Title:

Effect of the *rs2890498* polymorphism of the *OBPIIa* gene on the human ability to smell single molecules

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Abstract

Most odors of foods and drinks are mixtures of molecules. By means of the coupled Gas Chromatography-Olfactometry (GC-O) technique, single components of flavor mixtures can be separated, identified and verbally evaluated by subjects. The number of single molecules smelled by subjects during GC-O analysis (i.e., the number of odor-active compounds) was previously found to be linearly correlated with odor Threshold (T) score. Using the "Sniffin' Sticks" test, the same subjects were classified as normosmic or hyposmic. Hydrophobic odorants are captured and transported through the mucus layer by the odorant binding proteins (OBPs), particularly expressed in the olfactory cleft and associated with the olfactory function. In this study, subjects were genotyped for the *rs2590498* (A/G) polymorphism of the *OBPIIa* gene, whose major allele A is associated with a higher olfactory sensitivity as compared to the minor allele G. One-way ANOVA showed a significant effect of the genotype of the *OBPIIa* locus on the: a) T score; b) number of odor-active compounds smelled; c) intensity perceived when sniffing the complex odor of banana. In conclusion, the threshold olfactory performance, but also the individual ability to smell single molecules, can be attributed, partly at least, to the *rs2590498* polymorphism of the *OBPIIa* gene.

Keywords: human odorant binding proteins, gas cromatography-olfactometry analysis, threshold olfactory performance, odor-active compounds, intensity perceived, Sniffin' Sticks test.

1. Introduction

Odor perception begins in the olfactory epithelium when the odorants bind to the olfactory receptors (ORs) located on the ciliary ends of the olfactory sensory neurons (OSNs) [1-2]. The olfactory epithelium is covered with a thin layer of mucus rich in glycoproteins, which bind to large quantities of water offering physical and chemical protection from external agents [3-4]. In this region, known as perireceptor space, odorants bind to the odorant binding proteins (OBPs), which are highly expressed in the olfactory cleft 5-8]. Since most of volatile molecules are hydrophobic and need to cross the mucus barrier in order to reach and activate the olfactory receptors (ORs) and thus initiate the olfactory transduction process, some authors suggested that OBPs have the role of capturing odors and transporting them through the mucus to the OSN membrane [3,8-11]. In fact, substantial evidence from both vertebrates and invertebrates has shown that OBPs: a) transport odorants through the mucus layer to the ORs [12-15]; b) play a critical role in the process of odor discrimination [16] and receptor activation [17-18]; c) preserve the structure at the level of the carboxylic terminal of the polypeptide chain by defining a central apolar cavity (a calyx), whose function is to bind and transport the hydrophobic odorous molecules [19-22]. In the mucus of humans, the OBPIIa is the only OBP found [4,20] and its specific location at the level of the olfactory epithelium suggests its role as a "carrier" for odorants [20]. The *rs2590498* polymorphism of the gene coding for human OBPIIa has been associated with individual variations in the olfactory ortho and retronasal perception: subjects who were homozygous for the major allele A showed significantly higher olfactory performance than subjects that were heterozygous or homozygous for the minor allele G [23-25].

Another important aspect in the studies on olfaction is that most food and drink odors are mixtures of molecules, i.e. they consist of many volatile odorous compounds, only a few of which are sensorially significant. In the studies on food flavor one relevant problem is the identification of the compounds that strongly contribute to its aroma, the so-called odor-active compounds [26-29]. Gas Chromatography-Olfactometry (GC-O) is a combination of sensory and instrumental analysis, characterized by simultaneous chromatographic separation and odor evaluation by a human evaluator

[30], which allows to appraise the odor contribution of a single molecule to the overall aroma [28,31]. However, in this type of investigation, it should be kept in mind that individuals may show a normal, reduced or absent (general or specific) olfactory performance and hence be classified as normosmic, hyposmic or anosmic [32-35]. The reasons of this inter-individual variability are multiple and can be ascribed to disorders, personal experience, environmental and genetic factors [36-43]. From a recent study conducted with the GC-O technique and using the head space of the banana pulp as a complex stimulus, it emerged that of the 43 molecules found in the mixture, only 33 were "odor-active" (i.e. perceived by at least two individuals), as they were eluted from the chromatographic column [44]. These authors also found a linear and significant correlation between: a) the individual ability in the number of molecules identified (both total and smelling of banana) and the score obtained by each subject during the olfactory threshold test; b) the number of banana-smelling molecules perceived by each subject and the intensity reported for the complex banana odor [44].

Based on these considerations, the main goal of this study was to evaluate whether a relationship exists between: a) the olfactory status of the subject (both general and specific for threshold, discrimination and identification ability) and the *rs2890498* polymorphism of the gene encoding for OBPIIa; b) the ability of each subject to perceive single molecules during the GC-O analysis and the *rs2890498* polymorphism of the *OBPIIa* gene; c) the intensity reported for the complex banana odor and the polymorphism of the *OBPIIa* gene.

2. Materials and Methods

2.1 Subjects

The study was conducted on 52 healthy Caucasian subjects (41 women and 11 men), aged between 19 and 53 years, non-smokers and with a body mass index (BMI) between 18.5-24.99 Kg/m^2 (normal weight), recruited in Cagliari (Sardinia, Italy). All subjects had already been checked by their orthonasal olfactory performance and their ability to detect single odor-active compounds during the Gas Chromatography - Olfactometry (GC-O) analysis [44]. Briefly, by means of the Sniffin' Sticks

Extended Test [45] (SSET; Burghart Instruments, Wedel, Germany) we evaluated the orthonasal olfactory function of each subject, and using the score obtained during Threshold (T), Discrimination (D) and Identification (I) test, we classified subjects as normosmic or hyposmic according to Hummel et al. [46] (for detailed instructions visit the following link: https://www.uniklinikum-dresden.de/de/das-klinikum/kliniken-polikliniken-institute/hno/forschung/interdisziplinaeres-zentrum-fuer-riechen-und-schmecken/neuigkeiten/downloads). A visual analogue rating unit scale (VARU) was used by each subject to mark the intensity perceived for each odor smelled during the identification test [47]. During GC-O analyses, by means of a voice recording and digital signaling system connected to the PC (GERSTEL ODP recorder 3 for Windows 7), each subject evaluated intensity, duration and quality of compounds being eluted from the GC column, where 1 μL volume of banana head space was injected [44].

Before collecting saliva samples, they were informed about the time required, the aim of the research and the experimental procedure and, in agreement, they were asked to sign an informed consent. The study was conducted in accordance with the Declaration of Helsinki (1975) and approved by the local Ethical Committee (Prot. PG/2018/22 del 02.01.2018).

2.2 Genetic analysis

DNA was extracted from saliva samples by means of the QIAamp® DNA Mini Kit (QIAGEN S.r.l., Milan, Italy), respecting the manufacturer's instructions. Subjects were genotyped for the *rs2590498* (A/G) polymorphism of *OBPIIa* gene using a custom TaqMan® SNP Genotyping Assay (Applied Biosystems by Life-Technologies Italia, Europe BV) according to our previous investigations [23-25]. Briefly: forward PCR Primer GCCAGGCAGGGACAGA and Reverse PCR primer CTACACCTGAGACCCCACAAG were used; Two TaqMan probes were designed according to the *OBPIIa* gene (bold and underlined), probe/reporter 1: VIC-TCGGTGAC**A**TGAACC and probe/reporter 2: FAM–TCGGTGAC**G**TGAACC. After PCRs, the fluorescence of plates was read by the sequence detector system at 60 °C for 1 min and the results analyzed by allelic discrimination

of the sequence detector software (Applied Biosystems). The reactions included three positive controls (one for each genotype), two negative controls and two replicates.

2.3 Statistical analysis

One-way ANOVA was used to analyze: a) the effect of the *OBPIIa* genotype on the Threshold (T), Discrimination (D) and Identification (I) scores obtained by the subjects; b) the effect of the *OBPIIa* genotype on the number of odor-active compounds, both total-compounds (number of compounds that each subject perceived during GC-O analyses regardless of their quality, i.e. smelling of banana or other) and banana-compounds (number of odor-active compounds smelling of banana) detected by the subjects; c) the effect of the *OBPIIa* genotype on the intensity reported by the subjects for the banana-odor pen (i.e., the complex aroma of banana contained in the pen no. 5 that the subjects smelled during the identification test of the SSET). Post-hoc comparisons were conducted with the Fisher's least significant difference (LSD) test, unless the assumption of homogeneity of variance was violated, in which case Duncan's test was used [48-49]. Statistical analyses were performed using STATISTICA for WINDOWS (version 7.0; StatSoft Inc, Tulsa, OK, USA). P values < 0.05 were considered to be significant.

Differences on genotype distribution and allele frequencies at the *OBPIIa* locus between subjects classified as normosmic or hyposmic for the TDI olfactory status, and singularly for the T, D and I status, were analyzed using Fisher's method (Genepop software version 4.2; http://genepop.curtin.edu.au/genepop_op3.html) [50].

3. Results

All participants (n = 52) were genotyped for the *rs2590498* (A/G) polymorphism of the *OBPIIa* gene and the molecular analysis revealed that: 16 subjects were AA homozygotes, 14 subjects were heterozygous, while 22 of them were GG homozygotes. The mean value ± standard error (SE) of T, D and I score determined in participants according to genotypes of the *OBPIIa* locus is shown in

figure 1. One-way ANOVA revealed a significant effect of the *OBPIIa* locus genotypes on T score ($F_{2,49}$ = 6.682; p = 0.003), and post-hoc comparisons showed that subjects who were homozygous for the major allele A reached T scores that were statistically higher than those of heterozygous ones (p = 0.004; Fisher's LSD test) or homozygous for the minor allele G (p = 0.01; Fisher's LSD test). No effect of the *OBPIIa* locus genotype was found, instead, on the D score ($F_{2,49}$ = 2.438; p = 0.098) and I score ($F_{2,49}$ = 2.446; p = 0.097).

Table 1 shows the genotype distribution and allele frequency for the polymorphism of the *OBPIIa* gene according to TDI, T, D and I status. Fisher's method revealed significant differences based on the genotype distribution and on the allele frequency of the *OBPIIa* locus between subjects classified as normosmic and hyposmic on the basis of their TDI score (χ^2= 11.693, P = 0.003 and χ^2= 15.458, P < 0.001, for genotype distribution and allele frequency, respectively), T score (χ^2= 10.678, P < 0.005 and χ^2= 13.982, P < 0.001, for genotype distribution and allele frequency, respectively) and D score (χ^2= 7.530, P = 0.023 and χ^2= 10.134, P = 0.006, for genotype distribution and allele frequency, respectively). No significant difference based on the genotype distribution and on the allele frequency of the *OBPIIa* locus was found between subjects classified as normosmic and hyposmic on the basis of the I score.

In order to assess whether the ability to detect volatiles depends on the *OBPIIa* locus genotype, we tested the effect of the *rs2590498* (A/G) polymorphism on the number of molecules smelled by subjects during the GC-O analyses, by means of one-way ANOVA. The mean value ± standard error (SE) of the number of molecules detected by subjects according to their polymorphism of the *OBPIIa* gene, are shown in figure 2. In detail, the statistical analysis indicates a significant effect of the *OBPIIa* locus genotype on both number of total-molecules ($F_{2,49}$ = 6.639; p = 0.003) and banana-molecules smelled by subjects ($F_{2,49}$ = 6.379; p = 0.004). Pairwise comparisons showed that subjects who were homozygous for the major allele A perceived a higher number both of total-molecules and banana-molecules than subjects who were heterozygous (p = 0.009 for total-molecules and p = 0.002

for banana-molecules; Fisher's LSD test) or homozygous for the minor allele G ($p = 0.001$ for total-molecules and $p = 0.006$ for banana-molecules; Fisher's LSD test).

Finally, one-way ANOVA revealed a significant effect of the *OBPIIa* locus genotype on the intensity value attributed to the banana odor pen ($F_{2,49} = 8.328$; $p < 0.001$; Fig. 3) and post-hoc comparisons showed that the intensity perceived by subjects that were AA homozygotes was significantly higher than that perceived by heterozygous ($p = 0.006$; Fisher's LSD test) and GG homozygotes ($p < 0.001$; Fisher's LSD test).

4. Discussion

The first aim of this study was to evaluate whether a relationship exists between the individual olfactory performance and the *rs2590498* (A/G) polymorphism of the *OBPIIa* gene. In fact, recent studies have shown that one of the causes of physiological variability in olfactory sensitivity can be attrubuted, at least in part, to the phenotypic manifestation resulting from the allelic diversity of the gene that codes for "human odorant-binding protein" *hOBPIIa* [24]. The results we obtained show a significant relationship between the polymorphism of *OBPIIa* and T scores. In particular, we found that subjects who obtained a higher T score were homozygous for the major allele A, while those who were heterozygous or homozygous for the minor allele G reached significantly lower T scores. No relationship, instead, was found between the polymorphism of *OBPIIa* and the D and I scores. In agreement, we also observed that the AA genotype is associated with a normosmic olfactory status, while the AG and GG one is mainly associated with a hyposmic status, considering both the general olfactory status and the specific one for threshold. These results confirm data already present in the literature; in fact, a different distribution of the genotype was found among subjects classified as normosmic or hyposmic: the former showing a higher orthonasal and retronasal olfactory performance were associated with the genotype AA and the dominant allele A, while the hyposmic subjects showing a reduced performance olfactory were heterozygous AG or homozygous for the minor allele G [24-25].

Since the ability to perceive individual molecules depends on the olfactory status of the subject[44] which in turn depends on the polymorphism of *OBPIIa*, the second objective was to test whether the number of molecules perceived by each subject when eluted from the chromatographic column during GC-O analysis depends on the *rs2590498* (A/G) polymorphism of the *OBPIIa* gene. The results show that the ability to perceive single molecules, both total and smelling of banana, depends significantly on the *OBPIIa* genotype. In particular, once again, the subjects who perceived the highest number of molecules were homozygous for the major allele A and this number was significantly higher than that perceived by heterozygous or homozygous for the minor allele G. These data are in agreement with the fact that the number of molecules smelled by each subject is correlated with his/her TDI and T score, which depend on the *OBPIIa* polymorphism.

It has been reported that humans perceive odors in the environment in which they live in a rather variable way and this applies not only to the overall olfactory performance, but also to the ability to perceive specific odors and to the way they are perceived [32,51]. In fact, the perceived intensity and the pleasantness reported for some odors vary widely between subjects [35]. We previously found [44] that the intensity reported for the banana-odor pen is positively correlated with the number of banana-molecules that each subject perceives during the GC-O analyses, which in turn depends on the *OBPIIa* genotype; for this reason, the third objective was to verify whether a relationship exists between the perceived intensity for the banana-odor pen and the *OBPIIa* genotype. The results also show that AA homozygous subjects reported a significantly higher intensity than heterozygous and GG homozygous subjects.

In conclusion, the results of this study on the one hand confirm that the physiological variations of general and specific olfactory performance are, at least in part, attributable to the polymorphism of *OBPIIa* and, on the other hand, show that this polymorphism can also influence the ability to perceive individual molecules, helping to explain interindividual variations in the number of “odor-active” compounds for each individual. Furthermore, these findings also suggest the need to carry out further analyses with a greater number of subjects to better understand the role of OBPs also in the ability to

discriminate and identify odors, as already suggested in other studies [16]. In fact, even if the *OBPIIa* genotype does not seem to influence the score obtained by subjects during the Sniffin' Sticks test, it becomes significant about the distribution of the subjects classified as normosmic or hyposmic for discrimination and identification and the allelic frequency, as shown by the results of this and previous studies [24].

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Conflict of interest

There are no financial and personal relationships with other people or organizations that may lead to a conflict of interest.

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Figures legend

Figure 1. Effect of the *rs2590498* (A/G) polymorphism of *OBPIIa* gene on the olfactory performance.

Mean values ± s.e.m. of odor Threshold (T), odor Discrimination (D) and odor Identification (I) score according to genotypes of the *OBPIIa* locus. N = 52 (AA = 16; AG = 14; GG = 22). Different letters indicate significant differences ($p<0.005$; Fisher's LSD test subsequent to one-way ANOVA).

Figure 2. Effect of the *rs2590498* (A/G) polymorphism of *OBPIIa* gene on the number of odor-active compounds.

Mean values ± s.e.m. of the number of total-molecules and banana-molecules perceived by subjects during the GC-O analysis according to genotype of the *OBPIIa* locus. N = 52 (AA = 16; AG = 14; GG = 22). Different letters indicate significant differences ($p<0.01$; Fisher's LSD test subsequent to one-way ANOVA).

Figure 3. **Effect of the *rs2590498* (A/G) polymorphism of *OBPIIa* gene on the intensity perceived.**

Mean values ± s.e.m. of intensity perceived for banana-odor pen by subjects according to genotype of the *OBPIIa locus.* N = 52 (AA = 16; AG = 14; GG = 22). Different letters indicate significant differences ($p<0.01$; Fisher's LSD test subsequent to one.way ANOVA).

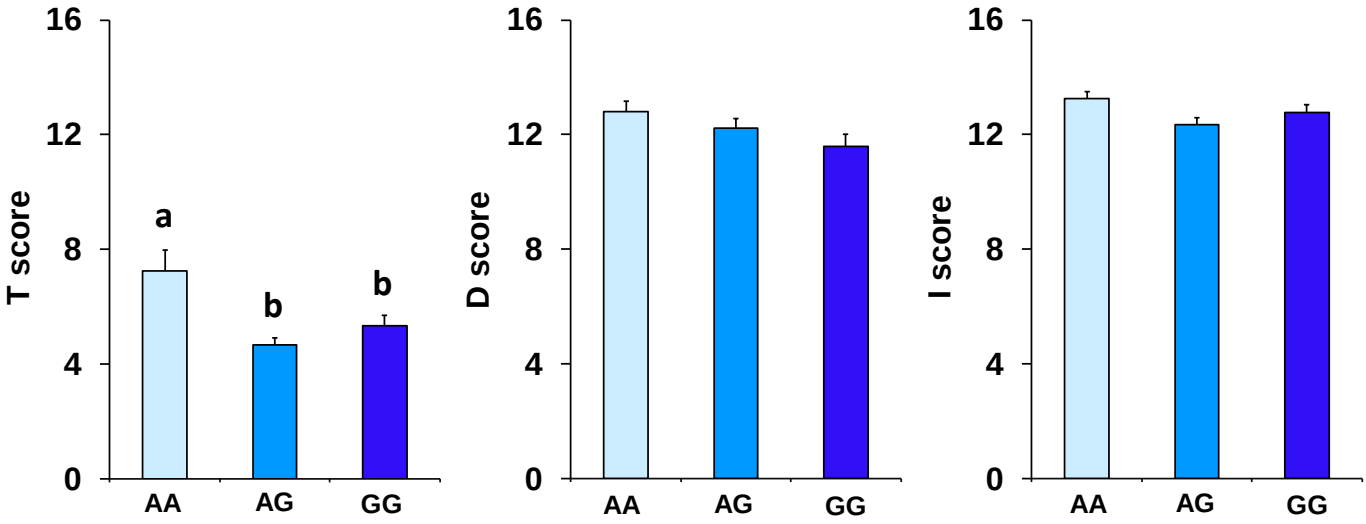
16
12
8
4
0
T score
a
b
b
AA
AG
GG
16
12
8
4
0
D score
AA
AG
GG
16
12
8
4
0
I score
AA
AG
GG

Figure 1

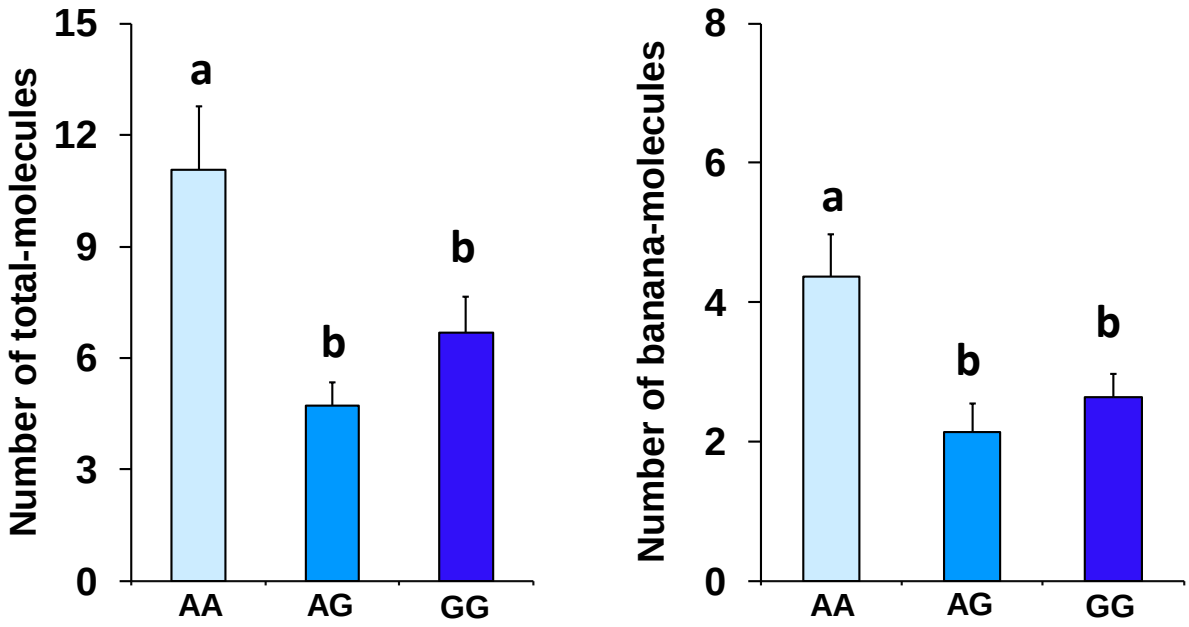
Number of total-molecules
15
12
9
6
3
0
a
b
b
AA
AG
GG
Number of banana-molecules
8
6
4
2
0
a
b
b
AA
AG
GG

Figure 2

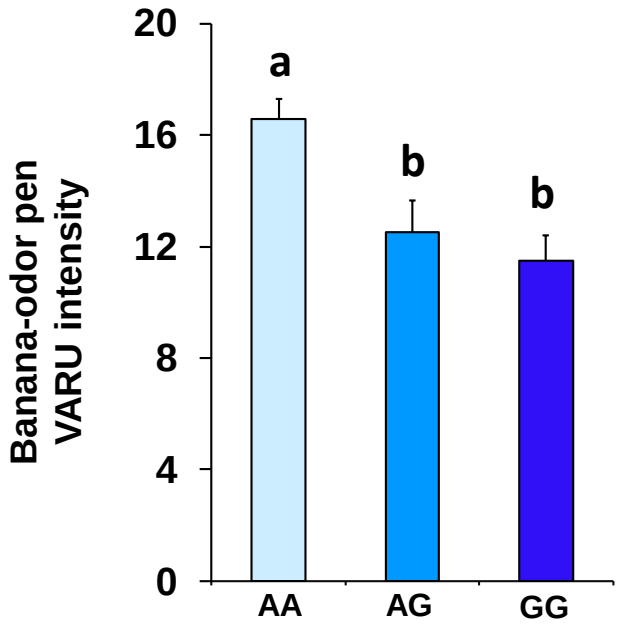

Figure 3

Table 1. Genotype distribution and allele frequencies of the *rs2590498* polymorphism of the *OBPIIa* gene (A/G) in subjects classified as normosmic or hyposmic on the basis of the TDI composite score obtained as the sum of results for Threshold, Discrimination and Identification, and also on the score obtained for T-test alone.

TDI	Normosmic n (%)	Hyposmic n (%)	P-value[a]
Genotype			0.003
AA	14 (51.85)	2 (8.00)	
AG	5 (18.52)	9 (36.00)	
GG	8 (29.63)	14 (56.00)	
Allele			< 0.001
A	33 (61.11)	13 (26.00)	
G	21 (38.89)	37 (74.00)	

T	Normosmic n (%)	Hyposmic n (%)	P-value[a]
Genotype			0.005
AA	13 (59.09)	3 (10.00)	
AG	2 (9.09)	12 (40.00)	
GG	7 (31.82)	15 (50.00)	
Allele			< 0.001
A	28 (63.64)	18 (30.00)	
G	16 (36.36)	42 (70.00)	

D	Normosmic n (%)	Hyposmic n (%)	P-value[a]
Genotype			0.023
AA	15 (34.09)	1 (12.50)	
AG	14 (31.82)	0 (/)	
GG	15 (34.09)	7 (87.50)	
Allele			0.006
A	44 (50.00)	2 (18.75)	
G	44 (50.00)	14 (81.25)	

I	Normosmic n (%)	Hyposmic n (%)	P-value[a]
Genotype			0.268
AA	16 (32.00)	0 (/)	
AG	14 (28.00)	0 (/)	
GG	20 (40.00)	2 (100)	
Allele			0.127
A	46 (46.00)	0 (/)	
G	54 (54.00)	4 (100)	

[a] P-value derived from Fischer's method.
Genotype AA: n = 16; Genotype AG: n = 14; Genotype GG: n = 22.
TDI = Threshold-Discrimination-Identification
T = Threshold; D = Discrimination; I = Identification.