nikon Coolpix L310 (14 megapixel) camera with optical zoom 21x, and the best digital image was analyzed by Adobe Photoshop CS2 version 9.0 software. The fungiform papillae in the stained area were identified and counted for each subject according to previously established criteria [36, 40, 53]. The density/cm^2 was calculated. Papillae were separately identified and counted by three trained observers who were blind to the PROP status of subjects. The final measurements were based on the consensus assessment of all observers.

2. 3. PROP bitterness ratings and taster status

PROP bitterness ratings and taster status of each subject was determined using the impregnated paper screening test, previously tested for validity and reliability [54]. The test is based on the ratings of two 2 paper discs, one impregnated with NaCl (1.0 mol/l) and the other with PROP solution (50 mmol/1). Intensity ratings for PROP or NaCl

were collected using the Labeled Magnitude Scale (LMS), a 100-mm scale anchored with the phrases “barely detectable” to “strongest imaginable” [55].

2. 4. Molecular analysis

Subjects were genotyped for polymorphism *rs2274333* (A/G) of the gustin (CA6) gene that results in a substitution of amino acid Ser90Gly, and for three single nucleotide polymorphisms (SNPs) at base pairs 145 (C/G), 785 (C/T), and 886 (G/A) of the *TAS2R38* locus that consist of three amino acid substitutions (Pro49Ala, Ala262Val, and Val296Ile). These substitutions give rise two common haplotypes, PAV and AVI, and three rare haplotypes, AAI, PVI and AAV. The DNA was extracted from samples of saliva using the QIAamp® DNA Mini Kit (QIAGEN) according to the manufacturer’s instructions. Purified DNA concentration was estimated by measurements at OD260. The gustin gene region including polymorphism *rs2274333*, and the two short region of the *TAS2R38* locus including the three SNPs of interest were amplified by PCR techniques.

To genotype gustin gene polymorphism *rs2274333*, a fragment of 253 bp was amplified with sense 5'TGACCCCTCTGTGTTCACCT3' and antisense 5'GTGACTATGGGGTTCAAAGG3' primers. DNA was amplified using EuroTaq thermostable DNA polymerase (EuroClone S.p.A. Italy). The amplification protocol consisted of an initial denaturation at 95°C for 5 min, followed by 30 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 over night. The sense primer binds within the gustin gene, from nucleotides 441-461. A single mismatch A/G changes the codon from CAG to CGG generating the nucleotide sequence GGCC recognized by HaeIII. The

presence of the G allele allowing the amplified to be cut into two fragments while the presence of the A allele is not cut. The digested fragments were electrophoresed on 2% agarose gel and stained with ethidium bromide. The PCR 50 bp Low Ladder DNA was used as Mr markers (GeneRuler™ -Thermo Scientific).

To determine haplotypes of the *TAS2R38* locus, PCR amplification was followed by restriction analysis using HaeIII enzyme for detection of the SNP at the 145 nucleotide position. Direct sequencing (using sense and antisense primers) identified the second and third SNPs, at the 785 and 886 nucleotide positions. The following primer set was used to amplify a fragment of 221 bp including the first of three SNPs: sense 5'-CCTTCGTTTTCTTGGTGAATTTTTGGGATGTAGTGAAGAGGC**G**G-3' antisense 5'-AGGTTGGCTTGGTTTGCAATCATC-3'. The sense 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. DNA was amplified using EuroTaq Thermostable DNA polymerase (EuroClone S.p.A. Italy). The amplification protocol consisted of initial denaturation at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 58°C for 35 s, and then extension at 72°C for 30 s. A final extension was carried out at 72°C for 10 min. For the analysis of the polymorphism G/C at position 143, the amplified samples were digested with HaeIII enzyme at 37°C over night. The digested fragments were electrophoresed on 2% agarose gel and stained with ethidium bromide. The PCR 100 bp Low Ladder DNA was used as Mr markers (GeneRuler™ -Thermo Scientific). Polymorphisms at the 785

and 886 nucleotide position were identified by a single PCR reaction using the sense primer 5'-TCGTGACCCCAGCCTGGAGG-3' and the antisense primer 5'-GCACAGTGTCCGGGAATCTGCC-3' delimiting a 298 bp fragment. DNA was amplified using EuroTaq Thermostable DNA polymerase (EuroClone S.p.A. Italy). The amplification protocol consisted of initial denaturation at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 67°C for 30 s, and then extension at 72°C for 30 s. A final extension was carried out at 72°C for 10 min. 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).

2. 5. Experimental procedure

The subjects were requested to abstain from eating and drinking for at least 2 h prior to testing that was carried out on two different visits. During the first visit, subjects were screened for PROP status. Each subject was asked to place the paper disk with the taste stimulus on the tip of the tongue for 30 seconds or until the disk was thoroughly wet with saliva, and then spit it out. Subjects were instructed to rinse with spring water at room temperature before and between tasting each paper disk. The interstimulus interval was set at 60 s. The order of taste stimulus presentation was NaCl first followed by PROP. After tasting each sample, the subject placed a mark on the LMS scale corresponding to his/her perception of the stimulus. Subjects were categorized as non-tasters if they rated the PROP disk lower than 13 mm on the LMS; they were categorized as super-tasters if they rated the PROP disk higher than 67 on the LMS. All others were classified as medium tasters [54] . NaCl ratings do not vary with PROP status in this method [56-58]. Therefore, NaCl ratings are used as a reference standard

to clarify the taster status of subjects who give borderline ratings to PROP. In practice, borderline ratings are rare in this method, occurring approximately 4% of the time [59]. Based on their taster group assignments, 31.87% of the subjects were non-tasters (n = 29); 36.26% were medium tasters (n = 33); and 31.87% were super-tasters (n = 29).

During the second visit, a sample (2 ml) of stimulated mixed saliva was collected from each subject between 01:00 and 03:00 PM. Subjects chewed a 3-cm square of parafilm (inert, odourless paraffin film) for 5-min and then expectorated the collected saliva into acid-washed polypropylene test tubes. The samples were stored at -80 °C until molecular analyses were completed as described previously.

2. 6. Statistical analyses

Genotype distribution and allele frequencies at the *TAS2R38* and gustin gene loci were compared between the group of Caucasians and the total sample and also according to PROP status using Fisher's method (Genepop software version 4.0; http://kimura.univ-montp2fr/~rousset/Genepop.htm) [60]. Genetic differences between the group of Caucasians and the total sample or across the three taster groups based on the distribution of the *TAS2R38* and gustin gene genotype combinations were tested by the Markov Chain method (Arlequin software version 3.1; http://cmpg.unibe.ch/software/arlequin3) [61]. Two-way analysis of variance (ANOVA) was used to examine PROP bitterness intensity ratings (50mM) and density of fungiform papillae (No./cm2) on the anterior part of the tongue across *TAS2R38* and gustin gene genotypes. Bonferroni and LSD tests were used for post-hoc comparisons. The Generalized Linear Model (GLM) procedure was used to analyze the contribution of the *TAS2R38* and gustin gene loci to PROP bitterness and density of fungiform papillae. The global R Squared offers a measure of this contribution The effects of

heterozygosity and homozygosity were analyzed for both genes, and the effect of interaction between the two genes was also included in the model. The Partial Eta Squared indicated the strength of the association of each independent variable (genotypes of *TAS2R38* and gustin gene loci) with the dependent variable (bitterness or density of fungiform papillae), after the effects of all other independent variables were accounted for. Individuals with rare haplotypes at the *TAS2R38* locus were excluded from the analysis in order reduce confounding factors.

Finally, Fisher's exact test was used to test for differences in *TAS2R38* genotype distribution within each gustin gene genotype group comparing the present sample with the genetically homogeneous sample from Sardinia analyzed by Calò et al. [42].

Statistical analyses were conducted using STATISTICA for WINDOWS (version 7.0; StatSoft Inc, Tulsa, OK, USA) and SPSS software (SPSS Inc. Headquarters, Chicago, IL, USA). p values ≤ 0.05 were considered significant.

3. Results

The present sample was ethnically mixed and similar to other populations previously investigated for PROP status at Rutgers University [26, 62]. The initial sample included ninety-one subjects who were Caucasian (n = 54), Asian (n = 29), Black (n = 5), or Hispanic (n = 3). Genotyping for the *TAS2R38* locus revealed that 23 subjects were PAV homozygous, 27 were heterozygous, and 30 were AVI homozygous. Eleven subjects had rare genotypes (1 PVI/AVI, 2 PAV/AAV, 2 PAV/PVI, 5 AAV/AVI and 1 AAI/AVI) and were eliminated from subsequent analyses. Thus, the final dataset included 80 subjects. Genotyping for the gustin gene showed that 30 subjects were homozygous AA, 37 were heterozygous and 13 were homozygous GG. No differences were found between the group of Caucasians (n = 54) and the total sample (n = 80) for the genotype distributions or the allele frequencies for *TAS2R38* or the gustin gene, or for the distribution of both genotype combinations ($p > 0.05$; Fisher's method and $p > 0.05$; Markov Chain method).

Genotype distributions and allele frequencies for polymorphisms of *TAS2R38* and the gustin gene according to PROP taster status are shown in **Table 1**. Molecular analysis of the *TAS2R38* polymorphisms showed that the three PROP taster groups differed statistically based on their genotype distribution and haplotype frequency ($\chi^2 > 50.00$; $p < 0.0001$; Fisher's test). Pairwise comparisons discriminated all groups from each other ($\chi^2 > 12.585$; $p \leq 0.0018$; Fisher's test). Super-tasters had a high frequency of diplotype PAV/PAV (57.69 %) and haplotype PAV (78.85 %), whereas non-tasters had a high frequency of diplotype AVI/AVI (81.48 %) and haplotype AVI (88.88). In medium tasters the two haplotypes had a similar frequency (48.15 % and 51.85 %, respectively). The three PROP taster groups were not different based on their genotype distribution and allele frequency for the gustin gene ($p > 0.75$; Fisher's test).

However, the three taster groups did differ statistically based on the combined distribution of *TAS2R38* and gustin gene genotypes ($p < 0.0001$; Markov Chain method) (**Table 2**). Post hoc comparison discriminated non-tasters from the other groups ($p \leq 0.024$; Markov Chain method). Homozygosity for the insensitive allele at the *TAS2R38* locus combined with the AA or AG genotype for gustin (AVI/AVI - AA and AVI/AVI - AG) was most frequent in non-tasters (33.33 % and 40.74 %, respectively). Homozygosity for the sensitive PAV haplotype which always combined with the gustin AA or AG genotype (PAV/PAV - AA and PAV/PAV - AG) was most frequent in super-tasters (23.08 % and 30.77 %, respectively), Medium tasters were more likely to heterozygous for both the PAV haplotype and AG genotype (PAV/AVI - AG) (22.22 %).

Figure 1 shows PROP bitterness intensity ratings (PROP 50 mM) and density of fungiform papillae (No./cm^2) on the anterior part of the tongue in individuals grouped by *TAS2R38* diplotypes, and gustin genotypes. Two-way ANOVA showed no significant interaction between the two loci on PROP bitterness ($F_{[4,71]} = 1.344$; $p = 0.262$), but a strong association between bitterness and *TAS2R38* diplotype, as expected ($F_{[2,71]} = 30.363$; $p < 0.000001$). Post-hoc comparisons showed that PROP bitterness ratings were lower in individuals with the AVI/AVI diplotype of *TAS2R38* than in individuals with PAV/PAV and PAV/AVI diplotype ($p < 0.000001$; Bonferroni test). No differences in bitterness intensity ratings were found among gustin genotypes ($p > 0.05$).

Two-way ANOVA also showed no interaction between the two loci on density of fungiform papillae ($F_{[4,71]} = 0.3279$; $p = 0.858$), but a strong association between density and gustin gene genotypes ($F_{[2,71]} = 4.9914$; $p = 0.0093$). No significant differences in the density of fungiform papillae related to *TAS2R38* diplotype were found ($p > 0.05$). However, pairwise comparisons showed that the density of fungiform papillae of

individuals with the gustin AA genotype was higher than that of individuals with the GG genotype (p = 0.0072; Bonferroni test). The papillae densities of heterozygous individuals were lower than that of AA individuals (p = 0.039, LSD test). Moreover, no correlation was observed between PROP bitterness intensity and fungiform papillae density (r = 0.163; p = 0.185; linear correlation analysis).

Figure 2 shows mean values (± SE) for PROP bitterness (upper graph) and density of fungiform papillae (lower graph) for each *TAS2R38* and gustin gene genotype combination. Bitterness intensity ratings were lower in those with two insensitive *TAS2R38* haplotype (AVI/AVI) but did not vary as a function of gustin genotypes despite the trend to decrease in the group of PAV/PAV diplotypes. On the other hand, the decrease of papillae density in each *TAS2R38* genotype group according to gustin locus was most evident and was significant in the PAV/PAV and PAV/AVI groups (p = 0.045 and p = 0.037, respectively; LSD test subsequent two-way ANOVA).

The Generalized Linear Model (GLM) procedure revealed the contribution of the *TAS2R38* and gustin gene loci in modulating PROP bitterness intensity. The Global R Squared for the PROP bitterness model was 0.578. In particular, the model showed significant associations between PROP bitterness and the presence of one or two sensitive haplotype in *TAS2R38* (Partial Eta Squared 0.323 and 0.514 in the PAV/AVI and the PAV/PAV groups, respectively; $p < 0.00001$). No associations were observed between PROP bitterness and gustin gene AA or AG genotypes (Partial Eta Squared 0.001 and 0.002, respectively; p = 0.808 and p = 0.690). Also, no significant interaction between the two loci was detected. The GLM procedure also revealed the contribution of the two loci in modulating the density of fungiform papillae, the global R Squared for the model was 0.157. This model showed a significant association between papillae density and the presence of the GG genotype for gustin (Partial Eta Squared 0.084; p =

0.013) and a modest association between density of fungiform papillae and the presence of the AG genotype for gustin (Partial Eta Squared 0.053; $p = 0.051$) No associations were observed between density of papillae and *TAS2R38* alleles, PAV /PAV or PAV/AVI (Partial Eta Squared 0.003 or 0.002; $p = 0.595$ or $p = 0.718$). Also for the density of fungiform papillae, no significant interaction between the two loci was found.

Finally, **Table 3** shows the genotype distributions of *TAS2R38* within each gustin gene genotype group in the present sample and in the genetically homogeneous sample, analyzed by Calò et al. [42]. Fisher's test showed that the present sample was significantly different from that of Calò based on the distribution of *TAS2R38* diplotypes within the AG and GG gustin gene genotypes ($\chi^2 = 9.364$; $p < 0.0092$ and $\chi^2 = 7.444$; $p < 0.024$). The percentage of subjects who were heterozygous at both loci (75 %) was higher in the genetically homogeneous population studied by Calò than that in the present study (29.73 %). Likewise, the percentage of individuals homozygous for the insensitive alleles at both loci was higher in the homogeneous population (73.3 %) than in the present study (23.07 %). The distribution of *TAS2R38* diplotypes within the gustin AA genotype group did not differ between the two populations ($\chi^2 = 5.40$; $p = 0.067$; Fisher's test).

4. Discussion

In a series of studies conducted in genetically, homogeneous cohorts we examined the influence of the gustin gene polymorphism, *rs2274333* (A/G) on PROP bitterness intensity and its role in modulating the activity of gustin (CAVI) protein, a trophic factor for cell growth and fungiform papillae maintenance. Padiglia et al. [44] first showed that PROP non-tasters were more likely to have the GG genotype at this locus in gustin than the other taster groups, and that the GG genotype was associated with structural changes and reduced functionality of the gustin protein. Subsequently, Calò et al. [42] showed that polymorphisms at the gustin gene and *TAS2R38* loci accounted for 60% of the variance in PROP bitterness intensity. Finally, Melis and coworkers [36] reported that papillae density was more closely related to the gustin gene locus than to the *TAS2R38* locus. In particular, the presence of the GG genotype in gustin was associated with the lowest papillae densities. In contrast, some features of papillae morphology such as size and distortion were strongly related only to gustin genotypes. Moreover, treatment of cells with either saliva from individuals with the AA genotype or the active iso-form of gustin protein (purified from the saliva of AA genotype individuals) led to greater cell proliferation and activity than similar treatments derived from individuals with the GG genotype [36]. The latter findings support the idea that the *rs2274333* (A/G) polymorphism is involved in fungiform papillae growth and maintenance.

Since population homogeneity can lead to over estimation of gene effects, a primary aim of the present work was to confirm gustin's role in PROP bitterness intensity and fungiform papillae density in a genetically diverse population. The present study was conducted in an ethnically-mixed sample in the U.S. that was typical of other cohorts studied in this laboratory [26, 62, 63]. The current data showed that PROP bitterness

was due to *TAS2R38* diplotypes, whereas the density of fungiform papillae was more closely associated with gustin genotypes. In particular, we found that the lowest responsiveness to PROP in non-tasters was strongly associated with the AVI nontasting variant of *TAS2R38* (89 %) and the highest PROP responsiveness in super-tasters was strongly associated with the PAV taster variant (79 %). This finding is consistent with previous reports showing that the PAV variant of theTAS2R38 receptor is necessary for perceiving high concentrations of PROP [29, 42]. On the other hand, a higher density of fungiform papillae was associated with the presence of the sensitive allele (A) in the gustin gene, but not *TAS2R38* haplotypes (figure 1). Moreover, a comparative analysis within each *TAS2R38* genotype group showed that the density of fungiform papillae decreased as the number of minor (G) alleles at the gustin locus increased.

GLM modeling of the current data also support the above associations. Results showed that the *TAS2R38* locus accounted for 57.8 % of the phenotypic variance in PROP bitterness intensity whereas the gustin polymorphism predicted 16 % of the variance in papilla density. The strength of the association we observed between the gustin polymorphism and fungiform papillae density was modest in the present study and in our previous work [36], but is consistent with the notion that the gustin gene may be only one of a constellation of genetic and non-genetic factors contributing to individual differences in PROP sensitivity and fungiform papillae density and morphology. Recent data point to variation in TAS2R38 receptor expression [64], variation in the levels of other salivary proteins (basic proline-rich proteins (PS-1, II-2) [41] and environment factors that may alter the taste system, such as smoking and otitis media [65].

Some recent reports have shown no relationships between gustin polymorphisms and PROP tasting or papillae density in genetically-diverse cohorts. Specifically, Genick

et al. [50] found no relationship between gustin and PROP phenotypes in a genome-wide association study conducted in Brazil [50]. Likewise, Feeney & Hayes [51] reported no associations between 12 selected polymorphisms in the gustin gene and PROP bitterness or papillae density in a U.S. cohort. Feeney and Hayes [51] did find a statistical relationship between two other gustin SNPs (rs3737665 rs3765964) and NaCl perception. However the differences across genotypic groups were small, raising questions about their biological relevance.

Feeney and Hayes [51] have suggested that our present and previous findings for gustin [36, 41, 42, 44] may be an artifact of the PROP screening methods we employed that utilize NaCl a reference standard. Presumably, if gustin polymorphisms alter NaCl perception in a meaningful way, then utilizing NaCl as a standard would confound study outcomes. Although two studies did use the 3-solution test which compares PROP to NaCl ratings [42, 44], we also used methods that do not utilize NaCl as a reference including threshold assessment of PROP taste sensitivity [36, 42], suprathreshold intensity ratings of PROP [36, 42] and (in the present study) a filter paper method which uses numerical cutoff scores for PROP taste intensity. The consistency of findings across these studies clearly suggests that the PROP screening procedure is not the source of differences across laboratories.

A more salient explanation for these discordant findings is that populations with different genetic admixtures were studied, and the presence of more extreme phenotypes in some populations relative to others may be driving the observed effects. Specifically, in the homogeneous population study by Calò et al. [42], a large majority (73%) of subjects homozygous for the less functional gustin iso-form (GG) were also homozygous for the non-taster variant of *TAS2R38*. The percentage GG-AVI/AVI individuals fell to 23% in the ethnically-mixed population studied here, and this

combination was relatively rare (14%) in the cohort studied by Feeney and Hayes [51]. The presence of minor alleles at both loci may be more important for determining the functionality of the gustin protein and its effects on papillae morphology than the frequency of the GG genotype alone, which was similar across all studies (16-21%).

In conclusion, our findings confirm the role of the gustin gene polymorphism, *rs2274333* (A/G), in fungiform papillae density in an ancestrally heterogeneous population. We speculate that the failure to replicate these findings in some studies may reflect population-based differences in the distribution of *TAS2R38* and gustin genotypes within the limited number of cohorts that have been investigated, rather than methodological shortcomings of individual studies. Future studies should examine the distribution of *TAS2R38* and gustin genotypes in population groups worldwide to better assess the role of these loci in taste perception and tongue anatomy.

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Figure legends

Fig. 1. PROP bitterness intensity ratings (50mM) and density of fungiform papillae (No./cm2) on the anterior part of the tongue in individuals with genotypes PAV/PAV, PAV/AVI and AVI/AVI of TAS2R38, and of individuals with genotypes AA, AG and GG of gustin (CA6) polymorphism rs2274333 (A/G). All values are means (± SEM). n=80. Different letters indicate significant differences in bitterness intensity across TAS2R38 allelic groups ($p \leq 0.00001$; Bonferroni test subsequent to two-way ANOVA).

* indicates a significant difference in papillae density between the AA and GG genotype for gustin (p=0.00723; Bonferroni test subsequent to two-way ANOVA).

Fig. 2. PROP bitterness intensity (50 mM) (upper graph), and density of fungiform papillae (No./cm2) (lower graph) according to TAS2R38 and gustin (CA6) loci. All values are means (± SEM). n=80.

* and § indicates significant differences (p=0.045 and p=0.037, respectively; LSD test subsequent two-way ANOVA).

Tables

Table 1. Genotype distribution and allele frequencies of polymorphisms of *TAS2R38* and the gustin (CA6) gene according to PROP taster status.

	Total		PROP status						*p*-value[a]
			Super-taster		Medium taster		Non-taster		
	n	%	n	%	n	%	n	%	
TAS2R38									
Genotype									
PAV/PAV	23	28.75	15	57.69	7	25.92	1	3.70	<0.00001
PAV/AVI	27	33.75	11	42.31	12	44.44	4	14.81	
AVI/AVI	30	37.50	0	0	8	29.63	22	81.48	
Haplotype									
PAV	78	45.62	41	78.85	26	48.15	6	11.11	<0.00001
AVI	87	54.37	11	21.15	28	51.85	48	88.88	
Gustin gene									
Genotype									
AA	30	37.50	10	38.46	9	33.33	11	40.74	0.755
AG	37	46.25	11	42.31	13	48.15	13	48.15	
GG	13	16.25	5	19.23	5	18.52	3	11.11	
Haplotype									
A	97	60.62	31	59.61	31	57.41	35	64.81	0.73
G	63	39.37	21	40.38	23	42.59	19	35.18	

[a] *p*-value derived from Fisher's method (n = 80).

Table 2. Distribution of the *TAS2R38* and gustin gene genotype combinations according to PROP taster status.

	Total		PROP status						*p*-value[a]
			Supertaster		Medium taster		Nontaster		
	n	%	n	%	n	%	n	%	
Genotype									
AVI/AVI-GG	3	3.75	0	0	1	3.70	2	7.41	<0.00001
AVI/AVI-AG	15	18.75	0	0	4	14.81	11	40.74	
AVI/AVI-AA	12	15.00	0	0	3	11.11	9	33.33	
PAV/AVI-GG	7	8.75	4	15.38	3	11.11	0	0	
PAV/AVI-AG	11	13.75	3	11.54	6	22.22	2	7.41	
PAV/AVI-AA	9	11.25	4	15.38	3	11.11	2	7.41	
PAV/PAV-GG	3	3.75	1	3.85	1	3.70	1	3.70	
PAV/PAV-AG	11	13.75	8	30.77	3	11.11	0	0	
PAV/PAV-AA	9	11.25	6	23.08	3	11.11	0	0	

[a] *p*-value derived from the Markov Chain method ($n = 80$).

Table 3. Comparison of genotype distributions of *TAS2R38* within each gustin gene genotype group in the present sample and in a genetically homogeneous population[a].

		Present Sample		Calò[b] et al. (2011)		
		n	%	n	%	*p*-value[a]
Gustin gene	***TAS2R38***					
AA						
	PAV/PAV	9	30.00	10	24.39	0.067
	PAV/AVI	9	30.00	23	56.09	
	AVI/AVI	12	40.00	8	19.51	
	Total	30	37.5	41	56.94	
AG						
	PAV/PAV	11	29.73	2	12.5	0.0092
	PAV/AVI	11	29.73	12	75.0	
	AVI/AVI	15	40.54	2	12.5	
	Total	37	46.25	16	22.22	
GG						
	PAV/PAV	3	23.07	2	13.33	0.024
	PAV/AVI	7	53.84	2	13.33	
	AVI/AVI	3	23.07	11	73.33	
	Total	13	16.25	15	20.83	

[a] *p*-values are from Fisher's Exact Test [b] (Calò et al. 2011).

Figure 1

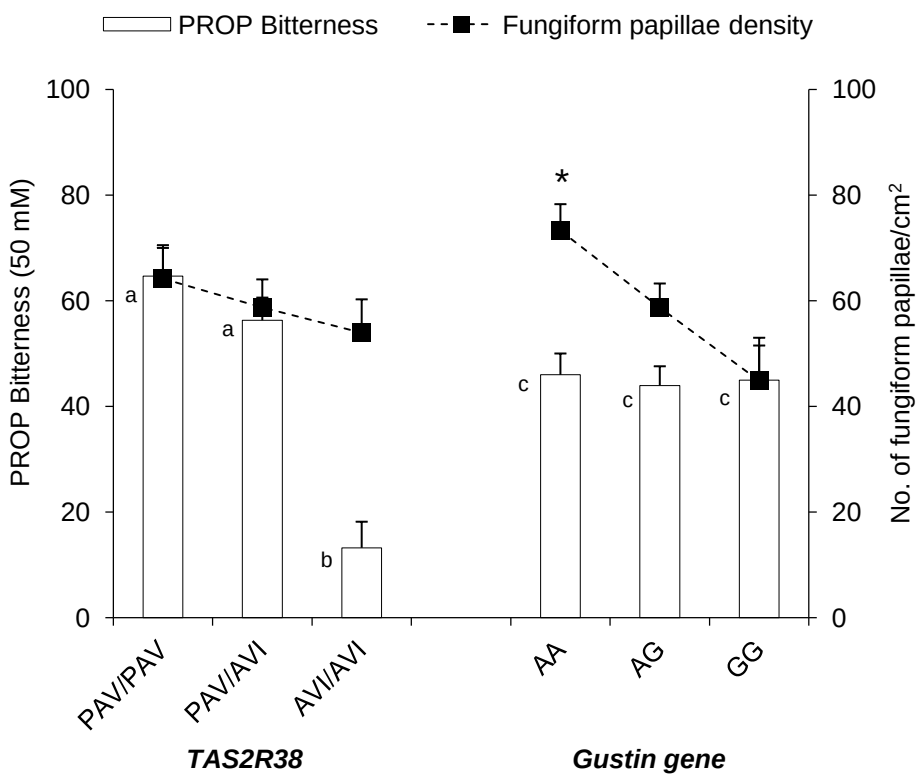
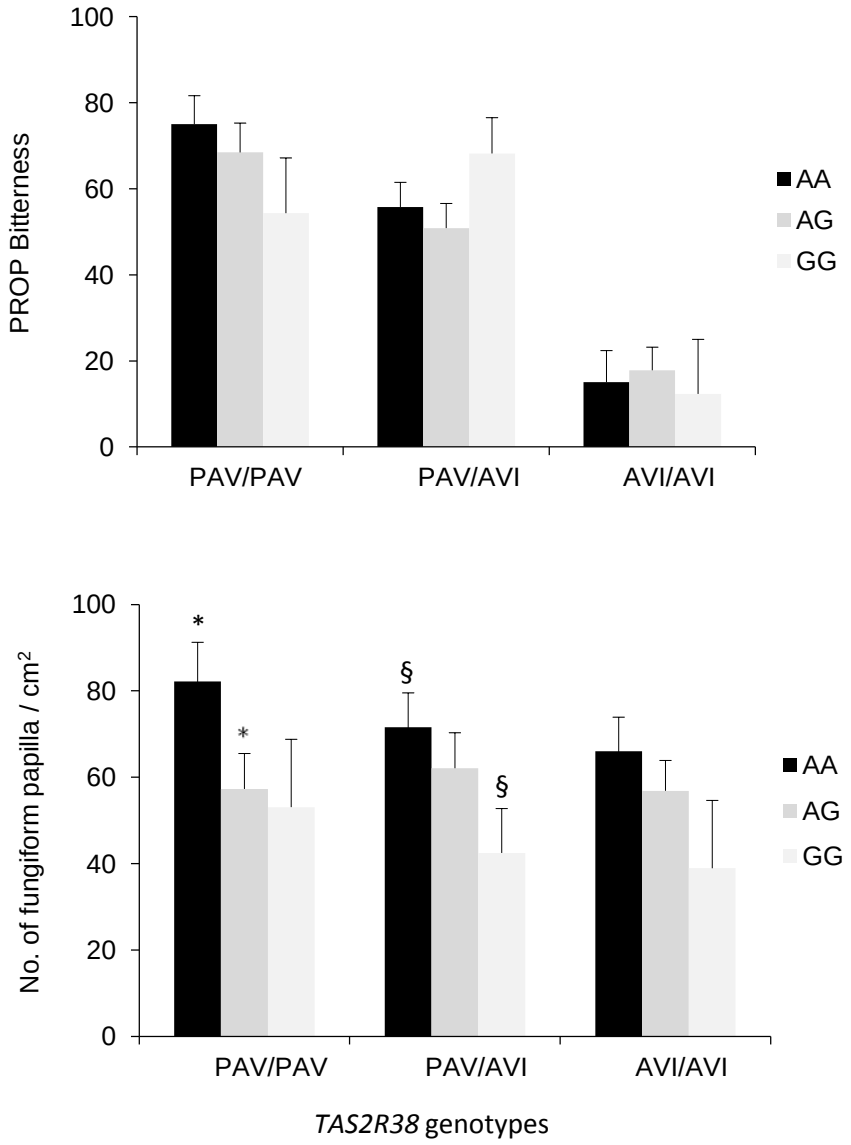

PROP Bitterness
Fungiform papillae density
PROP Bitterness (50 mM)
No. of fungiform papillae/cm²
100
80
60
40
20
0
a
a
b
c
c
c
*
PAV/PAV
PAV/AVI
AVI/AVI
AA
AG
GG
TAS2R38
Gustin gene

Figure 2


100
80
60
40
20
0
PROP Bitterness
PAV/PAV
PAV/AVI
AVI/AVI
AA
AG
GG
100
80
60
40
20
0
No. of fungiform papilla / cm²
*
*
§
§
PAV/PAV
PAV/AVI
AVI/AVI
AA
AG
GG
TAS2R38 genotypes