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Taste and Smell Physiological Mechanisms and Their Health Implications

BIO/09 – Physiology

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ABSTRACT

Taste and olfaction play a key role in individuals' behaviors, their interactions with the environment and memory processes, furthermore, they represent the most important factors influencing food preferences and therefore eating behavior, diet, metabolism, and health. Physiological variations or disorders in these two sensory modalities can have significant effects on the life's quality or constitute risk factors for the onset of metabolism disorders, worsening nutritional status, overweight and obesity, or pathogenesis of different diseases. The purpose of this work was to investigate the function of taste and olfaction and their mechanisms controlling individual variability, and therefore nutrition and health. Firstly, we evaluate the degree of the peripheral taste function activation in response to the six taste qualities by electrophysiological recordings from human tongue and characterized its variability in relation with PROP phenotype (the most known example of taste variability genetically determined) and fungiform papillae density. The results, by showing that each taste quality evoked a specific monophasic depolarization in the human tongue whose amplitude was associated with PROP phenotype and fungiform papillae density, provided important information about the cellular organization and function of the human peripheral taste system that can explain the individual variability across taste qualities. Specifically, the electrophysiological responses to oleic acid were associated with *rs1761667* SNP in the *CD36* gene allowing to better understand the mechanisms involved in the choice of fat-rich foods.

Secondly, to extend our comprehension on mechanisms involving the salivary proteome in determining the individual taste variations, we evaluated the role of salivary proteins in the development of astringency and in affecting BMI, in the context of PROP taster status and gender. Results showed that variation in the salivary protein composition (increases of the acidic proline-rich proteins, aPRPs),

related to PROP taster status and gender, could influence variation in astringency perception or drive possible unbalance food habits which could lead to obesity. Furthermore, to understand if taste and olfaction impairments can be significant risk factors that contribute to the pathogenesis of different diseases, we analyzed the perception for six taste qualities, olfactory performance, and specific taste/olfactory genes, in relation with BMI, in patients with inflammatory bowel disease (IBD) and studied taste and smell sensitivity in severe obese subject that underwent to a bariatric surgery. Results showed that taste and olfaction impairments, explained by the oral pathologies and microbiome variations known for IBD patients, and the high frequency of non-taster allele in *CD36* polymorphism (*r1761667*), can justify their typical dietary behaviors, and thus they may be significant risk factors that contribute to the pathogenesis of IBD. Results also showed an overall improvement in taste and olfactory performance, an increase in cognitive restraint, and a decrease in disinhibition and hunger after bariatric surgery, which were associated with PROP phenotype. These findings indicate that bariatric surgery can have a positive impact on olfactory and gustatory functions and eating behavior (with an important role of genetic), which in turn might contribute to the success of the intervention.

Finally, since deficits in olfaction and taste have also been associated to many health markers including neurodegenerative diseases and specifically are among the most frequent non-motor manifestations in Parkinson's disease (PD), we focused on reviewed the most relevant molecular and genetic factors involved in these impairments and their associations with the microbiota, with the aim to highlight that the basis of these dysfunctions are likely multifactorial and may include the same determinants responsible for other non-motor.

In conclusion, these results show that the function of taste and olfaction and their genetic and molecular mechanisms are involved in the individual physiological variability which, in turn, control different biological process, such as food preferences, diet, nutrition and pathophysiological mechanisms, and disorders

or modifications in the sensitivity, by affecting physiological functions, may constitute risk factors for pathogenesis of different diseases.

Keywords: taste, electrophysiology, PROP phenotype, gustatory function, oleic acid, salivary protein, astringency, obesity, olfactory function, IBD, Parkinson disease, bariatric surgery, smell

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CHAPTER 1

INTRODUCTION

Human senses work as the connection between environment and consciousness by interacting with the external stimuli and sending information to the brain. Among all senses, taste and olfaction strongly influence food choice, eating behavior and therefore the nutritional status and health of the individual [1, 2]. Taste discriminates harmful compounds from nutrients food. This ability allows to avoid ingesting toxic substances or spoiled food, that are perceived as bitter and sour, respectively, and favors the acceptance of sweet compounds like glucose, that represents a source of energy [3]. The sense of smell warns for noxious volatiles in the environments or toxic food and plays an important role in hedonic pleasure, as it is associated to flavor construction. Olfaction is also important in recognition and evaluating food edibility and enjoyment since the majority of flavor information are carried through the nose [4, 5]. The distinction between toxic and nutrient-containing food was an evolutionary essential when humans had to choose from a broad variety of natural sources in the environment. Also, the evaluation of taste in the mouth leads the ultimate acceptance of a food before its ingestion [1].

Gustatory system

Taste can recognize five sensory qualities: sweet, sour, salty, bitter and umami. Each of these is believed to represent different nutritional or physiological requirements or indicate potential dietary risk so the primary taste categories reflect complementary strategies to obtain essential nutrients and avoid harmful compounds [6, 7]. Sweet, umami, and salty are associated with specific classes of nutrients and they are perceived as good and pleasant at low and moderate concentrations but are avoided at high concentrations [8]. Sweet taste usually indicates the detection of soluble carbohydrates that serve as an energy source. The taste of umami is associated with the taste of L-glutamate and a few other L-amino acids, reflects a food's protein content [3] and is induced by monosodium glutamate (MSG), disodium inosinate (IMP), or disodium guanylate (GMP) [9]. Umami is often

described as “savory” or “meaty”, although many foods in addition to meat contain these compounds. Salty taste indicates the presence of sodium (Na^+) and other salts, such as lithium or potassium [10], it is essential for maintaining the homeostasis in the body [11, 12]. In contrast, bitter and sour are associated with compounds that are potentially harmful. Indeed, the perception of bitter taste is associated with different compounds including alkaloids (e.g., caffeine, strychnine, quinine, and glycosides). It is also considered innate aversion and, it is thought, to guard against consuming poisons or of substances that can be toxic or inhibit digestion. Similarly, sour taste is important for detection of acid (i.e., free protons or H+ ions) and can be useful to avoid ingesting excess acids and overloading the mechanisms that maintain acid–base balance for the body [3]. Although, acceptable at low concentrations, sour taste elicits a rejection response at higher concentrations and can be used to detect unripe fruits and spoiled foods [11, 13].

Numerous studies include free fatty acid in the list of basic tastes by demonstrating an important involvement of taste in fat detection [14-17]. Although dietary lipids are mostly triglycerides, free long-chain fatty acids released from dietary lipids during oral processing seem to be accountable for fat taste perception [18, 19]. Dietary fats were supposed to have no taste of their own, but rather to be sensed through their texture (viscosity, lubricity, moistness) and odorant properties. However, when these properties are masked, humans are still able to discriminate between fatty acid and control solutions. Besides being a source of energy, fats are also required for synthesize a wide range of biological active compounds involved with neural function, epithelial integrity, blood clothing, and immune function. It is reported a variation in free fatty acid detective threshold based on length and saturation [20].

Taste in humans arises with the activation of taste cells, where taste receptors interact with gustatory stimuli. Taste buds are aggregates of 50-100 polarized neuroepithelial cells [21, 22] that form compact, columnar pseudostratified “islands” embedded in the surrounding stratified epithelium of

the oral cavity [3]. The human gustatory system includes approximately 5000 taste buds in the oral cavity, mostly situated on the superior surface of the tongue. Isolated taste buds are also scattered on the surface of the palate and throat and on the epiglottis [23]. On the tongue, taste buds are grouped in specialized structures called gustatory papillae of the lingual epithelium. Three different morphological structures of taste papillae are arranged on the tongue: fungiform papillae (mushroom-shaped) are situated on the anterior two-thirds of the tongue and are more densely concentrated toward the tip; foliate papillae (leaf-shaped) on the lateral sides; circumvallate papillae on the posterior two-thirds (Figure 1). There are also filiform papillae located across the entire superior surface, but these do not contain taste buds (i.e., they are non-gustatory). Every taste bud consists of a single apical pore where microvilli of taste receptor cells (TRCs) come into contact with tastants present within the oral cavity.

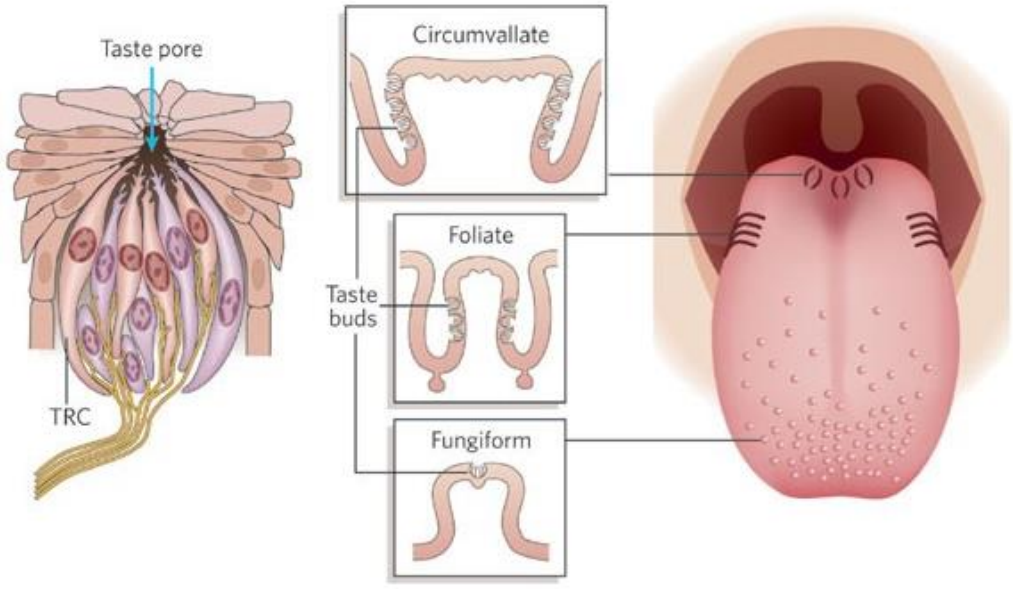

Figure 1. Lingual gustatory papillae and taste buds. Circumvallate papillae are found at the very back of the tongue and contain thousands of taste buds. Foliate papillae are present at the posterior lateral edge of the tongue and contain a dozen to hundreds of taste buds. Fungiform papillae contain one or a few taste buds and are found in the anterior two-thirds of the tongue. Taste receptor cells (TRC) project microvilli to the apical surface of the taste bud, where they form the 'taste pore', the site of interaction with tastants [24].

Each taste bud contains four cell types: Type I cells (or dark cell) are the most abundant cells in taste buds, with extended cytoplasm lamellae that surround

other cells. Since they appear to be involved in synaptic transmission and limiting the spread of transmitters, these cells are termed 'glial-like' [3]. In particular, the type I cells express different enzymes and transporters involved in neurotransmitter uptake from the extracellular space. GLAST, a transporter for glutamate, indicating that they may be involved in glutamate uptake [25], and NTPDase2, a plasma membrane–bound nucleotidase that hydrolyzes extracellular adenosine triphosphate (ATP) [26], have been found. Furthermore, type I cells may regulate the ionic environment [27, 28] by expressing ROMK, a K channel that may be involved in K^+ homeostasis within the taste bud [28]. During prolonged trains of action potentials elicited by intense taste stimulation, type I cells may serve to eliminate K^+ (see blue cell in Figure 2) that would accumulate in the limited interstitial spaces of the taste bud and lead to diminished excitability of Type II and III cells. Finally, type I cells may exhibit ionic currents implicated in salt taste transduction [29].

Type II (or light) cells are spindle-shaped cells with a large, round, clear nucleus. These cells were also renamed "receptor" cells [21] because of the presence, in the integral plasma membrane, of bitter, sweet, and umami receptors. Type II are considered the primary receptor cells in the taste bud [8, 21, 30-32]. As a matter of fact, G protein–coupled receptors (GPCR) with seven trans-membrane domains, specific for only one taste quality, are expressed [33]. Type II cells also express voltage-gated Na and K channels essential for producing action potentials, and secretion of ATP (yellow cell in Figure 2). In brief, Type II cells are "tuned" to sweet, bitter, or umami taste [34] but they do not appear to be directly stimulated by sour or salty stimuli [3].Every taste cell codifies for one specific taste receptor, which is located in apical microvilli in contact with the oral cavity.

Type III cells, which displays a slender profile, share many presynaptic neuron-like properties as they synapse with afferent sensory nerves (green cell in Figure 2) [21, 35]. These cells also express enzymes for the synthesis of at least two neurotransmitters and voltage-gated Ca^{2+} channels typically associated with

neurotransmitter release [21, 36] and showing depolarization-dependent Ca^{2+} transients. Like receptor cells, type III cells also are excitable and express a complement of voltage-gated Na^{+} and K^{+} channels to support action potentials [37-40]. In addition to these neuronal properties, they also respond directly to sour taste stimuli and carbonated solutions and are presumably the cells responsible for signaling these sensations [34, 41-43]. On the contrary to receptor cells, the presynaptic cells are not tuned to specific taste qualities but instead respond generally to sweet, salty, sour, bitter, and umami compounds [34].

Type IV cells are basal and a nonpolarized, presumably undifferentiated or immature taste cells (progenitor cells) [44]. Basal cells are small round cells at the base of the taste bud that are thought to be stem cells from which other cells are derived during cell turnover [35].

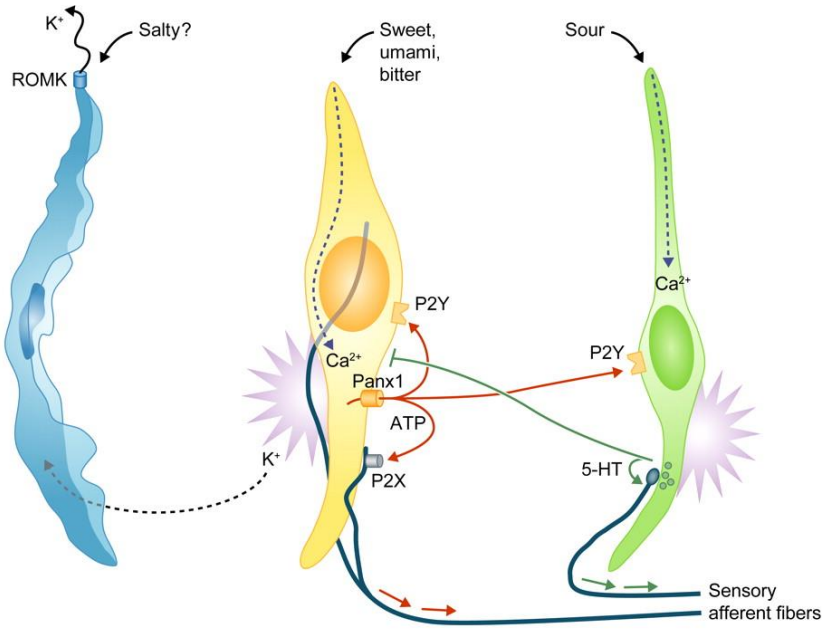

Figure 2. Schematic representation of the three major classes of taste cells and their communication patterns. Types I (blue), type II (yellow), and type III (green) taste cells [3].

The transduction pathways in the gustatory system involve a variety of mechanisms and appear to differ from mechanisms of the other senses [13, 35, 45]. Also, the six basic tastes are distinguishable in different pathways. Salty and sour taste sensations are both detected through ion channels. Sweet, bitter, and umami tastes, however, are detected by G protein-coupled taste receptors (GPCRs) with

seven transmembrane domains. In particular these qualities are detected by the two families of taste receptors TAS1R and TAS2R [46] located in the apical microvilli of Type II cells [30, 32, 33, 47-49]. The human TAS1R family contains just 3 genes, TAS1R1, TAS1R2, and TAS1R3. The sugar taste is identified by the receptor cells expressing the heterodimer TAS1R2/TAS1R3. A second class of receptor cells expresses the heterodimeric GPCR, TAS1R1/TAS1R3, which responds to umami stimuli, particularly the combination of l-glutamate and compounds that accumulate in many foods after hydrolysis of proteins [47, 48]. The bitter taste is identified by the TAS2R family of GPCRs [50]. There are 25 apparently functional TAS2R genes in humans encode members of the TAS2R family, whose products are responsible for bitter perception [50-52]. These taste receptors exhibit heterogeneous molecular receptive ranges: some are narrowly tuned to 2–4 bitter-tasting compounds, whereas others are promiscuously activated by numerous ligands [53]. Bitter sensing taste cells are known to functionally discriminate among bitter compounds [54]. This pattern of TAS2Rs expression, along with polymorphisms across the gene family, is thought to allow humans and animals to detect the vast range of potentially toxic bitter compounds found in nature [46]. Two GPCR have been documented to interact with fats, GPR40 and GPR120 (also known as free fatty acid receptors 1 and 4, respectively), but the most accepted theory identifies the multifunctional CD36 scavenger receptor to elicit elevation in intracellular Ca^{2+} levels when stimulated by fatty acid [55]. CD36 is localized in taste bud cells of circumvallate papillae but how the transporter couples to Ca^{2+} signaling is not yet known.

Taste receptors have similar signaling effectors: gustatory stimulus bonded at the taste receptors activates second messenger cascades to depolarize the taste cell. During sweet, umami, and bitter transduction, the G-protein subunit α-gustducin (Gα gustducin) participates in taste transduction (Figure 3A) [30, 33, 56-59]. The subunit Gγ13 is also involved in bitter taste transduction [60]. The pathway originates with the activation of GPCR [11]. Taking sugar taste into account, the

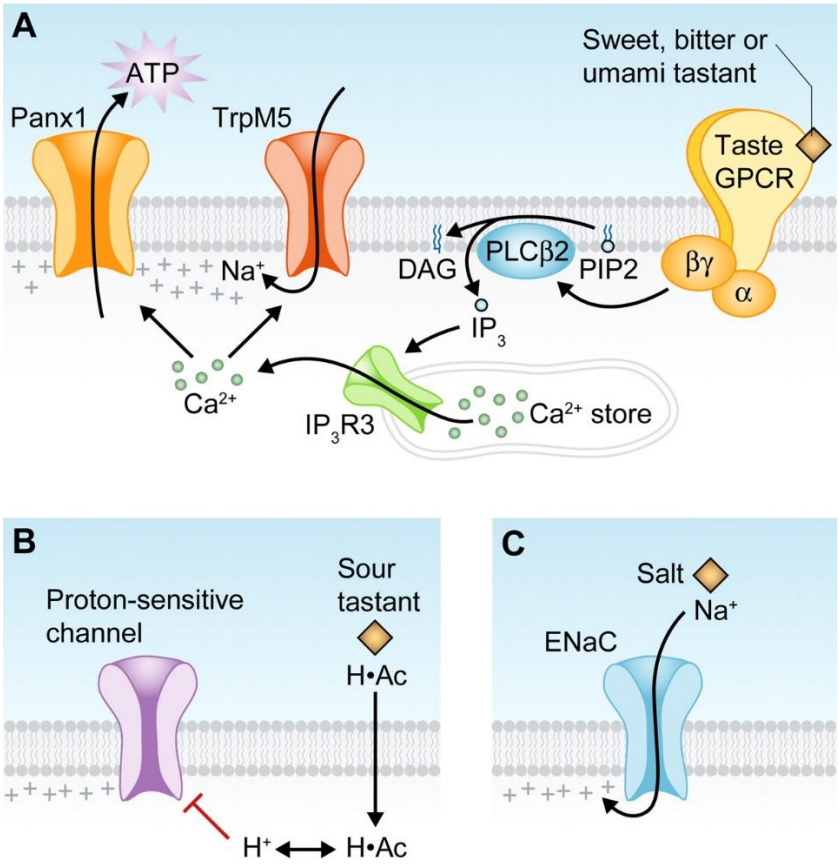

Figure 3. Mechanisms by which five taste qualities are transduced in taste cells for sweet, bitter and umami in receptor (Type II) cells (A); for sour in presynaptic (Type III) cells (B), for salty in Type I cells (C) [3].

activation of GPCR depolarizes the taste cell due to the activation of the intracellular second messenger cyclic Adenosine Monophosphate (cAMP), leading to transmitter release from taste cells, but how this happens is not known [61-63]. The other intracellular messenger involved are phospholipase Cβ2 (PLCβ2) and inositol 1,4,5-triphosphate (IP_3) [32, 64-67]. IP_3 receptors, located in the endoplasmic reticulum, release Ca^{2+} from intracellular stores. Increased intracellular Ca^{2+} activates transient receptor potential cation channel- subfamily M member 5 (TRPM5), which is a cation-permeable channel that allows the entry of sodium ions (Na^+), finally leading to cell depolarization [13, 66-69]. The combination of depolarization resulting from Na^+ and Ca^{2+} opens pannexin channels (Panx) in the taste-cell membrane, releasing ATP from the cell. The role of ATP is not completely clear. The ATP secreted from receptor cells in turn activates purinergic receptors on the sensory nerve fibers innervating the taste buds, thereby sending a signal to the brain [3]. At the same

time, Type II cells secrete ATP via paracrine pathways to excite adjacent presynaptic cells (Type III), stimulating them to release serotonin and/or norepinephrine [23,70] and eliciting afferent nerve output [13, 35].

Salty and sour taste transductions take place in the microvilli of taste cells and along the basolateral membranes [70]. Sour taste stimuli (acids) are detected by a small subset of cells such as presynaptic cells [34]. The hypothesis for sour taste receptors relies on non-selective cation channels formed by PKD2L1 and PKD1L3 [41, 71, 72]. This channel is sensitive to extracellular pH instead of cytoplasmic pH, which is known to be the direct stimulus for sour taste (Figure 3B) [42, 73]. There is evidence that the organic acids such as acetic acid, which are not fully dissociated at physiological pH values, can directly permeate through the plasma membrane of Type III cells, and acidify the cytoplasm and thereby elicit an electrical response. According to this mechanism, intracellular hydrogen ions inhibit or block a proton-sensitive K channel (normally function to hyperpolarize the cell). By a combination of direct intake of hydrogen ions (which itself depolarizes the cell) and the inhibition of the hyperpolarizing channel, sourness causes in the taste cell trigger action potentials and release neurotransmitter. The complete transduction pathways which detect sour taste are still not completely understood [3].

Taste buds detect Na salts by directly permeating Na^+ through apical ion channels. This ion channels are named amiloride-sensitive epithelial Na channel (ENaC) (Figure 3C) [11, 74-76]. Permeation of Na^+ determines the depolarization of taste cells. ENaC is also permeable to H^+ ions, so the transduction of substances that are perceived as sour is due to an input of these ions through amiloride-sensitive Na^+ channels. Sour and salty, in relation to their concentrations in the saliva, in part interfere with each other at peripheral [77]. The differences are observed in the ability to perceive the taste of different salts of Na^+ could also depend on the different permeability of the respective anions through the tight junctions and the consequent ability to affect other ion channels localized at the level of the basal lateral membranes of taste cells. Most of the transduction mechanisms determines

the depolarization of the membrane of the taste cell (receptor potential), which in turn determines increase of the concentration of Ca^{2+} for opening of voltage-dependent channels or for mobilization from intracellular stores. The increase in the Ca^{2+} causes the exocytosis of chemical mediator and the consequent transmission of the signal (make synapses) to the primary gustatory afferent fibers.

Three cranial nerves transmit sensory input from taste buds to the brain. The anterior two-thirds of the tongue and palate are innervated by facial nerve (cranial nerve VII). The taste buds of fungiform papillae and foliate papillae are innervated by the chorda tympani, a branch of the facial nerve [78-81]. The posterior third are innervated from the lingual branch of glossopharyngeal nerve (cranial nerve IX). The region around the throat, including the glottis, epiglottis, and pharynx, receive branches of the vagus nerve (cranial nerve X). In general, each fiber can respond to all gustatory qualities, although with a different intensity. The first synapse within the gustatory system is at the terminals of the sensory afferent fibers and individual synaptic cells [82]. The central axons of these primary sensory neurons in the respective cranial nerve ganglia project to rostral and lateral regions of the nucleus of the solitary tract in the medulla, which is also known as the gustatory nucleus of the solitary tract complex (Figure 4). From the gustatory nucleus, neurons project to the ventral posterior medial nucleus (VPM) of the thalamus. This nucleus projects to several regions of the cortex, including the anterior insula and frontal operculum (gustatory cortex) in the ipsilateral cerebral cortex. The gustatory cortex is responsible for conscious discrimination of gustatory stimuli [82-84]. Destruction of the insula causes ageusia, the total inability to perceive any compounds [85]. Finally, reciprocal projections connect the nucleus of solitary tract via the pons to the hypothalamus and amygdale. These projections presumably influence appetite, satiety and other homeostatic responses associated with eating. Projections from the gustatory cortex are also managed anteriorly to the dysgranular caudolateral region of the orbitofrontal cortex where they join with those from the visual and olfactory areas. It is here that the convergence of visual,

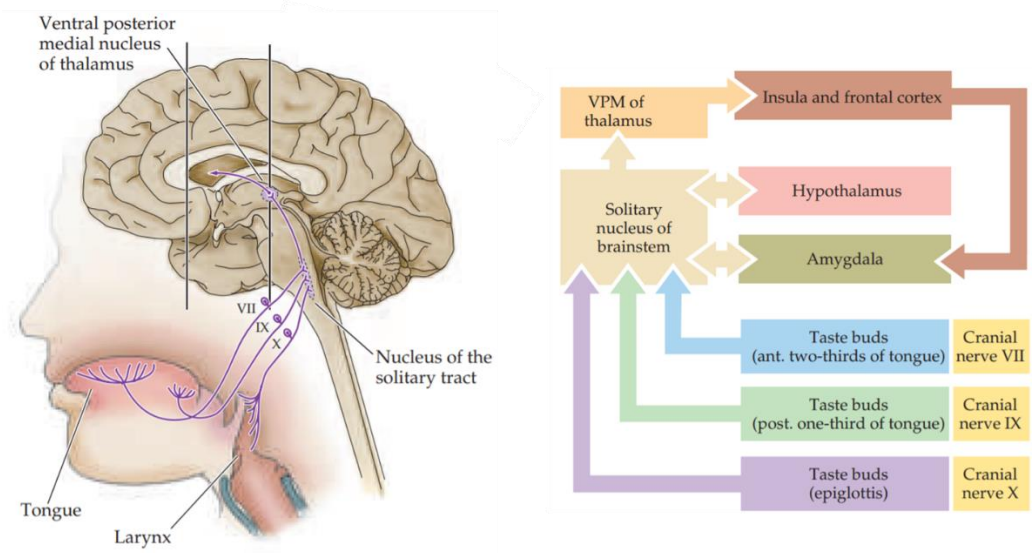

Figure 4. Organization of the human taste system. Left: drawing on the left shows the relationship between receptors in the oral cavity and upper alimentary canal, and the nucleus of the solitary tract in the medulla. The section on the right shows the VPM nucleus of the thalamus and its connection with gustatory areas of the cerebral cortex. Right: diagram of the basic pathways for processing taste information [86].

olfactory, and gustatory sensory input allows for an awareness of flavor, which is the combination of taste, olfaction, and somatosensory perception (such as texture and pain) [84, 87, 88]. Indeed, flavor is an integral part of food recognition, helping us to gauge whether a food is suitable to eat and its potential for providing hedonic pleasure and enjoyment.

Aside from the aforementioned taste qualities, additional sensory perceptions have been described. Pungency is the irritating, heat and/or pain sensation elicited by capsaicin from chili pepper, zingerone from ginger, and allyl isothiocyanate from horseradish. This compound seems to activate transient potential ion channels (TRPs) that are expressed in nociceptor located in the oral cavity and carried through the trigeminal nerve [89-92]. Additionally, astringency is an everyday sensation that is experienced with consumption of polyphenol-rich foods. It is marked by drying, roughing, and puckering of the oral surfaces [93] and is commonly associated with foods such as green tea, coffee, cocoa, berries, and red wine. The most widely accepted model for astringency is based on the interaction

between polyphenols and salivary proteins which form hydrogen bonds or hydrophobic interactions which evolves in insoluble precipitates [94-97].

Olfactory system

The sense of smell detects and discriminate a multitude of chemicals in identity and concentration through a variety of highly specialized receptors. The olfactory system has the function of localize and evaluate food, it has a role in communication (e.g., reproductive behavior, detection of fear-related cues, recognition of kin, recognition of disease) and in detection of danger. While consuming a food or drinking, volatile compounds reach the olfactory epithelium in the nose first by orthonasal stimulation, and while chewing, molecules from the food move via retronasal pathways [98].

Olfactory perception starts at the level of the pseudostratified olfactory epithelium in the roof of the nasal cavity. Human nose consists of three turbinate, curved bone projection that direct the air flow towards the olfactory epithelium. Olfactory Sensory Neurons (OSN), the sensory unit that perceive odors, are bipolar neurons that extends a dendrite to the luminal surface of the epithelium, where non-motile sensory cilia are enriched with olfactory receptors that captures chemical volatiles from the inhaled air. Their axon pass through the cribriform foramina projecting into the Olfactory Bulb (OB) (Figure 5) [98]. Together with 10 to 20 million of OSN, the olfactory epithelium includes non-neural cells that have a role in supporting olfactory system and in maintaining the integrity of the olfactory epithelium. In the apical layer, sustentacular cells and microvillar cells, which have microvilli rather than cilia, support the OSN playing a glial-like function by enwrapping OSN dendrites and metabolizing foreign substances that contact the epithelium, even though their function is not well understood. [99-101]. In the basal layer, ductal cells of Bowman's glands produce and secrete a specialized glycoprotein-rich mucus into the nasal cavity [102], protecting the epithelium from

drying out, providing trophic and functional support to neural cells, offering physical and chemical protection from external agents. The mucus is enriched with polysaccharides, immunoglobulins, and various enzymes. Bowman's glands also synthesize a number of olfactory-binding proteins (OBP) that facilitate transport of odorant to OSN [103]. Finally, basal stem cells in the basal layer are capable of differentiating into either supporting or sensory cells, giving the epithelium the ability to regenerate and replace themselves in a life-circle that lasts for 30-60 days, or upon injury [104, 105].

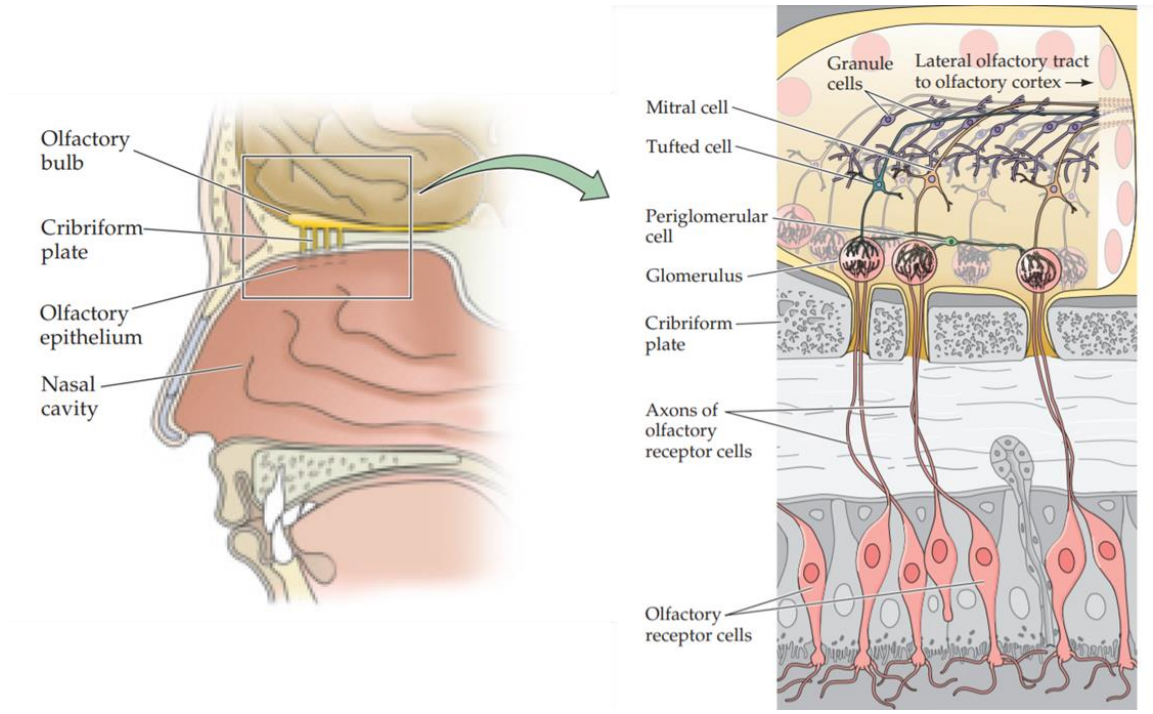

Figure 5. Organization of the human olfactory system. (A) Peripheral and central components of the primary olfactory pathway. (B) Diagram of the olfactory epithelium and of the Olfactory bulb [86].

Olfactory receptors (OR) are the chemoreceptors for odor detection in the nasal cavity. OR belong to the multigene family of GPCR, and they are characterized of seven-transmembrane domains. According to Human Genome Project, there are nearly 900 genes that codifies for OR, bur half of them are pseudogenes or non-functional genes [106]. There exist approximately 400 different OR that can be expressed by humans, thanks to the extensive genetic sequence diversity within the transmembrane domains. In particular, the major regions of divergence are those

that codify for the third, fourth and fifth transmembrane domains where the ligand binding site is located. The variation in the binding site allows to interact with a large range of odorant, and their identification and classification [107]. The reason for a large number of receptors may have the evolutionary explanation to provide a system that discriminate as many odors as possible.

Each OSN expresses just one specific OR in the membrane of its cilia, and each receptor detects limited number of odorant substances [108, 109]. Nevertheless, each OR is activated by a number of similar odorant structures, and each odorant binds to multiple receptors to generate specific activation pattern [110, 111]. Similarly to taste signal transduction, the binding of an odorant by the OR activates second messenger cascades to depolarize the olfactory neuron: the G-protein subunits $G\alpha_{olf}$ catalyzes the synthesis of cAMP, whose increased concentration leads to an influx of Na^+ and Ca^{2+} ions and depolarization of the neuron by opening a cyclic nucleotide-gated channel (Figure 6) [112, 113]. Subsequently, increasing concentration of cation trigger calcium-activated chloride channels, which elicits the efflux of Cl^- from the cell. The odor-induced depolarization spread through the neuron until it reaches the presynaptic terminal, where neurotransmitters are released [114].

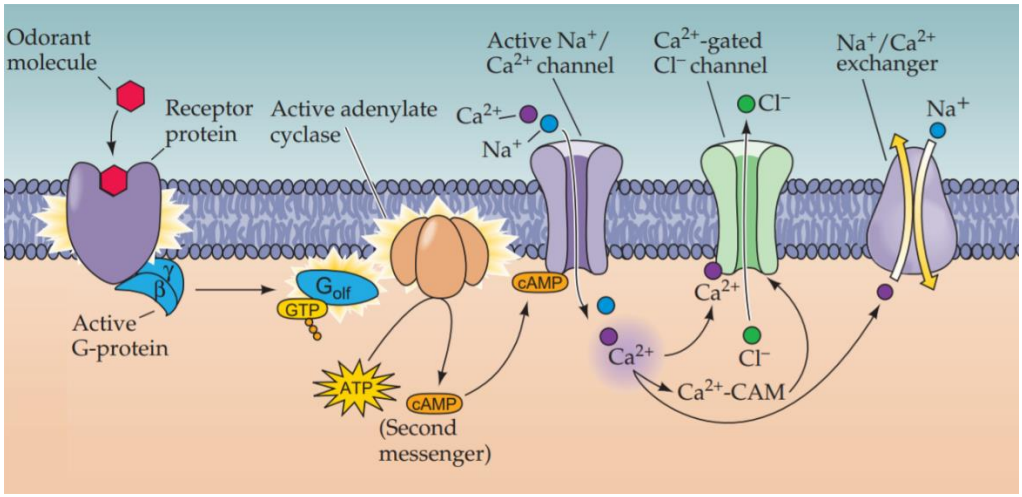

Figure 6. Signal transduction in the OSN. Representation of the receptors, enzymes, and ion channels—present in the olfactory cilia—that transduce activity of the odorant receptor (OR) into changes in membrane potential [86].

All OSN carrying the same OR converge in the same glomerulus in the bulb. Mitral cells in the OB combine the impulse from a glomerulus and send it to the primary Olfactory (or piriform) Cortex, involved in representing the intensity and identity of odors. OB takes has an important role in information processing and refining through two class of lateral inhibitory interneurons, periglomerular cells and granulate cells. The inhibition is specific to one glomerulus and inhibit principal neurons via dendrodentritic GABAergic synapses, or via dopaminergic synapses on axon of OSN (Figure 7) [115]. Besides it is believed that the interneurons increase sensitivity of each glomerulus, their function is still not clear. Olfaction is the only sensory signal that avoid the direct connection with the thalamus, and OB is linked to the cortex [116]. Only after had reached the olfactory cortex, primary projections go into orbitofrontal cortex (OFC) for odor processing and into the thalamus. The amygdala has close networks with primary olfactory areas. Indeed, among the stimuli from all sensory modalities that activates the area, amygdala nuclei respond more quickly to odor stimuli [117].

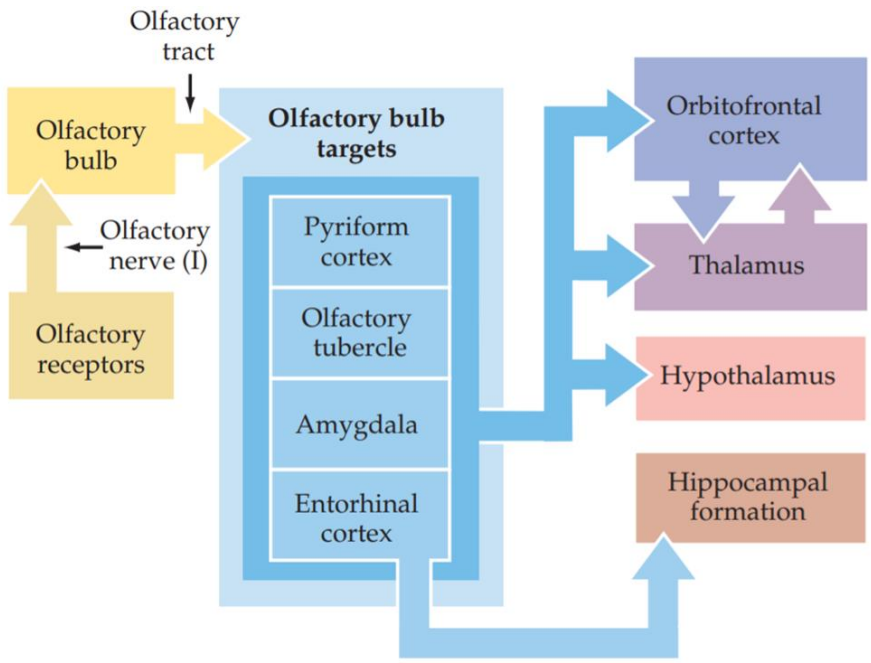

Figure 7. Diagram of the basic pathways for processing olfactory information [86].

Retronasal olfaction is the secondary pathway through odors are perceived in the nose. It plays a key role in flavor perception because of its additional interaction with other sensory modalities (i.e., taste and trigeminal sensation) [118]. Orthonasal sensation has a lower threshold [119], it is more efficient to identify suprathreshold [120] when compared to retronasal sensation, and they differ in the neuronal processing of either signal [121].

Odorant in the air flow that reaches the nose can dissolve in aqueous medium or be hydrophobic molecules that need to cross the mucus barrier in order to activate OSN. In the latter case, OBP are small water-soluble proteins which are supposed to transport hydrophobic odorants towards olfactory cilia by binding them reversibly. Three-dimensional structures of OBP are conservative, with numerous β-barrel along their sequence, and an α-calyx in the C-terminal which form a central non-polar cavity for molecules binding [122]. Also, OBP concentrate odorants in the mucus layer, and they seem to remove the perceived odorant for degradation. Although the role in olfactory perception has been documented, OBP exact function is uncertain. OBPIIa is the only binding-protein that was found in the human mucus [123]. The *rs2590498* (A/G) polymorphism of the gene is partially responsible for variation of olfactory performances both in terms of the ability to perceive complex odors and single molecules [124-126].

Individual variability of taste sensitivity

Taste sensitivity significantly varies in humans and the individual differences strongly influence food choice and satiety [127]. The physiologic role of taste variability could be related to evolutionary adaptation to specific environments to recognize substances potentially dangerous or necessary for bodily function [128]. For example, it is known that bitter taste plays a dual role in human nutrition as warning signal and as attractant. Some plants produce a large diversity of bitter-tasting compounds as protection against predation [129]. Since strong bitter taste is associated with the presence of toxic substances, the ability of humans to detect bitterness at low concentrations represents an important evolutionary adaptation for limiting or avoiding the consumption plant foods that could be harmful [127]. However, several classes of bitter polyphenols, such as tannins, catechins and anthocyanins (from grapes, tea, coffee, dark-colored fruit, citrus and chocolate), and glucosinolates from cruciferous vegetables [130] provide positive health benefits by acting as anti-bacterial and antioxidant [131].

The ability to taste the bitter thiourea compounds, phenylthiocarbamide (PTC) and 6-n-propylthiouracil (PROP) is a well-studied human trait [132]. Thiourea compounds contain the thiocyanate fraction (N-C=S) which is responsible of their bitter taste [133, 134] (Figure 8). The N-C=S group is also characteristic of glucosinolates and goitrin, naturally occurring substances commonly found in cruciferous vegetables such as broccoli, cabbage, cauliflower and Brussels sprouts (Brassica oleracea), and other plants of the Brassica family [135]. Goitrin has potent anti-thyroid properties and can be toxic when consumed in large quantities by populations at-risk for thyroid deficiency [136]. Moreover, these vegetables exhibit potent anti-cancer effects [135]. Depending on regional influences (over) ingestion of such vegetables can have positive as well as negative health effects for individuals which might lead to balancing selection for *TAS2R38* gene variants [101]. One interesting explanation for the persistence of this trait in humans is that

it served as an evolutionary adaptation to local eating environments [102]. Larger rejection of Brassica plants would provide survival advantages to those who were more sensitive to their bitter taste [103].

Individual variability in sensitivity to the bitter taste of PTC was first recognized by Fox more than eight decades ago [133]. Based on threshold methods, PROP sensitive and non-sensitive individuals are defined as tasters and non-tasters, respectively. The frequency of non-tasters varies among populations, from as low as 7% to more than 40% [137]. In the Caucasian population, the estimated frequency of non-tasters is 30% [138-142]. Bartoshuk [142, 143] first used the term "supertaster" to distinguish individuals who perceived PROP as extremely bitter from those who perceived PROP as moderately bitter. Although numerous studies support the classification of individuals into three phenotypic groups (non-tasters, medium tasters and super-tasters) [140, 144-151], other work suggests that PROP tasting may be a more continuous phenotype [144, 148, 152]. The ability to taste PROP is associated with haplotypes of the *TAS2R38* gene defined by three single-nucleotide polymorphisms that result in three amino acid substitutions (Pro49Ala, Ala262Val, and Val296Ile) [147, 153] (Figure 8). There are two common haplotypes: PAV is the dominant (sensitive) variant and AVI is the recessive (insensitive) one. Non-tasters are homozygous for the AVI haplotype, and it was assumed that supertasters were homozygous for the PAV haplotype and medium tasters were heterozygous for the PAV haplotype. However, studies have reported considerable genotypic overlap between the medium and super-taster groups [140, 147, 153] with substantial numbers of super-tasters carrying the PAV/AVI diplotype. Other work suggests that the presence of two PAV alleles (as opposed to one) confers no additional advantage for perceiving more bitterness intensity from PROP, at least in the suprathreshold (above threshold) range [148]. Thus, TAS2R38 genotypes do not completely explain the oro-sensory differences between medium and super-tasters. In fact, TAS2R38 genotype predicts the majority (55-85%) but not all of the

phenotypic variance in PROP threshold, implying that epigenetic factors may be involved in the expression of the trait [52, 127, 141, 148].

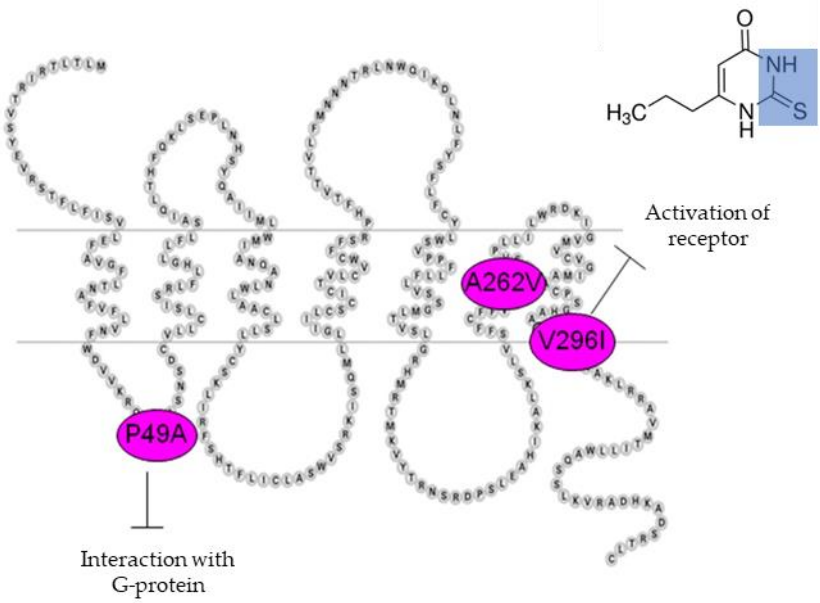

Figure 8. Schematic representation of TAS2R38 receptor and the three polymorphisms associated with different level of sensibility. Chemical structure of 6-n-propyltiouracil (PROP). The isothiocyanate chemical group is highlight in blue.

Indeed, evidence supporting the presence of modifying genes comes from studies using a variety of approaches (family segregation, family-based linkage, and genome-wide association studies [154-156]). Nevertheless, studies have consistently reported that super-tasters have a higher density of fungiform papillae on the anterior tongue surface when compared to the other groups [143, 157-159]. These anatomical differences could partially explain the greater oral responsiveness of supertasters to a range of oral sensations that are not mediated via bitter taste receptors. Recently, the role of the gustin (CA6) gene, which codifies for a trophic factor for taste bud development, has been studying as a function of PROP phenotype [160]. We showed that polymorphism *rs2274333* (A/G) of the gustin gene led to a modification gustin's primary structure which is crucial for zinc binding and full functionality of the protein [160]. The AA genotype (associated with a fully functional protein) was more frequent in super-tasters, whereas the GG genotype (associated with a disruption in the protein) was more frequent in non-tasters. These

data suggest that variation in gustin may be associated with differences in papillae densities and oral chemosensory abilities across PROP taster groups.

Contemporary studies in human nutrition have also revealed that PROP bitterness might also serve as a general marker for oral sensations and food preferences. This assumption is based on data showing that those who perceive PROP/PTC as more bitter are also more responsive than non-tasters to various oral stimuli, including other bitter-tasting compounds [142, 146, 161-165], sweet substances [166], chemical irritants [147, 167], and fats [144, 145]. Given the nutritional value of dietary lipids, the relationship between PROP bitterness intensity and acceptance or perception of fats is of particular interest. Several studies reported that PROP non-tasters had a lower ability to distinguish fat content in foods, showed a higher acceptance of dietary fat [144, 152, 168, 169] and consumed more servings of discretionary fats per day than did tasters [168]. These findings have led to the hypothesis of an inverse correlation between PROP status and body mass index (BMI) which is supported by several studies [140, 170-172]. However, PROP is not the only determinant in fat perception. It is known that the CD36 protein whose expression is controlled by the *CD36* gene and regulated by its allelic diversity have a key role. The exchange of A for G in the *rs1761667* SNP has been shown to decrease protein expression [173], and it is associated with a reduced oral ability to perceive fatty acids [17, 174, 175]. Ethnic-specific effects were also observed in one experiment where East Asians, but not Caucasians, with the AA genotype showed a reduced ability to perceive fatty acids [176]. The substitution of A for G in this SNP has also been shown to influence fat preference [177].

Some authors suggested that PROP-related sensory variations may be associated with olfactory function [125, 158], and that PROP tasting may affect the perception of foods via aromas or flavors [144, 178]. However, other reports show no associations between PROP taster status and these variables [158, 179-182]. This lack of consensus suggests that other factors contribute to feeding behavior, food perception and preference in PROP taster groups. For example, chemical-physical

composition of saliva can contribute to individual variations of taste [133, 139]. Salivary proteins play important role to affect sweet [183] salt [184], umami [185] taste, liking for fat and salt [186] and bitter acceptance [187]. Variations of the salivary proteome have been shown in human responses to bitter stimuli such as calcium nitrate, urea or quinine [188, 189]. Our laboratory has been studying the involvement of salivary proteins in the sensitivity in perceiving the prototypical taste stimulus PROP [190-192]. We demonstrated that PROP super-tasters have higher basal levels of two basic proline-rich proteins (bPRPs: Ps-1 and II-2), and PROP stimulation increase the levels of the same proteins with respect to basal levels, only in PROP super-taster subjects. In addition, the supplementation of amino acids of the sequence of these proteins (i.e. L-Arg and L-Lys) enhance the sensitivity for PROP and for other taste qualities [191-194]. The proposed mechanism that describes the permissive role of these proteins or amino acids in taste perception, depending on their concentration in saliva, indicates that they could act as "carriers" of tastants, by increasing their solubility in saliva and thus the availability to taste receptor sites [192].

Furthermore, salivary proteins seem to be implicated in evoking the astringency sensation [195-198]. These proteins are mainly identified as acidic proline-rich proteins (aPRPs), histatins (Hist), cystatins (S-Cyst) and statherin (Stath). Other proteins also participate in this phenomenon, such as mucins, glycosylated and bPRPs, which are mostly adsorbed onto oral surfaces and are essential in providing oral lubricity and protecting the salivary pellicle against damage and microbial insult [199, 200]. Interaction of polyphenols with these proteins also forms large aggregates, eventually eroding the protective lubricating layers. Together, these actions generate the astringency sensation [201, 202], which, in evolutionary terms, might serve as a warning cue against toxicity from over-consumption of these plant materials. The time course of astringency perception and oral recovery are poorly understood. It is known that astringency following oral stimulation takes anywhere from 100 s to 300 s or even longer to recede [93,

203-205], although the complementary work examining the salivary protein response over time is limited [206]. The intensity of an astringent sensation, its quality (i.e., the predominance of different sub-qualities such as drying, roughing, puckering) and time course depends on the type and concentration of the stimulus used [93, 207, 208]. Different types of polyphenols (e.g., grape seed tannins, catechins) as well as metal salts and organic acids have been used in astringency studies, but they produce different profiles of astringency perceptions [209, 210]. This diversity suggests that there may be more than one mechanism underlying the astringency response. However, our understanding of astringent sensation remains incomplete.

Taste and Olfactory Impairments controlling Nutrition and Health

Since taste and olfaction represent the most important factors influencing food preferences and therefore eating behavior and diet, disorders in these two sensory modalities can have significant effects on the life's quality [211]. Unbalanced eating habits can be consequences of a reduction of the gustatory and olfactory sensitivities or blunted brain reward activation in response to palatable food, which has been observed in people with overweight and obesity [212-217], as well as in pre-clinical models of obesity [218] which is a complex and multifactorial disease that originates from a combination of social, cultural, environmental, genetic, behavioral, metabolic and endocrinological factors. Obesity condition is correlated with eating habits characterized by higher preferences for energy dense foods, such as fats and sweets [219-222], which lead to greater consumption of these kind of foods [223-225]. Reductions of the taste sensitivity for sweet [226], umami [227], bitter and sour [225], and fatty acids [228], and impaired olfactory performance have been observed in people with obesity [225, 229-232]. Moreover, overall olfactory function in obese group, separately analyzed for threshold, discrimination, and identification ability, was shown to be lower than in control group [231].

However, bariatric surgery is considered the gold standard for the dealing of morbid obesity, leading to sustained body weight reduction, improvements in metabolic health and comorbidities, and decreased mortality [233-236]. The Roux-en-Y gastric bypass (RYGB) and sleeve gastrectomy (SG) represent the most effective long-term treatments for severe obesity and, respectively, account for 17.8% and 59.4% of all bariatric procedures [237]. Patients with obesity who undergo bariatric surgery report changes of taste, smell, appetite and food preferences [238-240]. Specifically, after undergoing surgery, patients report a preference for low calorie foods [241], reduced interest for sweet and high fat food [212, 242-247] and a specific aversion for sweet, high calorie foods and meats [239,

248, 249]. For example, while some authors found that patients became more sensitive to bitter and sour tastes (but not sweet or salty) following surgery [250], others found increased sensitivity to sweet [248, 251], but not bitter taste [248]. Yet, others found no changes in taste sensitivity or perceived intensity of sweetness, saltiness, or savoriness [246, 252]. Interestingly, using taste strip test in patients who mostly underwent SG, Holinski et al. found improved taste identification after 6 months of surgery [253]. A result that was replicated by Altun and collaborators in patients who were evaluated before and after 3 months of SG [254]. In comparison to surgery-related changes in taste function, less studies assess olfactory function, albeit there is discrepancy on study findings for smell as well. Using Sniffin Sticks, Holinskly and collaborators found that olfactory function improved by 6 months post-surgery to a level that was close to that of normal-weight subjects [253], and Hanci and colleagues found improvements of the threshold, discrimination and identification parameters of smell function [255]. However, Jurowich and colleagues found only an improvement of the threshold [256], and Enck and collaborators found no changes in either threshold, discrimination or identification [257]. Although these findings on the taste and smell changes following bariatric surgery are unclear and limited, they suggest that variations in gustatory and olfactory function could underly changes in eating choices [244], the reduction in the consumption of high-calorie foods and therefore, contribute to the success of the intervention.

In the past several decades, taste and smell disfunctions have been also associated to gastrointestinal inflammations like Inflammatory Bowel Disease (IBD), which is a chronic and relapsing inflammatory pathology of the gastrointestinal tract. The main clinical phenotypes are Crohn's disease (CD) and Ulcerative Colitis (UC). Accumulating evidence suggests that diet is one of the most significant risk factors that contribute to the pathogenesis of IBD [258]. Some studies found increased risk of IBD associated with the consumption of foods that are high in sugar, animal fats and oils, and protein [258-265]. On the other hand, high intake

of foods rich in fiber, including fruits and vegetables, has been shown to protect against IBD [259, 261, 262, 266-268]. In addition, it is known that some nutrients enhance intestinal barrier function and host immunity, which in turn protects against this disease [258]. Accordingly, diet recommendations that encourage the consumption of plant-based foods and de-emphasize excess consumption of sugar and animal foods may be a successful strategy for treatment of IBD. However, published data on taste changes in IBD patients are controversial [269-274]. Some authors have reported a reduction in overall taste function [274] or reductions limited to specific qualities [269, 272, 274], while others reported no taste impairments in these patients [273]. In addition, some studies reported that IBD patients with alterations of taste function show zinc deficiency [271, 272, 275, 276], which may be linked to the functionality of the zinc-dependent salivary enzyme gustin/CAVI [272]. Major gaps still exist in our understanding of taste mechanisms in IBD. Furthermore, recent studies have highlighted a relationship between olfactory function and autoimmune/inflammatory diseases such as rheumatoid arthritis, psoriasis, myasthenia gravis, Sjogren's syndrome and IBD [274, 277-281].

Additionally, smell and taste disorders accompany many neurodegenerative illnesses [282] and are important for their early and differential diagnosis. They are present with an incidence raging between 50 and 96% of patients with Parkinson's disease (PD) [283-285], which is a chronic neurodegenerative disorder with a prevalence of 1% in subjects aged 60–69, increasing to 3% in those over 80 years of age [286]. Pathologically, the disease is characterized by dopaminergic neuronal loss in the substantia nigra, and it is associated with intracellular inclusions, called Lewy bodies, in the neurons of affected brain regions. The Lewy bodies are intra-cytoplasmic eosinophilic deposits of a misfolded protein, α-synuclein which spreads to different regions of the brain in a prion-like fashion, giving rise to the successive non-motor and motor symptoms [287-289]. Clinically, PD is characterized by the presence of motor symptoms such as bradykinesia (slow movement), rigidity, tremor, postural instability (balance

problems), difficulty with walking, and coordination. In addition, PD is also characterized by the occurrence of several non-motor symptoms like sleep disturbances, apathy, anxiety, autonomic dysfunction, gastrointestinal dysfunction (such as nausea, dysphagia, abnormal salivation, constipation and defecatory dysfunction [290, 291]), cognitive impairment, olfactory and gustatory dysfunctions [292, 293]. Specifically, olfactory dysfunction is accepted to be an early biomarker of the disease since precedes the occurrence of clinical motor symptoms [294], with an incidence raging between 50 and 96% [283-285].

Aim of the study

The purpose of this PhD thesis was to investigate the function of taste and olfaction and their mechanisms controlling individual variability, and therefore nutrition and health.

Firstly, we evaluate the degree of the peripheral taste function activation in response to the six taste qualities by electrophysiological recordings from human tongue and characterized its variability in relation with PROP phenotype and fungiform papillae density (**chapter 2a**). We hypothesized that important information about the cellular organization and function of the human peripheral taste system could be obtained with the method of taste recordings from the tongue which are not influenced by factors interpreted at the level of the cortex and that the amplitude of electrophysiological responses was associated with PROP phenotype and fungiform papillae density. The electrophysiological responses to oleic acid were also characterized in relation with *rs1761667* SNP in the *CD36* gene with the aim of better understanding the mechanisms involved in the choice of fat-rich foods.

Secondly, to extend our comprehension on mechanisms involving the salivary proteome in determining the individual taste variations, we evaluated the role of salivary proteins in the development of astringency and in affecting food choice and BMI, in the context of PROP taster status and gender (**chapter 2b**). Our hypothesis was that variation in the salivary protein composition, related to PROP taster status and gender, could influence variation in astringency perception or drive possible unbalance food habits which could lead to obesity.

Furthermore, the third aim of this work was to understand if taste and olfaction impairments that can govern unbalanced food choices can be significant risk factors that contribute to the pathogenesis of different diseases (**chapter 3**). To this aim, we analyzed the BMI, perception for six taste qualities, olfactory performance and specific taste/olfactory genes in patients with IBD and studied

how a bariatric surgical treatment could modify taste and smell sensitivity and eating behavior in severe obese subjects.

In addition, we reviewed molecular and genetic factors involved in the PD-related smell and taste impairments, which also may represent risk factors associated with the disease.

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CHAPTER 2

PHYSIOLOGICAL BASIS OF INDIVIDUAL TASTE VARIABILITY

2a. Human tongue electrophysiological response as innovative and objective approach to evaluate the degree of the peripheral taste function activation

*Sollai G, Melis M, **Mastinu M**, Pani D, Cosseddu P, Bonfiglio A, Crnjar R, Tepper BJ, and Tomassini Barbarossa I. Human Tongue Electrophysiological Response to Oleic Acid and Its Associations with PROP Taster Status and the CD36 Polymorphism (rs1761667). Nutrients 2019. 11(2), 315, https://doi.org/10.3390/nu11020315*

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In this part of work, we present results on the degree of the peripheral taste function activation in response to the six taste qualities by electrophysiological recordings from human tongue and on characterization of its variability in relation with PROP phenotype and fungiform papillae density.

Materials and Methods

Participants

For this study, two groups were formed: Group 1 was composed of thirty-nine subjects (11 males, 28 females, age 28.31 ± 1.03 years), while Group 2 was of thirty-five volunteers (15 males, 20 females, age 28.6 ± 0.86 years). Volunteers were recruited according to public advertisement at Cagliari University. All volunteers were non-smoking Caucasian, originally from Sardinia, Italy. Although the sample size was not pre-determined by statistical analysis, the number of subjects was comparable to the one already employed in electrophysiological procedures which assess the degree of activation of the receptor under study [1]. The following selecting criteria were used: subjects had normal body mass index (BMI) ranging from 20.2 to 25.2 kg/m^2; none had food allergy, were dieting, or taking drugs that might affect overall sensory perception. To exclude any taste impairment, their taste function for the four basic tastes was screened by the taste strip test (Burghart Messtechnik, Wedel, Germany). Both groups were formed as to have three similar equal-sized PROP-taster subgroups, matched for gender and age. However, for the Group 2 it was not possible to construct equal sample size within each of the *CD36* genotype/phenotype subgroups due to the high frequency of AG heterozygotes at the *rs1761667* SNP in the gene among Caucasian, as reported in 1000 Genomes (dbSNP Short Genetic Variations, 2017). After an explanation of the experimental procedure, subjects read and signed an informed consent form. This study was conducted following the latest revision of the Helsinki Declaration, and all procedures have been approved by the Ethical Committee of the University

Hospital Company (AOU) of Cagliari, Italy. The trial was registered at ClinicalTrials.gov (identifier number is UNICADBSITB-1).

Experimental Procedure

Subjects were separately tested in two morning sessions on two consecutive days. They were requested to be present in the testing room 15 min before the session in order to adjust to the regular room conditions (23–24 °C; 40–50% relative humidity), and they were always asked to abstain from eating, drinking (except water) using oral care products or chewing gum for 2 h prior the testing. To prevent taste sensitivity modification due to the estrogenic phase, women were tested around the sixth day of the menstrual cycle. During the first session, they were classified for PROP Taster Status, while the electrophysiological response to taste stimuli was recorded and density of fungiform papillae was tested on the second day. After the first visit, a sample of whole saliva (2 mL) was collected from each subject belonging to Group 2 in an Eppendorf tube, and immediately stored at -80 °C for the molecular analysis described below.

The study design is shown in Figure 1.

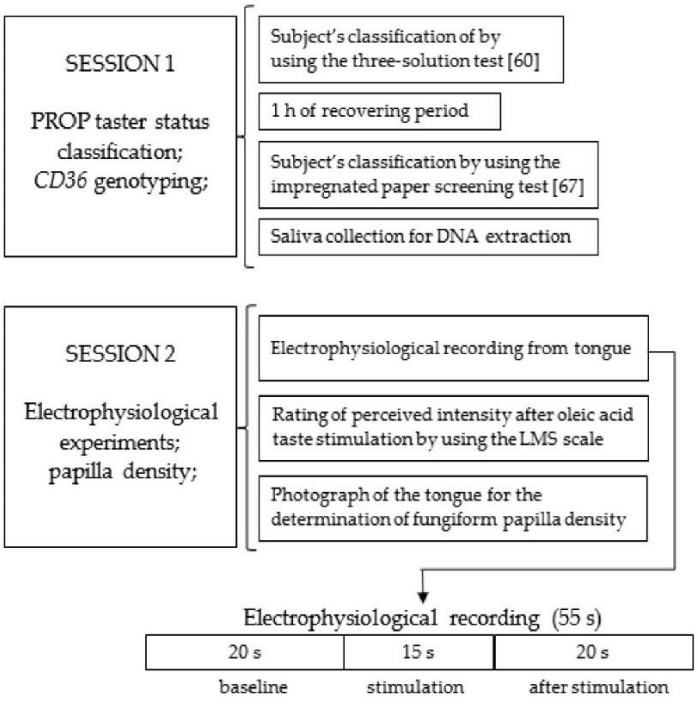

Figure 1. A graphic diagram representing the study design.

PROP Taster Status Classification

Subjects were classified for their PROP taster status using two scaling methods. Firstly, they were all assessed using the three-solution test according to Tepper et al. 2001 [2]. The test request to rate the perceived intensity of three suprathreshold sodium chloride (NaCl; 0.01, 0.1, 1.0 mol/L) (Sigma-Aldrich, Milan, Italy) and PROP (0.032, 0.32, and 3.2 mmol/L) (Sigma-Aldrich) solutions by using the Labeled Magnitude Scale (LMS) [3]. The LMS is a 100-mm semi-logarithmic scale in which seven labeled verbal descriptors are positioned along the length of the scale. The verbal labels and their positions on the LMS are: barely detectable, 1.4 mm; weak, 6.1 mm; moderate, 17.2 mm; strong, 35.4 mm; very strong, 53.3 mm; and strongest imaginable, 100 mm. The LMS gives subjects the freedom to evaluate the perceived taste intensity for a taste stimulus comparing it to the strongest imaginable oral stimulus ever experienced in life.

The stimuli were diluted in spring water. The solutions were prepared the day before the session, stored at 4 °C and left at room temperature 1 h prior the test. Solutions (10 mL samples) were presented in a random order. Subjects were demanded to wash their mouth with spring water after every solution and the interstimulus time was set at 1 min. Subjects who gave lower intensity ratings to PROP than to NaCl were classified as PROP non-tasters, those who gave higher ratings to PROP than to NaCl were classified as super-tasters, while those who gave overlapping ratings to both PROP and NaCl were classified as medium tasters. NaCl was used as a control because taste intensity to NaCl does not change with PROP taster status in this test. After 1 h of break, the classification of each subject as belonging to a PROP taster group was confirm by mean of the impregnated paper screening test [4, 5]. Briefly, two paper disks, one impregnated with PROP solution (50 mmol/L) and the second with NaCl (1.0 mol/L) were sequentially presented by placing the paper disk on the tip of the tongue for 30 s. Subjects were instructed to taste the paper disk as if it was a candy, then spit it out. The perceived intensity

ratings were collected by using the LMS. Subject who rated the PROP disk lower than 15 mm on the LMS were categorized as non-tasters; those who rated the PROP disk higher than 67 on the LMS were categorized as super-tasters; all others were classified as medium tasters. Volunteer who scored doubtful results were excluded for the study. The classification results for Group 1 and Group 2, which were documented by three-way ANOVA are shown in Table 1 together with the basic anthropometric of the PROP taster groups.

		Age (years)	**Female/Male**
Group 1	Super-Taster (n = 10)	27.20 + 2.07	7/3
	Medium Taster (n = 15)	28.87 + 1.87	9/6
	Non-Taster (n = 14)	28.43 + 1.75	12/2
Group 2	Super-Taster (n = 10)	25.2 + 2.6	6/4
	Medium Taster (n = 13)	29.2 + 1.8	7/6
	Non-Taster (n = 12)	26.5 + 2.6	7/5

Table 1. Anthropometric features of subjects classified for PROP taster status among Group 1 and Group 2.

Electrophysiological recordings

Differential electrophysiological recordings from the tongues of volunteers were performed between two silver electrodes according to Sollai et al. 2017 [1]. As shown in Figure 1, Electrode 1 in contact with the ventral surface was a silver wire (0.50 mm) (WPI Sarasota, USA) with a curved distal end to form a small ball (5 mm dia) in order to obtain good electrical contact and make the electrode harmless with respect to possible injury or irritation of the sublingual mucosa. Electrode 2 was placed in perfect adhesion with the dorsal surface on the left side of the tip of tongue. It was made by depositing a silver film (100 nm thick) on a thin (13 μ m) polyimide layer (Kapton©), by means of an evaporation technique in high vacuum. A film (2 μ m thick) of insulating and biocompatible material (Parylene C) was deposited by chemical vapour deposition in vacuum, in order to cover both sides of

the electrode except for a circular area (A) at its distal end which, after positioning the electrode, allowed free access to the tongue surface. This circular hole (6 mm dia) was the area where taste stimuli were delivered, and the density of fungiform papillae was calculated. The material's thickness allowed a perfect adhesion on a dry tongue. A third disposable electrode placed on the skin of the subject's left cheek, an electrically neutral position with respect to the observed phenomenon, was used as a ground terminal of the instrument. The biopotentials were bipolarly recorded with the polygraph Porti7, which is a portable physiological measurement system for human use (TMS International B.V., The Netherlands). Porti7 is a Class IIa medical device whose channels with high-input impedance ensures that the current drained from the subject is minimal and the perturbations in the signal are limited. The signal recording started after reaching a stable baseline, and a stimulus was delivered in the circular area which gives access to the tongue. The recorded signals were digitalized and sent to the PC for a real-time visualization, pre-processing and recording through the Polybench software. For each subject, the recording lasted 55 s: 20 s baseline, 15 s during taste simulation, and 20 s after stimulation, i.e., after the paper disk was removed. Subsequently, the original signal was filtered to reduce high frequency interfere, and extract in a smoother trend that kept its accuracy, using Matlab (The MathWorks, Inc.). Clampfit 10.0 software (Molecular Devices, Sunnyvale, CA, USA) was used to determine amplitude values (mV) in response to taste stimuli at 0.1, 2.5, 5, 10 and 15 s with respect to baseline. The rate of potential variation (mV/s) was also calculated at the same time intervals.

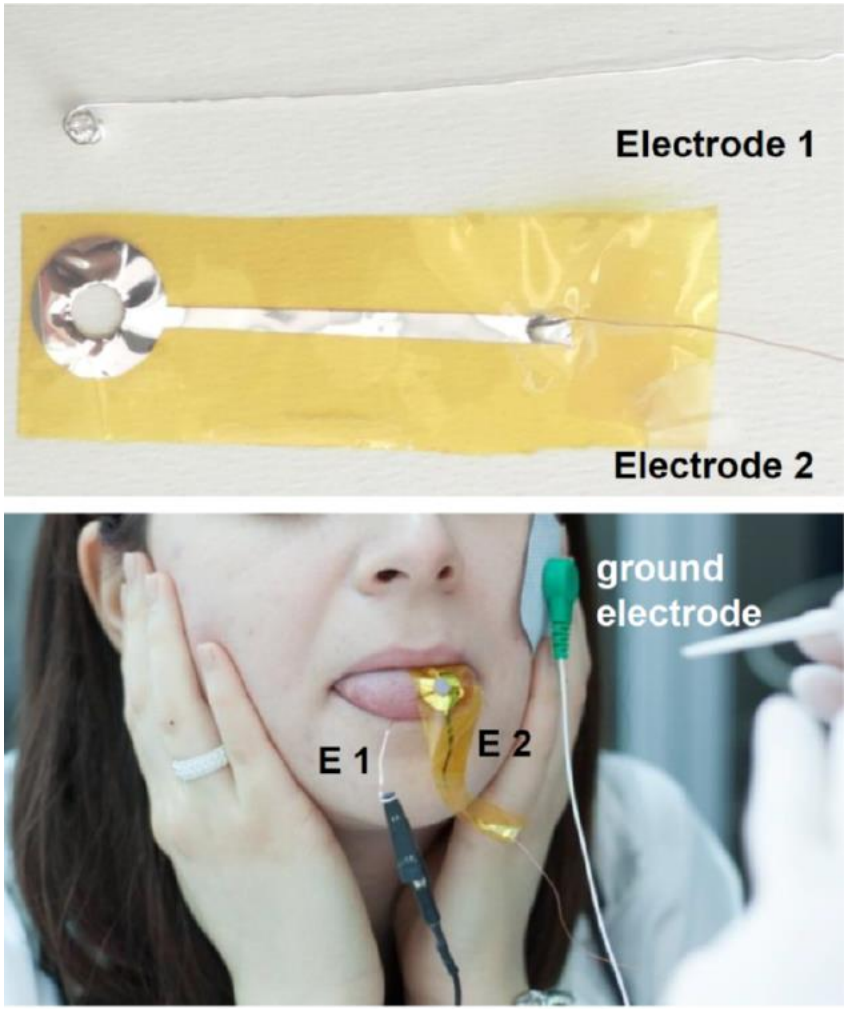

Figure 2. Photographs of the two electrodes and their application in the tongue

Taste Stimulation

Taste stimuli were delivered by placing paper disk (6 mm dia.) impregnated with 30 μL of solutions on the circular area of the tongue surface which was left free by the hole of the second electrode. The paper disk was removed after 15 s. Subjects belonging to Group 1 tasted 30 μL of undiluted oleic acid and solution of sucrose (200 mM), NaCl (200 mM), citric acid (5.2 mM), caffeine (10 mM), and monosodium glutamate (MSG) (160 mM), chosen to represent the five primary taste qualities, while subjects belonging to Group 2 only tasted 30 μL of undiluted oleic acid. Solutions, diluted in spring water, were prepared 1-2 days before each session, stored in refrigerator, and presented at room temperature. The concentration for each stimulus was selected based on preliminary tests. Each subject tasted each

stimulus in a double-blinded and counterbalanced order, with an interstimulus interval set at 1 h. Finally, the perceived intensity of the chemosensory perception was rated by using the LSM. Dry paper disks were used as control to verify whether the potential variation was influenced by a mechanical stimulation.

Density Assessment of Fungiform Taste Papillae

Fungiform papillae density was assessed in the same area where the taste stimuli were delivered, according to Melis et al. 2013 [6]. This region was chosen to provide the most reliable measurement of papilla density in high correlation with the papillae total number [7]. The tongue was dried from the saliva, and the left side of the anterior surface was colored by using a blue food dye (E133, Modecor Italiana, Cuvio, Italy). After removing the excess of dye, a series of photographs were taken using a Canon EOS D400 (10 megapixels) camera with a lens (model: EF-S 55–250 mm). The digital images were analyzed using Adobe Photoshop 7.0 software. The fungiform papillae were separately identified and counted by three trained operators who did not know about the PROP taster status of the subjects. Finally, the number was calculated as density/cm^2.

Molecular Analysis

Subject belonging to Group 2 they were genotyped for *rs1761667* (G/A) single nucleotide polymorphism (SNP) of *CD36* gene. DNA was extracted from saliva samples using the QIAamp® DNA Mini Kit (QIAGEN S.r.l., Milano, Italy) according to the manufacturer's instructions. Purified DNA concentration was estimated by measurements at an optical density of 260 nm. A polymerase chain reaction (PCR) was employed, followed by analysis with restriction enzyme (HhaI) of the fragments obtained according to Banerjee et al. 2010 [8]. Digested fragments were separated by electrophoresis on a 2% agarose gel and the bands of DNA were visualized by ethidium bromide staining and ultraviolet light to mark the deletion.

Statistical Analysis

Statistical analyses were conducted using STATISTICA for WINDOWS (version 10; StatSoft Inc., Tulsa, OK, USA). Data were verified for the assumptions of normality, homogeneity of variance, and sphericity if applicable. All data are presented as mean value ± Standard Error of the Mean (SEM).

Differences of mean values of the signal amplitude (mV) and the potential variation rate (mV/s) at 2.5, 5, 10, and 15 s, which were evoked by the six taste qualities in subjects belonging to Group 1, and by oleic acid stimulation in those belonging to Group 2, were analysed using Repeated-measures ANOVA. The same differences were also analyzed across PROP taster status, and across *CD36* genotype for Group 2. For Group 1, differences in perceived intensity rating for the six taste qualities in super-tasters, medium tasters, and non-tasters were compared using two-way ANOVA while one-way ANOVA was used across PROP taster groups and *CD36* genotype groups, to compare mean values of the perceived taste intensity of oleic acid stimulation in Group 2. One-way ANOVA was also used to compare differences in density of fungiform papillae according to PROP taster status. Post-hoc comparisons were conducted with the Fisher LDS test, unless the assumption of homogeneity of variance was violated, in which case the Duncan's test was used.

Linear correlation analysis was applied to investigate the relationship between the density of fungiform papillae and perceived intensity in response to oleic acid taste stimulation. Linear correlation analysis was also used to elucidate the relationships between signal amplitude (mV) and biopotential variation rate (mV/s) with density of fungiform papillae, or perceived taste intensity of oleic acid.

In order to test CD36 genotype distribution and allele frequencies according to PROP taster status for subjects belonging to Group 2, Fisher's method (Genopop software version 4.0) [9] was used. p values < 0.05 were considered significant.

Results

The differential electrophysiological recordings from the human tongue by mean of two electrodes, allowed the measurement of monophasic bioelectrical potential changes in response to taste stimulations, with respect to the baseline. Thus, the analysis of the waveform of bioelectrical potentials showed that taste stimulations evoked positive monophasic potentials characterized by a faster initial rise followed by a slower phase, which continued for the whole duration of stimulation, while NaCl stimulation evoked depolarizing (negative) monophasic potential variations. However, each stimulus determined a characteristic time course of the potential change during stimulation and the amplitude values grandly varied among subjects. Figure 3 shows examples of electrophysiological recordings in a super-taster subject. Dry paper stimulation did not evoke any potential change.

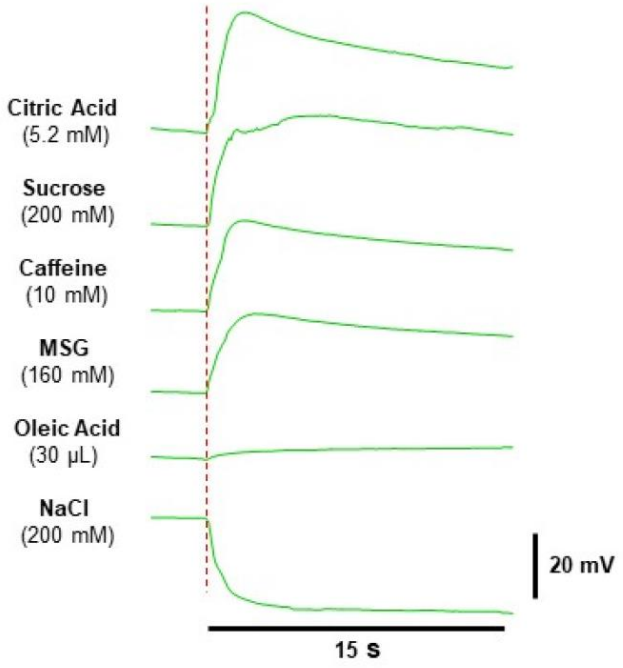

Figure 3. Examples of electrophysiological recordings in a super-taster subject in response to 30 µL of citric acid, sucrose, caffeine, NaCl, and monosodium glutamate solutions or oleic acid. The very first data point on the left side of the electrophysiological recordings represents the baseline.

Figure 4 shows the mean values of the potential amplitude (mV) and of potential change rate (mV/s) determined after 0.1, 2.5, 5, 10, and 15 s from the

stimulus application in response to the six taste qualities in subjects belonging to Group 1.

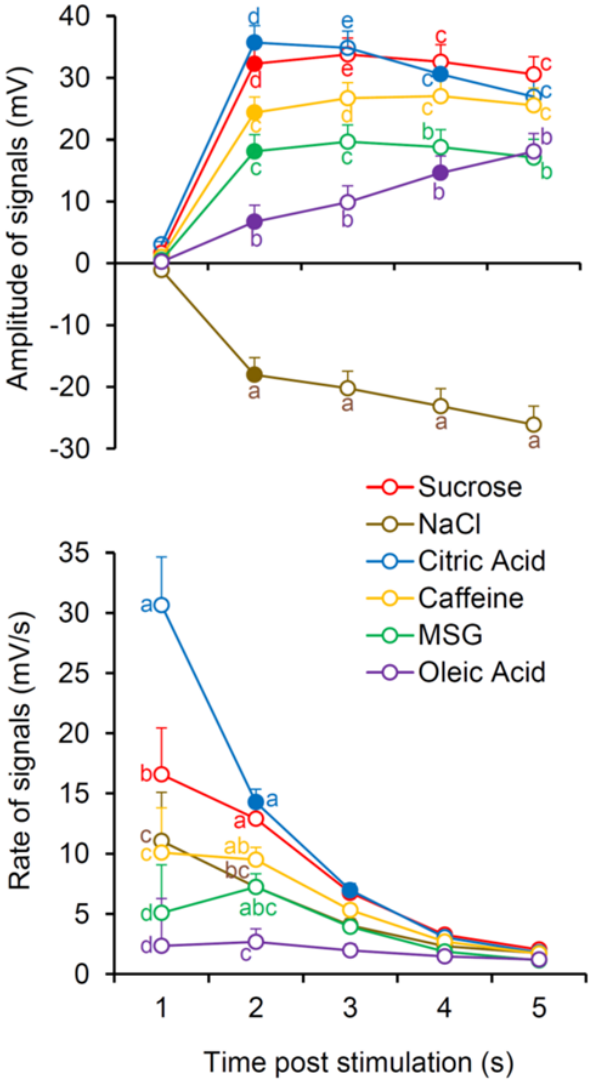

Figure 4. Time course of potential amplitude (mV) and of potential change rate (mV/s) of bioelectrical signals in response to taste stimulation with six taste qualities. Data (mean values ± SEM) determined after 0.1, 2.5, 5, 10, and 15 s (expressed in numbers from 1 to 5 respectively) after application of taste stimulation are shown. Group 1, n = 39. Solid symbol indicates significant difference with respect to the previous value of the corresponding stimulus ($p \leq 0.0016$; Fisher LDS after repeated-measures ANOVA). Different letters indicate a significant difference with respects to another stimulus at the same time ($p \leq 0.041$; Fisher LDS, after repeated-measures ANOVA).

Repeated-measures ANOVA revealed that the time course of the amplitude and rate of potential change during stimulation time depended on taste stimulus

(amplitude: $F_{20,820} = 37.840$; $p < 0.0001$ and rate: $F_{20,820} = 5.5235$; $p < 0.0001$). Post hoc comparison showed that all stimuli produced substantial potential changes, with respect to baseline, after 2.5 s from stimulus application ($p \leq 0.0016$; Fisher LDS). The biggest positive potential changes were recorded in response to sucrose and citric acid ($p < 0.0001$; Fisher LDS), which the latest significantly decreased at 10 s ($p = 0.0388$; Fisher LDS); caffeine and MSG evoked intermediate changes ($p < 0.0001$; Fisher LDS), and together with sucrose did not change during stimulation time ($p > 0.05$). Oleic acid response was the lowest positive potential change which slowly continues to increase (significantly at 10 s; $p = 0.0198$; Fisher LDS) until the end of stimulation. ($p = 0.0016$; Fisher LDS). Negative potential changes were recorded in response to NaCl ($p < 0.0001$; Fisher LDS) whose did not change during the stimulation time ($p > 0.05$). Analysis of the potential rate revealed that citric acid evoked the quickest potential change at 0.1 s ($p < 0.0001$; Fisher LDS), which rapidly decreased during the recording. The lowest values were observed with MSG and oleic acid ($p \leq 0.026$; Fisher LDS) which stayed unchanged over time.

The time course of same values of the potential amplitude (mV) and of potential change rate (mV/s), stratified for PROP taster status, recorded in subjects belonging to Group 1 after stimulation of the six taste qualities are reported in Figure 5. Time course of the amplitude of bioelectrical signals during stimulation time depended on taste quality and PROP taster status of subjects ($F_{40,772} = 2.1256$; $p < 0.0001$ Repeated-measures ANOVA). After 2.5 s from stimulation, all stimuli evoked significant potential changes in all subjects ($p \leq 0.00013$; Fisher LDS), except for oleic acid in the non-taster group ($p > 0.05$). PROP super-tasters showed larger amplitudes than other PROP taster groups. Larger signal amplitudes in super-tasters in response to NaCl and sucrose at 2.5, 5, 10, and 15 s ($p \leq 0.042$; Fisher LDS), as well as larger signal amplitudes in response to MGS and citric acid than those of other taster groups at 2.5 and 5 s ($p \leq 0.038$; Fisher LDS) were recorded. Super-tasters

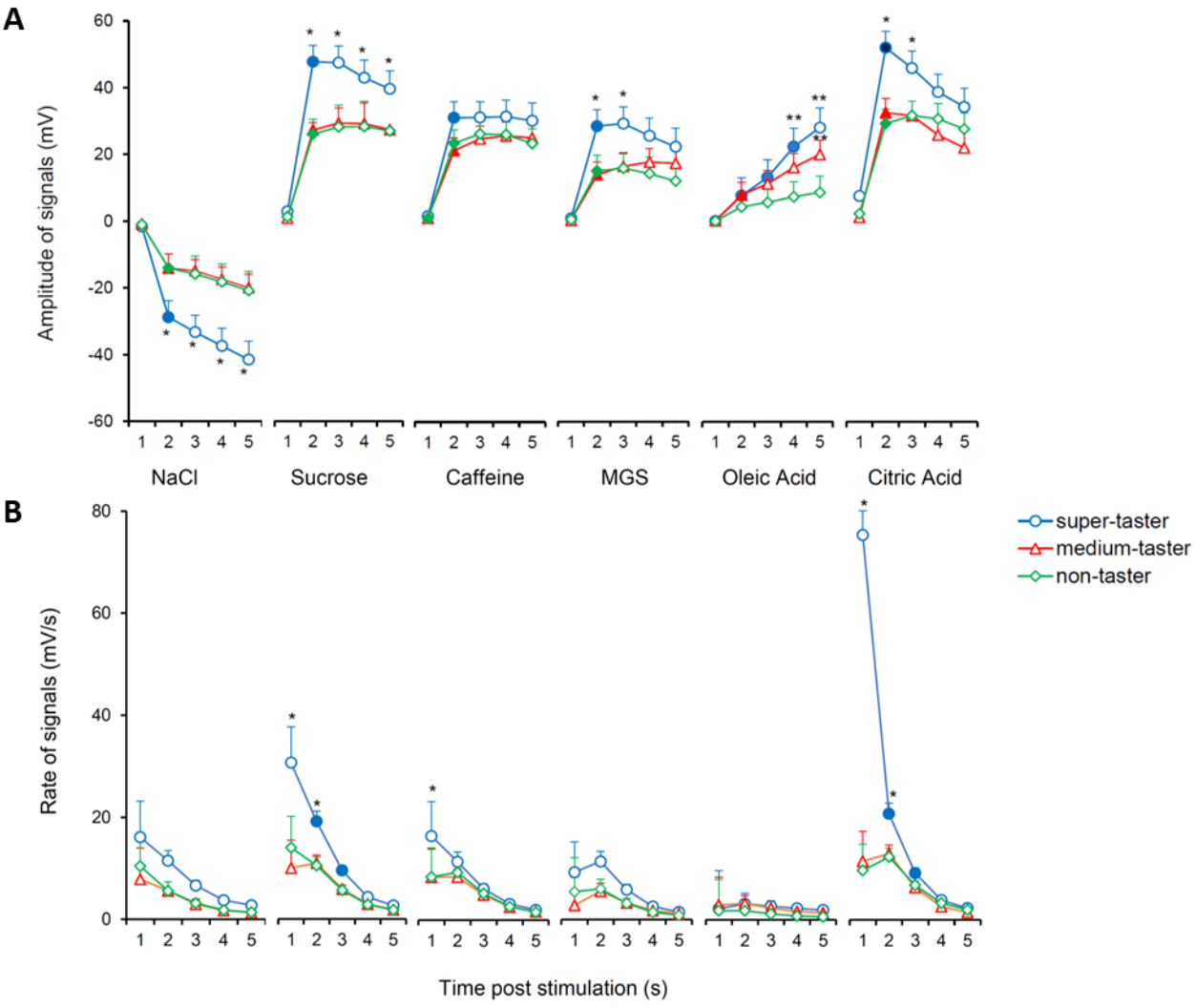

Figure 5. Time course of potential amplitude (mV) (A) and change rate (mV/s) (B) of bioelectrical signals in response to taste stimulation with six taste qualities according to PROP taster status. Data (mean values ± SEM) determined after 0.1, 2.5, 5, 10, and 15 s (expressed in numbers from 1 to 5 respectively) after application of taste stimulation. Group 1, n = 10 super-tasters, n = 15 medium tasters, and n = 14 non-tasters. Solid symbol indicates significant difference with respect to the previous value of the corresponding group ($p \leq 0.011$; Fisher LDS, after repeated-measures ANOVA). * Indicates a significant difference with respects to the corresponding values of other taster groups ($p \leq 0.042$; Fisher LDS, after repeated-measures ANOVA). ** Indicates a significant difference with respects to the corresponding values of non-tasters ($p \leq 0.036$; Fisher LDS or Duncan's test, after repeated-measures ANOVA).

belonging to Group 1 also showed larger signal amplitudes in response to oleic acid than those of non-tasters at 10 and 15 s ($p \leq 0.019$; Fisher LDS) and medium tasters showed higher values for oleic acid relative to non-tasters at 15 s ($p = 0.036$; Fisher LDS), while no difference in response to caffeine was found ($p > 0.05$) (Figure 5A).

Repeated-measures ANOVA revealed that the time course of the rate of bioelectrical signals during stimulation time depended on taste quality and PROP taster status of subjects ($F_{40,772} = 5.541$; $p < 0.00001$). PROP super-tasters showed a quicker hyperpolarization with respect to the other taster groups, at 0.1 and 2.5 s, in response to sucrose and citric acid ($p \leq 0.0427$; Fisher LDS); this same effect was only seen for caffeine at 0.1 ($p \leq 0.0239$; Fisher LDS) (Figure 5B).

Among Group 2, molecular analysis at the *rs1761667* polymorphism of CD36 gene found 6 AA homozygous (3 males, 3 females), 20 heterozygous (9 males, 11 females,), and 9 GG homozygous subjects (3 males, 6 females). PROP taster groups did not differ statistically based on genotype distribution and haplotype frequency of the CD36 gene ($\chi^2 > 0.665$; $p < 0.71$; Fisher's test). Figure 6 shows examples of positive monophasic potentials evoked after oleic acid stimulation in representative subjects belonging to Group 2 divided for their PROP taster status and genotypes of the CD36 gene.

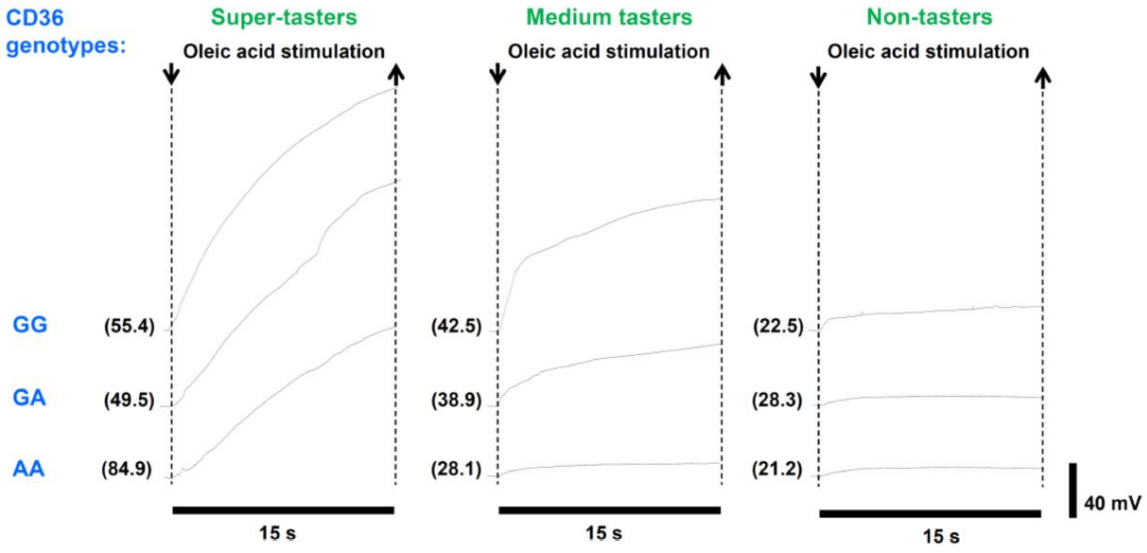

Figure 6. Examples of electrophysiological recordings in response to oleic acid (30 μL) taste stimulation in representative super-tasters, medium tasters, and non-tasters with different genotypes of the CD36 gene. The very first data point on the left side of each electrophysiological recording represents the baseline. Numbers within parentheses on the left of each trace indicate the density of fungiform papillae (No./cm^2) of each subject calculated in the small circular area of the tongue where oleic acid stimulation was applied.

The lowest value of amplitude (0.64 mV) was measured in a non-taster volunteer with the AA genotype in the CD36 polymorphism while the maximum value (91.99 mV) was determined in a super-taster volunteer with the GG genotype in the CD36 polymorphism.

Mean values of the potential amplitude and change rate of signals recorded in response to oleic acid taste stimulation in subjects belonging to Group 2 genotyped for the *rs1761667* SNP of the *CD36* gene are shown in Figure 7. Amplitude and rate of signals determined in GG homozygous were higher than those of subjects with the AA genotype ($p \leq 0.043$; Fisher LDS test subsequent one-way ANOVA). No differences between heterozygous and homozygous volunteers were found ($p > 0.05$).

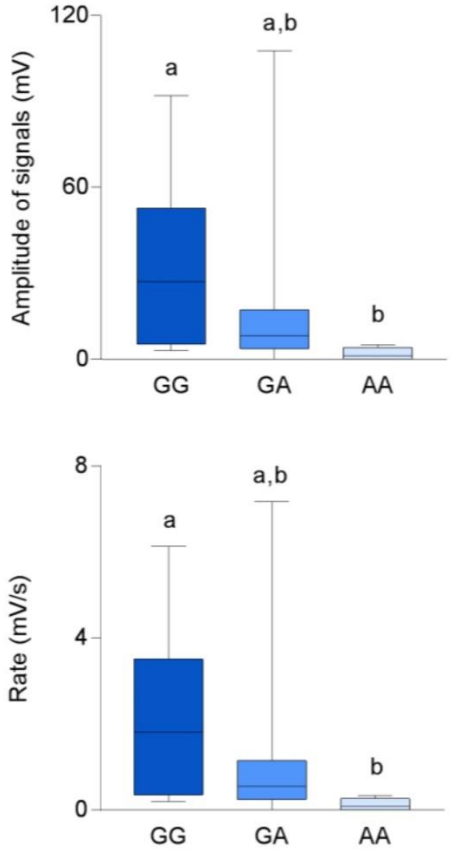

Figure 7. Box-and-whisker plots showing the minimum, first quartile, median, third quartile, and maximum of each data set of amplitude and rate of signals evoked in subjects belonging to Group 2 with genotypes GG (n = 9), GA (n = 20) and AA (n = 6) of CD36 by oleic acid (30 μL) taste stimulation. Different letters indicate a significant difference ($p \leq 0.05$; Fisher LDS or Duncan's test subsequent one-way ANOVA).

Mean values of potential amplitude and change rate, determined after 2.5, 5, 10, and 15 s after the application of oleic acid taste stimulation according to PROP taster status and CD36 polymorphisms, are shown in Figure 8. The time course of the hyperpolarization amplitude and variation rate were different in volunteers with different PROP phenotypes or CD36 genotypes. Specifically, a significant increase of the potential amplitude during the stimulation up to 15 s was recorded in super-tasters and medium tasters ($p < 0.001$ and $p = 0.004$; Fisher LDS or Duncan's test subsequent repeated measured ANOVA), and a similar increase was detected in subjects with GG genotype in the CD36 gene ($p \leq 0.001$; Fisher LDS or Duncan's test subsequent repeated measured ANOVA), but only for a duration of 10 s in heterozygous subjects ($p = 0.025$; Fisher LDS test subsequent repeated measured ANOVA) whereas no changes in non-tasters or in subjects with AA genotype was detected ($p > 0.05$). The change rate decreased (at 10 s and 15 s) in super-tasters and it rapidly in medium tasters ($p \leq 0.019$; Fisher LDS or Duncan's test subsequent repeated measured ANOVA), while it diminished only at 5 s in non-tasters ($p = 0.049$; Fisher LDS test subsequent repeated measured ANOVA). Also, the change rate decreased in subjects with GG genotype in the CD36 gene ($p \leq 0.037$; Fisher LDS or Duncan's test subsequent repeated measured ANOVA), only at 10 s in heterozygous subjects ($p = 0.005$; Fisher LDS test subsequent repeated measured ANOVA) and at 15 s in volunteers with the AA genotype ($p > 0.05$).

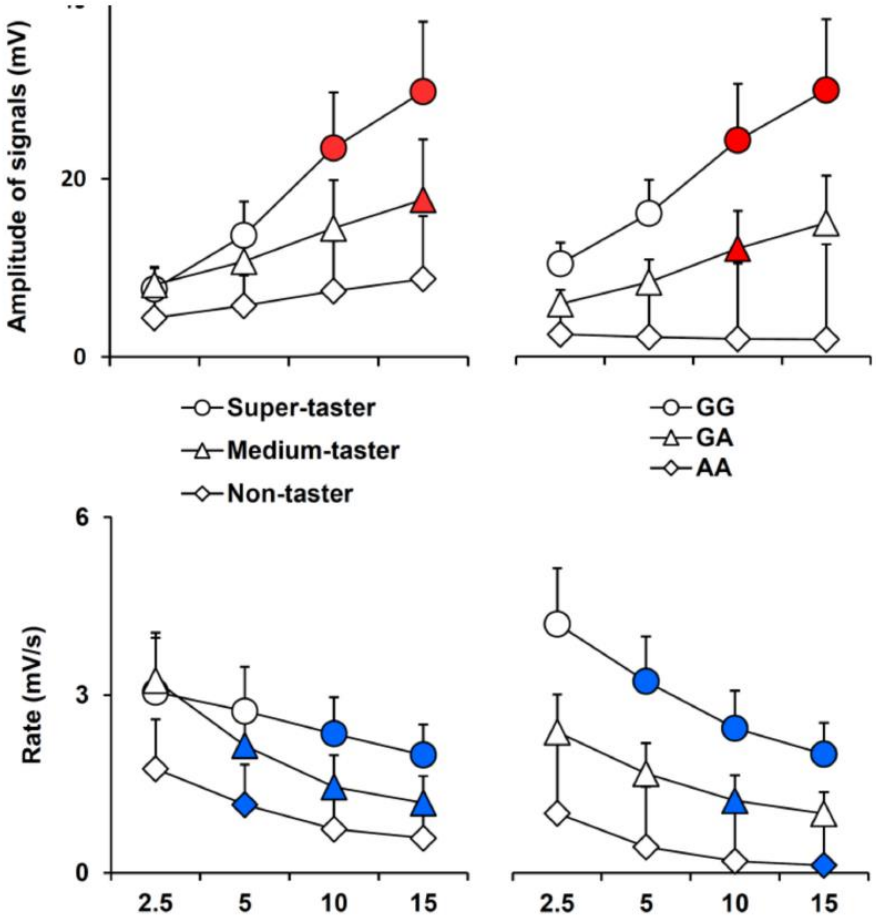

Figure 8. Time course of potential amplitude (mV) or rate (mV/s) of the signal across PROP taster status or CD36 polymorphism groups during stimulation time. Data (mean values ± SEM) are determined after 2.5, 5, 10, and 15 s from the application of oleic acid (30 μL). n = 10 super-tasters, n = 13 medium tasters and n = 12 non-tasters; n = 9 volunteers with genotypes GG in CD36, n = 20 GA genotypes and n = 6 AA genotypes. Solid symbols (red for amplitude of signals and blue for rate) indicate a significant difference with respect to the previous value of the corresponding group ($p \leq 0.05$; Fisher LDS or Duncan's test, after repeated measures ANOVA across PROP taster groups or CD36 genotype of volunteers).

Figure 9 shows the mean values of the rating of the perceived intensity for the six taste qualities given by Group 1 according to PROP taster status. PROP super-tasters gave statistically significant higher intensity ratings to all stimuli with respect to the other taster groups ($p \leq 0.019$; Fisher LDS, subsequent to two-way ANOVA), except for oleic acid for which the ratings of super-tasters were higher than those of non-tasters only ($p = 0.0436$; Fisher LDS, subsequent to two-way ANOVA). No significant difference related to PROP taster status was found for

NaCl. The association between intensity ratings to oleic acid stimulation and Prop taster status was confirmed in Group 2, but the perceived intensity was higher in the super-taster subjects than in non-taster or medium-taster ones ($p \leq 0.046$; Duncan's test after one-way ANOVA; $F_{2,32} = 3.138$; $p = 0.054$).

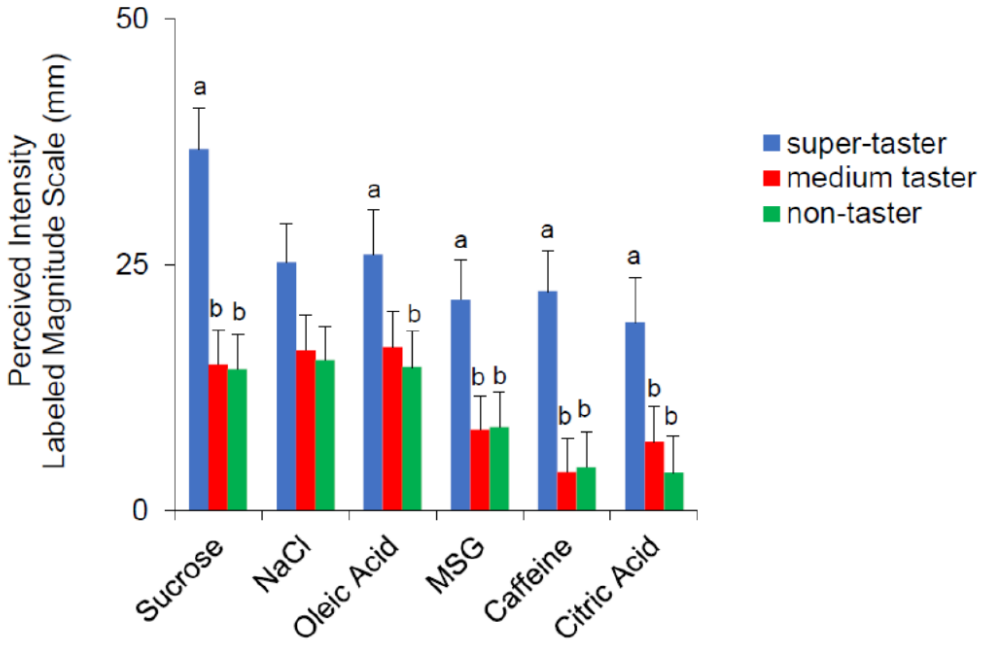

Figure 9. Perceived intensity ratings for the six taste qualities in super tasters (n = 10, medium tasters (n = 15), and non-tasters (n = 14) belonging to Group 1. Different letters indicate a significant difference ($p \leq 0.0436$; Fisher LDS, after repeated-measures ANOVA).

Figure 10 shows mean values ± SEM of the rating of the perceived intensity for oleic acid given by subjects belonging to Group 2 genotyped for the *rs1761667* SNP of *CD36* gene. Subjects with the GG genotype gave intensity ratings higher than volunteers with the AA genotype ($p = 0.047$ Fisher LDS test). No differences between the heterozygous and homozygous volunteers were found ($p > 0.05$).

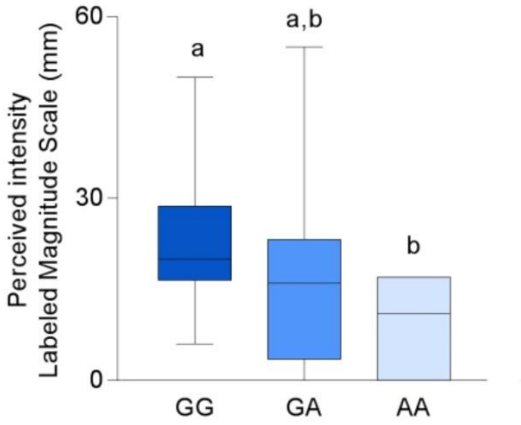

Figure 10. Box-and-whisker plots showing the minimum, first quartile, median, third quartile, and maximum of each set of perceived intensity data evoked by taste stimulation with oleic acid (30 μL) and in subjects belonging to Group 2 with genotypes GG (n = 9), GA (n = 20) and AA (n = 6) of CD36. Different letters indicate a significant difference (p = 0.047 Fisher LDS test, subsequent one-way ANOVA).

The mean values of density of fungiform papillae determined in super-tasters, medium tasters, and non-tasters belonging to Group 1 are shown in Figure 11. One-way ANOVA showed that the density of fungiform papillae varies with PROP taster status ($F_{2,33}$ = 13.105; p = 0.00006). PROP super-tasters had a higher density than medium tasters (p = 0.021; Fisher LDS test), who showed higher values than non-tasters (p = 0.0072; Fisher LDS test). In Group 2, same statistical differences in fungiform papillae density according to PROP taster status were found ($p \leq 0.032$; Duncan's test after one-way ANOVA; $F_{2,32}$ = 18.712; $p < 0.001$;). No differences in fungiform papillae density related to the *CD36* polymorphism were found ($p < 0.05$).

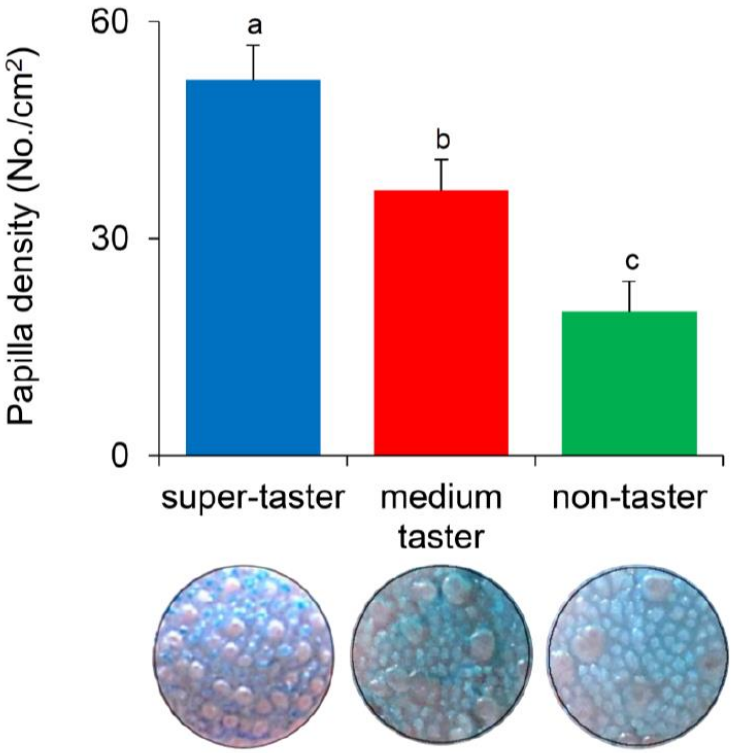

Figure 11. Fungiform papilla density in super-tasters (n = 10), medium tasters (n = 15), and non-tasters (n = 14) belonging to Group 1. Different letters indicate a significant difference ($p \leq 0.021$; Fisher LDS test subsequent one-way ANOVA). Photographs of the dyed area of the tongue where the fungiform papillae were counted in a representative super-taster, medium taster and non-taster are shown.

Linear correlation analysis on data collected from Group 2 revealed that density of fungiform papillae measured in the same circular area of the tongue where the oleic acid stimulus was delivered, was linearly correlated with the signal amplitude (r = 0.394; p = 0.028), with hyperpolarization rate, (r = 0.410; p = 0.019), as well as with perceived taste intensity (r = 0.477; p = 0.005) whereas no correlation between taste intensity and electrophysiological parameters was found ($r < 0.251$; $p > 0.06$).

Discussion

The first aim of this study was to characterize the electrophysiological responses evoked in taste buds on a localized area of the human tongue by stimulation with the six taste qualities, as an objective and quantitative measure of the degree of activation of the peripheral taste system generated by each taste stimulus. Taste stimulations evoked negative monophasic potentials, which represent a measure of the summated voltage change resulting from the response of stimulated taste cells. These signals are also similar to those recorded from the olfactory epithelium [10, 11], where the electrical activity has been reported as the summated generated potential by the population of stimulated olfactory neurons [12]. Interestingly, we found that each taste quality evoked a characteristic monophasic voltage change with a specific time course during taste stimulation. In fact, a comparative analysis of values of potential amplitude and of potential change rate, determined during stimulation, revealed that the waveform of the signal depends on the taste quality of stimulus. Although the bioelectrical activity derived by all stimuli was represented by a monophasic potential change characterized by a fast-initial variation followed by a slow decline, NaCl was the only stimulus that evoked negative potential variations, while all other stimuli evoked positive potential changes, which nevertheless were differed from one another. On the other hand, the control stimulations were ineffective in evoking this response.

These biopotential variations recorded in response to all six qualities might be explained with the difference type of taste cells activated and in transduction mechanisms across taste qualities. Sweet, bitter, umami, and fat stimuli directly activate type II cells, which express specific receptors and, via ATP released during their tastant-induced stimulation, indirectly activate the adjacent type III cells, which instead directly respond to sour stimuli [13, 14]. Sucrose and citric acid generated the largest hyperpolarization mostly in the first part of the recordings. However, signal amplitude for citric acid decreased significantly after 10 s, while

for sucrose it did not change until the end of the stimulation. The responses to these two stimuli also differed for the hyperpolarization rate. For citric acid, the hyperpolarization arose very quickly, while after sucrose it was slower, with values that were half those evoked by citric acid. Caffeine and MSG evoked signals with intermediate amplitudes in the first part of the recordings, but afterward the signal amplitude distinguished the response to these two stimuli. Differences in the hyperpolarization rate also allowed us to distinguish the electrophysiological responses to these two stimuli; intermediate values were found in response to caffeine, while very slow signals were recorded in response to MSG. Finally, the lowest and slowest hyperpolarization was recorded in the responses to oleic acid, in which the signal amplitude reached values comparable to those of other stimuli only at the end of stimulation. This slow electrophysiological activation in response to oleic acid could depend on the high surface tension of this molecule that determines its slow diffusion toward the cell that detects it. The rapid hyperpolarization evoked by citric acid (which was particularly evident in super-tasters who have a high density of papillae), compared to that elicited by other stimuli (sucrose, caffeine MGS, oleic acid) may be explained by differences in the transduction mechanisms across stimuli [15]. Organic acids permeate through the membrane, acidify the cytoplasm and intracellular H+, by blocking a proton-sensitive K+ channel, and depolarize the cell membrane. On the other hand, the transduction mechanisms of sucrose, caffeine, MGS, or oleic acid are mediated by GPCRs, causing activation and diffusion of second messengers, which need more time to depolarize the cell.

The negative potentials we observed in response to NaCl stand in contrast to those of all other taste stimuli (which showed positive potentials). Although the taste bud cells involved in the salty taste transduction have not been conclusively identified [15, 16], salty is the only taste quality known to be transduced by some Type I cells, which may exhibit depolarizing ionic currents due to direct Na+ permeation through membrane ion channels [17]. However, we cannot exclude that

the salty stimulus may undergo an ionic dissociation directly recorded by the measuring electrode, thus superposing with the bio-signal itself. Future studies will clarify this issue.

Our results shed additional light on the validity of using the PROP phenotype as a biomarker of general taste perception. We found a direct relationship between PROP sensitivity and the electrophysiological response evoked by the six taste qualities in the buds of a localized area of human tongue. Specifically, the largest and quickest responses were recorded in PROP super-taster subjects, who had the highest density of fungiform papillae in the same area of the tongue where stimulations were applied during the recordings. Smaller and slower responses were observed in medium taster and non-tasters, who had lower densities of papillae in the same area of the tongue. Results were confirmed in Group 2. The analysis of the time course of parameters defining the waveform of signals evoked by NaCl, sucrose, caffeine, MGS, and citric acid revealed that the super-taster phenotype, which was associated with the highest density of papillae, confers additional advantage already in the initial phase (2.5 s) of the response, which is certainly the most significant one to induce a behavioral response. Super-tasters showed a more prompt and intense response to sucrose and citric acid, a more intense one to NaCl and MGS, and a more rapid one to caffeine, as compared to medium tasters and non-tasters.

Importantly, the differences in the electrophysiological responses to sucrose, caffeine, MGS, oleic acid and citric acid that we recorded in the PROP taster groups agree with the intensity ratings given by these subjects during oral stimulations, indicating that our bioelectrical measurements are consistent with common human psychophysical observations. It is noteworthy that the amplitude of electrophysiological response to NaCl by super-tasters was larger than those for medium tasters and non-tasters, however, the perceived intensities reported by subjects did not vary with PROP taster status. This is consistent with the psychophysical procedures for the classification of subjects by PROP taster status,

which use NaCl as standard control showing that the rating of perceived intensity for this stimulus does not vary with the PROP phenotype of subjects [2]. It is also worth highlighting that we found lack of agreement between signal amplitude and perceived intensity for caffeine, relatively to PROP taster groups. The perceived intensities for caffeine reported by super-tasters were higher than those for medium tasters and non-tasters, while the response amplitude did not vary with PROP taster status, though signals for super-tasters were faster.

The super-taster phenotype was important to elicit a large potential change in the last part of the response to oleic acid. The signal amplitude values slowly increased during stimulation in super-tasters, while no changes in the whole-time course of recordings were found in medium tasters and non-tasters. This extended activation evoked by oleic acid in super-tasters, having a higher density of papillae, may reflect the high surface tension of the molecule that determines its slow diffusion toward the taste bud cells. These results strongly support previous psychophysical experiments showing a direct relationship between fat perception and PROP taster status [20-25], that can be linked to differences in the density of papillae across the three PROP taster categories [6, 26-29]. It is important to acknowledge, however, that some studies report weak or no associations between PROP taster status and papillae density [30, 31]. As pointed out by Tepper et al. [22] and Dinnella et al. [31], personal factors such as age, gender, smoking, bodyweight, and modified genes can influence papillae density as well as PROP tasting, potentially undermining interrelationships between PROP, taste perceptions, and papillae density. Moreover, the differences in the screening procedures can cause inconsistencies among studies [22]. This topic deserves further investigation.

In Group 2 we also observed a relationship between the values of the hyperpolarization amplitude and rate, and the *rs1761667* polymorphism of the *CD36* gene. In agreement with evidence showing that the presence of the homozygous AA genotype at this location of the *CD36* gene is characterized by reduced protein expression [32] and low taste sensitivity to fats [23, 33-35], we

measured the lowest amplitude values and biopotential variation rates in volunteers carrying two A alleles who verbally reported the lowest values of perceived intensity. Likewise, we measured the highest bioelectrical values in volunteers with the GG genotype who perceived the highest intensity of oleic acid and intermediate values in heterozygous volunteers who reported intermediate values of perceived intensity. As expected, no variations in the density of papillae related to *CD36* genotypes were found. The analysis of the time course of the responses showed that the amplitude of the signal increased during stimulation, mostly in the last portion of the response, and this effect was most prominent in PROP tasters who had a higher number of papillae, and in volunteers having at least one G allele in CD36, which is known to be associated with an increase of receptor expression [36]. On the contrary, signal amplitude did not change in PROP non-tasters who had a low number of papillae and in volunteers homozygous for the non-tasting (AA) form of this polymorphism in CD36, which is associated with reduced protein expression [36]. In addition, the hyperpolarization rate rapidly decreased across the whole-time course of recordings in volunteers with two tasting (GG) alleles, who showed at 2.5 s after stimulus onset, values about twice as high as those of the volunteers with only a single G allele. In turn, the hyperpolarization rate slowly decreased in both heterozygous (GA) volunteers and in volunteers with two non-taster variants in *CD36*. All these results suggest that the presence of the tasting variant in the specific receptor is the most important condition to elicit a prompt response and, in addition, turns out to be the most important condition to evoke an intense perception when the volunteers have a high number of fungiform papillae in their tongue.

All in all, psychophysical measures have played a critical role in understanding human chemosensory experiences, by permitting the determination of taste responses at the CNS level, which can influence food choices and eating behaviors. Nevertheless, because taste intensity measures are subjective and sensitive to reporting bias, they are less useful for gaining insights into taste

mechanisms, particularly those rapid responses that occur within the initial few seconds of oral exposure to a stimulus. The present work helps to fill gaps in knowledge of these processes by combining traditional behavioral methods with our electrophysiological recording method. Moreover, the presence of the tasting variant in the specific receptor is the most important condition to elicit a prompt response and, in addition, turns out to be the most important condition to evoke an intense perception when the volunteers have a high number of fungiform papillae in their tongue.

The direct relationships that we found between the parameters describing the waveform of signals and those defining PROP phenotype indicate that the electrophysiological recording technique is a simple and reliable method for the objective measure of the activation of peripheral taste function which can be of great help to resolve controversial opinions on the role of PROP phenotype in taste perception, food preferences, and nutrition [37].

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2b. Effect of salivary proteins

*Yousaf NY, Melis M, **Mastinu M**, Contini C, Cabras T, Tomassini Barbarossa I, Tepper BJ. Time Course of Salivary Protein Responses to Cranberry-derived Polyphenol Exposure as a Function of PROP Taster Status. Nutrients 2020. 12(9), 2878. https://doi.org/10.3390/nu12092878*

Melis M, **Mastinu M***, Pintus S, Cabras T, Crnjar R, Tomassini Barbarossa I. Differences in Salivary Proteins as a Function of PROP taster status and Gender in Normal Weight and Obese Subjects. Molecules 2021. 26(8), 2244. https://doi.org/10.3390/molecules26082244*

In this part of work, we present results on analysis of mechanisms involving the salivary proteome in determining the individual taste variations. Specifically, here present an evaluation of the role of salivary proteins in the development of astringency and in affecting food choice and BMI, in relation with of PROP taster status and gender.

Methods

Participants

For this study, two groups were formed. Group 1 was composed of sixty healthy adults between 18-45 years of age, recruited at the Rutgers University (NJ, USA). Selection criteria included general suitability (e.g., demographics, health information) and familiarity with chosen astringent stimuli. They had to have consumed such products within the last 2 years. Volunteers were screened for PROP taste responsiveness as described below; only PROP non-tasters (n = 29; females = 15; males = 14) and super-tasters (n = 31; females = 16; males = 15) were admitted in the study into groups balanced for gender, whereas medium tasters were excluded. Mean participant age was 22.0 ± 0.6 years.

Group 2 was composed of one hundred and eighteen volunteers divided into two clusters based on the body mass index, resulting in a random classification by gender and age: Obese (OB) who had a BMI ranging from 30 to 50 kg/m^2 (n = 57; females = 34, males = 23; age ranging from 19 to 72) and Normal Weight (NW) who had a BMI ranging from 18 to 25 kg/m^2 (n = 61; female = 40, males = 21; age ranging from 20 to 67). They were recruited in the city of Cagliari (Italy) through public advertisement.

For both groups, exclusion criteria included major metabolic disorders (diabetes, kidney disease, etc.), pregnancy, lactation, food allergies, and the use of medications that interfere with taste or smell functions. OBs with metabolic disorders were also excluded. After an explanation of the experimental procedure, all subjects read and signed an informed consent form. The study was approved by

the Rutgers University Arts and Sciences Institutional Review Board (Approval#13-309M) and by the ethical committee of AOU of Cagliari.

Experimental Procedure

Subjects were separately tested in the morning. Subjects selected for Group 1 underwent to two sessions while experiment for Group 2 was designed as a single day of tasting. They were always requested to be present in the testing room 15 min before the session in order to adjust to the regular room conditions (23–24 °C; 40–50% relative humidity), and they were always asked to abstain from eating, drinking (except water) using oral care products or chewing gum for 2 h prior the testing. To prevent taste sensitivity modification due to the estrogenic phase, women were tested around the sixth day of the menstrual cycle. Additionally, subjects volunteered for Group 1 were asked to refrain from consuming any alcoholic beverages and any astringent foods for approximately 24 h and 8 h respectively prior to each session. A list of such foods was provided to them.

Subjects selected for Group 1 were classified for their PROP taster status and completed questionnaires on demographic information during the first session. During session 2, subjects provided saliva samples by spitting in a plastic cup, which was immediately stored at 4 °C until further processing. First, a sample of resting saliva was collected. After a resting period set at 5 min, subjects switched in their mouth and entirely swallowed 20 mL of one of the two astringent stimuli and provided a saliva sample at 5 and 10 minutes. Two astringent oral stimuli were selected: Cranberry Juice (CJ) and Cranberry-derived polyphenol extract (CPE). After a 20 min break, the subject was provided with the second astringent stimulus and followed the same procedure for saliva collection as above. The order of presentation (CJ first or CPE first) was randomized across subjects. The experimental procedure is schemed in Figure 1.

CJ was made from frozen cranberries donated by Ocean Spray Cranberries Cooperative (Chatsworth, NJ). 300 g of berries were defrosted, washed, and cooked

on a stovetop for 10 min with 648 mL of spring water. Lately, the mixture was filtered and cooled. CPE solution was made using a carrier-free powdered extract (Ocean Spray Cranberries, Inc., Lakeville, MA, USA) added to spring water at a final concentration of 0.75 w/v g/L. Stimuli were prepared the day before testing and refrigerated at 4 °C until serving at room temperature.

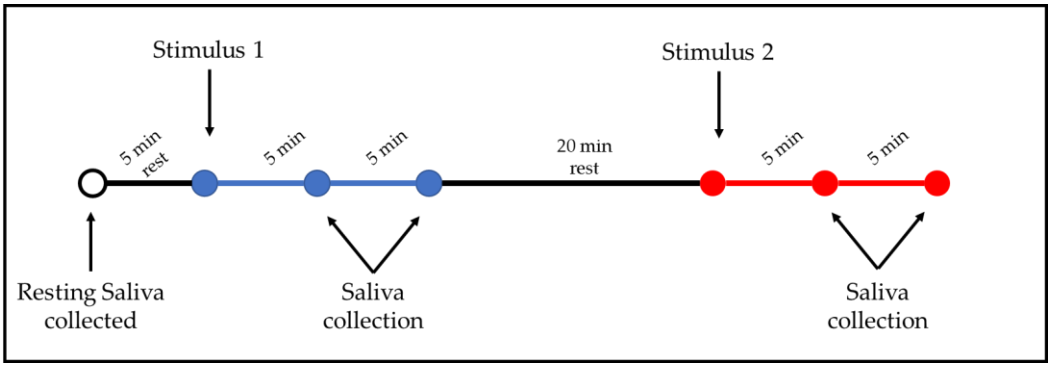

Figure 1. Saliva collection timeline

During the single session, subjects volunteered for Group 2 were asked to provide a sample of unstimulated whole saliva, which was collected in an Eppendorf tube (1 mL) and processed as described below. Subsequentially, weight (kg) and height (m) were measured to calculate BMI (kg/m^2). Finally, subjects were classified for their PROP taster status.

PROP Taster Status Classification

Subjects were classified for PROP taster status via the impregnated paper screening test. This procedure requires the evaluation of perceived intensity after tasting one filter paper disk impregnated with 1.0 mol/L of NaCl (Sodium Chloride, Sigma-Aldrich, Milan, Italy) and another impregnated with 50 mmol/L of PROP (6-n-propyl–2-thiouracil, Sigma-Aldrich, Milan, Italy) for 30 s, by using the label magnitude scale (LMS). Subjects rinse their mouth with spring water in between tasting the paper disks and interstimulus time was set at 1 min. Subjects were classified as non-tasters if they rate the PROP disk < 15 mm on the LMS; they are categorized as super-tasters if they rate the PROP disk > 67 on the LMS. Subjects who rated PROP disk between 15 and 67 mm were classified as PROP medium-

tasters and were excluded if they had volunteered for Group 1. Ratings for NaCl disk were used to classify volunteers who gave borderline ratings for PROP disk.

Saliva Treatment

Saliva sample collected at each time point (at resting; 5 and 10 min after the first stimulus; 5 and 10 min after the second stimulus) from subjects belonging to Group 1 was transferred in two microcentrifuge tube (0.5 mL per tube) immediately after collection: one tube was prepared for High Performance Liquid Chromatography- low resolution - Electrospray Ionization - Ion Trap - Mass Spectrometry (HPLC-ESI-MS) analysis while the other was prepared for amylase quantification. Saliva samples collected from subjects belonging to Group 2 were only prepared for HPLC-ESI-MS analysis as follow.

For HPLC-ESI-MS analysis, saliva samples were treated with acqueous solution of trifluoroacetic acid (0.2%; Sigma-Aldrich, Milan, Italy) in a 1:1 v/v ratio in order to inhibit salivary proteases and stabilize the sample. The acidic supernatant, obtained after centrifugation at 8000x g at 4 °C for 15 min of the samples, was separated from the pellet and stored at -80 °C. For the immunoblot procedure, a protease inhibitor cocktail solution [1 tablet/1.4 mL of cOmplete® Protease Inhibitor Cocktail (Roche Diagnostics, Indianapolis, IN, USA) + 175 mM NH_4HCO_3 (Ammonium Bicarbonate, Sigma-Aldrich, St. Louis, MO, USA)] was added to saliva in a 1:2 *v/v* ratio. During the treatment, these samples were maintained constantly at 4 °C then stored at -80 °C until further analysis.

HPLC-ESI-IT-MS Analysis

The three proteoforms (PRP-1, PRP-3 and P-C) belonging to the family of aPRPs, the three peptides (Hist 1, Hist 5 and Hist 6) belonging to the family of Hist, the eight proteoforms (Ps-1, P-J, P-H, P-F, P-D, II-2, IB-8a and IB-1) belonging to the family of bPRPs, and the five proteoforms (Cyst S, Cyst S1, Cyst S2, Cyst SA and Cyst SN) belonging to the family of S-Cyst were identified and quantified in each saliva sample, by HPLC-ESI-IT-MS (ThermoFisher, San Jose, CA, USA) according

to our previous studies, which had applied the same analytical condition [1-3]. Additionally, the P-Ko proteoform belonging to the family of bPRPs and the proteoforms belonging to the family of Stath (di-phosphorylated, monophosphorylated and non-phosphorylated) were also searched in Group 2 while PB peptide was only searched in Group 1. The measurements were performed by a Surveyor HPLC system connected by a T splitter to a diode-array detector and to an LCQ Advantage mass spectrometer provided with an ESI source (ThermoFisher Scientific, San Jose, CA, USA). Vydac (Hesperia, CA, USA) C8 with 5 μm particle diameter was used as the chromatographic column (dimensions 150×2.1 mm). Briefly, 30 μL of acidic soluble fraction corresponding to 15 μL of saliva were injected. Salivary proteins were eluted using a linear gradient. The T splitter permitted 0.20 mL/min to flow toward the diode array detector and 0.10 mL/min to flow toward the ESI source. The photodiode array detector was set at 214 and 276 nm. Mass spectra were collected every 3 ms in the positive ion mode in the range 300–2000 m/z. Average mass values (Mav), obtained by deconvolution of averaged ESI-MS spectra automatically performed by using MagTran 1.0 software (Amgen Inc., Thousand Oaks, CA, USA) [4], and elution times of proteins/peptides were compared with those determined in previous studies [5, 6] and with the theoretical ones available at the UniProtKB/Swiss-Prot human database (http://us.expasy.org/tools). The quantification of each protein and peptide was based on the area of the HPLC-ESI-IT-MS extracted ion current (XIC) peaks. The XIC analysis reveals the peak associated with the peptide of interest by searching, along the total ion current chromatographic profile, the specific multi-charged ions generated by the protein. Since the area of the ion current peak is related to concentration under constant conditions, it may be used to perform relative quantification of the same analyte in different samples. Table 1 shows the salivary proteins and peptides analyzed in each of the salivary samples collected using the HPLC-low resolution-ESI-IT-MS technique according to Cabras [2].

Table 1. List of salivary proteins and peptides quantified by HPLC-low resolution-ESI-MS.

Protein (Swiss-Prot Code)	Experimental Average Mass (Da) ± SD (Theoretical)	Elution Time (min ± 0.5)
Acidic proline-rich phosphoproteins family (aPRPs):		
P-C peptide (P02810)	4370.9 ± 0.4 (4370.8)	13.6-14.5
PRP-1:		
-PRP-1 type di-phosphorylated (P02810)	15515 ± 2 (15514-15515)	22.9-23.3
-PRP-1 type mono-phosphorylated	15435 ± 2 (15434-15435)	23.9-24.3
-PRP-1 type non-phosphorylated	15355 ± 2 (15354-15355)	24.2-24.7
-PRP-1 type tri-phosphorylated	15595 ± 2 (15594-15595)	22.6-22.9
PRP-3:		
-PRP-3 type di-phosphorylated (P02810)	11161 ± 1 (11161-11162)	23.3-23.8
-PRP-3 type mono-phosphorylated	11081 ± 1 (11081-11082)	23.8-24.2
-PRP-3 type non-phosphorylated	11001 ± 1 (11001-11002)	24.8-25.1
-PRP-3 type di-phosphorylated Des-Arg$_{106}$	11004 ± 1 (11005-11006)	23.5-23.8
Histatin family (Hist):		
Hist-1(P015515)	4928.2 ± 0.5 (4928.2)	23.3-23.8
Hist-1 non-phosphorylated	4848.2 ± 0.5 (4848.2)	23.4-23.8
Hist-6 (P15516)	3192.4 ± 0.3 (3192.5)	14.0-14.4
Hist-5 (P15516)	3036.5 ± 0.3 (3036.3)	14.2-14.7
Basic proline-rich protein family (bPRPs):		
Ps-1	23460 ± 3 (23459.0)	17.0-18.0
P-J	5943.9 ± 0.5 (5943.6)	14.1-14.7
P-H (P02812/P04280)	5590.2 ± 0.5 (5590.1)	15.0-15.5
P-F (P02812)	5843.0 ± 0.5 (5842.5)	14.3-14.8
P-D (P010163)	6949.5 ± 0.7 (6949.7)	15.2-15.8
II-2:		
- II-2 (P04280)	7609 ± 1 (7609.2)	18.7-19.1
- II-2 non-phosphorylated	7529 ± 1 (7529.2)	19.5-19.8
- II-2 Des-Arg$_{75}$	7453 ± 1 (7453.0)	18.8-19.2
IB-8a:		
- IB-8a (Con1+)	11888 ± 2 (11887.8)	17.1-17.8
- IB-8a (Con1-)	11898 ± 2 (11896.2)	17.1-17.8
IB-1:		
- IB-1 (P04281)	9593 ± 1 (9593.4)	18.8-19.3
- IB-1 non-phosphorylated	9513 ± 1 (9513.4)	19.4-19.7
- IB-1 Des-Arg$_{96}$	9437 ± 1 (9437.2)	19.0-19.4
P-Ko	10434 ± 1 (10433.6)	16.0-16.4
Statherin family (Staths)		
Stath di-phosphorylated (P02808)	5380.0 ± 0.5 (5379.7)	28.9-29.5
Stath mono-phosphorylated	5299.9 ± 0.5 (5299.7)	28.7-29.1
Stath non-phosphorylated (P02808)	5220.5 ± 0.5 (5219.7)	28.4-28.8
Cystatin family (S-Cyst)		
Cyst S non-phosphorylated (P01036)	14,186 ± 2 (14185)	36.5-37.1
Cyst S mono-phosphorylated (S1)	14,266 ± 2 (14265)	36.6-37.1
Cyst S di-phosphorylated (S2)	14,346 ± 2 (14345)	36.8-37.2
Cyst SN (P01037)	14,312 ± 2 (14313)	34.8-35.2
Cyst SA (P09228)	14,347 ± 2 (14346)	38.4-38.9
PB	5972.9 ± 0.5 (5792.7)	29.4-30.5

Alpha Amylase Quantification

In resting and stimulated saliva samples provided by subjects belonging to Group 1, alpha amylase was searched and quantified. To normalize protein levels for dot blot analysis, Bicinchoninic Acid (BCA) Protein Assay Kit (Sigma-Aldrich, St. Louis, MO, USA) was used to quantify total protein content. Consequently, the saliva samples treated with cOmplete protease inhibitor cocktail were first diluted with Tris Buffered Saline (TBS: 20 mM Tris-HCl pH 7.6, 150 mM NaCl) so that each diluted sample would have the same amount of total protein content (adjusted to 0.38 μg/μL). Then, the concentration of alpha amylase in the individual salivary samples was estimated semi-quantitatively by using dot-blot technique, where the protein samples were spotted directly onto a PVDF membrane (0.2 μm pore size; Immun-Blot® PVDF Membrane, Bio-Rad Laboratories, Inc., Italy). Specifically, 1 μL of all samples was spotted in triplicate onto pre-wetted (methanol for 1 min; TBS for 2 min) PVDF membrane. Therefore, the membrane was: blocked with blocking agent 5% of BSA (Bovine serum albumin, Sigma Aldrich) in TBS-T buffer (20 mM Tris-HCl pH 7.6 + 150 mM NaCl + 0.05% Tween 20) for 1 h; incubated for 1 h with primary antibody (dilution 1:1000; Amylase G-10: sc-46657-Santa Cruz Biotechnology, Inc.) in 5% of BSA in TBS-T buffer; washed three times for 5 min with TBS-T buffer; incubated for 1 h with secondary antibody (dilution 1:5000; Rabbit anti-Mouse IgG, Secondary Antibody, HRP ThermoFisher Scientific); washed three times for 5 min with TBS-T; incubated for 5 min with ECL substrate (Clarity Western ECL Substrate, Bio-Rad, Laboratories, Inc, Italy) for fluorescence signal development. Finally, the membrane was captured on the Chemidoc MP Imaging System (Bio-Rad, Hercules, CA, USA).

An amylase fraction was used as a standard in 4 increasing concentrations (0.01, 0.02, 0.05, 0.1 μg/μL) after been purified, from a sample of whole saliva, in an Ultimate 3000 Micro HPLC apparatus (Dionex, Sunnyvale, CA, USA), analyzed in HPLC-ESI-MS, lyophilized and quantified with BCA protocol. Individual signals of

samples were determined as intensity values, which were transformed in values of concentration by using the standard as a reference. The final concentration (µg/µL) is express as mean of the triplicates and the acceptable coefficient of variation (CV%) was set as < 15%.

Statistical Analysis

Repeated-measures ANOVA (between subjects' factor: order; within-subjects factor: treatment, order × treatment) was used to determine potential effects of stimulus order presentation (CJ or CPE) on protein families as well as alpha-amylase. The same resting values were used for CJ and CPE. In case data normality was not respected, Friedman's ANOVAs (non-parametric test) were performed to understand the effect of stimulation on the individual protein levels in saliva of subjects belonging to Group 1. Finally, post hoc comparisons for the individual proteins were done following Bonferroni corrections. Two-way ANOVA was used to evaluate differences in BMI between NW and OB according to gender and PROP taster status, and to compare differences in the basal levels of salivary proteins in NW and OB while three-way ANOVA was used to evaluate differences in protein levels related to gender and PROP taster status in NW and OB. Post hoc comparisons were performed with the Fisher's least significant difference (LSD) test. The Fisher's exact test was used to compare the number of NW and OB subjects lacking a protein. Since the XIC peak areas of the His-5 and His-6 peptides, as well as those of the three proteoforms of the family of Staths, revealed a similar trend in all subjects, they were considered and analyzed and shown in the figures as a sum, labeled Hist 5-6 and Staths, respectively.

Statistical analyses of the protein data obtained for Group 1 data were performed with XLSTAT Statistical and Data Analysis Solution (Addinsoft 2020, New York, NY, USA) and SAS 9.4 Analytical Software (Cary, NC, USA) whereas statistical analyses for Group 2 data were conducted with STATISTICA for WINDOWS (version 10; StatSoft Inc., Tulsa, OK, USA). Normality testing was done

using the Anderson–Darling test while homogeneity of variances was tested using Bartlett's test. p values ≤ 0.05 were considered significant and data reported in the figures are mean value ± Standard Error of the Mean (SEM).

Results

By the HPLC-ESI-IT-MS analysis we searched for differences in the extracted ion current (XIC) peak areas of the proteoforms and peptides belonging to the family of aPRPs, Hist, bPRPs, Staths and S-Cyst, and of PB in the stimulated/unstimulated saliva between subjects. An example of an HPLC profile (total ion current) of the acidic-soluble fraction of unstimulated saliva of a representative PROP super-taster is shown in Figure 2. The range of elution time where the protein/peptides were searched are indicated according to their families.

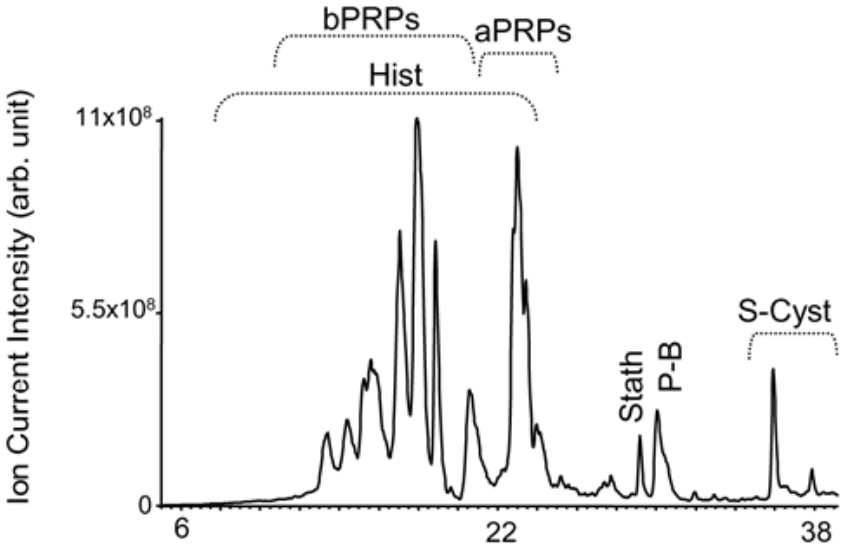

Figure 2. Example of HPLC-MS total Ion Current profile of an acidic-soluble fraction from un-stimulated saliva of a representative super-taster.

Levels of protein families before and after stimulation with CJ and CPE are shown in Figure 3. Stimulation with CJ and CPE affected levels of aPRPs ($F_{4,225}$ = 24.96, p < 0.000). aPRP levels were higher relative to baseline at 5 min and remained elevated at 10 min after stimulation with CJ (p < 0.000). The same pattern was observed after stimulation with CPE; levels were higher in comparison to baseline

at 5 min ($p < 0.000$) and remained elevated at 10 min after stimulation ($p < 0.000$). A similar trend was observed on other protein families (bPRPs, Stath, Hist and S-type cystatins), besides not significant ($p > 0.05$).

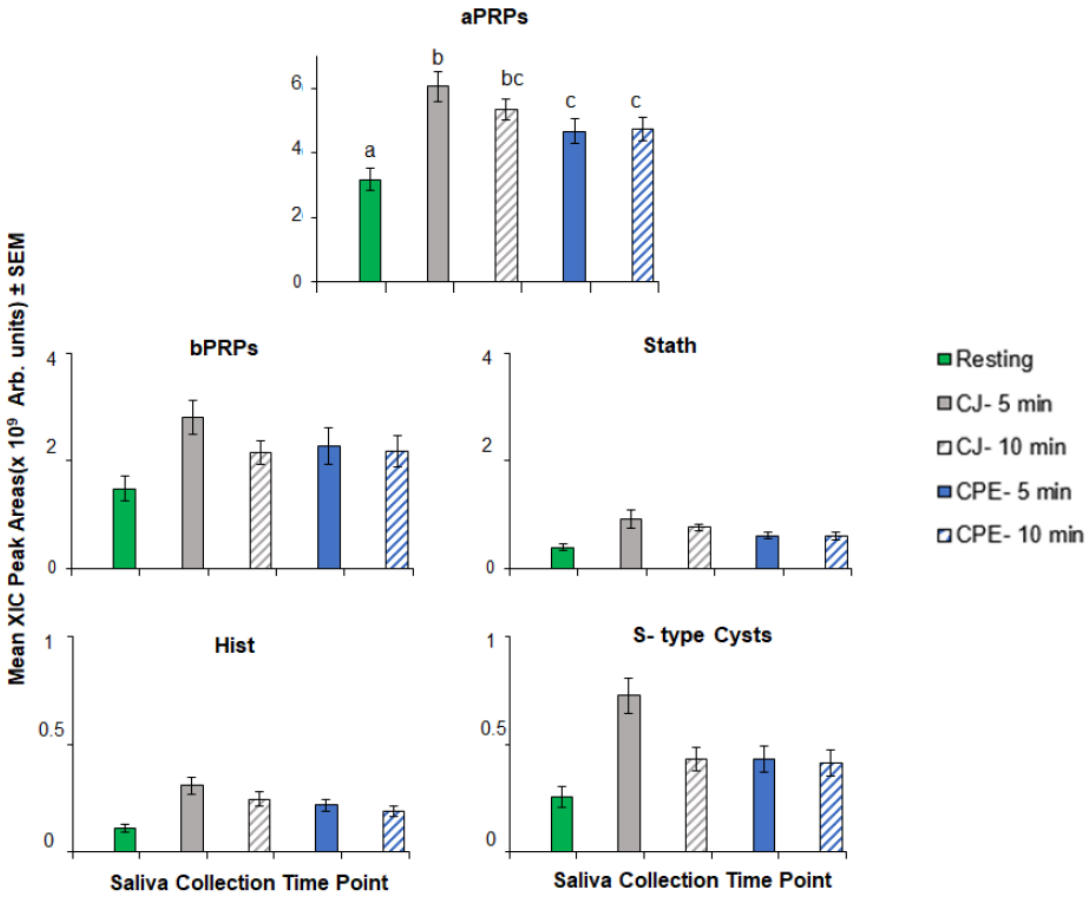

Figure 3. Extracted ion current (XIC) peak areas (mean values ± SEM x10^9) in arbitrary units for salivary protein families following stimulation with cranberry juice (CJ) and cranberry-derived polyphenol extract (CPE) (n = 60). Values with different superscript letters (a, b etc.) differ at $p < 0.05$.

However, there was a significant gender*taster*treatment effect ($F_{4,224} = 2.60$, $p = 0.037$) on bPRPs, as shown in Figure 4. In particular, there were significant differences in bPRP levels across the time course in super-taster subjects with CJ ($F_{4,116} = 3.20$, $p = 0.015$) (Figure 3a). Super-taster males had higher levels at 5 min than they had at resting after CJ and CPE ($p = 0.007$), which were not statistically different at 10 min from levels at previous times. Female super-tasters showed a small increase at 5 min, only after CJ stimulation. In comparisons by gender, male super-

tasters had higher peak bPRP levels (at 5 min) in comparison to female super-tasters (p = 0.018). Within non-tasters, no effect of PROP taster status was seen after stimulation with either CJ or CPE (Figure 13b).

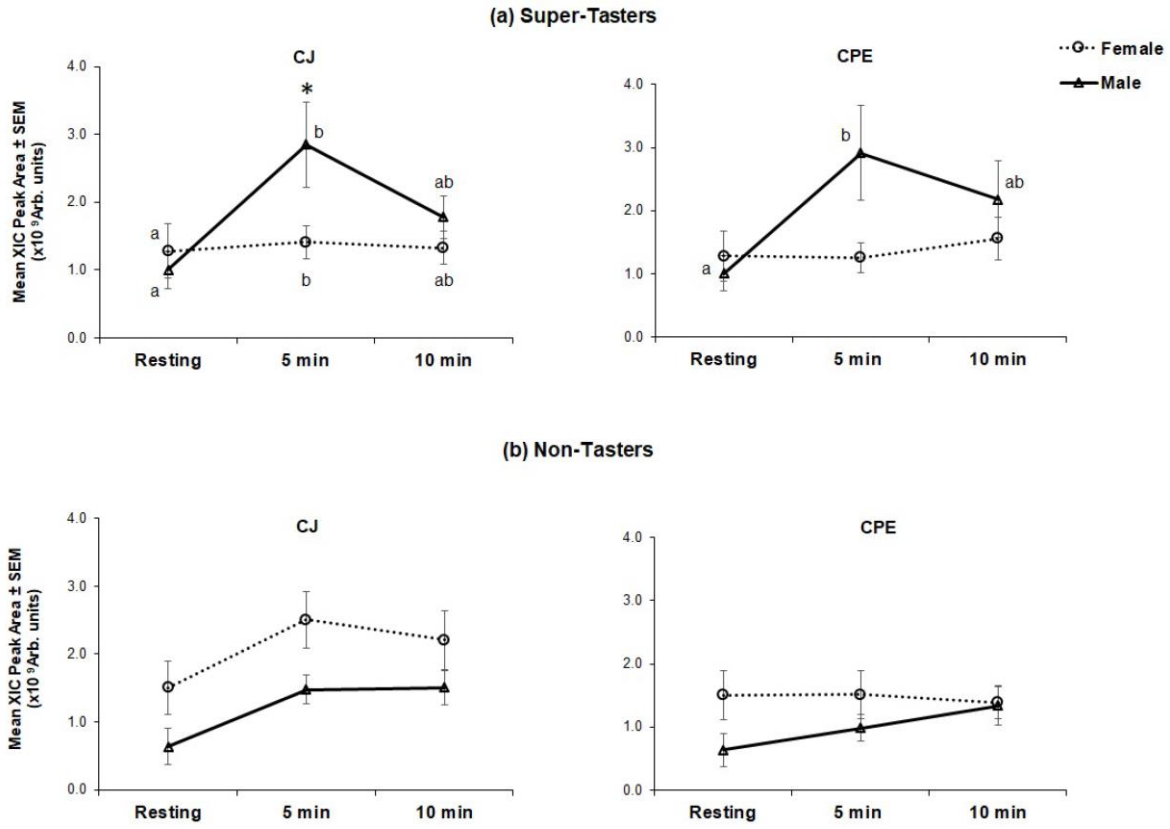

Figure 4. Mean extracted ion current (XIC) peak areas ± SEM (x10^9 arbitrary units) total bPRP (basic proline-rich protein) levels in taster × gender subgroups are shown for super-taster (a) and non-taster subjects (b). Left panels show response to stimulation to CJ, while right panels show response to CPE. Different letters show significant difference in protein levels across the time course in each subgroup; * shows differences between males and females at each time point. Non-taster females (n = 15), non-taster males (n = 14), super-taster females (n = 16) and super-taster males (n = 15).

Figure 5 show the effects of CJ stimulation on the levels of individual proteins. CJ stimulation was effective for levels of all proteins at 5 min ($p \leq 0.002$) except for PD and Ps-1 (bPRP family). P-C, PRP-1, PRP-3 (aPRP family), PF and PJ (bPRP family), Hist 1 and Hist 5+6 (Hist family) and PB rose after stimulation with CJ at 5 min and remained elevated after 10 min. Figure 14 also shows that, PH, IB-8a, II-2 and IB-1 (bPRP family), Cyst S and Cyst SA (S-Cyst family) were statistically

elevated only at 5 min. Three proteoforms of the S-Cyst family (Cyst S1, S2, SN) rose to peak levels at 5 min, trended downward, but remained elevated compared to baseline at 10 min.

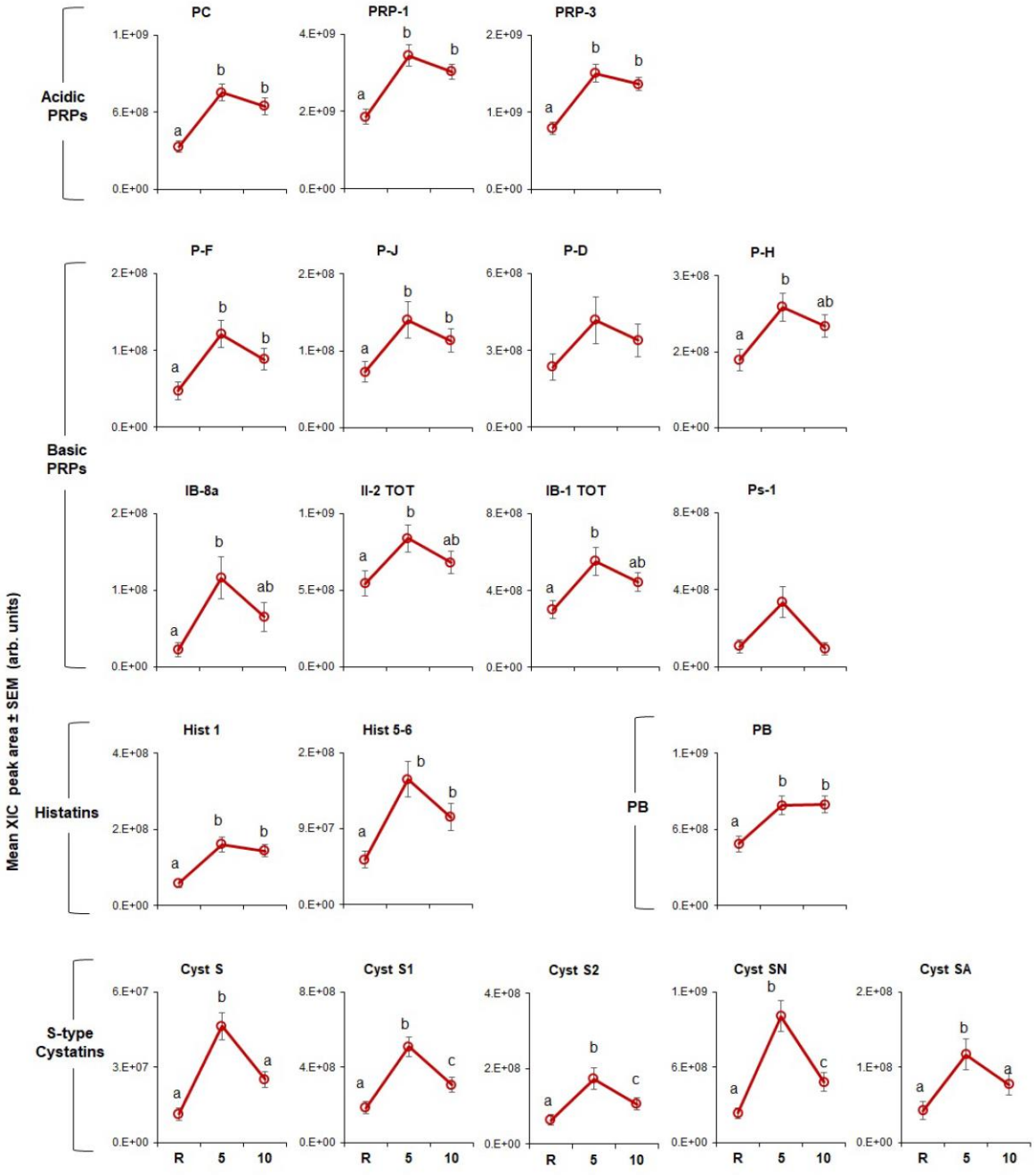

Figure 5. Effect of stimulation with CJ on levels of individual salivary proteins. Mean extracted ion current (XIC) peak areas SEM (108 arbitrary units) of individual proteins measured at resting (R), 5 min (5) and 10 min (10) after stimulation (n = 60). Means within protein type with different superscript letters (a, b, etc.) are different at $p < 0.002$.

The effects of CPE on individual proteins were less strong than they were for CJ ($p \leq 0.002$), as shown in Figure 6. Levels of all three aPRPs and of Hist 1 (Hist family) were higher at 5 min and remained elevated at 10 min after stimulation with CPE. Levels of Cyst S1, S2 and SN were only elevated at 5 min. In contrast, Hist 5+6, Cyst S and Cyst SA and all bPRPs were unaffected by stimulation ($p > 0.05$).

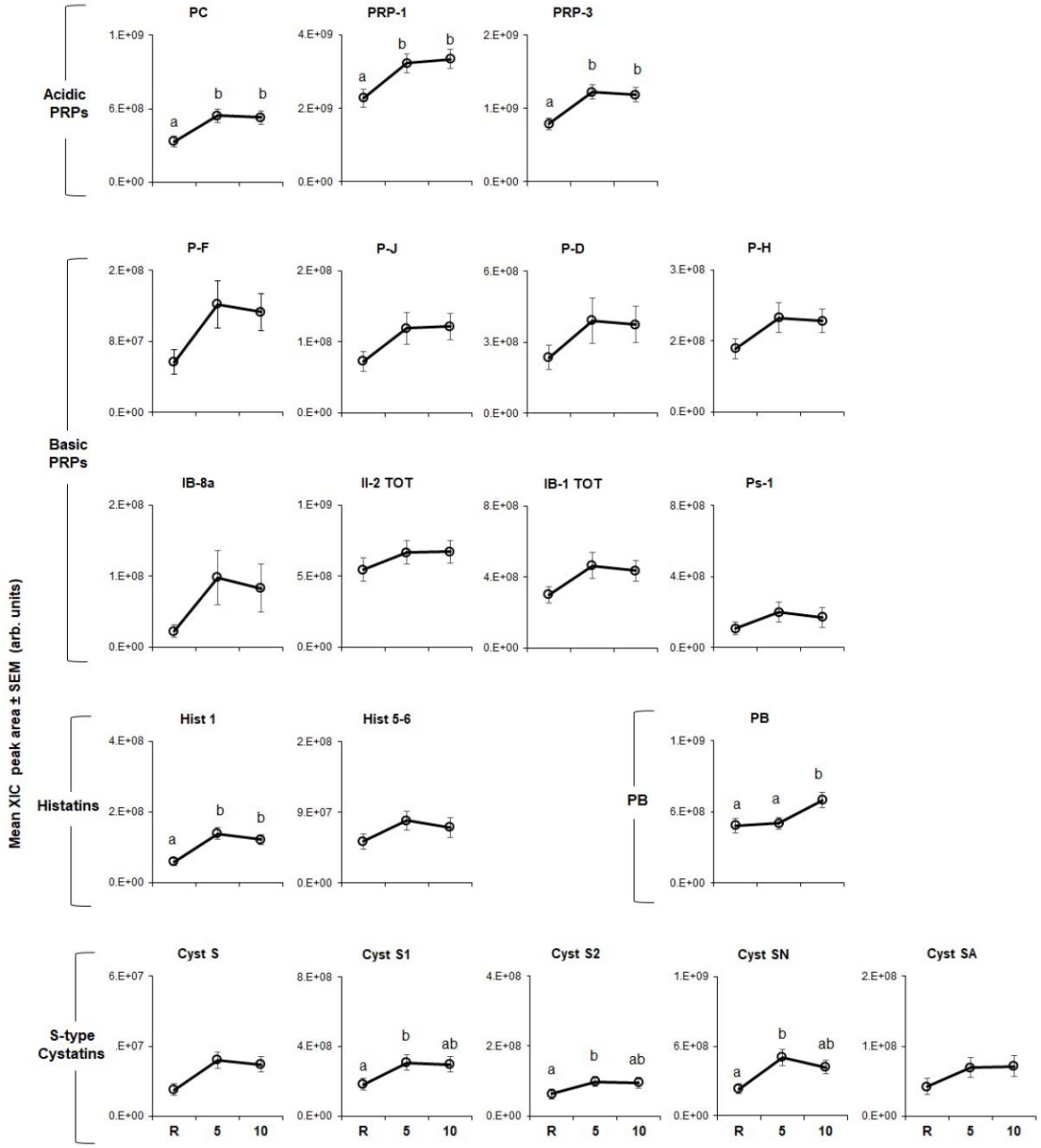

Figure 6. Effect of stimulation with CPE on levels of individual salivary proteins. Mean extracted ion current (XIC) peak area SEM (108 arbitrary units) of individual proteins measured at resting (R), 5 min (5) and 10 min

(10) after stimulation (n = 60). Means within protein type with different superscript letters (a, b) are different at $p < 0.002$.

Analysis of immunoblotting results on levels of alpha-amylase detected in saliva of subjects from Group 1, at resting levels, and at 5 and 10 min after CJ/CPE stimulation, revealed no overall main effect in response to stimulation ($p > 0.05$ after repeated-measure ANOVA). However, there was a significant taster × treatment interaction, in response to stimulation ($F_{4,224} = 5.95$; $p = 0.001$). As shown in Figure 7, PROP super-tasters had higher levels of amylase than non-tasters at 5 ($p < 0.014$) and 10 min ($p < 0.000$) after exposure to CJ. No effect of stimulation was seen with CPE. Finally, there was no effect of stimulus presentation order on levels of alpha-amylase over the time course ($p > 0.05$).

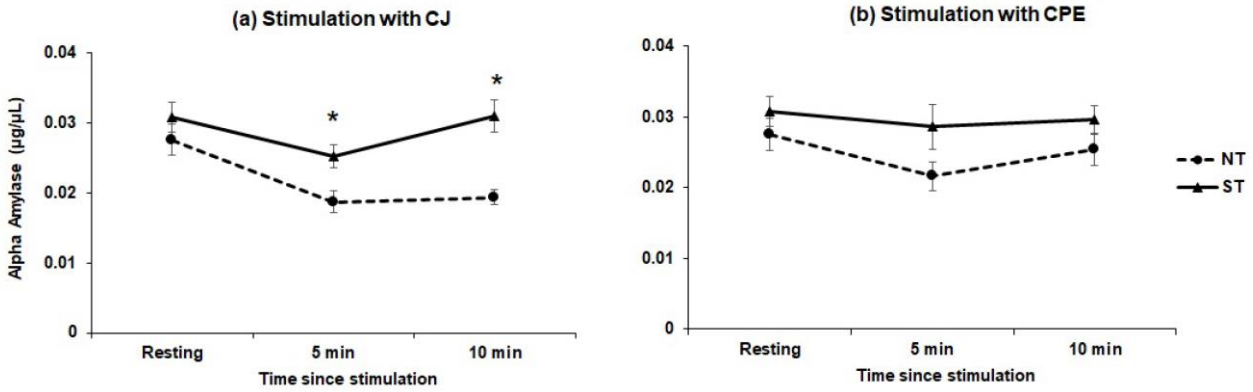

Figure 7. Effect of stimulation on alpha-amylase levels (g/L) after stimulation with CJ (a) and CPE (b). * indicates a difference between non-tasters and super-tasters ($p \leq 0.014$) at a given time point. Non-tasters (n = 29) and super-tasters (n = 31).

Figure 8 shows mean values of BMI in NW and OB belonging to Group 2 according to gender and PROP taster status. A significant two-way interaction of gender × NW/OB status ($F_{1,14} = 17.142$; $p < 0.00007$) and of the PROP taster status × NW/OB status ($F_{2,112} = 4.713$; $p = 0.011$) on BMI values was found. Post hoc comparison showed that the BMI of NW males was significantly higher than that of NW females, while the BMI of males was lower than that of females in OB ($p \leq 0.017$; Fisher's least significant difference (LSD) test). Post hoc also showed that the BMI of super-taster OB was significantly higher than that of medium taster and non-taster OB ($p \leq 0.002$; Fisher's test LSD). No significant difference in BMI related to PROP taster status was found in NW, although an opposite trend was evident ($p > 0.05$).

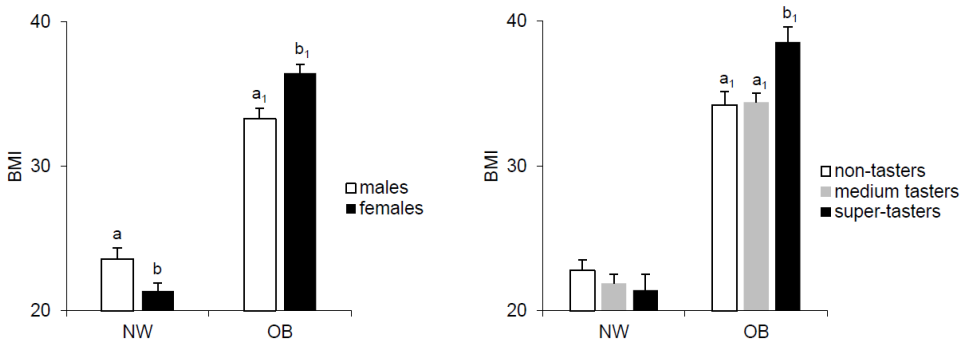

Figure 8. Mean values (± SEM) of body mass index (BMI) in normal weight (NW) (n = 61) and obesity (OB) (n = 57) according to gender (NW: male n = 21; female n = 40. OB: male n = 23; female n = 34) and to PROP taster status (NW: non-taster n = 22; medium taster n = 29; super-taster n = 10. OB: non-taster n = 14; medium taster n = 32; super-taster n = 11). a,b = significant difference within NW; a_1,b_1 = significant difference within OB ($p \leq 0.017$; Fisher's least significant difference (LSD) test .

By the HPLC-ESI-IT-MS analysis we investigated differences in the extracted ion current (XIC) peak areas of the proteoforms and peptides belonging to the family of aPRPs, Hist, bPRPs, Staths and S-Cyst in the unstimulated saliva of NW and OB belonging to Group 2. Figure 9 shows the XIC peak areas of the proteoforms belonging to the family of aPRPs, the peptides belonging to the family

of Hist, the proteoforms belonging to the family of bPRPs, the Staths and the proteins belonging to the family of S-Cyst in the unstimulated saliva of NW and OB. Levels of Ps-1 protein in OB were higher than that in NW (p = 0.00079; Fisher's test LSD subsequent to two-way ANOVA). Remarkably, the Ps-1 was undetected in 19 NW and 23 OB (χ^2 = 1.09; p = 0.30).

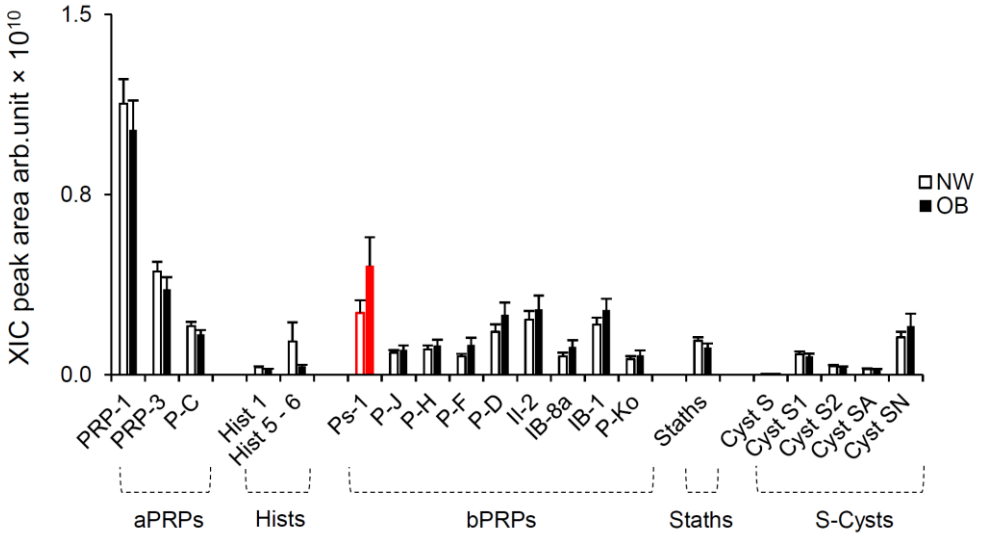

Figure 9. Mean values (±SEM) of the extracted ion current (XIC) peak areas of the analyzed proteins in unstimulated saliva of NW (n = 61) and OB (n = 57). Red color indicates a significant difference between NW and OB (p = 0.00079; Fisher's test LSD).

The XIC peak areas of the same proteins measured in unstimulated saliva of NW and OB females (upper graph) and males (lower graph) are shown in Figure 10. Three-way ANOVA revealed a significant interaction of gender × protein type × NW/status on the salivary protein levels ($F_{20,2394}$ = 3.2908; p < 0.00001). Post hoc comparison revealed that unstimulated saliva of OB males had higher levels of PRP-1, Ps-1, II-2 and IB-1 than NW males ($p \leq 0.038$; Fisher's test LSD) (lower graph). Post hoc comparison also showed that the levels of PRP-1, Ps-1 and II-2 of OB males were higher than those measured in OB females ($p \leq 0.013$; Fisher's test LSD) and the levels of PRP-1 in unstimulated saliva of NW males were lower than those measured in NW females (p = 0.000001; Fisher's test LSD). The unstimulated saliva of all females had PRP-1 and PRP-3 proteins, while some were lacking Hist 5-6 (4

NW and 5 OB), Ps-1 (13 NW and 12 OB) and II-2 (1 NW and 3 OB). No difference was found between NW and OB females ($\chi 2 < 1.437$; $p > 0.231$).

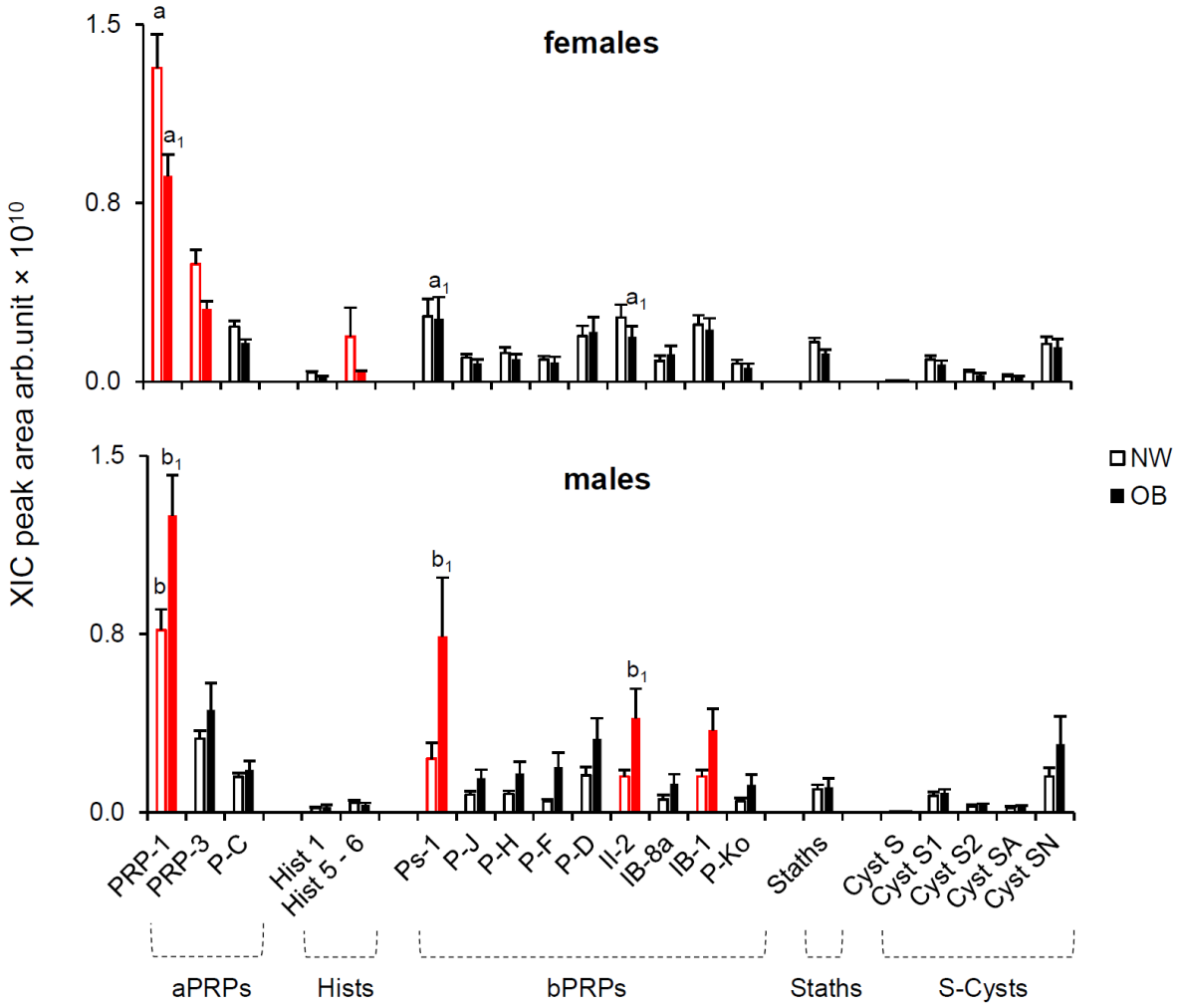

Figure 10. Mean values (±SEM) of the XIC peak areas of the analyzed proteins in unstimulated saliva of NW and OB according to gender. Males: NW $n = 21$, OB $n = 23$; females: NW $n = 34$, OB $n = 40$. Red color indicates a significant difference between NW and OB ($p \leq 0.013$; Fisher's test LSD). a_1, b_1 = significant difference between females and males with obesity; a, b = significant difference between NW females and males ($p \leq 0.013$; Fisher's test LSD).

Figure 11 shows the XIC peak areas of the same proteins measured in unstimulated saliva of NW and OB according to their PROP taster status. Levels of Ps-1 and Cyst SN were significantly higher in the PROP non-taster OB, with respect to non-taster NW ($p \leq 0.032$; Fisher's test LSD subsequent to three-way ANOVA)

and that of the Hist 5-6 was significantly lower in the PROP super-taster OB, with respect to super-taster NW ($p \leq 0.00039$; Fisher's test LSD subsequent to three-way ANOVA). No difference between NW and OB was observed in medium tasters ($p > 0.05$). OBs who were classified as non-tasters showed higher levels of Ps-1 than OBs who were super-tasters ($p \leq 0.000013$; Fisher's test LSD subsequent to three-way ANOVA). NWs who were classified as super-tasters had higher levels of Hist 5-6 than NWs who were non-tasters ($p = 0.00009$; Fisher's test LSD subsequent to three-way ANOVA). Several NW and OB subjects of the PROP taster groups were lacking Hist 5-6 (super-tasters: 2 NWs and 3 OBs and non-tasters: 4 NWs and 3 OBs), Ps-1 (super-tasters: 2 NWs and 4 OBs and non-tasters: 9 NWs and 6 OBs) and Cyst SN (super-tasters: 1 NW and 2 OBs and non-tasters: 5 NWs). No difference was found between NW and OB super-tasters or non-tasters ($\chi 2 < 0.687$; $p > 0.47$).

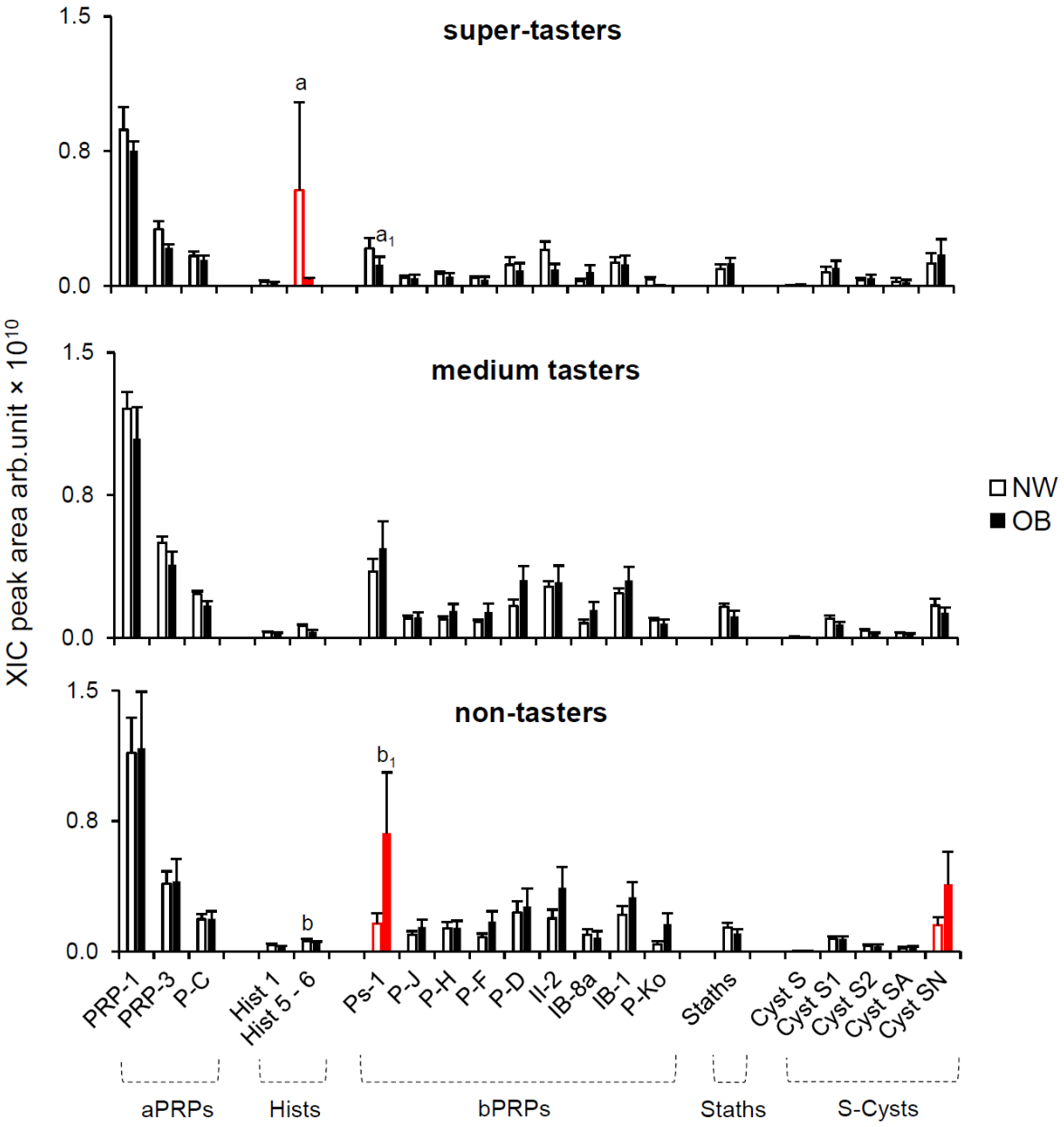

Figure 11. Mean values of the XIC peak areas of the analyzed proteins in unstimulated saliva of NW and OB according to PROP taster status. Non-tasters: NW n = 22, OB n = 14; medium tasters: NW n = 29, OB n = 32 and super-tasters: NW n = 10, OB n = 11. Red color indicates a significant difference between NW and OB ($p \leq 0.032$; Fisher's test LSD). a, b = significant difference between NW super-tasters and non-tasters; a_1, b_1 = significant difference between OB super-tasters and non-tasters ($p \leq 0.013$; Fisher's test LSD).

Discussion

The results of the conducted experiments provide novel findings of interindividual differences in the salivary proteome that may influence the development of astringency sensation in the mouth. Here we also showed, for the first-time, significant variations of taste-related salivary proteins, between NW and OB, which were differently associated with gender and PROP sensitivity.

Primarily, we examined salivary responses to astringent stimuli with respect to major salivary protein families and individual protein sub-types within those families. We found that oral stimulation with CJ and CPE determined a robust increase in aPRPs but not the other protein families, whose increase was not significant. This agrees with our previous findings [3] with the same set of proteins showing that aPRPs rose robustly after oral stimulation with CJ. Another time-course study conducted by Brandao and co-workers [7] also reported that aPRPs were most responsive to stimulation with condensed tannins, while bPRP levels were unaffected. Interestingly, we observed the same outcomes for aPRPs, even though the latter study had a smaller sample size (triplicates of n = 4), used pooled saliva samples and repeated oral stimulation prior to beginning the time course. Despite these methodological differences, all three studies support an important role for aPRPs in the salivary response to oral polyphenols. Together, these findings seem to conflict with the earlier literature suggesting that bPRPs were primarily responsible for oral astringency due to their highest binding affinity to tannins [8, 9]. However, recent competitive in vitro assays showed that aPRPs and histatins, mostly found in the mobile phase of saliva, are first in line to interact with polyphenols (in comparison to other proteins such as mucins or gPRPs, which are mostly adsorbed onto mucosal or dental surfaces) particularly at lower phenolic concentrations [10]. Presumably, all the astringent stimuli may not be cleared from the mouth upon swallowing. It is possible that interaction of a protein with residual polyphenols in saliva may occur [11, 12], leading to persistent soluble aggregate

formation, which do not coalesce and precipitate, initiating the so-called 'second step' of protein–polyphenol interaction [8, 13].

An important observation in our study was that CJ was a more effective stimulus for protein responses than CPE, both at the family level as well as for individual protein sub-types. Essentially, all protein sub-types were significantly elevated by CJ, except PD and Ps-1 belonging to bPRPs. In comparison, the CPE effects on other protein types were less consistent than what we observed for CJ. CPE did not increase any of the individual bPRPs and only affected some of the S-type cystatins and histatins. Additionally, we noticed that individual S-type cystatins, (e.g., Cyst S1, S2 and SN) returned to baseline at the end of 10 min after CJ but not after CPE. Although CPE is a carrier-free flavor ingredient that contains a mixture of various flavonoids, especially anthocyanins, flavanols and proanthocyanidins, which have all been shown to interact with salivary proteins [14-16], CJ also contains highly acidic and contains pectin. Pectins have been shown to hinder the complex formation between salivary proteins and polyphenols [17]. Acidic stimuli elicit saliva release, mainly from the parotid gland, which leads to an elevated level of proteins, such as proline-rich proteins in the oral cavity [18, 19]. Notably, bPRPs are only released from the parotid gland, where they make up 23% of the total protein secreted [20]. This could be an explanation for why bPRP levels rose after CJ but not to CPE stimulation. Recent work studying mixtures of proteins in vitro has shown that depending on the size of protein–polyphenol aggregates formed by other salivary proteins such as aPRPs, interactions between phenolic stimuli and bPRPs may be impeded [15]. It is possible that CPE formed aggregates with other salivary proteins, which prevented the interaction between bPRPs and CPE. Finally, a large component of our understanding of protein–polyphenol interactions come from purified salivary protein fractions, whereas the present study analyzed whole saliva, which is a mixture of many proteins. These factors alone or in combination could have influenced the higher levels of proteins following stimulation with CJ in contrast to CPE.

The present study also examined the role of PROP taster status in salivary protein responses to CJ with the goal of replicating the earlier findings of Melis and co-workers [3] and potentially extending these findings to CPE. In the latest work, Melis et al. showed PROP-specific effects of CJ on two sub-types of aPRPs (PRP-1 and PRP-3) and one of the Cystatin sub-types (Cyst-SN). Specifically, levels of these proteins rose after CJ stimulation in medium-tasters and super-tasters, but no increases were observed in non-tasters. The present findings diverge from earlier results as we observed no PROP-related effects on either aPRP or Cystatin sub-types. However, we did observe a taster by gender interaction of stimulation on the bPRP protein family only in super-tasters. Generally, levels of bPRPs among male super-tasters rose significantly higher than those of female Super-tasters at 5 min after stimulation. CPE stimulation also raised bPRPs for male super-tasters, but these levels were not significantly higher than those of female super-tasters. The reasons for the discrepant findings between studies are presently unclear. Nevertheless, an important difference is that Melis et al. [3] measured protein levels at 1 min after stimulation, whereas the current study examined protein levels at 5 and 10 min. The dominance of different salivary proteins at different time points (i.e., aPRPs and cystatins at 1 min and bPRPs at 5 min after stimulation) suggest the sequential involvement of proteins at different stages of astringency development and that PROP-taster status may be an important marker at each stage of this process. Although methodologically challenging, repeated saliva collections at short intervals directly following CJ exposure may reveal important dynamics of the early protein response that would clarify our understanding of PROP effects as well as other individual differences in salivary function. Future studies should address this question. Interestingly, Melis et al. [3] also found a gender dichotomy in their astringency perception experiment. Together, these data suggest a gender-specific role for PROP status that may link oral astringency perception with protein responses. Greater recognition of these potential differences may be important for interpreting the results of future sensory studies and proteomic analyses.

Salivary amylase plays a key role in the oral digestion of starches by hydrolyzing complex carbohydrates to smaller sugars [21] and its expression has been shown to be influenced by high-fat and high-tannin diets in animal models [22, 23]. To our knowledge, salivary amylase has not been measured in studies investigating the involvement of salivary proteins in astringency responses over a time course. We found no main effect of either CJ or CPE on amylase levels. However, super-tasters had higher levels of amylase in response to CJ exposure than did non-tasters. Individual differences in salivary amylase activity are well known and may be related to *AMY1* gene copy number [79] in humans. Whether salivary amylase activity also varies with PROP taster status should be investigated in future studies as amylase has important implications in digestion, nutrient absorption and most recently, texture perception [24-27].

Seminal work by Dinnella and colleagues [11, 12] on individual variation in the astringency response utilized total protein concentrations (D values) in correlation with sensory responses to classify individuals into low or high responders to astringency. In the current study, we targeted specific proteins and peptides, which enabled us to offer insights into a selected individual difference, i.e., interindividual variation in salivary proteins associated with PROP taster status. Conducting a time-course of astringent sensations in PROP-classified subjects could supplement our understanding of the sensory relevance of these differences. Examples of this could include use of temporal dominance of sensations or other time-intensity measures to track differences in the experience of astringent sub-qualities, which may be important for understanding this complex sensation.

We also investigated whether the salivary proteome composition, which can contribute to differences in taste perception, may be considered an important factor in driving unbalanced food behavior, which could lead to obesity. The results showed that variations of taste-related salivary proteins, between NW and OB, differently associated with gender and PROP sensitivity. Interestingly, BMI values were associated with gender and PROP sensitivity with an opposite trend in OB and NW. NW males showed higher values than NW females, while OB males showed lower values than OB females and, as already shown in previous studies [28, 29], OB who were classified as super-tasters had a higher BMI than the other two PROP taster groups, while an opposite trend was found in NW [29]. A summary of principal results is shown in Figure 12.

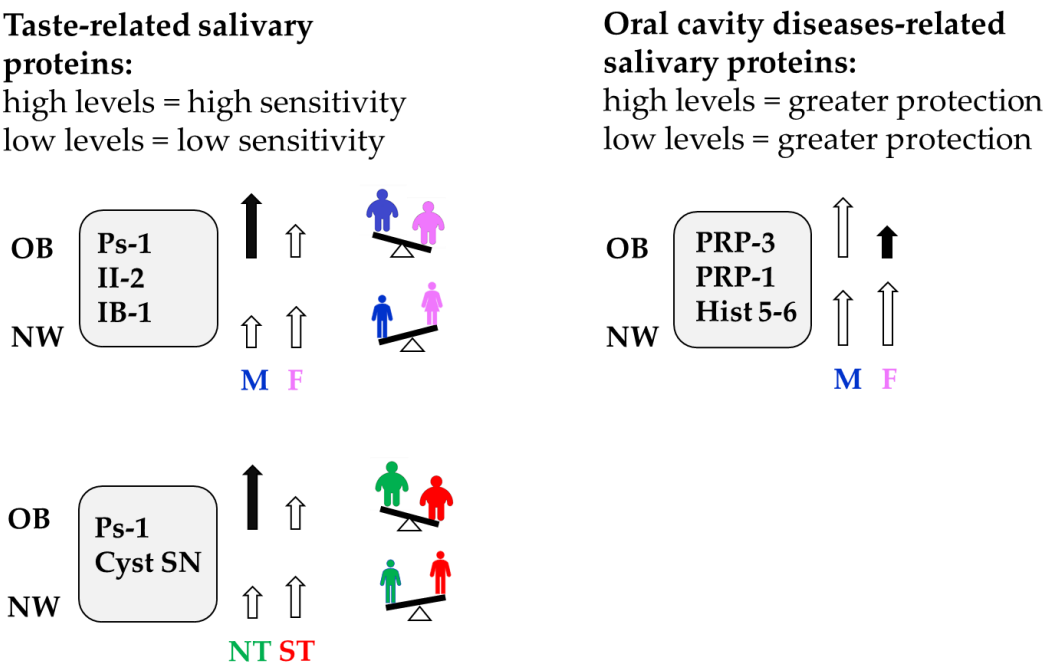

Figure 12. Picture showing a summary of findings on differences between NW and OB in salivary protein levels as a function of gender and PROP taster status. The height of arrows indicates the quantified level of proteins.

HPLC-ESI-IT-MS analysis of unstimulated saliva of NW and OB allowed us to reveal that OB had higher levels of Ps-1 protein than NW. Since this protein and the amino acids highly represented in its sequence (L-Arg and L-Lys) have been

shown to facilitate perception of hydrophobic molecules (i.e., PROP or oleic acid) by increasing their solubility in aqueous media [30, 31], our finding seems to indicate that OB with higher levels of Ps-1 protein may find a greater reward in fat food intake than NW.

The differences of salivary proteome that we found between OB and NW depend on gender: Ps-1, II-2 and IB-1, all proteins of bPRPs family, which had already been associated with higher taste perception [2], were lower in NW males than OB males and the latter had higher levels of Ps-1 and II-2 proteins than OB females. These results seem to indicate that a greater reward in response to palatable foods associated with obesity is specific for males. Differently, OB females who present lower levels of these proteins, and thus lower taste sensitivity, might be expected to eat more, as confirmed by their higher BMI. This result is consistent with data showing that a reduced oral perception strongly correlates with increased preferences for high-fat or energy foods [32], which lead to a higher consumption of these kinds of foods [33, 34], a higher BMI [9,62,71] and a greater adiposity [35].

Our data also showed a strong effect of gender on the levels of PRP-1 and PRP-3 proteins of the aPRP family. It is known that PRP-1 and PRP3 have a strong affinity for the tooth mineral hydroxyapatite and can inhibit calcium phosphate precipitation, playing a role in the enamel pellicle formation, tooth protection and calcium homeostasis [36-38]. Previous studies also reported that peptides belonging to Hist family display an antifungal activity and inhibit bacterial enzymes involved in periodontal disease [39, 40]. In addition, the occurrence of periodontitis was found to be more prevalent in OB females [41], who showed lower levels of Hist 5-6 than NW females. Our results showed that the levels of PRP-1, PRP-3 and Hist 5-6 were higher in NW than OB females, thus suggesting an increased probability of occurrence of caries and periodontitis in OB females according to studies that emphasize a high presence of dental disease in OB, especially in females [42-44]. The low levels of the PRP-1, together with the lower levels of Ps-1 and II- 2, that we found in OB females let us speculate that diseases in the oral cavity create an

adverse environment for the dissolvement of food chemicals in saliva and for the interaction between tastants and taste receptors, eventually affecting overall taste perception.

The chemical interaction between taste stimuli and salivary proteins has been shown to explain the big phenotypic differences in the PROP taste perception [1, 2, 30, 45]. Specifically, high levels of Ps-1 and II-2 proteins are associated with high PROP sensitivity, while low levels with low sensitivity. In addition, low sensitivity in NW is associated with higher preference and lower discrimination for foods high in calories [46-48] and this leads to an unbalance of food habits affecting nutritional status and BMI [34]. In addition, the oral supplementation of Ps-1 protein, and that of amino acids (L-Arg and L-Lys), has been shown to modify the perception of PROP and the five taste qualities [1, 2, 30, 49]. Differently, in OB we found that the phenotype with low sensitivity showed higher levels of Ps-1 with respect to that with high sensitivity according to its higher BMI values. It is interesting to note that OB non-tasters also had higher levels of Ps-1 protein than NW non-tasters and this proves their higher sensitivity.

Noteworthy, proteins of the bPRP family are secreted in saliva exclusively by parotid glands [5, 38] and gland activity is affected by gender and obesity status. Inoue et al. showed that young NW females have smaller parotid and submandibular gland sizes as compared to males [50]. Moreover, Bozzato et al. [51] described a deposition of adipocytes in parotid glands of OB, but not in submandibular glands, with a correlation between BMI and gland size. This allowed us to speculate that the bPRPs synthesis and secretion depend on gender and nutritional status.

Our results also show that OB who were classified as non-tasters had higher levels of Cyst SN than NW non-tasters. Hyposensitive subjects for the bitterness of caffeine express higher salivary levels of Cyst SN than hypersensitive subjects, and it was overexpressed in infants that accepted a bitter solution [52, 53]. In vitro experiments on human submandibular gland cells showed an overexpression of

Cyst SN after caffein stimulation [54] and in vivo experiments after cranberry juice, a model stimulus for astringency [3]. In addition, Cyst SN is overexpressed in sensitive subjects for oleic acid (C18:1) [55]. These considerations support the hypothesis that OB non tasters having high levels of Ps-1 and Cyst SN may have an increased taste sensitivity compared to NW non-tasters.

On the whole, results show novel insights on the role of salivary proteome as a factor driving the greater propensity for body weight excess of females or that associated with higher PROP sensitivity, which have been already shown [29, 56]. However, further studies should investigate the effect of the oral supplementation with Ps-1 protein or amino acid L-Arg on taste perception in OB to test the hypothesis that this mechanism may alter taste response related to foods for the development of a sex or taste-specific approach for weight loss treatment. In addition, the oral modification of specific salivary components might find application also by solving the increased predisposition for all diseases, which are related with unbalanced food habits in subjects with reduced taste [29, 57-71].

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CHAPTER 3

RELATIONSHIPS BETWEEN TASTE AND OLFACTORY FUNCTIONS, NUTRITIONAL STATUS, AND HEALTH

Melis M, **Mastinu M***, Sollai G, Paduano D, Chicco F, Magrì S, Usai P, Crnjar R, Tepper BJ, Tomassini Barbarossa I. Taste Changes in Patients with Inflammatory Bowel Disease: Associations with PROP Phenotypes and polymorphisms in the salivary protein, Gustin and CD36 Receptor Genes. Nutrients 2020, 12(2), 409. https://doi.org/10.3390/nu12020409*

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*Melis M, Haehner A, **Mastinu M**, Hummel T, Tomassini Barbarossa I. Molecular and Genetic Factors Involved in Olfactory and Gustatory Deficits and Associations with Microbiota in Parkinson's Disease. International Journal of Molecular Sciences 2021, 22(8), 4286. https://doi.org/10.3390/ijms22084286*

In this part of work, we present results aimed to understand if impairments of taste and olfaction driving unbalanced food choices can be significant risk factors that contribute to the pathogenesis of different diseases. Specifically, we analyzed the perception for six taste qualities, olfactory performance, specific taste/olfactory gene polymorphisms and BMI, in patients with inflammatory bowel disease (IBD) and studied how a bariatric surgical treatment could modify taste and smell sensitivity and eating behavior in severe obese subjects.

Methods

Participants

For this study, two groups were formed. Group 1 was composed of one hundred and ninety-nine Caucasian volunteers divided into two clusters based on their clinical status. The first was represented by IBD patients (n = 100; females = 46, males = 54; age 51.2 ± 1.41 year), recruited at the clinic of the Gastroenterology Unit of the University Hospital Company (AOU) Monserrato (Cagliari, Italy), and included CD (n = 44) and UC patients (n = 56); the second cluster included healthy control (HC) subjects (n = 99; females = 40, males = 59; age 47.3 ± 1.42 year) recruited to match for age (χ^2 = 4.33, p = 0.12) and gender (χ^2 = 0.64, p = 0.43). All volunteers performed the olfactory sensitivity screening whereas only 97 IBD patients (females = 44, males = 53; age 51.38 ± 1.5 year) and 62 HC subjects (females = 36, males =26; age 48.79 ± 3.06 year) completed the taste sensitivity measurement. All patients enrolled (both CD and UC patients), were diagnosed by means of Crohn's Disease Activity Index (CDAI) and Partial Mayo Score (PMS) as far as clinical examination, endoscopy and radiology. They had a disease in remission and were treated with mesalazine or 5-ASA agents or monoclonal antibodies against TNF-α for their disease. BMI in IBD patients was stable and did not tend to change over time, due to the condition of disease remission.

Group 2 was composed of sixty-eight Caucasian volunteers, recruited at the Bariatric surgery Center, G. Brotzu Hospital (Cagliari, Italy). Fifty-one of them (females = 36, males = 15; age 43.5 ± 1.5 y; BMI: 43.0 ± 0.8 kg/m^2, range 33.1–59.2 kg/m^2) who were scheduled to undergo either sleeve gastrectomy (SG) (n = 21), Roux-en-Y gastric bypass (RYGB) (n = 26) or mini gastric bypass (n = 4), participated in this study, while seventeen subjects left the study after surgery and were excluded from the analysis. Prior to enrolment, volunteers were interviewed by a multidisciplinary team with surgical, nutritional, and psychological expertise.

For all volunteers belonging to both groups, exclusion criteria include major metabolic illnesses (diabetes, kidney disease, etc.), pregnancy, lactation, food allergies, and the use of medications that interfere with taste or smell functions, head trauma, sinusitis or nasal septum disorders. Patients who had any systemic diseases associated with the pathological condition and those who had undergone a former gastrointestinal surgery were not included. After an explanation of the experimental procedure, all subjects read and signed an informed consent form. Study procedures were carried out in accordance with the Helsinki Declaration and approved by the ethical committee of AOU of Cagliari.

Experimental Procedure

Subjects were separately tested in the morning (from 9 to 11 am) in a well-ventilated room with controlled-environmental parameters (23–24 °C; 40–50% relative humidity), where they were asked to stay 15 min before the beginning of the testing. They were always asked to abstain from eating, drinking (except water), smoking, using oral care products or chewing gum for 12 h prior the testing. Controls and patients were asked if they had a cold or had any allergic reactions, in which case they were discarded. All subjects were also requested not to wear perfumes.

Subjects belonging to Group 1 completed the experimental procedure in one session while those belonging to Group 2 were tested in three separate visits:

before bariatric surgery (T0) and one month (T1) and six months (T2) after surgery. The same experimental procedures were carried out in all three visits. All completed a battery of sensory tests to assess their PROP taster status, their taste sensitivity for the six taste qualities (sweet, salty, sour, bitter, umami and fat) and olfactory function. Participants were evaluated for cognitive control of eating behaviors by the 3-Factor Eating Questionnaire (TFEQ) [1] which assesses three characteristics of eating behavior: dietary restraint, disinhibition, and perceived hunger.

In the first visit, a sample of whole saliva (2 mL) from subjects belonging to Group 1 was collected into an Eppendorf tube, and a sample of blood (4 mL) from subjects belonging to Group 2 was collected and rapidly centrifuged. Biological samples were stored at −80 °C until the molecular analyses described below were completed. Weight and height were measured for each subject in order to calculate the BMI (Kg/m^2).

PROP Taster Status Classification

Subjects were classified for PROP taster status via the impregnated paper screening test. This procedure requires the evaluation of perceived intensity after tasting one filter paper disk impregnated with 1.0 mol/L of NaCl (Sodium Chloride, Sigma-Aldrich, Milan, Italy) and another impregnated with 50 mmol/L of PROP (6-n-propyl–2-thiouracil, Sigma-Aldrich, Milan, Italy) for 30 s, by using the label magnitude scale (LMS). Subjects rinse their mouth with spring water in between tasting the paper disks and interstimulus time was set at 1 min. Subjects were classified as non-tasters if they rate the PROP disk < 15 mm on the LMS; they are categorized as super-tasters if they rate the PROP disk > 67 on the LMS. Subjects who rated PROP disk between 15 and 67 mm were classified as PROP medium tasters. Ratings for NaCl disk were used to classify volunteers who gave borderline ratings for PROP disk.

Sweet, Salty, Sour, Bitter and Umami Taste Sensitivity Assessment

Taste sensitivity to sweet, sour, salty and bitter was examined by using the Taste Strip Test (TST, Burghart Company, Wedel, Germany) [2]. Briefly, sixteen filter paper strips impregnated with four concentrations of each taste quality (sweet: 0.05, 0.1, 0.2, or 0.4 g/mL of sucrose; sour: 0.05, 0.09, 0.165, or 0.3 g/mL of citric acid; salty: 0.016, 0.04, 0.1, or 0.25 g/mL of NaCl; bitter: 0.0004, 0.0009, 0.0024, or 0.006 g/mL of quinine hydrochloride) were presented to each subject in a pseudo-randomized manner (from the lowest concentrations to the highest). Subjects placed each paper strip on the tongue and identified, from a list of four descriptors (sweet, sour, salty, and bitter), the taste quality they perceived. Each correct answer was rated 1 and the maximum score for the whole TST was 16 (4 per each taste quality). A subject was considered normogeusic if he/she scored ≥ 9, hypogeusic or ageusic if he/she scored < 9 (total taste score below the 10th percentile). Similarly, subjects were tested for their sensitivity for the umami taste by asking them to identify umami after perceiving four filter papers impregnated with 10 μL of monosodium glutamate (MSG) solution (0.0017, 0.0085, 0.0170 and 0.0338 g/mL). Solutions were prepared 1-2 days before testing and refrigerated at 4 °C until serving at room temperature. Each correct identification was rated as 1 and the maximum score was 4. The interstimulus interval was set at 60 s and before each stimulation subjects rinsed their mouths with spring water.

Oleic Acid Assessment

Oleic acid threshold was estimated by using a 3-Alternative Forced Choice (3-AFC) implemented by Melis et al. [3]. Subjects were presented with three paper filter disks: 2 were impregnated with 10 μL of mineral oil (control) whereas 1 with the amount of oleic acid under evaluation. After subjects had placed the disk on the tip of their tongue, kept it in the mouth for 10 s and spit it out, they were requested to identify the odd sample among the triplet. Oleic acid samples were tested without nose clip and in ascending order, from the lowest concentration (0.0015 μL) to the

highest (pure). The oleic acid concentration was increased after a single incorrect response and decreased after two correct responses in a row. The concentration at which subjects correctly identified the odd sample was reported as the detection threshold. Participants rinsed their mouths after each triplet. The time between triads was 1-2 min. Twenty subjects belonging to Group 1 were not able to distinguish paper disks impregnated with pure oleic acid from those of controls. These subjects were excluded from the oleic acid thresholds and CD36 molecular analyses.

Olfactory Assessment

Orthonasal olfactory function assessments of each participant were evaluated by using the standardized "Sniffin' Sticks" test battery (SSET; Burghart, Wedel, Germany) [4] including three subtests for olfactory threshold (T-test), olfactory discrimination (D-test) and olfactory identification (I-test). In the test, internationally recognized for its validity and reliability, odors are delivered by using felt-tip pens: after removing the cap, the pen tip was positioned under the nose for 3 sec, approximately 2 cm away from the nostrils, and slightly moved from left to right nostril and backwards.

T-test had 16 triplets of pen available, each of them consisting of two pens containing a solvent and the third soaked in a growing concentration of n-butanol. Triplets were presented in ascending order until the subject identified, for two consecutive times in the same triplet, the odd pen containing n-butanol (first reversal). The experiment was concluded when the seventh reversal was reached, and the threshold score was given by the average of the last four reversals, ranging between 1 and 16 points.

Additionally, D-test had 16 triplets of pen, each formed by two pens containing the same odor and one soaked with a different one. Pens of a triplet were presented in a randomized order and subjects were asked to identify the pens with the dissimilar odor. Within triplets, intervals were approximately 3 s whereas

intervals were 20–30 s between-triplets. Importantly, during the T-test and D-test, the subject was also blindfolded to prevent them from identifying the pen containing the odor.

In order to determine the identification of odors, 16 pens were presented singularly, which contained as many familiar odors to the subjects. Each pen was associated with 4 descriptors, expressed verbally and visually before delivering the stimulus, from which the subject was required to choose. The scores for D-test and I-test corresponds to the number of correct discrimination and identification from 0 to 16, respectively. The sum of the scores obtained with the T-test, D-test and I-test gave the total Threshold, Discrimination and Identification (TDI) score, by which the subjects were classified for their general olfactory performance as normosmic or hyposmic.

Patients belonging to Group 2 only performed the I-test in all three visits. The subject identification score (I-test score) corresponds to the number of correct identifications and ranged from 0 to16. The classification of each participant as normosmic or non-normosmic take into account their age and gender [5]. The cut off values for normosmia for those in the age group 36–55 y were: 11 for men and 12 for women. For those older than 55 y, the values were nine for both sexes.

Molecular Analysis

DNA was extracted from saliva or blood samples by using the QIAamp® DNA Mini Kit (QIAGEN S.r.l., Milan, Italy) according to the manufacturer's instructions. Its concentration and purity were estimated by measurements at an optical density and the wavelength of 260 nm and 280 nm, respectively, with an Agilent Cary 60 UV–Vis Spectrophotometer (Agilent, Palo Alto, CA, USA).

All subjects which participated to the study were genotyped for the single nucleotide polymorphism (SNP) *rs1761667* (G/A) of *CD36* gene, and for the *rs2590498* (A/G) SNP of the *OBPIIa* gene. A 190-bp fragment of *CD36* gene containing the SNP was amplified by PCR techniques with forward 5'-

CAAATCACAATCTATTCAAGACCA-3′ and reverse 5′-TTTTGGGAGAAATTCTGAAGAG-3′ primers. DNA was amplified using EuroTaq thermostable DNA polymerase (EuroClone S.p.A., Pero, MI, Italy). The amplification protocol included an initial denaturation at 95 °C for 5 min, followed by 30 cycles of denaturation at 95 °C for 30 s, annealing at 56 °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. The fragments including the SNP of CD36 were digested by the restriction enzyme (*HhaI*) according to Banerjee et al. 2010 [6]. Electrophoresis on a 2% agarose gel was used to separate the products of digestion. DNA bands were visualized by staining with ethidium bromide and ultraviolet light to mark the deletion. PCR 50 bp Low Ladder DNA was used as a marker of molecular mass (Gene Ruler™-Thermo Scientific, Waltham, MA, USA). The *OBPIIa* gene was amplified using custom TaqMan®SNP Genotyping Assay (Applied Biosystems by Life-Technologies Italia, Europe BV). The PCR reactions were run in duplicate in a StepOnePlus™ instrument (Applied Biosystems) using the following two primers: sense primer 5′-GCCAGGCAGGGACAGA-3′ and the antisense primer 5′-CTACACCTGAGACCCCACAAG-3′. Two TaqMan probes were drawn according to the OBPIIa gene (bold and underlined), probe/reporter 1: VIC-TCGGTGAC**A**TGAACC and probe/reporter 2: FAM-TCGGTGAC**G**TGAACC. After the PCR runs, the fluorescence of plates was read (60 °C for 1 min) in the sequence detector system, and the results were analyzed by allelic discrimination by the sequence detector software (Applied Biosystems). Replicates and positive and negative controls were included in all reactions.

Additionally, subjects belonging to Group 1 were also genotyped for the single nucleotide polymorphism (SNP), *rs2274333* (A/G) of gustin gene located in the exon 3 that results in a substitution of amino acid Ser90Gly. The gustin gene was amplified by PCR techniques as describe above. Amplified samples of the fragments of 253 bp including the SNP were digested with restriction enzyme (*HaeIII*) according to Padiglia et al. [7].

Finally, subjects belonging to Group 2 were also genotyped for the following SNPs: the three SNPs, *rs713598*, *rs1726866*, and *rs10246939* of *TAS2R38*, which result in three amino acid substitutions (Pro49Ala, Ala262Val, and Val296Ile) and give rise to two major haplotypes, PAV (the dominant taster variant) and AVI (the non-taster recessive one) and three rare haplotypes (AAI, AAV, and PVI); molecular analyses were performed by using TaqMan SNP Genotyping Assay (C_8876467_10 assay for the rs713598; C_9506827_10 assay for the rs1726866 and C_9506826_10 assay for the rs10246939; C_8314999_10 assay for the rs1761667) according to the manufacturer's specifications as described above.

Statistical Analysis

Data were checked for the assumptions of homogeneity of variance and normality. One-way ANOVA was used to compare the differences in total taste score of the whole TST, taste score of each taste quality, oleic acid threshold and BMI between IBD patients and HC subjects who performed the gustatory part of the study. IBD patients are represented as a single panel because no differences were found between CD and UC patients for all statistical analyses. One-way ANOVA was used to analyze the effect of the health status (HC or IBD) of the subjects, who performed the olfactory measurement, on the score obtained with the T-test, D-test, I-test and their sum TDI.

Separate repeated measures ANOVAs were used in patients belonging to Group 2 to compare the differences in total taste score of the whole TST, taste score of each taste quality (sweet, sour, salty, bitter and umami), oleic acid detection threshold, odor identification score (I-test score), PROP bitterness intensity, BMI values and three factors of the TFEQ across time—i.e., before (T0), and at one month (T1) and six months (T2) after surgery. Data were also separately analyzed according to CD36 and OBPIIa polymorphisms or PROP taster status. We ran the same data analyses including gender or type of surgery (SG vs. RYGB including mini bypass) in the model. A main effects ANOVA was used to assess the first order

(noninteractive) effects of multiple categorical independent variables. When the sphericity assumption was violated, we used the Greenhouse–Geisser correction or Huynh–Feldt correction to modify the degrees of freedom.

Two-way ANOVA was used to analyze the differences in total taste score of the whole TST, taste score of each taste quality and in BMI, between IBD patients and HC subjects according to PROP taster status and to the rs2274333 (A/G) polymorphism of the gustin gene, and to compare differences in oleic acid threshold between two groups according to PROP taster status and the rs1761667 polymorphism of CD36. Analysis of covariance (ANCOVA) was also used to control for differences in BMI between IBD patients and HC subjects that could influence the taste scores. Specifically, one-way ANCOVA (controlling for BMI) was used to compare differences in total taste score of the whole TST, taste score of each taste quality and oleic acid threshold between two groups; two-way ANCOVA (controlling for BMI) was used to analyze the differences between two groups in total taste score and taste score of each taste quality according to PROP taster status and the rs2274333 (A/G) polymorphism of the gustin gene; two-way ANCOVA (controlling for BMI) was also used to analyze the differences in oleic acid threshold between two groups according to PROP taster status and the rs1761667 polymorphism of CD36. ANCOVA confirmed all associations and results are shown in the figures. Post hoc comparisons were performed with the Fisher's least significant difference (LSD) test, except the assumption of homogeneity of variance was violated, in this case the Duncan test was used. P values were adjusted by Bonferroni correction (adjusted P = P · number of groups being compared).

Two-way ANOVA was also used to test for a significant interaction between health status (HC or IBD) × OBPIIa genotype on the T, D and I scores, while two-way multivariate analysis of variance (MANOVA) was used to analyze differences of the T, D and I scores (within factors) according to the OBPIIa genotype and health status of the subjects (HC or IBD) (between factors).

Multiple linear regression was applied to determine the relative contribution of T, D and I scores as predictor variables of TDI score, in both HC subjects and IBD patients.

Differences between IBD patients and HC subjects, who performed the gustatory part of the study, on genotype distribution and allele frequency of the rs2274333 (A/G) polymorphism of the gustin gene and the polymorphism of the CD36 gene were compared by using the Fisher method (Genepop software version 4.2; http://genepop.curtin.edu.au/genepop_op3.html). Fisher method was also used to identify differences in genotype distribution and allele frequencies at the OBPIIa locus between HC subjects and IBD patients who performed the olfactory test, and to highlight differences in genotype distribution and haplotype frequency of the *TAS2R38* locus tested at T0, T1 and T2 according to PROP taster status.

Fisher's exact test was used to analyze differences in frequency related to PROP taste status between two groups, and in the TDI, T, D and I olfactory statuses between HC subjects and IBD patients. Finally, Fisher's Exact Test was used to analyze differences in the number of patients belonging to Group 2 classified as PROP super-tasters, PROP medium tasters and PROP non-tasters in each sampling time (at T0, T1 and T2).

Statistical analyses were conducted using STATISTICA for Windows (version 10; StatSoft Inc., Tulsa, OK). The significance level was set at $p < 0.05$ and data reported in the figures are mean value ± Standard Error of the Mean (SEM).

Results

Taste scores

Figure 1 shows mean values of the total taste score for the TST determined in IBD patients (n = 97) and HC subjects (n = 62) who completed the taste sensitivity measurement; the same data are also shown according to PROP taster status and the *rs2274333* (A/G) polymorphism of the gustin gene. One-way ANCOVA controlling BMI showed that the total taste score of HC subjects was higher than that of IBD patients ($F_{1,156}$ = 4.789; p = 0.0301) (Figure 1A).

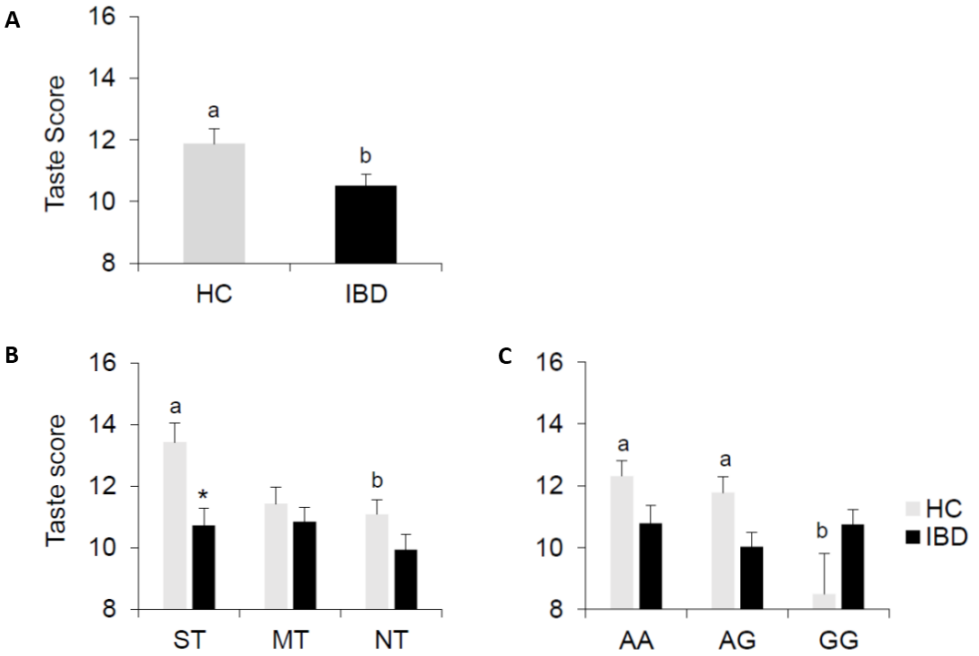

Figure 1. Means (±SEM) values of the total taste score of the whole TST determined in IBD patients (n = 97) and HC subjects (n = 62) (A). The same data are shown for each PROP taster group (B) and for each genotype group of the *rs2274333* (A/G) polymorphism of gustin gene (C). Different letters indicate a significant difference (A: $F_{1,156}$ = 4.789; p = 0.0301, one-way ANCOVA; B/C: $p \leq 0.039$, Duncan test adjusted by Bonferroni correction subsequent two-way ANCOVA). * indicate a significant difference with respect to the corresponding value of HC subjects (p = 0.0075; Duncan test adjusted by Bonferroni correction subsequent two-way ANCOVA).

HC subjects who were classified as super-taster had higher total taste scores than IBD patients classified as super-tasters (p = 0.00756; Duncan's test adjusted by Bonferroni correction subsequent to two-way ANCOVA), and higher than those of HC subjects classified as non-tasters (p = 0.026 Duncan's test adjusted by Bonferroni

correction subsequent to two-way ANCOVA) (Figure 1B). TST data according to PROP taster status was not different among IBD patients ($p > 0.05$).

HC subjects who carried the AA and AG genotypes of the gustin gene polymorphism were higher than those of the HC subjects with the GG genotype ($p < 0.039$; Duncan's test adjusted by Bonferroni correction subsequent to two-way ANCOVA), whereas TST of IBD patients related to the gustin gene polymorphism showed no significant differences ($p > 0.05$) (Figure 1C). Also, no differences in total taste scores between CD and UC patients were found ($p > 0.05$) (data not shown).

The percentage of subjects who completed the taste sensitivity measurement, and who were classified as normogeusic or hypogeusic/ageusic in IBD patients differed with respects that determined in HC subjects ($\chi 2 = 6.243$, $p = 0.0125$). Specifically, 74% ($n = 72$) of IBD patients were normogeusic, and 26% ($n = 25$) were hypogeusic/ageusic, while 90% ($n = 56$) of HC subjects were normogeusic, and 10% ($n = 6$) were hypogeusic/ageusic. Also, IBD patients and HC subjects did not differ by PROP taster status classification ($\chi 2 = 0.598$, $p = 0.741$).

Table 1 shows genotype distribution and allele frequency of the gustin gene polymorphism in IBD patients ($n = 97$) and HC subjects ($n = 62$) who completed the taste sensitivity measurement. Fisher's test revealed that the two clusters did not differ in genotype distribution ($\chi 2 = 1.265$, $p = 0.531$) and allele frequency ($\chi 2 = 1.285$, $p = 0.526$) of the gustin gene polymorphism.

Polymorphisms	IBD		HC		*p*-Value
	n	%	*n*	%	
Gustin gene					
Genotype					
AA	53	54.6	29	46.8	0.531
AG	36	37.1	28	45.2	
GG	8	8.3	5	8	
Allele					
A	142	73.2	86	69.4	0.526
G	52	26.8	38	30.6	

Table 1. Genotype distribution and allele frequency of polymorphisms of Gustin in IBD patients ($n = 97$) and HC subjects ($n = 62$) . p derived from Fisher's method.

Mean values (± SEM) of the taste scores for sweet, sour, salty, bitter and umami determined in IBD patients and HC subjects are shown in Figure 2; the same data are also presented according to PROP taster status and the *rs2274333* (A/G) polymorphism of the gustin gene. One-way ANCOVA revealed that the taste score of IBD patients for sweet, salty, bitter and umami was lower than that of HC subjects (sweet: $F_{1,156} = 18.640$, $p = 0.00003$; salty: $F_{1,156} = 6.1010$, $p = 0.01459$; bitter: $F_{1,156} = 21.686$, $p = 0.00001$; umami $F_{1,156} = 10.804$, $p = 0.00126$), but was higher in IBD patients for sour ($F_{1,156} = 36.663$, $p < 0.00001$) (Figure 2A). Results showed in HC subjects a significant effect of PROP taster status only on bitter scores with super-tasters having higher values than medium tasters and non-tasters ($p < 0.0207$; Duncan's test adjusted by Bonferroni correction subsequent to two-way ANCOVA), though a similar trend was observed for all qualities. No significant differences related to PROP taster status were found in IBD patients (Figure 2B). Post hoc analysis also revealed the following effects of disease on taste score: medium tasters and non-tasters IBD patients had lower sweet scores than corresponding HC subjects ($p < 0.016$; Fisher's LSD test adjusted by Bonferroni correction subsequent to two-way ANCOVA); medium taster and non-taster IBD patients had higher sour scores than corresponding HC subjects ($p < 0.0288$; Duncan's test adjusted by Bonferroni correction subsequent two-way ANCOVA); super-taster and non-tasters IBD patients had lower bitter scores than corresponding HC subjects ($p < 0.015$; Duncan's test adjusted by Bonferroni correction subsequent to two-way ANOVA) (Figure 2B).

Two-way ANCOVA on the same data according to the gustin gene polymorphism revealed a significant interaction of the participants' group (IBD patients/HC subjects) × gustin gene genotype group on the taste score relative to sweet ($F_{2,152} = 4.6693$; $p = 0.0179$) (Figure 2C). Specifically, post hoc comparison showed that HC subjects with A allele (AA and AG) had higher sweet scores than GG HC subjects ($p < 0.010$; Fisher's LSD test adjusted by Bonferroni correction subsequent to two-way ANCOVA). IBD patients with AA and AG genotypes had lower scores for sweet, bitter and umami than those of the corresponding HC

subjects, while scores for sour were higher in the IBD patients than HC subjects, (p < 0.026; Fisher's LSD test adjusted by Bonferroni correction subsequent to two-way ANCOVA) (Figure 2C). No differences between CD and UC patients were found (p > 0.05).

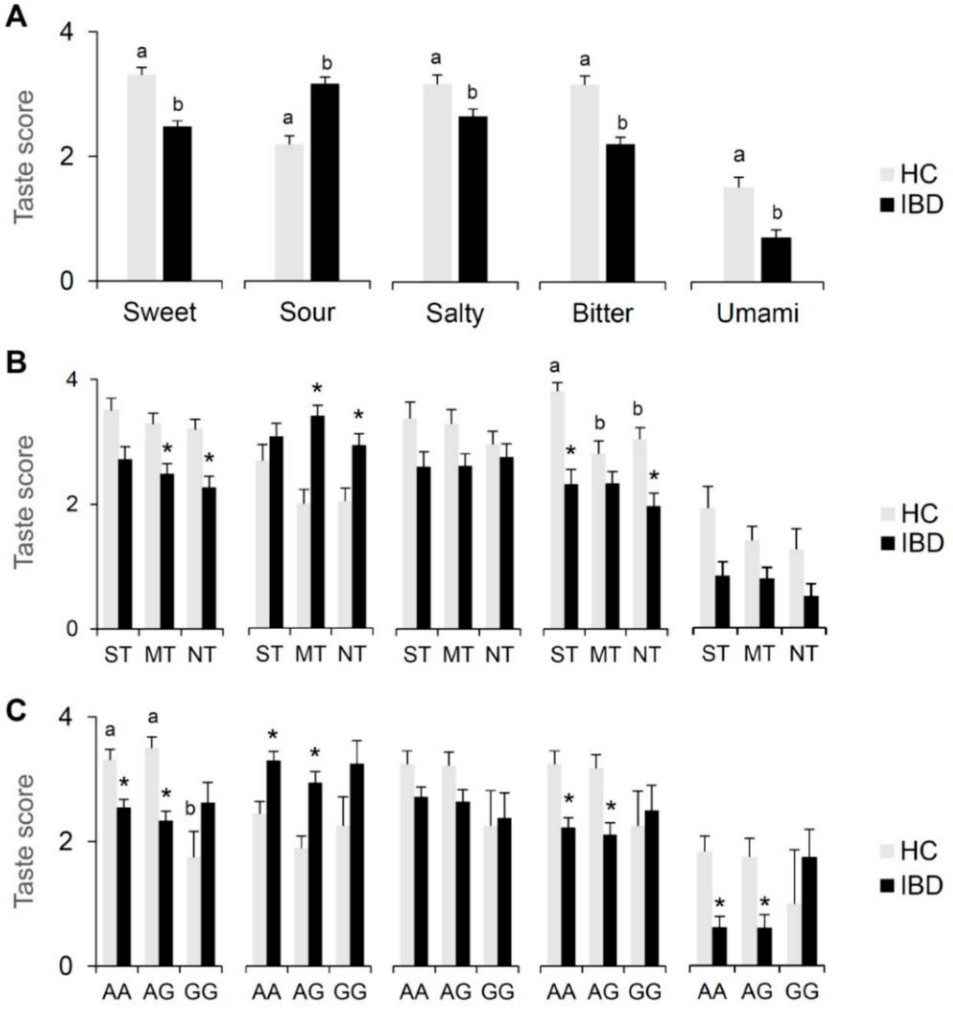

Figure 2. Mean values (±SEM) of the taste score relative to sweet, sour, salty, bitter and umami determined in IBD patients (n = 97) and HC subjects (n = 62) (A). The same data are shown for each PROP taster group (B) and for each genotype group of the rs2274333 (A/G) polymorphism of gustin gene (C). Different letters indicate a significant difference (A: $F_{1,156} \geq 6.1010$, $p \leq 0.01459$, one-way ANCOVA; B: $p \leq 0.0207$; Duncan's test adjusted by Bonferroni correction subsequent two-way ANCOVA; C: $p \leq 0.0297$; Fisher LSD test adjusted by Bonferroni correction subsequent two-way ANOVA). * indicate a significant difference with respect to the corresponding value of HC subjects (B: $p \leq 0.0288$; Fisher LSD test or Duncan's test adjusted by Bonferroni correction subsequent two-way ANOVA; C: $p \leq 0.026$; Fisher LSD adjusted by Bonferroni correction test subsequent two-way ANOVA).

The taste score changes associated with bariatric surgery-induced weight loss are shown in Figure 3. The mean values of the total taste score for the whole TST and of that relative to sweet, sour, salty, bitter and umami determined before (T0), one month (T1) and six months (T2) after bariatric surgery are shown in Figure 3A. Data of the total taste score of the whole TST are shown also according to the *rs2590498* polymorphism of *OBPIIa* gene in Figure 3B and for each PROP taster group determined at T2 in Figure 3C. Molecular analysis identified 15 subjects were homozygous AA for the *rs2590498* SNP of the *OBPIIa* locus, 12 were heterozygous, and 24 were homozygous GG.

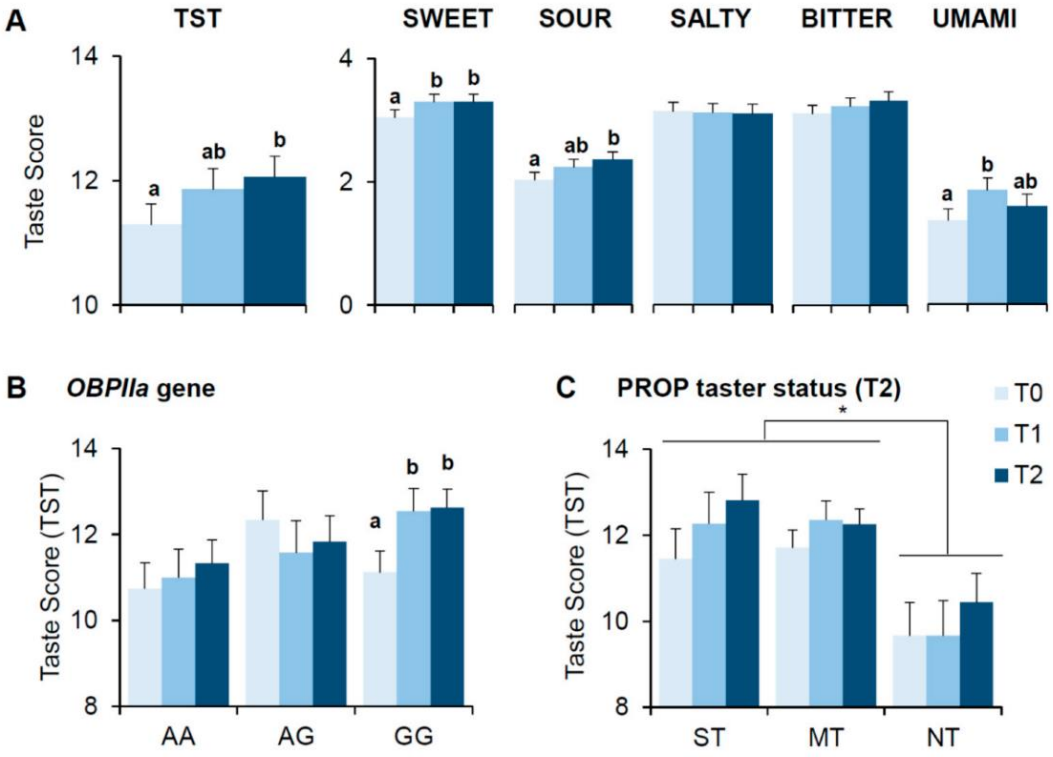

Figure 3. Taste perception scores determined before (T0), one month (T1) and six months (T2) after bariatric surgery. Means (± SEM) values of the total taste score of the whole Taste Strip Test (TST) and of that relative to sweet, sour, salty, bitter and umami (n = 51) (A). Data of the total taste score of the whole TST are shown according to the rs2590498 polymorphism of OBPIIa gene (genotypes AA: n = 15; genotypes AG: n = 12; genotypes GG: n = 24) (B) or PROP taster status determined at T2 (super-tasters: n = 11; medium tasters: n = 31; non-tasters: n = 9) (C). Different letters indicate a significant difference ($p \leq 0.048$, Fisher's test LSD subsequent repeated measures ANOVA). * indicate a significant difference between values of tasters and non-tasters ($p \leq 0.027$ Fisher's test LSD subsequent repeated measures ANOVA).

The repeated measures ANOVA showed that TST score varied with the time factor (T0, T1 and T2) ($F_{1.84,91.85}$ = 3.509; p = 0.038) and post hoc comparison showed that the total taste score determined at T2 was higher than that determined at T0 (p = 0.0122, Fisher's test LSD). Changes in sweet, sour and umami scores contributed the most to the TST changes across time (sweet: $F_{1.78,88.94}$ = 2.978; p = 0.059; sour: $F_{2,100}$ = 3.38; p = 0.038; umami: $F_{2,100}$ = 2.995; p = 0.054). Post hoc comparison showed that while sweet and umami scores increased already at T1 ($p \leq 0.045$, Fisher's test LSD), the sour score increased only at T2 (p = 0.0118, Fisher's test LSD). No differences in salty and bitter scores were found ($p > 0.05$) (Figure 3A).

Repeated measures of ANOVA also showed that the changes in TST across time were associated with the OBPIIa gene polymorphism ($F_{4,96}$ = 2.836; p = 0.0284) (Figure 3B). Specifically, the total taste score of participants who carried the GG genotype increased already at T1 ($p < 0.0011$, Fisher's test LSD), while no differences in the TST across time were found in participants who carried the AA or AG genotypes ($p > 0.05$). Differently, the change of the TST observed with the time factor (T0, T1 and T2) did not associate with PROP taster status of participants. However, a significant main effect of the PROP taster status on the total taste score was found ($F_{2,148}$ = 10.762; p = 0.00004), such that super-tasters and medium tasters had higher scores than non-tasters ($p \leq 0.000074$, Fisher's test LSD) (Figure 3C). No other difference related to PROP taster status was found ($p > 0.05$). There were no significant differences of total taste score or scores relative to each taste quality related to gender or type of bariatric surgery ($p > 0.05$; data not shown).

The mean values of the score for sweet, sour, salty, bitter and umami taste perception determined before (T0), one month (T1) and six months (T2) after bariatric surgery are shown according to the *rs2590498* polymorphism of *OBPIIa* gene or PROP taster status in Figure 4 . Repeated measures of ANOVA showed that the changes in the sweet and sour scores across time were associated with *OBPIIa* locus (sweet: $F_{3.66,87.94}$ = 3.169; p = 0.020; sour: $F_{4,96}$ = 4.107; p = 0.0041) . The sweet scores determined at T1 and T2 in the participants who carried the GG genotypes were

higher than that determined at T0 ($p \leq 0.027$, Fisher's test LSD), while no differences in participants who carried the AA or AG genotype were found ($p > 0.05$). The sour score determined at T2 in the participants who carried AG and GG genotypes was higher than those determined at T0 ($p \leq 0.0038$, Fisher's test LSD), while no differences in participants who carried AA genotype were found ($p > 0.05$). There were no significant interactions between *OBPIIa* locus and changes in taste scores for salty, bitter or umami across time ($p > 0.05$).

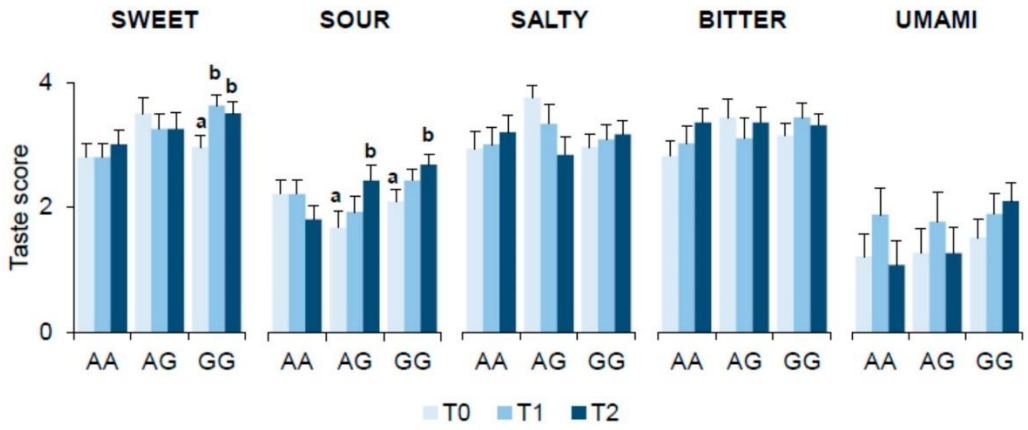

Figure 4. Taste perception scores relative to sweet, sour, salty, bitter and umami determined before (T0), one month (T1) and six months (T2) after bariatric surgery ($n = 51$). Means (± SEM) values are shown according to the rs2590498 polymorphism of *OBPIIa* gene (genotypes AA: $n = 15$; genotypes AG: $n = 12$; genotypes GG: $n = 24$). Different letters indicate a significant difference ($p \leq 0.048$, Fisher's test LSD subsequent repeated measures ANOVA).

Oleic acid Threshold

Figure 5 shows mean values of the oleic acid threshold (μL) determined in IBD patients and HC subjects; data are also shown according to PROP taster status and the *rs1761667* (A/G) polymorphism of CD36. One-way ANCOVA showed that the oleic acid threshold of IBD patients was higher than that of HC subjects ($F_{1,136} = 44.779$, $p < 0.000001$) (Figure 5A). Post hoc comparison showed that the oleic acid threshold of IBD patients was higher than that of HC subjects in both medium tasters and non-tasters ($p < 0.00025$; Duncan's test adjusted by Bonferroni correction subsequent to two-way ANCOVA). Also, the oleic acid threshold in IBD patients was higher in each CD36 genotype group compared to these same genotype groups

in HC subjects ($p < 0.017$; Duncan's test adjusted by Bonferroni correction subsequent to two-way ANCOVA) (Figure 5B,C). No differences of the oleic acid threshold between CD and UC patients were found ($p > 0.05$).

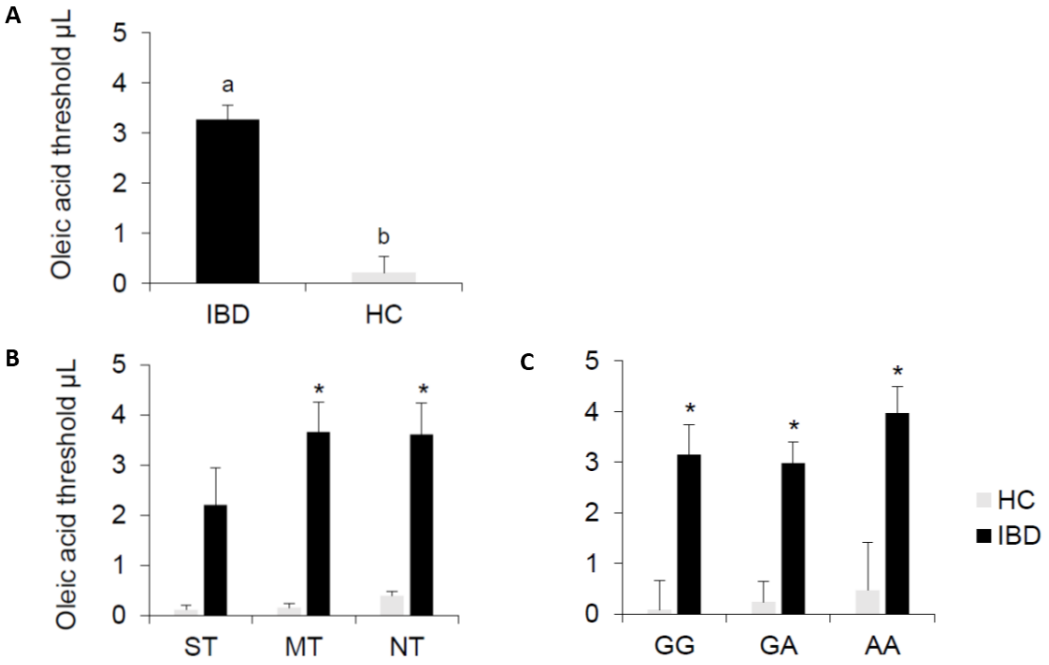

Figure 5. Means (± SEM) values of the oleic acid threshold (µL) determined in IBD patients ($n = 97$) and HC subjects ($n = 62$) (A). The same data are shown for each PROP taster group (B) and for each genotype group of the rs1761667 (A/G) polymorphism of CD36 gene (C). Different letters indicate a significant difference (A: $F_{1,136} = 44.779$; $p < 0.000001$, one-way ANCOVA). * indicate a significant difference with respect to the corresponding value of HC subjects (B/C: $p \leq 0.017$, Duncan test subsequent adjusted by Bonferroni correction two-way ANOVA).

Table 2 shows genotype distribution and allele frequency of the *rs1761667* polymorphism of *CD36* gene in IBD patients ($n = 97$) and HC subjects ($n = 62$) who completed the taste sensitivity measurement. Fisher's test revealed that the two clusters were different in genotype distribution ($\chi2 = 6.001$, $p = 0.049$) and allele frequency ($\chi2 = 6.099$, $p = 0.047$) of the *rs1761667* polymorphism of *CD36* gene. Specifically, the genotype AA and allele A were more frequent in IBD patients, while genotype GG and allele G were more frequent in HC subjects.

Polymorphisms	IBD		HC		*p*-Value
	n	%	*n*	%	
CD36					
Genotype					
GG	18	23.7	19	30.6	0.049
AG	36	47.4	36	58.1	
AA	22	28.9	7	11.3	
Allele					
G	72	47.4	74	59.7	0.047
A	80	52.6	50	40.3	

Table 2. Genotype distribution and allele frequency of polymorphisms of *CD36* gene in IBD patients (n = 97) and HC subjects(n = 62) who completed the taste sensitivity measurement. p derived from Fisher's method.

Figure 6 shows mean values of the oleic acid threshold determined before (T0), one-month (T1) and six months (T2) after bariatric surgery (Figure 6A). The repeated measures ANOVA showed that oleic acid threshold varied with the time factor (T0, T1 and T2) ($F_{1.8,90.06} = 6.028$; $p = 0.0047$). Post hoc comparison showed that the oleic acid threshold determined at T2 was lower than those measured at T0 and T1 ($p \leq 0.043$, Fisher's test LSD). Molecular analysis at the SNP (*rs1761667*) of the CD36 locus identified 14 subjects who were homozygous GG, 23 who were heterozygous, and 14 who were homozygous AA. When results were analyzed according to the rs1761667 polymorphism of CD36, the decrease in the oleic acid detection threshold observed after bariatric surgery did not depend on the CD36 locus, and all genotype groups showed the same trend—i.e., oleic acid thresholds were decreased at T2 compared to T0 (Figure 6B). No significant differences of oleic acid threshold related to gender or type of bariatric surgery were found ($p > 0.05$; data not shown).

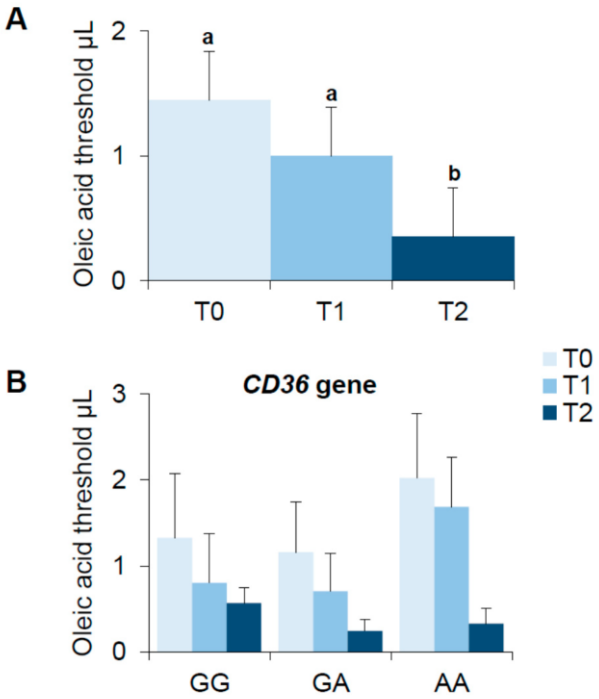

Figure 6. Means (±SEM) values of the oleic acid threshold (µL) determined before (T0), 1 month (T1) and 6 months (T2) after bariatric surgery (A). The same data are shown for each genotype of the rs1761667 (A/G) polymorphism of CD36 gene (genotypes GG: n = 14; genotypes GA: n = 23; genotypes AA: n = 14) (B). (n = 51). Different letters indicate a significant difference ($p \leq 0.043$, Fisher's test LSD subsequent repeated measures ANOVA).

PROP Tasting Effect

PROP tasting changes associated with bariatric surgery-induced weight loss are shown in Figure 7. The repeated measures ANOVA showed that PROP bitterness intensity ratings increased after the bariatric surgery ($F_{2,100}$= 10.724; p = 0.00006) (Figure 7A). Post hoc comparison showed that the PROP intensity ratings determined at T1 and T2 were higher than that measured at T0 ($p \leq 0.016$; Fisher's test LSD), and the intensity rating determined at T2 was lower than that determined at T1 ($p = 0.031$ Fisher's test LSD). No difference related to gender or type of bariatric surgery was found ($p > 0.05$; data not shown).

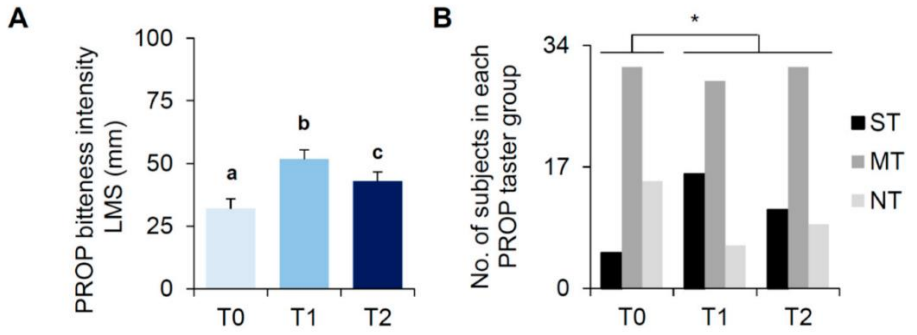

Figure 7. 6-N-propylthiouracil (PROP) tasting before (T0), one month (T1) and six months (T2) after bariatric surgery. Means (± SEM) values of PROP bitterness intensity ratings (50 mM) (A) and numbers of subjects classified as super-tasters (STs), medium tasters (MTs), and non-tasters (NTs) (B). (n = 51). Different letters in (A) indicate significant difference ($p \leq 0.037$, Fisher's test least significant difference (LSD) subsequent repeated measures ANOVA). * in (B) indicates a significant difference ($p = 0.0078$; Fisher's exact).

Similarly suggesting a shift towards increased PROP sensitivity after surgery, the proportion of participants who were classified as PROP super-tasters, medium tasters, and non-tasters at T0 differed to those at T1 and T2 ($\chi 2 > 7.72$; $p < 0.0073$; Mc Nemar test) (Figure 7B). Specifically, super-tasters increased after surgery (T0: 9.8%, T1: 31.4%, T2: 21.6%), non-tasters decreased after surgery (T0: 29.4%, T1: 11.8%, T2: 17.7%), while medium tasters did not change after surgery (T0: 60.78%, T1: 56.86%, T2: 60.78%). Medium tasters were younger than super-taster and non-tasters at T0 ($p < 0.044$). No other differences in age of subjects belonging to each PROP taster group were found ($p > 0.05$).

The genotype distribution and haplotype frequency for SNPs of TAS2R38 according to PROP taster status determined at T0, T1, and T2 are shown in Table 3. PROP taster groups differed statistically on the basis of the genotype distribution ($\chi2 > 12.439$; $p < 0.0019$) and haplotype frequency ($\chi2 > 11.927$; $p < 0.00257$) at T0, T1 and T2. Post hoc comparison also showed that the non-taster group differed from the other ones at all time of assessments (genotype: $\chi2 > 8.45$; $p < 0.014$; haplotype: $\chi2 > 11.188$ $p < 0.0037$; Fisher's method), while no difference between super-tasters and medium tasters was found ($p > 0.05$; Fisher's method). The genotype AVI/AVI and haplotype AVI were more frequent in non-tasters while the genotype PAV/AVI was more frequent in super-tasters and medium tasters. The prediction of PROP taster groups by genotype and haplotype at TAS2R38 varied with the time of assessment (i.e., T0, T1 and T2). Participants with a PAV haplotype were more likely to be classified as a super-taster after (T1 or T2) than before (T0) surgery ($\chi2 = 10.28$; $p < 0.0058$; Mc Nemar test) and subjects with the AVI haplotype were more likely to be classified as a non-taster before than after surgery ($\chi2 = 8.236$; $p < 0.016$; Mc Nemar test). The statistical differences with and without inclusion in the analysis of participants with rare haplotype were the same.

	PROP Status						*p*-Value
	Super-Taster		Medium Taster		Non-Taster		
TAS2R38	*n*	%	*n*	%	*n*	%	
T0							
Genotype							
PAV/PAV	1	25	9	32.1	0	0	0.00098
PAV/AVI	2	50	13	46.4	4	26.7	
AVI/AVI	1	25	6	21.4	11	73.3	
Haplotype							
PAV	4	50	31	55.4	4	13.3	0.0003
AVI	4	50	25	44.6	26	86.7	
T1							
Genotype							
PAV/PAV	4	26.7	6	23.1	0	0	0.0019
PAV/AVI	8	53.3	11	42.3	0	0	
AVI/AVI	3	20	9	34.6	6	100	
Haplotype							
PAV	16	53.3	23	44.2	0	0	0.0026
AVI	14	46.7	29	55.8	12	100	
T2							
Genotype							
PAV/PAV	4	40	6	21.4	0	0	0.0004
PAV/AVI	6	60	12	42.9	1	11.1	
AVI/AVI	0	0	10	35.7	8	88.9	
Haplotype							
PAV	14	70	24	42.9	1	5.6	0.000004
AVI	6	30	32	57.1	17	94.4	

Table 3. Genotype distribution and haplotype frequency of TAS2R38 single nucleotide polymorphisms (SNPs) according to PROP taster status before (T0), one month (T1) and six months (T2) after bariatric surgery. *p*-value in derived from Fisher's method (Genepop software version 4.2) ($n = 47$) (participants with rare haplotype are not included in the analysis).

Olfactory score

Figure 8 shows the mean values of the total TDI olfactory score obtained from each population considered: HC subjects and IBD patients. One-way ANOVA showed that the TDI score obtained by HC subjects was significantly higher than that obtained by IBD patients ($F_{1,197} = 22.75$; $p < 0.001$).

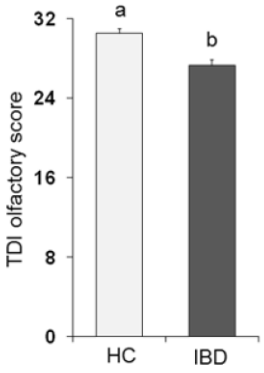

Figure 8. Mean (± SEM) values of the Threshold, Discrimination and Identification (TDI) olfactory score determined in healthy control (HC) subjects (n = 99) and inflammatory bowel disease (IBD) patients (n = 100). Different letters (a and b) indicate a significant difference ($p < 0.001$, Duncan's test).

The percentage of subjects who were classified as normosmic or hyposmic for their TDI olfactory status in HC subjects differed from that determined for IBD patients ($\chi 2 = 5.499$, $p = 0.019$). In detail, 60.61% ($n = 60$) of HC subjects were normosmic, and 39.39% ($n = 39$) were hyposmic, while 44% ($n = 44$) of IBD patients were normosmic and 56% ($n = 56$) were hyposmic.

Stepwise forward multiple regression models for TDI score revealed that the relative contribution of each subtest to the TDI score was significant for both HC subjects and IBD patients, albeit in a different way. Indeed, in HC subjects, the major contributor to the model was the T score (52.05%), secondly the D score (20.90%) and finally the I score (24.20%). Instead, in IBD patients, the major contributor to the model was the score obtained with the D-test (70.33%) and, to follow, the T (17.11%) and I (12.88%) scores.

The mean values of the T, D and I scores determined in HC subjects ($n = 99$) and IBD patients ($n = 100$) are shown in Figure 9A. The same data are presented

according to the *rs2590498* polymorphism of the *OBPIIa* gene (A/G) (Figure 9B). One-way ANOVA showed that the olfactory score obtained by HC subjects was significantly higher than that obtained by IBD patients for the T-test ($F_{1,197} = 6.02$; $p = 0.015$), D-test ($F_{1,197} = 24.28$; $p < 0.001$) and I-test ($F_{1,197} = 8.88$; $p = 0.003$) (Figure 9A). Two-way MANOVA revealed a significant interaction of the health state × OBPIIa genotypes on the T, D and I scores ($F_{6,382} = 2.18$; $p = 0.04$). In detail, pairwise comparisons showed that individuals who were homozygous for the major allele A exhibited T (both in HC subjects and IBD patients) and D scores (only in IBD patients) that were statistically higher than those of heterozygous individuals (T score: $p < 0.01$; D score: $p < 0.001$; Fisher's LSD test) or homozygous for the minor allele G (T score: $p < 0.001$; D score: $p < 0.001$; Fisher's LSD test). In addition, we found that IBD patients who were heterozygous or GG homozygous reached significantly lower T ($p = 0.024$; Fisher's LSD test) and D ($p < 0.001$; Fisher's LSD test) scores than HC subjects, and IBD patients who were heterozygous reached significantly lower D scores than HC subjects ($p < 0.001$; Fisher's LSD test) (Figure 9B). No other differences between HC subjects and IBD patients according to the *OBPIIa* genotype were found.

Table 4 shows genotype distribution and allele frequency of the *rs2590498* polymorphism of the *OBPIIa* gene (A/G) in IBD patients ($n = 100$) and HC subjects ($n = 99$) who completed the olfactory sensitivity measurement. Fisher's test revealed that the two clusters did not differ in genotype distribution ($\chi 2 = 0.14$, $p = 0.935$) and allele frequency ($\chi 2 = 0.16$, $p = 0.924$) of the *OBPIIa* gene polymorphism.

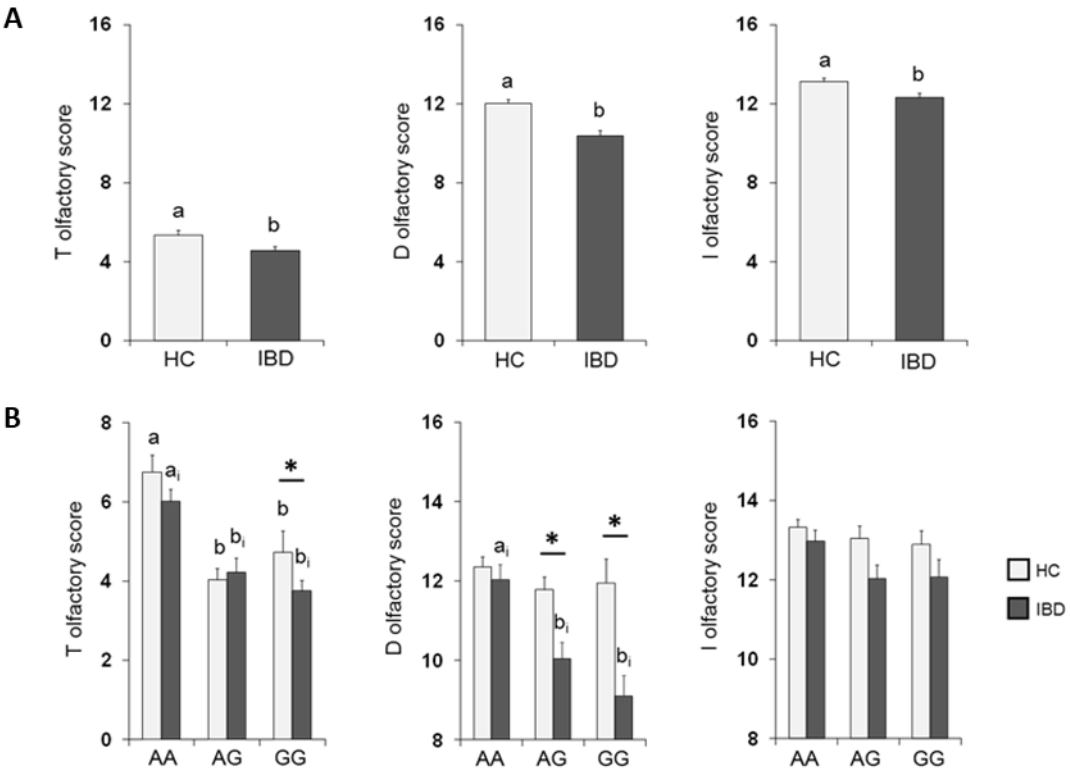

Figure 9. Mean (± SE) values of the T, D and I olfactory score in HC subjects and IBD patients (A), and according to genotypes of the OBPIIa locus (HC $n = 99$; 36 AA, 17 AG, 46 GG; IBD $n = 100$; 30 AA, 28 AG, 42 GG). (A) Different letters indicate a significant difference ($p \leq 0.015$ after Fisher's LSD or Duncan's test subsequent one-way ANOVA). (B) Different letters indicate a significant difference: a-b for HC subjects ($p < 0.001$; Fisher's LSD test); a_i-b_i for IBD patients (T score $p < 0.01$, Fisher's LSD test; D score $p < 0.001$, Duncan's test). (*) indicates a significant difference with respect to the corresponding value of HC subjects (T score GG: 0.024, Fisher's LSD test; D score AG: $p < 0.001$, GG: $p < 0.001$, Duncan's test).

Polymorphisms	IBD		HC		*p*-Value
	n	%	*n*	%	
OBPIIa					
Genotype					
AA	30	30.0	36	36.4	0.935
AG	28	28.0	17	17.2	
GG	42	42.0	46	46.4	
Allele					
A	88	44.0	89	45.0	0.924
G	112	56.0	109	50.0	

Table 4. Genotype distribution and allele frequency of the rs2590498 polymorphism of the *OBPIIa* gene (A/G) in IBD patients ($n = 100$) and HC subjects ($n = 99$). *p* derived from Fisher's method.

Table 5 shows the distribution of the HC subjects and IBD patients classified as normosmic or hyposmic based on their Threshold (T), Discrimination (D) and Identification (I) olfactory status. Fisher's method evidenced that the percentage of HC subjects who were classified as normosmic or hyposmic for their D olfactory status differed from that determined in IBD patients ($\chi 2 = 7.27$, $p = 0.007$). Specifically, 85.86% ($n = 85$) and 14.14% ($n = 14$) of HC subjects were, respectively, normosmic or hyposmic, while in the case of IBD patients, 70% were classified as normosmic and 30% as hyposmic. No differences in percentage between subjects classified as normosmic or hyposmic on the basis of T and I olfactory status were found.

	Group	**IBD**	**HC**	***p*-Value**
Variable	**Olfactory Status**	***n* (%)**	***n* (%)**	
T	Normosmic	65 (65.00)	57 (57.58)	0.282
	Hyposmic	35 (35.00)	42 (42.42)	
D	Normosmic	70 (70.00)	85 (85.86)	0.007
	Hyposmic	30 (30.00)	14 (14.14)	
I	Normosmic	88 (88.00)	91 (91.92)	0.358
	Hyposmic	12 (12.00)	8 (8.08)	

Table 5. Distribution of the healthy control (HC) subjects and IBD patients classified as normosmic or hyposmic based on their Threshold (T), Discrimination (D) and Identification (I) olfactory status.

Olfactory performance of group 2 was analyzed before, one-month and six months after bariatric surgery. The olfactory function of participants improved after the surgery. Figure 10 shows mean values of the odor identification score determined before (T0), one month (T1) and six months (T2) after bariatric surgery (Figure 10A). The repeated measures ANOVA showed that the I-test score varied with the time factor (T0, T1 and T2) ($F_{2,100} = 9.104$; $p = 0.00023$), with higher values determined at T1 and T2 with respect to T0 ($p \leq 0.00084$, Fisher's test LSD). Analysis of the same data according to OBPIIa locus showed that the increase in I-test scores observed after bariatric surgery was not associated with the OBPIIa locus ($p > 0.05$), and all genotype groups showed improvement of olfactory function after bariatric

surgery (Figure 7B). There were no differences in I-test scores determined before or after surgery between participants who underwent the different types of bariatric surgery or related to gender ($p > 0.05$; data not shown).

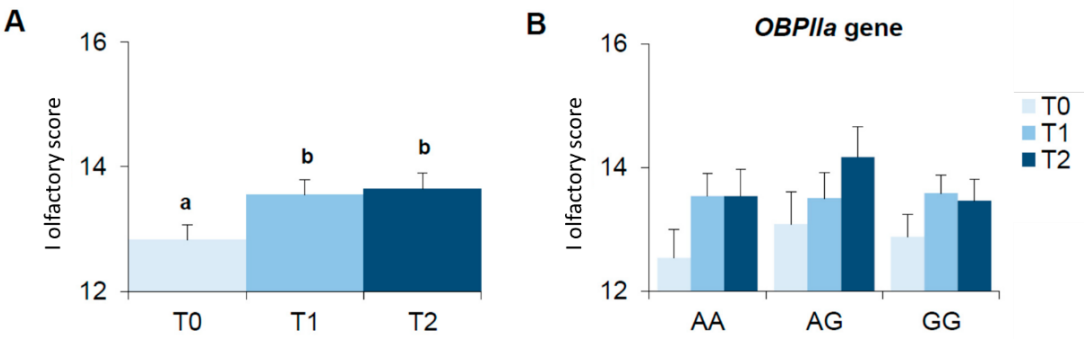

Figure 10. Means (± SEM) values of the odor identification score (I-test score) determined before (T0), one month (T1) and six months (T2) after bariatric surgery ($n = 51$) (A). The same data are shown for each genotype of the rs2590498 polymorphism of OBPIIa gene (genotypes AA: $n = 15$; genotypes AG: $n = 12$; genotypes GG: $n = 24$) (B). Different letters indicate a significant difference ($p \leq 0.0056$, Fisher's test LSD, subsequent repeated measures ANOVA).

BMI effect

Figure 11 shows mean values for BMI determined in IBD patients ($n = 97$) and HC subjects ($n = 62$) who performed the taste assessment part of the study. The same data are also shown according to PROP taster status and the *rs2274333* (A/G) polymorphism of the gustin gene. One-way ANOVA showed that BMI of IBD patients was higher than that of HC subjects ($F_{1, 157} = 27.459$, $p < 0.00001$) (Figure 11A). Post hoc comparison showed that BMI of IBD patients was higher than that of HC subjects in each PROP taster group ($p < 0.045$; Duncan's test adjusted by Bonferroni correction subsequent to two-way ANOVA) (Figure 11B). Post hoc comparison also showed that IBD patients who carried the AA and AG genotype at this gustin gene polymorphism had higher BMI than those of HC subjects with similar genotypes ($p < 0.0051$; Fisher's test LSD adjusted by Bonferroni correction subsequent to two-way ANOVA) (Figure 11C). In contrast, the BMI of IBD patients who had the GG genotype did not significantly differ from that of HC subjects with GG genotype ($p > 0.05$). No differences in BMI values between CD and UC patients were found ($p > 0.05$).

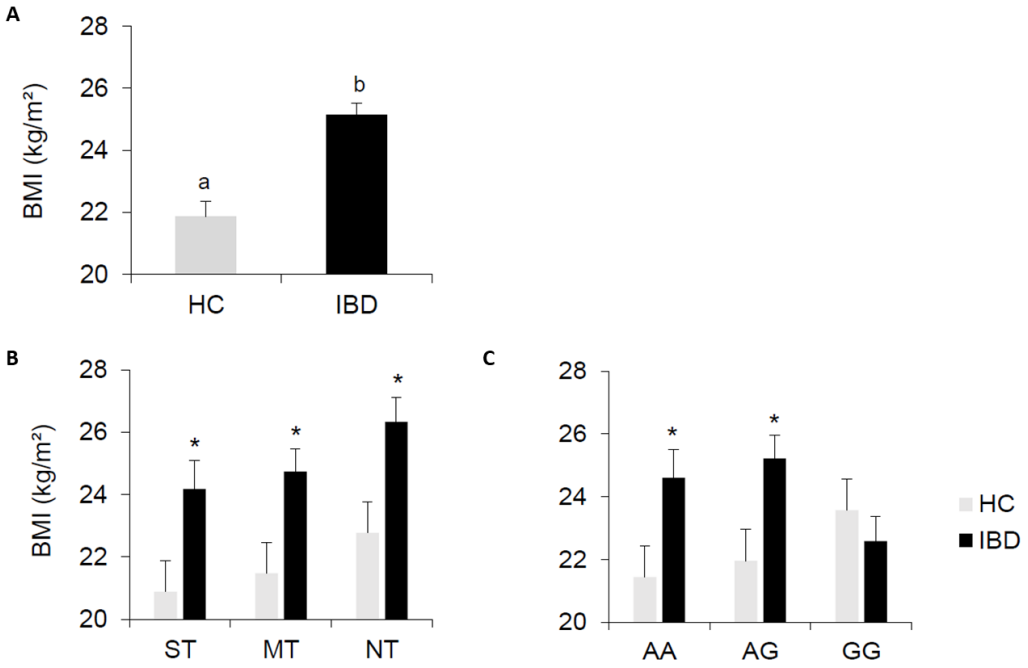

Figure 11. Mean (± SEM) values of body mass index BMI (kg/m²) determined in HC subjects (n = 62) and IBD patients (n = 97) (A). The same data are shown for each PROP taster group (B) and for each genotype group of the rs2274333 (A/G) polymorphism of gustin gene (C). Different letters indicate a significant difference (A: $F_{1, 157}$ = 27.459, p < 0.00001, one-way ANOVA). * indicate a significant difference with respect to the corresponding value of HC subjects (B: $p \leq 0.045$, Duncan's test adjusted by Bonferroni correction subsequent two-way ANOVA; C: $p \leq 0.0051$, Fisher LDS test adjusted by Bonferroni correction subsequent two-way ANOVA).

The mean values of BMI determined in HC subjects (n = 99) and IBD patients (n = 100), who performed the olfactory assessment part of the study, are shown in Figure 12A. One-way ANOVA revealed that the BMI of HC subjects was significantly lower than that of IBD patients ($F_{1,197} = 18.44$; $p < 0.001$). The same data are also shown according to their overall olfactory status (TDI status) (Figure 12B) and to the *rs2890498* polymorphism of the *OBPIIa* gene (Figure 12C). Two-way ANOVA highlighted significant interactions of the health state × TDI olfactory status ($F_{1,195} = 4.84$, $p = 0.029$); post-hoc comparisons revealed that individuals who were hyposmic showed a BMI higher than those who were normosmic (HC $p = 0.032$; IBD $p < 0.001$; Fisher's LSD test) and that IBD patients who were hyposmic had a higher BMI than hyposmic HC subjects ($p < 0.001$; Fisher's LSD test), while no difference was observed between normosmic HC and IBD individuals ($p > 0.05$;

Fisher's LSD test). Two-way ANOVA also revealed a significant interaction of health state × OBPIIa genotype on BMI ($F_{2,193} = 4.05$, $p = 0.018$). For HC subjects, post-hoc comparisons indicated that subjects who were homozygous for the A-allele exhibited a lower BMI than subjects that were homozygous for the G-allele ($p < 0.001$; Fisher's LSD test) or heterozygous ($p = 0.008$; Fisher's LSD test). In addition, pairwise comparison revealed that IBD patients who were homozygous for the G-allele or heterozygous exhibited a higher BMI than patients that were AA homozygous ($p < 0.001$; Fisher's LSD test). The BMI of HC subjects who had the GG or AG genotype was significantly lower than that of IBD patients with the same genotype (GG genotype: $p < 0.001$; AG genotype: $p = 0.017$; Fisher's test LSD).

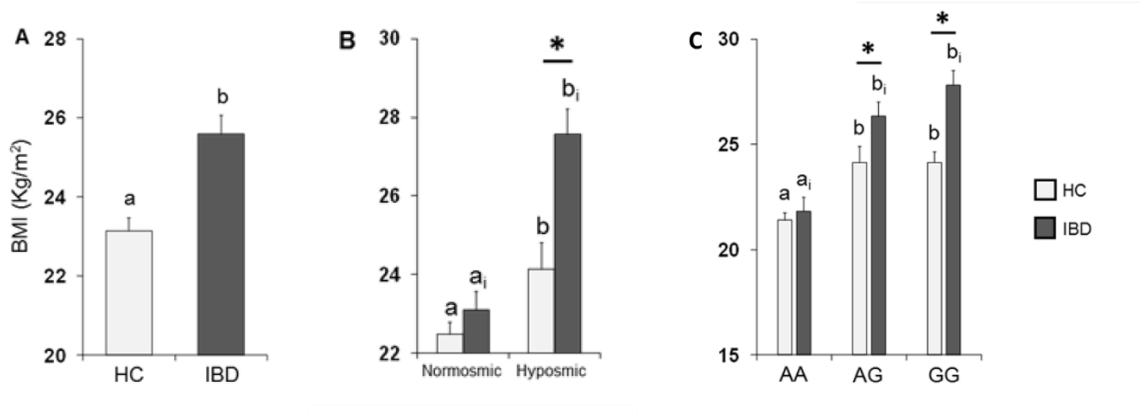

Figure 12. Mean (± SEM) values of BMI determined in HC subjects ($n = 99$) and IBD patients ($n = 100$) (A). The same data are shown according to their overall TDI olfactory status (B), and to genotypes of the *OBPIIa* gene (HC: 36 AA, 17 AG, 46 GG; IBD: 30 AA, 28 AG, 42 GG) (C). (A) Different letters indicate a significant difference between HC and IBD ($p < 0.001$; Fisher's LSD test). (B) Different letters indicate a significant difference between normosmic and hyposmic individuals within the same population (HC subjects or IBD patients) (a and b for HC: TDI status $p = 0.032$; a_i and b_i for IBD: TDI status $p < 0.001$; Fisher's LSD test). (*) indicates a significant difference between HC subjects and IBD patients within the same olfactory status (normosmic or hyposmic individuals) ($p < 0.001$; Fisher's LSD test). (C) Different letters indicate a significant difference: a-b for HC subjects ($p < 0.01$; Fisher's LSD) test; a_i-b_i for IBD patients ($p < 0.001$; Fisher's LSD test). (*) indicates a significant difference with respect to the corresponding value of HC subjects (AG: $p = 0.017$; GG: $p < 0.001$).

Repeated measures ANOVA showed that BMI of patients belonging to Group 2 significantly varied with time ($F_{1.42,71.09} = 420.28$, $p < 0.00001$).

Eating Habits effect

Figure 13A shows mean values of the scores of the 3-Factor Eating Questionnaire (TFEQ) of Stunkard and Messick [1], assessed before (T0), one month (T1), and six months after surgery (T2). The same data are shown for each PROP taster group determined at T2 (Figure 13B). The repeated measures ANOVA showed that the values of restraint, disinhibition and hunger varied with the time factor (T0, T1 and T2) (restraint: $F_{2,100} = 7.353$, $p = 0.0011$; disinhibition: $F_{1.24,59.52} = 52.908$, $p < 0.00001$; perceived hunger: $F_{1.51,72.74} = 48.461$, $p < 0.00001$) (Figure 13A). Post hoc comparison showed that, compared to score values attained at T0, values of restraint increased ($p < 0.05$; Fisher's test LSD) and values of disinhibition and perceived hunger decreased ($p < 0.000001$; Fisher's test LSD) at T1 and T2 (Figure 13A).

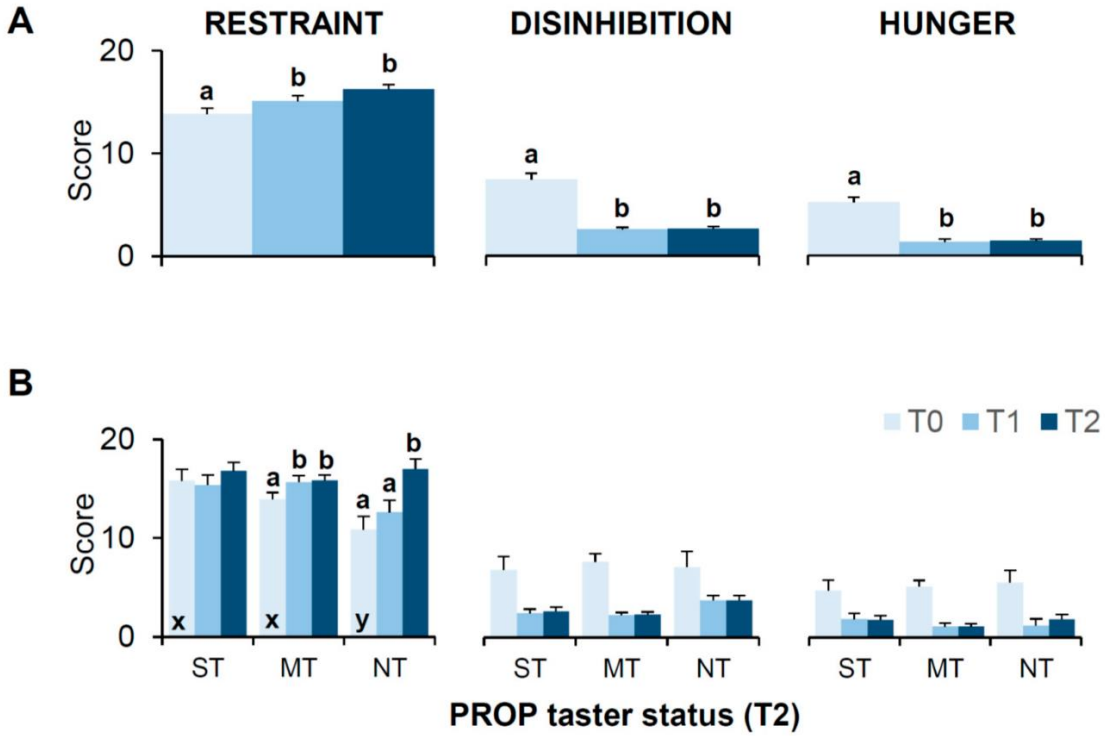

Figure 13. Mean (± SEM) values of the scores of the Three-Factor Eating Questionnaire (TFEQ) determined before (T0), one month (T1), and six months (T2) after bariatric surgery (A). The same data are shown for each PROP taster group determined at T2 (super-tasters ST: $n = 11$; medium tasters MT: $n = 31$; non-tasters NT: $n = 9$) (B). Significant differences are indicated by different letters: a and b are used to denote differences within sampling time (T0, T1 or T2), while x and y are used to denote differences with respect to the corresponding value of other groups. For all comparisons ($p \leq 0.027$, Fisher's test LSD subsequent repeated measures ANOVA).

The repeated measures ANOVA showed that the restraint scores determined at the three sampling times were associated to participant's PROP taster status ($F_{3.76,86.41}$ = 2.688, p = 0.039). Post hoc comparison showed that compared to PROP tasters, PROP non-tasters had lower restraint scores at baseline (T0) ($p \leq 0.025$; Fisher's test LSD). Post hoc comparison also showed that the restraint scores of medium tasters increased already at T1 with respect to T0 ($p \leq 0.027$, Fisher's test LSD), while those of non-tasters increased only at T2 ($p \leq 0.0045$, Fisher's test LSD) (Figure 13B). No differences in super-tasters were found related to sampling time ($p > 0.05$). No effect of PROP taster status on disinhibition and hunger scores was found ($p > 0.05$).

There were no differences in the restraint or perceived hunger scores related to types of bariatric surgery or gender ($p > 0.05$; data not shown), although, overall, women had a basal level of disinhibition that was higher than that of men ($F_{1.24, 58.53}$ = 4.169, p = 0.0037; $p \leq 0.0040$, Fisher's test LSD).

Discussion

The main goals of this part of the study were to evaluate the impact of IBD on the taste and olfactory perception, and how they change in obese subjects which had gone through a bariatric surgery. In detail, the study compares gustatory and olfactory function of IBD patients with that of a group of healthy control subjects matching for age and gender. Moreover, the study evaluates smell and taste performances before and after a surgical treatment for severe obesity.

First, the results we obtained show that overall taste intensity, as well as intensity to specific qualities such as sweet, salty, bitter and umami, were reduced for the IBD patients. In contrast, sour taste was higher in IBD patients than in HC subjects. Together, these results are consistent with data reported by Steinbach et al. [8]. It is worth highlighting that, in the present study, the taste parameters were analyzed controlling for differences in BMI between IBD patients and HC subjects. The present study also documented, for the first-time, reduced taste perception by IBD patients for umami and fat which are the taste qualities related to appetitive responses to protein- and lipid-rich food sources, respectively.

The overall reduction in taste in IBD patients can be well understood in the light of the manifold oral pathologies observed in these patients [9] which can be specific or non-specific manifestations. Moreover, the symptoms that are associated with these oral pathologies (specially acidic taste, taste changes, changes in the tongue and dry mouth) can be caused by iron, zinc, or vitamin deficiencies due to rectal bleeding and intestinal malabsorption linked to IBD, or its pharmacological treatments [10-12]. It is also well known that zinc deficiency (regardless of its cause) is associated with taste dysfunction and losses in taste acuity [13]. On the other hand, we observed increased acid taste in IBD patients which could also be related to disturbances in gustin CAVI. This salivary protein is a zinc dependent enzyme that, among other functions, regulates pH balance of the saliva. Low salivary pH promotes the growth of infectious microbes over beneficial ones. The shift in the

composition of the oral microbiome observed in IBD patients is associated with increased generation of bacteria-derived acid metabolites and greater risk of oral disease [14]. Therefore, we speculate that disruptions in the levels or in the functionality of gustin CAVI with a consequent reduction of its capacity to neutralize bacteria-produced acids may play a role in the increased sour taste we observed in these patients. These results fit well with data on the role of gustin CAVI in oral health, showing that variations in the gustin gene are associated with the presence of aciduric and acidogenic species which are promoted at low pH [15]. Previous studies in IBD patients indicate low levels of zinc that may be insufficient to activate the gustin enzyme [16-19]. Our observations further suggest that zinc deficiency in combination with gustin CAVI changes may play a role in the oral dysbiosis in IBD.

Our findings indicate a significant direct relationship between overall taste function or bitter taste and PROP taster status only in HC subjects, who displayed a similar trend also for the other qualities. Differently, we have not found a specific effect of PROP taster status on taste function in IBD patients that may be explained by oral pathologies. This suggests that IBD patients who are PROP super-tasters do not have taste advantages over PROP non-tasters.

Particular attention should be given to the marked decrease of fat perception that we found in IBD patients with respect to HC subjects. This reduction does not depend on the PROP taster status or the *r1761667* polymorphism of *CD36* gene. in HC subjects we previously showed a direct relationship between fatty acid perception and PROP taster status or the polymorphism of *CD36* locus [3]. These data seem inconsistent with respect to what we found in this study. However, the wide differences we observed between HC subjects and IBD patients dampened the effects of the two genetic factors in HC subjects, when the two groups were analyzed together. In fact, if data of the two groups were analyzed separately it would be possible to highlight the effect on fat perception of PROP taster status (with PROP super-tasters displaying higher values than non-tasters), or *CD36* gene (with GG

subjects showing higher values than AA subjects) in HC subjects, but not in IBD patients. Our results also show a higher frequency of IBD patients with genotype AA and allele A in CD36 locus, as compared to HC subjects. This could explain the low sensitivity for fat that we found in these patients since the genotype AA and allele A are associated with low expression of the protein [20, 21] or decreased perception to fat [3, 22-24].

Additionally, we demonstrated that the general olfactory sensitivity of IBD patients is significantly impaired when compared with HC subjects, as shown by the lower performance they reached in the total TDI score and by the high number of hyposmic subjects. Since the TDI olfactory status depends on the abilities of olfactory threshold, odor discrimination and odor identification, we investigated which of them were compromised. The results we obtained show that IBD patients reached T, D and I values that were significantly lower than those obtained by HC subjects. The lower olfactory threshold scores (which means that individuals showed a higher olfactory threshold) we observed in IBD patients, as compared to controls, can be explained by the elevated levels of tumor necrosis factor (TNF-α) which were identified in the blood and intestinal mucosa of IBD patients. High levels of TNF-α have been reported to cause the loss of mature olfactory sensory neurons (OSN) in the olfactory epithelium [25-27], thus affecting the olfactory threshold which, to some degree, represents the peripheral olfactory function [28-30]. While a threshold impairment has already been observed in a previous study on the olfactory performance of IBD patients [8], these are the first results highlighting an impairment in odor discrimination and odor identification, higher-order olfactory functions, that require a more pronounced involvement of cognitive factors [28, 29].

Individuals exhibit a physiological variability in their olfactory function due to environmental and genetic factors [31-35]. Recent studies on different groups of healthy subjects have shown that this variability can be, at least partially, determined by the *rs2590498* (A/G) polymorphism of the *OBPIIa* gene, both in terms

of the ability to perceive complex odors and single molecules [35-37]. In particular, these authors found that individuals who were homozygous for the major allele A showed a lower olfactory threshold than heterozygotes and homozygotes for the minor allele G. The results of this study show that both IBD patients and HC subjects who were homozygous AA achieved higher T-scores than heterozygous and homozygous GG and surprisingly, among IBD patients, those with the AA genotype also achieved higher scores than those with genotype AG and GG in the D-test. These results agree with previous studies that report that OBPs play an important role both in carrying odorous molecules, generally lipophilic, through the mucus layer to the ORs, and in odor discrimination [38-41]. Patients with GG genotype reached a significantly lower T-score than controls with the same genotype, while no difference was observed between patients and controls with at least one A allele. This suggests that the lowest T-score obtained by patients is mainly determined by GG homozygous ones. Additionally, the D olfactory performances of IBD patients carrying two sensitive alleles (AA) were not different from those of HC subjects. Similarly, recent evidence has shown that women with PD who are AA homozygous exhibit a better olfactory performance than heterozygous or GG homozygous PD women and that their olfactory scores are not different from those of HC subjects [42].

In our study, we wanted to compare the body weight of IBD patients (by calculating the BMI) with that of HC subjects, also in relation to their PROP taster status, genotype of the *rs2274333* (A/G) polymorphism of gustin gene, olfactory status and *rs2590498* (A/G) polymorphism of the *OBPIIa* gene. Results showed that BMI was higher in IBD patients as compared to HC subjects whereas there were no specific effects of PROP taster status or the *rs2274333* (A/G) polymorphism of the gustin gene on BMI. Even if the BMI was marginally higher in subjects who were non-tasters as compared to those who were medium tasters and super-tasters. It is interesting to note that the relationship between the gustin gene polymorphism and BMI appears to be opposite in HC and IBD patients. Melis et al [43] previously

demonstrated higher cell culture production of gustin CAVI from the saliva of healthy normal weight individuals with the AA genotype compared to gustin CAVI from saliva of individuals with the GG genotype. However, Lamy and co-workers [44] showed that the level of gustin CAVI was elevated in morbidly obese women relative to normal weight controls. Based on these observations, one can speculate that AA and AG individuals, who are associated with a higher BMI among IBD patients, produce more gustin CAVI. Finally, we found that both controls and patients who were homozygous for the A-allele exhibited a lower BMI than individuals who were homozygous for the G-allele or heterozygous, and patients with at least one G-allele showed a higher BMI than controls.

Olfactory sensitivity plays a role in food choices and intake, and its impairment affects eating behavior [28, 45-47]. Individuals with olfactory dysfunction tend to experience a lower reward from food, report that food is less flavorful and less enjoyable, and compensate for this deficiency by changing their feeding habits (e.g., eating saltier, sweeter, more spicy foods) and by decreasing their intake of low-fat foods [46, 48, 49]. A similar eating behavior has been observed in IBD patients, who increase their consumption of sucrose and refined carbohydrates and reduce that of fruits and vegetables [18, 50]. Both individuals with olfactory dysfunction and IBD patients may display variations in body weight [8, 46, 49, 51]. The results we obtained show that: first, the BMI of hyposmic IBD patients was significantly higher than that of hyposmic HC controls, while no statistical difference was found between normosmic IBD and normosmic controls; second, the BMI of normosmic was lower than that of hyposmic individuals in both HC subjects and IBD patients. Based on these findings, we speculate that olfactory impairment may be considered a more important factor than the disease in causing an increase in BMI. These results are in agreement with previous studies that have shown that obese adults show reduced olfactory sensitivity [52] and that a negative correlation exists between body weight and orthonasal olfactory ability [53]. The sense of smell participates in the cephalic phase responses to food, which play a

direct role in regulating meal size [54], by acting on appetite and satiety [47, 48, 55, 56]. Several studies have shown that a reduced olfactory sensitivity determines a reduced response of the cephalic phase, with a consequent delay in reaching the sense of satiety and an increase in the duration of the meal: this leads to an over-feeding of gratifying and palatable foods that causes an increase in body weight [57-59]. Finally, the expression of olfactory receptors (ORs) has been found in the enterochromaffin cells of the gut, and their activation by odors leads to the release of serotonin that affects gut motility and increases satiety [60, 61]; this means that an increase in their threshold leads to a lower release of serotonin, a reduced sense of satiety, a higher intake of calories and longer duration of meals [8].

These findings revealed that in IBD patients, the presence of at least one sensitive allele (A) of the *rs2590498* (A/G) polymorphism of the *OBPIIa* gene prevents the decline in the olfactory threshold compared to controls. On the other hand, the presence of at least one G allele is sufficient to impair odor discrimination, and on the other, it increases the BMI of patients compared to controls.

The comparative analysis performed in the patients undergoing bariatric surgery, allowed us to demonstrate that the surgery is associated with improved taste and smell identification, as well as with weight loss and improvement of body composition. Interestingly, we found an overall improvement of taste sensitivity to PROP bitterness and general taste sensitivity, but we found that taste score improvements were mostly explained by increase identification scores for sweet, sour and umami with no changes in bitter of quinine or salty taste. That taste identification scores overall improved at both 1- and 6-months post-surgery, agrees with results from previous authors that also used the taste strip test to assess taste function in bariatric population [62, 63]. The lack of a non-surgical control group that was also evaluated three consecutive times, does not allow us to control for learning effects, which could potentially contribute to the observed improvement of taste identification post-surgery. However, we believe this to be unlikely given

that the value of the total taste score that we measured after six months of surgery approached values that are close to those determined in healthy normal weight subjects [63, 64]. Although there are controversial data, some studies indicate that an increase of taste sensitivity may associate with a decrease of preferences of related foods [65-74]. Therefore, the increase of ability for sweet that we found after bariatric surgery may determine a shift forward a reduced intake of high-calorie foods contributing to the success of the intervention. On the other hand, since umami taste is related to appetitive responses to protein [75], the increase of identification scores for umami that we found after surgery, could explain the reduction of preferences for protein-rich food that are reported by subjects after surgery, which drastically reduce the consumption of this kind of foods [76].

Our results also documented, for the first-time, an increased in the sensitivity to detect a fatty acid. Interestingly, the increase in PROP sensitivity after surgery was not only evident by patients report of increased bitterness intensity ratings, but also by the increased number of subjects that were classified as super-tasters at the expense of those classified as non-tasters. Differently, Hubert and colleagues did not find variation on frequency of the PROP taster categories between the pre- or post-surgery groups [77], which might be likely because they used a cross-sectional study design unlike the current study which used a longitudinal design. In other words, we found that subjects with the PAV variant were more likely classified as a super-taster after than before surgery and subjects with the AVI variant were more likely classified as a non-taster before than after surgery. We also observed that many of the AVI/AVI subjects could detect PROP after surgery. Together these results suggest that the improvement of PROP bitter sensitivity after surgery is supported by a mechanism different to that mediated by TAS2R38 receptor. Although variants in *TAS2R38* account for most of the PROP phenotype variance, other genetic and non-genetic modifiers exist. For example, previous research suggest that other receptors in the T2R family [78] and other non-bitter receptor genes [79-81] modify PROP taste ability. Previous research also

suggests other modifiers, including receptor cell number and density [43, 82, 83], development and disease [83-85].

It is worth highlight the indirect association that we found between the *OBPIIa* (A/G) locus and the variations in the overall taste sensitivity or sensitivity to sweet and sour after surgery. Olfactory performance assessments and bioinformatics data suggested that the presence of the mutation in this locus decreases the expression of OBPIIa protein in the olfactory epithelium [42]. Our results showed that only the carriers of G allele showed after surgery an increase of the overall taste sensitivity and sweet and sour taste. This observation leads us to speculate that the improvement of taste sensitivity after surgery would be more effective in subjects who have a minor expression of OBPIIa protein. Future studies will have to explore this. Contrary, the changes in the overall taste sensitivity or sensitivity to single taste qualities after surgery was not depended by PROP taster status of subjects. However, we observed a main effect of the PROP taster status on the total taste score and on bitter score, such that taster subjects (super-tasters and medium tasters) had higher scores than non-tasters at each time point. These findings fit with data showing a greater general taste sensitivity in tasters than non-tasters [3, 72, 86-92].

Our results also showed that the obese subjects of this study had a higher fat threshold (about 3- fold) with respect to that determined in normal weight subjects in an our previously study [3]. In addition, we found a significant improvement of fat sensitivity after surgery determining threshold values which became after six months similar to those observed in normal weight subjects (0.22 μL) [3]. These results may explain the drastic reduction of preferences for high fat-foods that have been shown after bariatric surgery. Contrary to expectation, the positive effect of bariatric surgery on fatty acid taste was independent of *CD36* locus. All genotype groups had the same trend that oleic acid thresholds are decreased after surgery, though the effect seems more evident in subjects homozygous for the non-taster variant (AA). This could be due to the fact that the

fat taste sensitivity is individual feature complex and other factors can be involved especially in subjects with overweight or obesity. It is known that habitual diet and BMI could influence taste sensitivity [93-95]. In addition, it is known that the CD36 expression in papillae decreased in high-fat diet-induced obese rats [96] and the exposure to, or restriction from, dietary fat can modulated taste sensitivity [94].

Consistently with previous studies [63, 97, 98] our results showed that the odor identification improved after bariatric surgery indicating as improvement of olfactory function of these patients. The increased olfactory sensitivity associated with weight loss and improvement of body composition that we found after surgery is consistent with data that have identify the olfactory bulb (OB) as a brain region, outside of the traditional hypothalamic pituitary, endocrine axis and show that the increased ability of OB neurons (via modulation of Kv1.3 channel) contributes to the improvement of metabolic function and energy consumption [99]. Since it has been shown an olfactory role in the modulation of PROP and oleic acid sensitivity [36], the improvement of smell sensitivity could explain, at least in part, the increase in PROP and oleic acid sensitivity that we find not to be related to *TAS2R38* or *CD36*. Surprisingly, we did not find a specific effect of *OBPIIa* locus on changes of odor identification after surgery given that similar trends for all genotypes was found.

Finally, our results showed that subjects with obesity, especially tasters, had high scores in cognitive restraint factor before surgery. In our study, scores for the restrained factor were much higher (≥ 10, median value) than those previously reported in the literature [100, 101]. We hypothesize that these high scores are due to the educative training these subjects received before surgery, which were designed to re-establish a correct eating habit. Furthermore, a conscious control of eating was a fundamental inclusion criterion for being qualified for the surgery since it has already been associated to a long-term weight loss success [102-105]. Consistently with previous works [77, 106-110], our results also showed an increase of cognitive restraint and a decrease of disinhibition and perceived hunger after surgery. These findings seem indicate that bariatric surgery can have a positive

effect on cognitive control of eating behavior turn to contribute to the success of the intervention. In fact, a neuro-imaging study indicated that bariatric surgery-related decreases in preference for unhealthy foods and increases in preference for healthy foods arise from changes in the network of frontoparietal control, which involves cognitive control of food sensations, while failed to find involvement of reward-related brain regions [111]. The increase of restraint after surgery was associated with PROP phenotype of subjects. Non-tasters and medium tasters showed increased values after surgery, while no significant changes in super-tasters were found. These observations lead us to speculate that non-tasters and medium tasters, compared to super tasters, might need a higher restraint to be able to control their incorrect eating behavior dictated by their lower taste sensitivity. Further investigation is needed to clarify this issue.

Finally, since deficits in olfaction and taste have also been associated to many health markers including neurodegenerative diseases and specifically are among the most frequent non-motor manifestations in Parkinson's disease (PD), we focused on reviewed the most relevant molecular and genetic factors involved in these impairments and their associations with the microbiota, with the aim to highlight that the basis of these dysfunctions are likely multifactorial and may include the same determinants responsible for other non-motor.

Taste impairments in PD

Over recent years, the link between taste dysfunctions and neurodegenerative disorders have increasingly been recognized. Several studies evaluated gustatory function in PD patients [112-118], but reporting inconsistent results. This may because they were carried out by using small sample size or different assessment methods: Whole Mouth Test (WMT), supra-threshold taste solutions sprayed into the oral cavity [119]; Taste Strip Test, (TST), patients had to identify the taste they perceived for each taste strip [2, 120] and electrogustometry

(EGM), rapid measure of taste threshold by using electric current as stimulus) [121, 122].

Despite the different tests adopted by the research groups, it is generally reported that taste can be affected in PD patients by showing persistent, but slight and stable taste impairments [123]. In particular, most of the studies identified a reduced taste sensitivity with an estimated frequency between 9 and 27% [115, 116, 118, 124]. Shah et al. [115], using EGM, found that about 27% of PD patients had an impaired taste function. Taste thresholds measured in the front and back of the tongue were higher in PD patients, than in healthy controls (HC), suggesting significant deficits in CN VII and CN IX. Deeb et al. [118] by using EGM showed that about 22% of PD patients had impaired taste function. Kim et al. [116] by using TSTs reported a decrease in the ability to identify tastants in female but not in male PD patients when compared to HC. Cecchini et al. [117] reported difference between PD patients and HC in taste performance assessed by the TST, but not by WMT. In fact, only the TST score was significantly lower in PD patients than HC. The reason of the fact that WMT do not show reduction of taste could be due to the use of stimuli at supra-threshold concentration, which are not able to capture slight impairment of taste function.

Doty et al. [125] studied whole-mouth (WMT) and regional taste perception of early-stage PD patients and HC matched on the basis of age, sex, and race. They reported that the WMT scores were lower in the PD patients than in controls (for all four taste stimuli), and the intensity ratings for the weaker concentrations of all stimuli, except caffeine, tended to be higher in the PD patients than in HC. This last finding is in accord with the findings of Sienkiewicz-Jarosz and co-workers who demonstrated that, in the WMT test, PD patients rated quinine [112] and sucrose as more intense than HC [113]. Moreover, Doty et al. [125] using regional tests showed that subjects tended to better identify and rate the stimuli as more intense on the front than in the back of the tongue with respect to controls. These findings suggest that the suprathreshold measures of taste function are influenced by PD which

differentially influences taste function on CN VII and CN IX. These results are not observed if the taste techniques are limited to WM. In addition, in the same study [125] EGM was not able to observe differences between the PD patients and controls. In addition, a specifically reduced identification of sweet [126], salty or bitter stimuli was found [125]. Despite the slightly controversial results, it appears that taste is affected in PD, although less frequently than smell. However, future investigations are necessary to explore the causes of taste impairments related to PD.

It is interesting to note that the taste loss has been related mostly to the advanced stages of the disease [118], whereas reports on prodromal presentation are rare. Pont-Sunyer and colleagues [127] observed that the time of the taste loss onset varied between 2 and 10 years before diagnosis. Taste loss was present before the onset of motor symptoms in more than 70% of PD patients, providing evidence for a very early onset of taste loss, which is comparable to that of olfactory impairments. Therefore, the evaluation of the taste function may be used in combination with that olfactory as a potential marker of PD. Having said that, it is also understood that a deficit in gustatory function can occur as a consequence of olfactory loss, possibly as a consequence of a missing amplification of taste through the sense of smell [128-130].

The role of taste and smell receptors in PD has been investigated showing that the cortical olfactory receptors (ORs) and the TAS2Rs are altered in PD patients [131]. Olfactory receptors OR2L13, OR1E1, OR2J3, OR52L1, and OR11H1 and taste receptors TAS2R5 and TAS2R50 were downregulated, whereas TAS2R10 and TAS2R13 were upregulated, at premotor and parkinsonian stages, in the frontal cortex area 8 of the brains in PD patients [131]. These findings support the idea that ORs and TA2SRs in the cerebral cortex may have physiologic functions that are affected in PD patients. The identification of altered regulation of OR and TAS2R in PD patients, suggests the study of the chemical signaling system of the brain to understand the mechanisms involved in the occurrence of the neurodegenerative

diseases. Future studies will have to point out whether the altered TAS2R may play a role in the inflammatory mechanisms associated with the initiation of misfolding of the α-synuclein cascade.

Other than individual differences in PTC/PROP tasting and food linking patterns TAS2R38 SNPs dictate individual differences pathophysiology [132], such as susceptibility, severity, and prognosis of upper respiratory infection, rhinosinusitis and biofilm formation in chronic rhinosinusitis patients [133-140], development of colonic neoplasm [141-143], taste disorders [64], and neurodegenerative diseases [144]. Moberg and colleagues were the first that examine PTC sensitivity in PD patients and HC to determine whether taster status can be a marker for PD. They showed significant differences in the distribution of taster and non-taster subjects between the PD patients HC. They showed that only 44% of PD patients could detect the bitterness of PTC, as compared to 75% of HC [145]. Cossu et al. [144] confirmed the result showing a reduced of PROP taste sensitivity in PD patients compared to HC. Specifically, a decreased perceived taste intensity and reduced ability to recognize bitter-taste quality was found. They also showed an increase in the frequency of the PD patients classified as PROP non-tasters (54.13%) and a decrease in frequency of PD patients classified as PROP super-tasters (8.25%) compared to HC. Furthermore, the results showed that the homozygous genotype for the tasting variant of TAS2R38 (PAV) was uncommon in PD patients, only 5% of them carried this genotype, whereas most of them carried the non-taster form (AVI). These results seem to indicate that individuals who have a couple of tasting haplotypes (PAV/PAV) at TAS2R38 may be at lower risk of developing PD, with respect to those with the haplotype (AVI). Therefore, the latter might represent a prodromal genetic marker for the identification of early pre-degenerative changes that could be instrumental to understand the origin of this disorder. Thus, studying the PROP phenotype and genotype may represent a new, simple way to identify increased predisposition for PD.

PD has been associated with the dysbiosis of gut microbiota [146] and imbalance in gut microbiota plays an important role in worsening of disease [147-150]. Specific taste receptors, expressed in the lower gastrointestinal tract (GI), respond to change of the composition of gut microbiota, detecting bacterial molecules, including quorum-sensing molecules and potential toxins and regulate immune responses against pathogens [132, 151-153]. In particular, it is known that when TAS2R38 expressed in the enteroendocrine cells of the gut is activated by bacterial molecules, increase the release of β-defensin (an anti-microbial compound) [132] and a peptide hormone termed cholecystokinin (CCK). This hormone can limit the absorption of dietary toxins [154, 155], inhibit feeding behavior and gastric function [156-158] and it can also play a key roles in regulating the immune response to antigens and bacterial toxins [159]. Thus, the response of TAS2R38 represents an important defense of the organism in contrasting the noxious effects in the gut lumen.

Vascellari et al. [160] showed that the composition of the gut microbiota was different across genotypes of TAS2R38 in PD patients. Specifically, a decrease in bacteria alpha-diversity with a predominant reduction of *Clostridium* genus was associated with AVI/AVI genotype, compared to the PAV/PAV genotype. It is important to mention that some members of *Clostridium* genus produce toxin [161], while other members confers beneficial effects which has a multitude of metabolic function in the GI tract, such as modulation of gastrointestinal motility, barrier integrity and immune response [161-163]. Therefore, a decrease in the abundance of helpful-*Clostridium* molecules associated to a high frequency of the form of TAS2R38 receptor at a low affinity for the ligands might determine, in PD, a decrease in the activation of protective signaling-molecules involved in the regulation of the immune response. This factor could affect different cellular processes which are impaired in PD, thereby contributing to the development of gut dysbiosis [160].

Smell impairments in PD

Impaired olfaction has been associated with a variety of age-related neurodegenerative conditions that impair cognitive and motor function, including PD [164, 165], Alzheimer's disease [166], and Huntington's disease [167]. Smell loss may therefore be considered an important contribution to the diagnosis of neurodegenerative diseases. In PD, olfactory loss has been extensively studied and is now widely acknowledged as one of the major non-motor symptoms of the disease which precedes the occurrence of clinical motor symptoms [168]. Olfactory disturbances are found in around 90% of patients with PD [169] and have been considered as a supportive criterion in clinical PD diagnosis according to the International Parkinson´s Disease and Movement Disorder Society diagnostic criteria [170]. The majority of PD patients with smell loss are already functionally anosmic or severely hyposmic at the time of testing regardless of the type of olfactory test being used for diagnosis. Wenning et al. [171] presented data suggesting that olfactory function is differentially impaired in distinct Parkinsonian syndromes. They reported a preserved or mildly impaired olfactory function to be more likely for atypical parkinsonism such as multiple system atrophy, progressive supranuclear palsy, or corticobasal degeneration, whereas markedly pronounced olfactory loss appeared to suggest PD. Similar results were reported by Müller et al. [172] and Krismer et al. [173]. In dementias, the loss of smell is usually very severe. This applies to Lewy Body Disease (LBD), where significant olfactory deficits were found [174, 175] which does not allow differentiation from PD. Similar olfactory deficits have been shown in AD. In a meta-analysis by Mesholam [166] olfactory deficits in patients with AD and PD were relatively uniform although there was a trend toward better performance in AD patients on threshold tests compared to odor identification tests. Smell loss can already be observed in patients with mild cognitive impairment [176] and is associated with the progression from MCI to AD [177]. Huntington´s disease patients present with moderate hyposmia affecting

olfactory detection threshold, odor discrimination and odor identification [167]. Deficits in odor identification are prevalent prior to diagnosis of HD [178]. In patients with cerebellar ataxia, olfactory impairment was found in Friedreich's ataxia [179] and spinocerebellar ataxias [180-182]. Mild olfactory impairment has also been demonstrated in motor neuron disease [183, 184].

Support for the existence of a prodromal phase of PD, including a long pre-motor phase, comes from imaging, neuropathology, and various clinical or epidemiological surveys. Loss of smell is recognized as a very early non-motor symptoms of PD and has been suggested as a possible biomarker [185]. Several population-based studies already pointed out the association between unexplained smell loss and later development of PD. Data of a large, thoroughly diagnosed patient cohort study of a Smell & Taste Clinic suggest a 10 % rate of PD development among patients with diagnosed idiopathic olfactory loss [186]. The duration of the hyposmic phase prior to PD diagnosis is still a matter of debate. In many previous studies investigating the prospective risk for PD in relation to baseline [187-189] follow-up periods ranged from 2 to a maximum of 8 years. We could demonstrate that the olfactory dysfunction frequently precedes the PD motor symptoms by more than 10 years [186]; other authors assumed that this period may last up to decades [190]. In a follow-up study of patients with idiopathic REM sleep behavior disorder who phenoconverted to PD or dementia, olfactory loss was the first marker to develop, with predicted onset >20 years before phenoconversion [191].

Further, current study results indicate a correlation between olfactory function and progression of the disease as measured by motor and other non-motor symptoms. An association between disease severity and smell loss [118, 192-195] and a disease duration-related progression of olfactory loss [194] might suggest the use of olfactory function as potential marker of PD progression. This was confirmed by an imaging study using Dat-SPECT, indicating that a more pronounced olfactory dysfunction was associated with greater loss of nigrostriatal dopamine neurons [196]. Also, non-motor symptoms like cognitive impairment, depression, anxiety

and sleep disturbances which are typically related to PD severity are associated with the degree of olfactory loss [192, 196, 197]. The close correlation between smell function and cognitive impairment is reflected by the results of the Parkinson's Progression Markers Initiative study [198] which indicated that olfactory loss is one of the strongest clinical predictors of cognitive impairment in the first 2 years after PD diagnosis. Decline in cognition seems to be linked to progressive cholinergic denervation in PD as described by Bohnen et al. [199] who found a positive correlation between odor identification performance and forebrain cholinergic pathway integrity in PD patients.

The olfactory system could be one of the peripheral sites where PD first develops [200]. However, there is little and inconsistent information on changes at the olfactory periphery. While α-synuclein aggregates (Lewy bodies and neurites) have been described in the olfactory bulb (OB) at early neuropathological stages of the disease, α-synuclein was not detected in olfactory epithelium biopsies of PD patients [201], it was found however, in olfactory cells in PD autopsy cases [202]. Further, in-vivo examinations of the olfactory epithelium revealed histological changes comparable to other causes of smell loss [201] which suggest non-specific peripheral changes in the olfactory system in PD. On the OB level, PD seems to differ from other causes of olfactory loss. In aetiologies involving peripheral olfactory loss, such as postinfectious or sinonasal smell disorders [203, 204] but also in more central pathologies above the level of the OB like depression [205], schizophrenia [206], and temporal lobe epilepsy [207] a clear and consistent correlation between olfactory function and OB volume can be observed suggesting that smell loss is associated with a measurable OB volume loss. In PD however, despite of the severity of olfactory impairment it remains a matter of debate whether PD patients present with decreased OB volumes compared to age-matched controls. So far, a number of recent studies have reported conflicting results: while some studies [208, 209] reported an overall reduction of the OB volume in PD, the vast majority of studies [210-213] question any OB volume differences between PD and

HC. This is in line with findings of an increased number of olfactory dopaminergic periglomerular cells in PD patients [214, 215] which might underlie hyposmia in PD patients. However, in central regions related to both primary (piriform cortex, amygdala) and secondary integrative (orbitofrontal cortex) olfactory processing a significant atrophy was found in PD which correlated with olfactory performance [216-218]. This might suggest that central olfactory areas in PD seem to represent the degree of disease progression whereas this correlation is not seen in peripheral olfactory structures.

The causes of olfactory dysfunction in PD are poorly understood, but it is supposed they are related with both peripheral and central olfactory impairments [219]. The mechanisms implicated in the smell impairments in PD may involve neuropathological alterations and/or dysfunctions caused by alteration in the neurotransmitter levels [220]. The importance of these mechanisms is addressed in the successive paragraphs.

The olfactory system is one of the earliest brain regions involved in PD before involvement of the nigrostriatal pathway [200, 221]. The α-synuclein deposition, predominant component of Lewy bodies [222, 223], have been identified before in the olfactory bulb, anterior olfactory nucleus, and several areas of olfactory cortices of PD patients [224, 225], than in the substantia nigra [200, 226]. The α-synuclein pathology seems appear before in the olfactory nerve layer and then it spread to the central olfactory structures [227]. However, the involvement of the olfactory epithelium on olfactory loss in PD have not well been defined. In fact, no significant difference was found by immunohistochemical markers for α-synuclein between PD patients and HC [201, 228]. These findings suggested that the changes in the olfactory function in PD may be due to processes associated with formation of Lewy bodies in the central olfactory areas and not in the peripheral ones [165, 229]. The α-synuclein pathology has been revealed across the central olfactory system, including the anterior olfactory nucleus, cortical nucleus of the amygdala, piriform cortex, olfactory tubercle, entorhinal cortex, and orbitofrontal cortex [229,

230]. In particular, the Lewy pathology in the anterior olfactory nucleus of the olfactory bulb is correlated with neuronal loss [231]. Furthermore, the olfactory nerves were grossly atrophic in all PD patients [231]. The cortical nucleus of the amygdala, which has major olfactory connections, have more α-synuclein pathology and neuronal loss than other nuclei in the amygdala [232], consequently its volume is reduced by 20% [232]. Moreover, the reduced volume in the amygdala and piriform cortex inversely correlates with olfactory deficits, suggesting that neural loss in these regions could play a role on the olfactory impairments of PD [216, 217].

Several neurotransmitter systems are altered in PD and most of them have been associated with olfactory loss, including dopaminergic, cholinergic and serotoninergic systems. Dopamine has long been known to play a key role in the pathogenesis of PD. Some studies suggested that the olfactory dysfunction of PD patients could reflect damage to dopaminergic cells [233]. As a matter of fact, correlations between odor identification tests (UPSIT scores) and a decrease in dopamine transporter activity in the striatum, substantia nigra and hippocampus in PD patients have been found [118, 234, 235]. However, the use of dopaminergic replacement therapy has no effect on olfactory test scores [236, 237]. Nevertheless, it is still not known whether changes in dopamine activity are directly associated with olfactory loss or whether there is an unknown common underlying mechanism. Acetylcholine levels are also altered in PD. It is known that acetylcholine release and activation of its receptors facilitate olfactory learning, memory, and odor discrimination [238-241]. Thus, cholinergic deficits may be responsible, at least in part, for the olfactory dysfunction in PD. It has been found that in PD the Lewy bodies and neuronal loss in the substantia nigra occur simultaneously with accumulation of the α-synuclein deposition in cholinergic neurons of the basal forebrain [200, 242-244]. Furthermore, the nucleus basalis, a main cholinergic nucleus with projection to olfaction-related brain regions, is significantly damaged in PD [245-247]. In addition, Bohnen and colleagues found

positive association between odor identification performance and acetylcholinesterase activity in PD patients [199]. Serotonin is another neurotransmitter with a possible role in the pathogenesis of olfactory dysfunction in PD. It arises from the raphe nuclei, which send projections to the olfactory bulb [248-250]. In PD patients, Lewy pathology is found in the raphe nuclei [251], parallel to marked depletion of serotonin in the olfactory bulb and other areas of the olfactory system [252-254], while a relative protection of serotonin was found in other diseases without important olfactory impairments [255]. Although the evidence is not conclusive so far, these studies suggest that changes in levels of some neurotransmitters may be implicated in the olfactory loss in PD.

Polymorphisms of specific genes coding for membrane receptors or odorant binding proteins (OBPs) (carrier proteins that vehicle the molecules toward receptor sites [256]), have been reported as mechanisms that result in the functional variations of olfactory function [32, 36, 82, 257]. Recently, the polymorphism rs2590498 (A/G) of the OBPIIa gene has been shown to affect retronasal [36] and olfactory [35] perception. Subjects with the A allele were generally more sensitive than those with the G allele**.** Moreover, bioinformatics data suggested that the presence of the mutation in this locus decreases the expression of OBPIIa protein in the olfactory epithelium [42]. The same polymorphism affected the olfactory performance of woman with PD [42]. Specifically, the olfactory performance of women with PD carrying two sensitive alleles (AA) was higher than that of women with PD with at least one insensitive allele (G) and of all men with PD. Interestingly, the olfactory scores of the AA genotype women with PD were not different from those of HC participants. These findings indicate that the AA homozygous condition in this locus preserves the olfactory function of women with PD, but not that of men. Furthermore, these results indicate that the smell dysfunction related to PD may occur, at least in part, at a peripheral level. Therefore, OBPIIa locus may provide a mechanism to determine the risk factor for olfactory deficits in woman with PD at the molecular level.

Despite, the associations between PD and the olfactory dysfunction and the composition of gut microbiota are unequivocal [258, 259], results on a role of the nasal microbiome in olfactory dysfunction in PD are not conclusive. A recent study has shown that there are no significant differences in the nasal microbiome composition between PD patients and HC [260]. However, the incapacity to collect samples in the olfactory cleft did not rule out the existence of differences in the microbial composition around the olfactory neuroepithelium. In addition, high spatial variability of microbial communities in the nasal cavity can exist [261]. Also another study did not find consistent difference in the nasal microbiota composition between PD patients and HC, even though a high interindividual variability was observed, with sex as the strongest factor [262]. Future studies in which samples of nasal microbiome are collected in the olfactory cleft are needed to understand its role in olfactory dysfunction of PD patients.

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CHAPTER 4

CONCLUSIONS AND FUTURE DIRECTIONS

The primary aim of this work was to study the physiological mechanisms implicated in human taste variability. To specifically analyze the peripheral taste function evoked in taste buds, we used an objective electrophysiological recording technique from the human tongue to measure directly and quantitatively the degree of activation of the peripheral gustatory system in response to taste stimuli. The electrophysiological recording from the tongue is a highly reliable and non-invasive method that allows us to obtain data that are not affected by the individual's subjective confounding factors differently by the common psychophysical approaches. The latter in fact imply highly subjective evaluations that, albeit of simple application, may produce measurement errors that account for up to 20% of phenotypic variance [1] and they are less useful for gaining insights into taste mechanisms, particularly those rapid responses that occur within the initial few seconds of oral exposure to a stimulus. Results showed that each taste quality can elicit a specific and characteristic electrophysiological response in taste buds, that was represented by a monophasic potential change characterized by a fast-initial variation followed by a slow decline, consistent with our current understanding of the biological mechanisms of taste transduction and cell-to-cell communications. In fact, the waveform of the signal depended on the taste quality of stimulus. In addition, our data provide the first direct and objective demonstration of the role of PROP phenotype in individual variability of general taste perception, and the role of *rs1761667* polymorphism in *CD36* in the perception of oleic acid. Specifically, the largest and quickest responses were recorded in PROP super-tasters, who had the highest density of fungiform papillae in the same area of the tongue where stimulations were applied during the recordings. Also, amplitude and rate of signals determined in GG homozygous were higher than those of subjects with the AA genotype.

The present results help to fill gaps in knowledge of these processes by combining traditional behavioral methods with our electrophysiological recording

method. The latest can be used to obtain objective data on individual taste sensitivity and understand human chemosensory experiences, free of personal bias.

In addition, we evaluated how the salivary proteome changes after everyday astringent stimulations and the differences in their basal levels between a healthy and a clinical condition (obesity). By using a time-course approach, we found that an everyday oral stimulation determined a robust increase in aPRPs but not the other protein families. The study has also provided novel insights into how PROP taster status may influence variation in astringency perception, i.e., via differential involvement of specific proteins, which may ultimately guide selection of polyphenol-rich foods. This variation was specific for male super-taster. Studies in progress are analyzing the effect of daily exposure to astringent stimuli on taste perception, salivary proteins, and oral microbiota, also as a function of PROP status.

Furthermore, the results showed novel insights on the role of salivary proteome as a factor driving the greater propensity for body weight excess of females or that associated with higher PROP sensitivity. At basal levels, OB showed higher levels of Ps-1 protein than NW, and in particular this difference was significant in OB males along with other proteins of bPRPs family, which had already been associated with higher taste perception. High levels of Ps-1 protein and Cyst SN were found in OB non-tasters, who had lower BMI than OB super-tasters. These new understandings on the role of salivary proteins as a factor driving the specific weight gain of OB females and super-tasters, suggest the use of specific proteins as a strategic tool modifying taste responses related to eating behavior. Since our research demonstrated that the supplementation of amino acids of the sequence of taste-related proteins enhance the sensitivity for PROP [2, 3] and for other taste qualities [4], further studies should examine how this oral supplementation can alter taste perception in subjects with metabolic syndromes like obesity.

Finally, we wanted to understand if taste and olfaction impairments can be significant risk factors that contribute to the pathogenesis of different diseases.

Results of the study between IBD patients and a healthy population suggest that impairment in the taste and smell functions might determine an imbalance food intake leading to a develop of IBD. In our cohort, the damage to the taste system was so severe in IBD patients that the genetic effect cannot be observed. On the contrary, a genetic effect on the olfactory function impairment function was found in IBD patients. Therefore, the increased BMI found in IBD patients, that was not related to PROP phenotype or gustin genotype depended on olfactory status and on genotypes of the *OBPIIa* locus. Our findings show that impaired olfactory threshold, odor discrimination and odor identification in IBD patients may partly contribute to delay the satiety sensation and increase the duration of a meal, resulting in over-feeding on gratifying and palatable foods that cause an increase in body weight. In addition, the high frequency of non-tasting form of CD36 receptor, which has not been previously described associated with IBD, may substantiate the fact that disruption of fat perception in IBD patients may represent a risk factor for this disease. Elevated sour taste in IBD patients may interfere with food enjoyment and may represent a separate quality of life issue in this disease.

Our findings also showed that bariatric surgery improved olfactory and gustatory functions in obese subjects. Increases in sweet, umami and fat perception, together with increased cognitive restraint and decreased disinhibition and hunger, may contribute to the decrease in the preference and consumption of foods high in calories, sugar, fat, and protein therefore contributing to the loss of weight, which was significant overtime. *OBPIIa* gene polymorphisms and the heritable variation in PROP taste sensitivity can play important roles in the bariatric surgery-induced changes of taste function and cognitive control of eating behavior. Further investigation is needed to clarify this issue.

Finally, we summarized the knowledge about smell and taste disorders in PD, describing the most relevant molecular and genetic factors involved in the PD-related smell and taste impairments. The limited data available suggest that the basis of the olfactory and gustatory dysfunction related to PD are likely

multifactorial and may include the same determinants responsible for other non-motor symptoms of PD. It is known that in many of the patients, taste loss accompanies the smell dysfunction, thus testing these two sensory functions together would help clinicians in the early diagnosis of PD enhancing the predictive value for diagnosis of disease.

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