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VARIATIONS OF TASTE PERCEPTION AND POSSIBLE ASSOCIATION WITH BMI IN HEALTHY SUBJECTS: A FUNCTIONAL AND GENOMIC APPROACH

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CHAPTER 1
INTRODUCTION

1. INTRODUCTION

1.1 Taste system

Taste is an important source of sensory input and it influences the palatability and the degree of acceptance of foods or drinks.

It is part of the sensory system, that provides us with an internal representation of the outside world through chemical (taste and olfaction) and physical (mechanical, sound, vision and temperature) information.

There are six different tastes known to date: bitter, sweet, sour, salt, umami and fat.

Each of these six taste qualities has a particular meaning: bitter taste protects from ingestion of potentially toxic substances, sour taste avoids ingestion of spoiled foods, sweet taste allows to identify energy nutrients, salt taste leads to intake of sodium and ions for maintenance of salt and water balance, umami taste recognizes aminoacids and indicates flavor of monosodium glutamate (mainly present in meat and aged cheeses) (Robino et al., 2014).

From an evolutionary perspective, originally the sense of taste had the essential role to ensure that individuals and species survive and adapt to the environment, in which they live. For example, the sweet taste perception originated for recognizing sugars, that are the main source of energy; instead the bitter taste perception originated for recognizing potentially toxic compounds including especially those of vegetable origin.

But over the millennia the sense of taste has lost this function, remaining however the primary sensitive discriminant for food choices: in any case it is considered an important aspect that influences body mass and, therefore, obesity.

The anatomical and functional units for taste detection are *taste receptor cells (TRCs)*, that are assembled into taste buds, that contain about 50-150 TRCs and are distributed across three different papillae of the tongue and palate epithelium. There are *circumvallate papillae*, located at the very back of the tongue; *foliate papillae*, located at the posterior lateral edge of the tongue and *fungiform papillae*, located in the anterior two thirds of the tongue (Figure 1) (Chandrashekar et al., 2006).

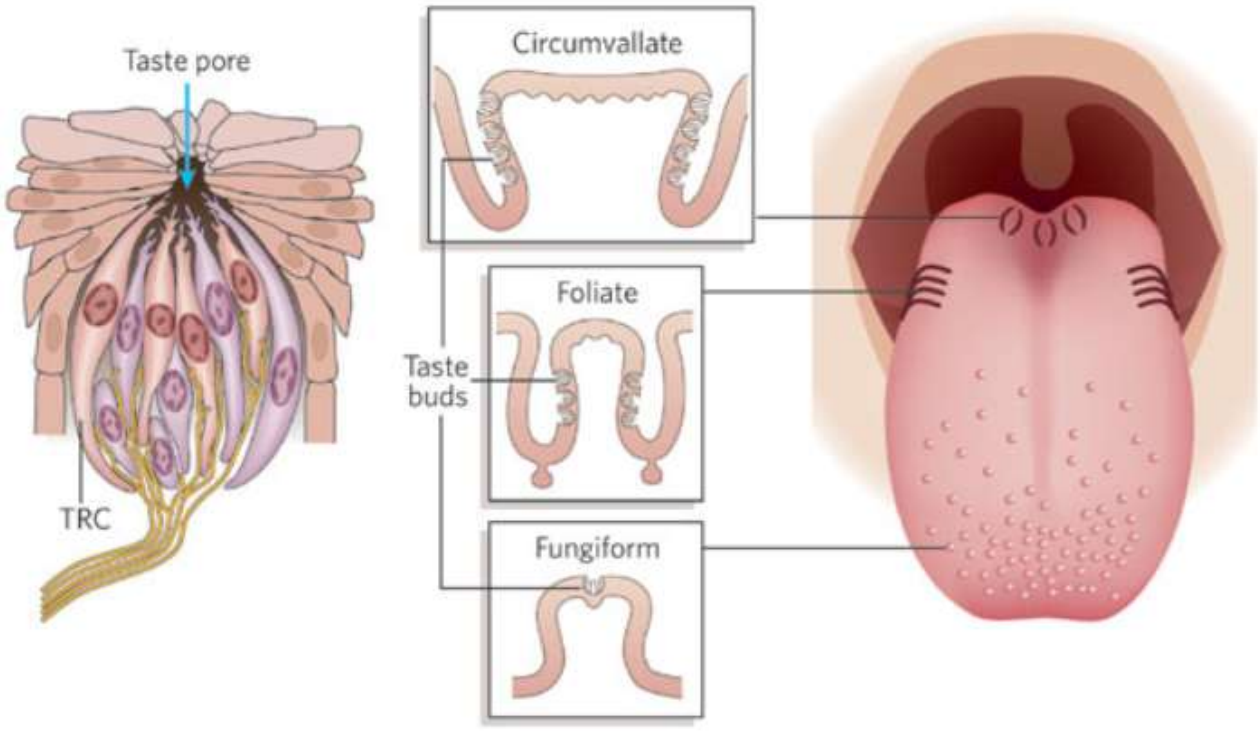

FIGURE 1. *Taste-receptor cells, buds and papillae* (Chandrashekar et al., 2006).

In addition, molecular and functional studies of recent years have shown that the ability to perceive different tastes is not localized in areas of the tongue specific for a taste rather than for another taste, but all the taste qualities are perceived in all areas of the tongue ((Hoon et al., 1999), (Nelson et al., 2001), (Adler et al., 2000), (Nelson et al., 2002), (Huang et al., 2006)).

Humans generally have about 5000-10000 taste buds located on the tongue surface, palate and epiglottis: as mentioned above, the taste buds consist of 50-100 neuroepithelial cells named TRCs inside of the epithelium of the oral cavity (Loper et al., 2015).

These neuroepithelial cells are distinguished in three types displayed in the following figure (Figure 2): type I cells, type II cells and type III cells ((Chaudhari and Roper, 2010), (Doty, 2012), (Lindemann, 2001)).

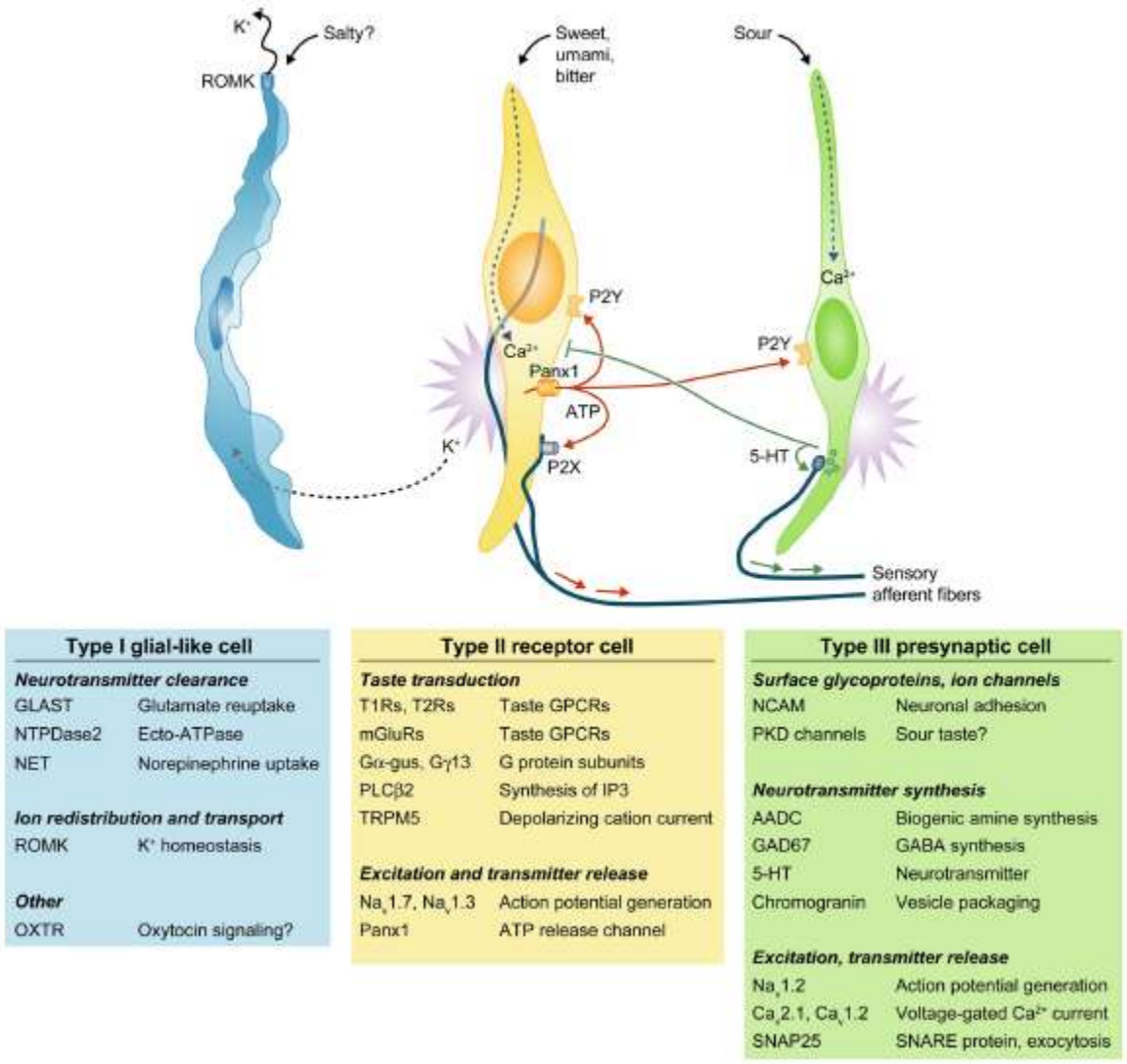

FIGURE 2. *Types of taste receptors cells.*

This classification incorporates ultrastructural features, patterns of gene expression and the functions of Types I, II and III taste cells (Chaudhari and Roper, 2010).

The most abundant cells are *type I cells*: they have cytoplasmic lamellae that envelop other taste cells; express a plasma-membrane-bound nucleotidase, which degrades extracellular adenosine triphosphate (ATP) and restrict neurotransmitter spread; regulate the extracellular ionic environment within the taste buds; terminate synaptic transmission and, thus, have a "glial-like function" ((Kinnamon et al., 1985), (Lindemann, 1996), (Bartel et al., 2006), (Vandenbeuch et al., 2013), (Dvoryanchikov et al., 2009)).

The *type II cells* are considered the "taste receptor cells": they express only plasma membrane G-

protein-coupled receptors for sweet, umami and bitter taste ((Roper, 2013), (Yang et al., 2000)); they express voltage-gated sodium and potassium channels that mediate the secretion of ATP as a function of action potential firing rate (Murata et al., 2010).

Finally, the *type III cells*, known as "presynaptic cells", express voltage-gated calcium channels associated with neurotransmitter release, enzyme for serotonin and γ-amino butyric acid as uptake transporters for biogenic amines (Roper, 2013); they release serotonin, γ-amino butyric acid and norepinephrine ((Huang et al., 2008), (Cao et al., 2009)).

When the chemical constituents of food interact with receptors on taste cells, the taste stimuli is transduced: in fact these taste cells inform about the identity, concentrations and pleasant or unpleasant quality of food, ensuring that the gastrointestinal system receives food by salivation and swallowing reflexes. Conversely, the somatic sensory receptors from trigeminal and other sensory cranial nerves to the thalamus and somatic sensory cortices inform about the temperature and texture of food (Purves et al., 2001).

The organization of the taste system consists of peripheral receptors and some central pathways.

The peripheral receptors are the taste cells on taste buds localized on the dorsal surface of the tongue, soft palate, pharynx and the upper part of the esophagus: these taste cells make synapses with primary sensory axons that run in the facial nerve (VII cranial nerve), glossopharyngeal nerve (IX cranial nerve) and vagus nerve (X cranial nerve) for innervating the taste buds respectively in the tongue, palate, epiglottis and esophagus (Figure 3).

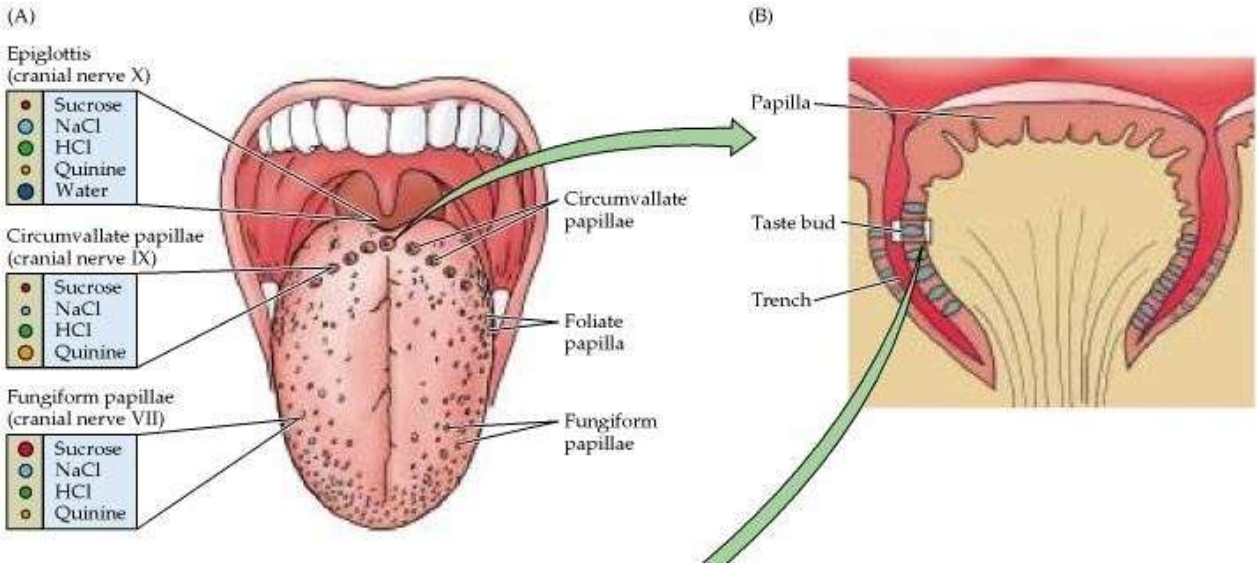

FIGURE 3. *(A) Distribution of taste papillae on the dorsal surface of the tongue. Different responses to sweet, salty, sour, and bitter tastants recorded in the three cranial nerves that innervate the tongue and epiglottis. The size of the circles representing sucrose, NaCl, HCl, quinine, and water*

corresponds to the relative response of the papillae to these stimuli. (B) Diagram of a circumvallate papilla showing location of individual taste buds (Purves et al., 2001).

The central axons of these primary sensory neurons in the respective cranial nerve ganglia project to the nucleus of the solitary tract in the medulla and from here to the thalamus and, finally, to gustatory areas of the cortex (Figure 4 and Figure 5).

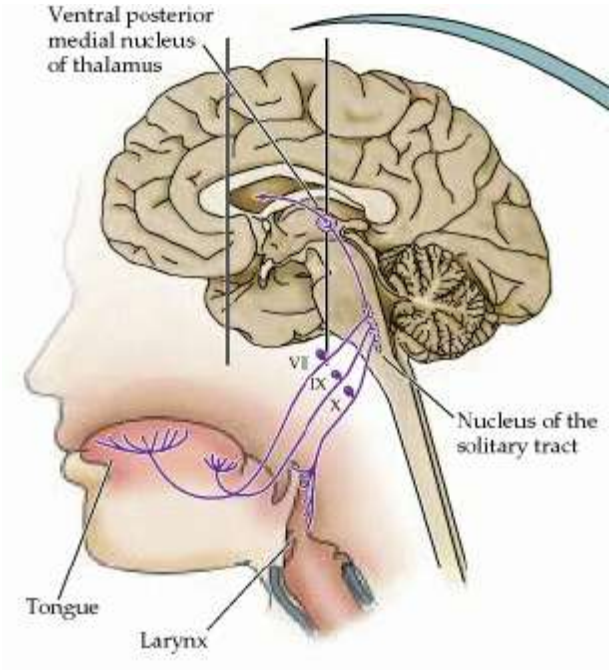

FIGURE 4. *Drawing above shows the relationship between receptors in the oral cavity and upper alimentary canal, and the nucleus of the solitary tract in the medulla (Purves at al., 2001).*

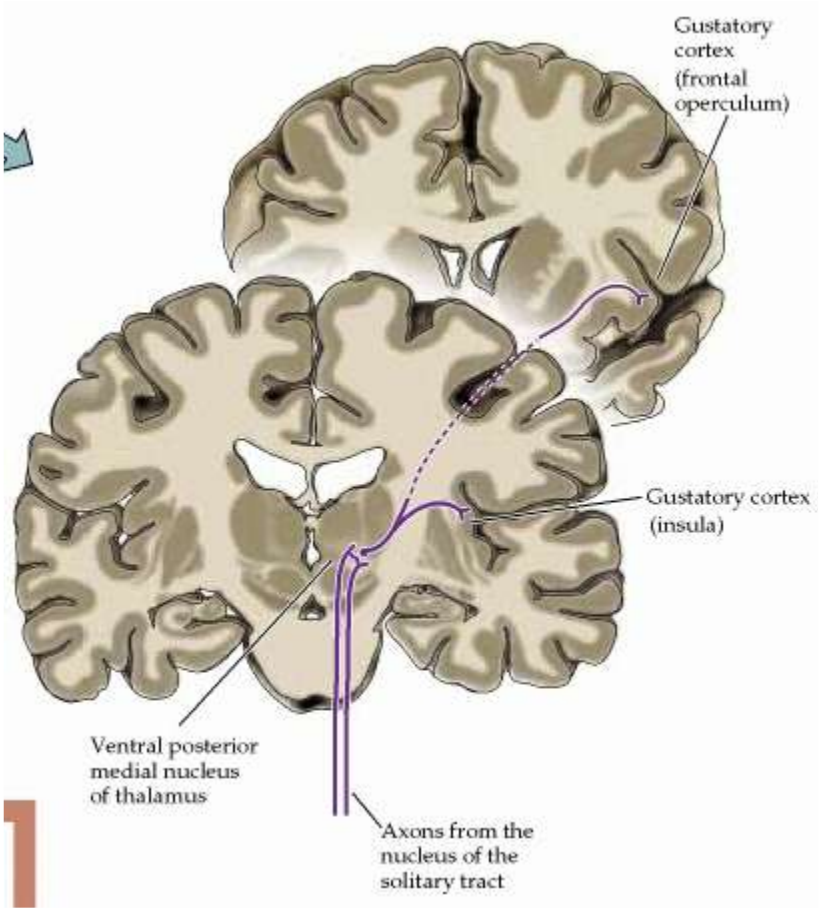

FIGURE 5. *The ventral posterior medial nucleus of the thalamus and its connection with gustatory regions of the cerebral cortex (Purves at al., 2001).*

The central pathways, instead, are shown in the following figure (Figure 6).

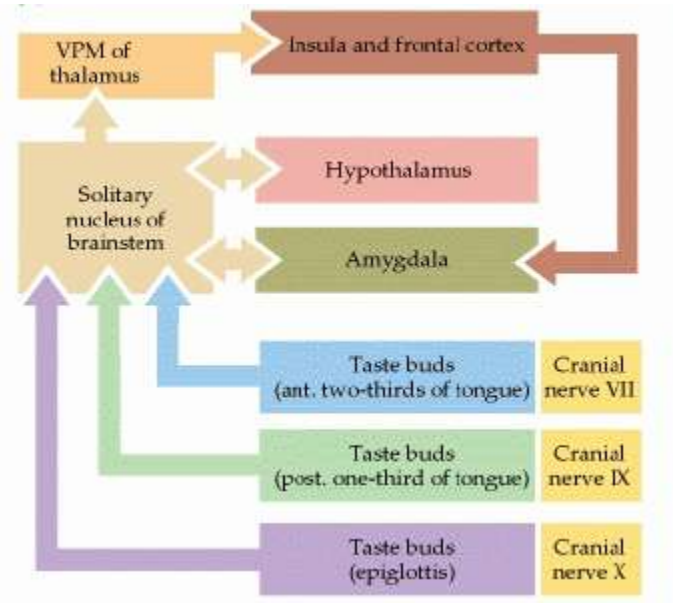

FIGURE 6. *The taste information pathways (Purves et al., 2001).*

1.2 Taste receptors

In past years, the findings on taste receptors have allowed us to determine the genetic individual predisposition in taste perception, which is what allows us to appreciate some foods more than others. Different tastes are perceived on the tongue, specifically on taste buds contained in taste papillae, through receptors located on the surface of TRCs.

We report in the following table (Table 1) all taste receptors with their signal transduction mechanism (Robino et al., 2014).

TASTE	RECEPTOR(S)	SIGNAL TRANSDUCTION
Bitter	T2Rs	G - protein - coupled receptors activation
Sweet	T1R2/T1R3	G - protein - coupled receptors activation
Umami	T1R1/T1R3	G - protein - coupled receptors activation
Salt	ENaC - TRPV1	Ion – channels
Sour	PKD2L1 - PKD1L3	Ion – channels
Fat	CD36	Fatty acid transporter

TABLE 1. *Taste receptors and corresponding signal transduction mechanism (Robino et al., 2014)*.

All genes currently known as taste receptors or involved in the mechanism of signal transduction are listed in detail below:

- TAS2R or T2R genes encode for 25 bitter taste receptors located on chromosomes 12, 7 and 5 ((Behrens et al., 2007), (Adler et al., 2000), (J Chandrashekar et al., 2000));
- TAS1R2 or T1R2 and TAS1R3 or T1R3 genes encode for two subunits of the heterodimeric receptor involved in the perception of sweet taste ((G Nelson et al., 2001), (X. Li et al., 2002));
- TRPV1 and SCNN1A, SCNN1B, SCNN1G and SCNN1D genes, which are four subunits of the sodium-specific amiloride-sensitive epithelial sodium channel (EnaC), are involved in the perception of salty taste (Dias et al., 2013);
- PKD2L1 and PKD2L3 genes encode ion channel proteins and form a sour taste receptor ((Chen et al., 2011), (Huang et al., 2006), (Ishimaru et al., 2006));
- TAS1R1 and TAS1R3 genes, which form heterodimeric G protein–coupled receptor ((Nelson et al., 2002); (Zhao et al., 2003)), and MGLUR1 and MGLUR4 genes, which are metabotropic glutamate receptors, function as an umami taste receptors ((Shigemura et al., 2009), (Chaudhari et al., 2009); (Yasumatsu et al., 2012));

- CD36 gene, a glycoprotein which belongs to the scavenger receptors family, and GPR120 and GPR40 genes, 2 LCFA-specific receptors expressed on taste cells, may play a role in oral fat detection and preference of fat taste and may act as an oral fat sensor ((Keller, 2012), (Galindo et al., 2012), (Fukuwatari et al., 1997), (Laugerette, 2005));
- TRPM5, GNAT3, IP3, PLCB2, PDE1A, PDE1B, PDE4B, GLUT2, AC= ASAH1, DRD2 are several genes that are involved in taste signal transduction: in particular, salt and sour tastes directly act on membrane ion channels; instead sweet, umami and bitter tastes use transduction mechanisms mediated by G protein - coupled taste receptors ((Clapp et al., 2008b), (Kim et al., 2006), (Ruiz-Avila et al., 1995a), (Rossier et al., 2004), (Bachmanov et al., 2011), (Schiffman et al., 1994), (Zhang et al., 2003), (Purves et al., 2001)).

1.3 Relationship between genetic variation in taste receptors and variation in taste perception

Genetic variations in taste receptor genes induce variations in taste perception: these genetic variations are well known especially in relation to bitter, sweet and umami tastes ((Kim and Drayna, 2005), (Mainland and Matsunami, 2009), (Shigemura et al., 2009)).

In particular, the majority of studies focused on variation of *bitter taste*, that is the variation of TAS2R38 gene: this gene determines the perception of bitter compounds containing thiocyanate group (N-C=S) such as phenylthiocarbamide (PTC), 6-n-propylthiouracil (PROP) or glucosinolates and goitrine, substances found in cruciferous vegetables and in other plants of the Brassicaceae family such as broccoli, cabbage and cauliflower (Bufe et al., 2005). In the general population the subjects can be classified as "non-taster", if they do not perceive these compounds; "medium-taster", if they perceive them and "super-taster", if they perceive them with extreme sensitivity (Guo and Reed, 2001). The status of “taster” and “non-taster” are characterized respectively by the PAV (proline-alanine-valine) and AVI (alanine-valine-isoleucine) form, that differ in three different SNPs (single nucleotide polymorphisms).

Seventy-five percent (75%) of the Caucasian population is taster, instead the remaining 25% is non-taster (Bartoshuk et al., 1994). However, the genetic variations in the TAS2R38 gene explain only 55-80% of the variability in the perception of PTC and PROP compounds, because there are other genetic and environmental factors (Kim et al., 2003).

A study of Fushan (Fushan et al., 2009) identified that variations in promotor region of TAS1R3 gene are associated with a decrease of *sweet taste* perception.

A minor perception of *umami taste* is induced by genetic differences of TAS1R3 gene; conversely a major perception is induced by genetic differences of TAS1R1 gene (Shigemura et al., 2009).

Salt taste different perception is caused by variations in genes encoding for TRPV1 channel and for beta-subunits of ENaC channel (Dias et al., 2013).

Finally, in relation to *fat taste*, genetic variations of CD36 gene produce variations in perception of fat molecules of foods (Keller, 2012).

1.4 Relationship among health, nutrition and taste perception

The health status of a person, according to the preamble of the Constitution of the World Health Organization (WHO), is positively defined as "a state of complete physical, mental and social well-being and not merely the absence of disease or infirmity" and "one of the fundamental rights of every human being without distinction of race, religion, political belief, economic or social condition" (Constitution of the World Health Organization, 1948).

The health-promotion is carried out through strategies of disease-prevention to reduce morbidity and mortality and to improve quality of life (Slawson et al., 2013).

The concept of prevention is articulated in primary prevention, actions that prevent the disease risk factors; secondary prevention, interventions that focus on early detection and prompt intervention of health issues or diseases and tertiary prevention, approaches that prevent disease complications.

In this context, nutrition assumes a central role in primary, secondary and tertiary level.

In fact, the dietary consumption may affect the development of many complex diseases.

There are several complex diseases, in which food choices have a significant impact.

There is a link between excess body weight, and therefore *overweight/obesity*, and *type 2 diabetes*; *cancer of the breast*, *endometrium*, *ovaries*, *colon*, *rectum* and *kidney*; *hypertension*; *coronary artery disease*; *stroke*; *asthma*; *gallbladder disease*; *osteoarthritis* (Guh et al., 2009).

Risk factors for *cardiovascular and cerebrovascular disease*, that are dyslipidemia, glucose intolerance, hypertension and obesity, can be reduced by dietary intervention (Roger et al., 2012).

Hypertension, low high-density lipoprotein, cholesterol and high triglyceride levels are predictive of *type 2 diabetes,* thus to improve dietary patterns can be very useful for preventing or delaying the onset of this disease ((Wilson et al., 2007) (Gillies et al., 2007)).

About common *cancers* such as colon, breast, uterine, esophageal and renal cancers, excessive adiposity, poor dietary patterns and physical inactivity are considered risk factors; therefore dietary interventions are important for primary prevention of these pathologies ((Prentice et al., 2006), (Prentice et al., 2007)).

Among these complex diseases, obesity has a social and health impact so important to contend the world record for cause of death with malnutrition and infectious diseases ("Obesity: preventing and managing the global epidemic. Report of a WHO consultation.," 2000): to define obesity, it is possible to resort to both radiological and laboratory techniques able to determine body composition, such as Dual Energy X-ray Absorptiometry (DEXRA) and bioelectrical impedance, or anthropometric

measurements including body mass index (BMI), calculated by dividing a person's weight (kg) by his height (cm^2).

In Table 2 the values of BMI and corresponding phenotypes according to the definitions developed by the WHO are listed ("Obesity: preventing and managing the global epidemic. Report of a WHO consultation.," 2000).

BMI	**Classification**
< 18.50	Underweight
18.50–24.99	Normal weight
25.00–29.99	Overweight (Pre-obese)
30.00–34.99	Obese class I
35.00–39.99	Obese class II
≥ 40.00	Obese class III

TABLE 2. *Classification of adults according to BMI (WHO, 2000).*

In summary, food choices and dietary habits may influence the health status and the risk of complex diseases.

But how much we eat and what we eat depends on different factors including the ability to perceive the various tastes.

Some studies established a relationship between genetic variation, taste perception and health status: genetic variations, as far as taste receptors are concerned, may in fact influence the degree of acceptance of a substance and participate in defining the dietary preferences and, consequently, energy and nutrient intake. Finally, the synergistic action of food intake and metabolism is expressed in outlining the health status of each person, in particular with regard to the risk of developing some complex diet-related diseases such as diabetes and obesity (Garcia-Bailo et al., 2009a) (Figure 7).

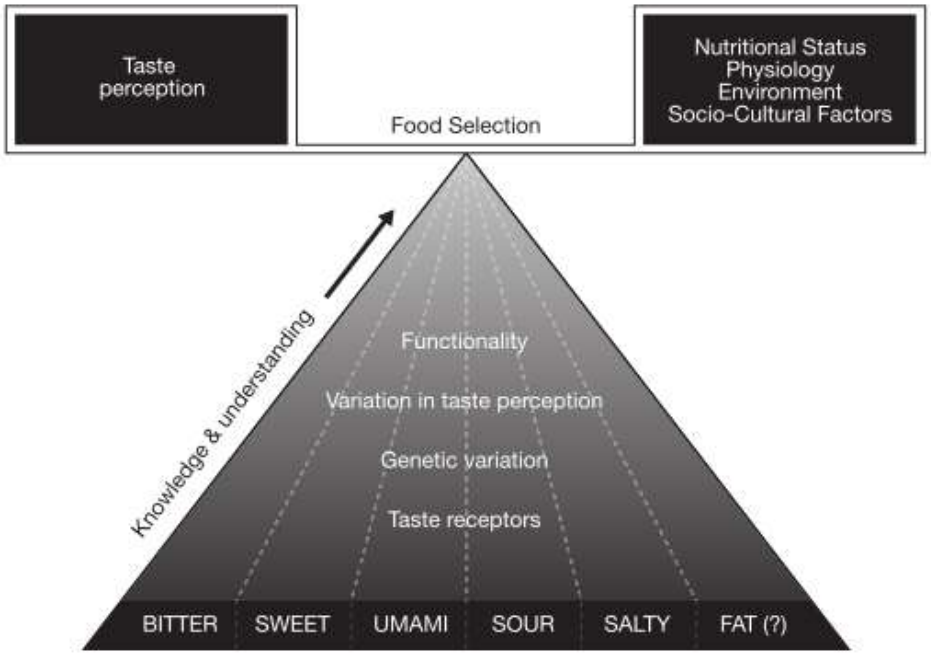

FIGURE 7. *How genetic variations influence the health status of a person, determining different perception of foods, food preferences and, therefore, intake of food (Garcia-Bailo et al. 2009).*

However, food selection depends not only on genetic factors, but also on other factors like nutritional status, physiology, environment and sociocultural factors (Figure 8).

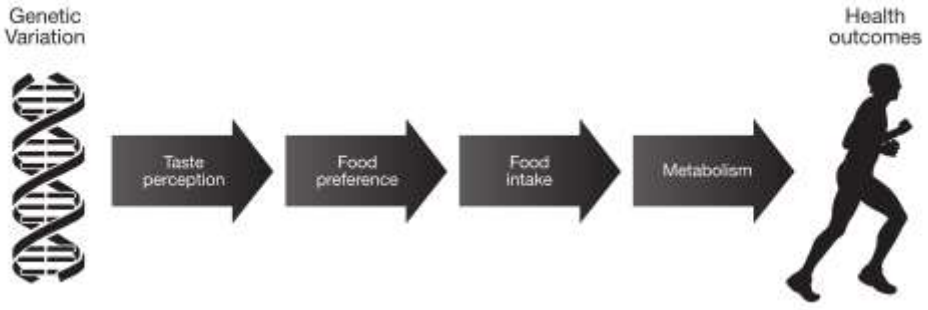

FIGURE 8. *Food selection is the outcome of the balance between genetic variation of taste receptors and other factors such as nutritional status, physiology, environment and sociocultural factors (Garcia-Bailo et al. 2009).*

In conclusion, since it is known that taste receptor genes and those involved in signal transduction influence food choices and therefore the BMI, association studies involving the polymorphisms of these genes could identify a relationship between food intake behavior and risk of complex diseases such as obesity.

1.5 Complex disease and association study

The definition of complex or multifactorial disease is due to the fact that, unlike the Mendelian or single-factor disease, it is characterized by a complex interaction between a certain number of genetic factors, each with a small role in susceptibility to disease, and environmental factors that are predisposing or protective with regard to the onset of the disease; it has, moreover, a small impact on the phenotype, because each gene has a low penetrance, and it is not easily recognizable by a pedigree, so its study is mainly based on statistical models.

The method most commonly used is the association study, that aims to identify, in a population, a correlation between the alleles of one or more genetic markers, and the phenotype. Of particular importance within these studies is to choose a high number of markers located pervasively in the genome: the most commonly used are the Single Nucleotide Polymorphisms (SNPs), variations of the DNA sequence relative to a single nucleotide and present in the general population with a frequency of at least 1% (Figure 9).

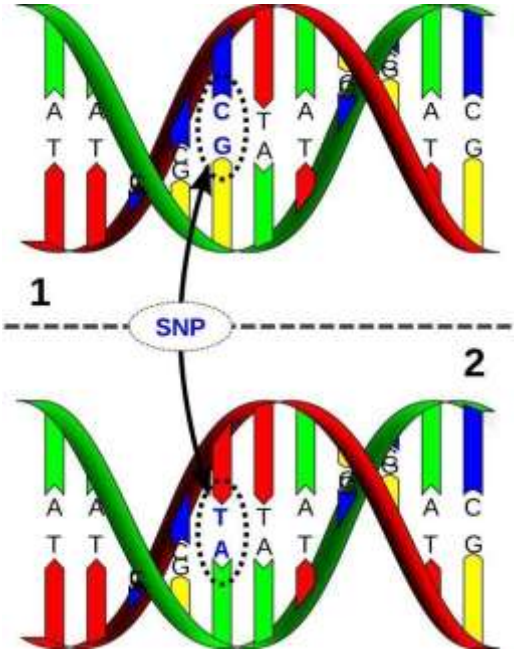

FIGURE 9. *Representation of a SNP in two subjects: in the subject "1" there is the nucleotide C in a particular position of genome; in the subject "2", in the same genomic position, there is the nucleotide T.*

To date, almost all the SNPs that have been identified, have two allelic variants and are very common in our genome. The SNPs, however, are distributed in a non-random mode (the frequency of polymorphic markers is higher in non-coding than in coding regions). About 80% of SNPs associated with phenotypic traits are located in non-coding regions of the genome. In fact "only 12% of SNPs associated with traits are located in, or occur in a tight linkage disequilibrium with, protein-coding regions of genes" and "approximately 40% of trait-associated SNPs fall in intergenic regions, and another 40% are located in non-coding introns" (Manolio, 2010). (Linkage disequilibrium is defined as "the non-random association of alleles at tightly linked markers" (Cardon and Abecasis, 2003).) For all these reasons, genotyping arrays mainly consist of markers of non coding regions.

In the last 10 years, the Genome-Wide Association Study (GWAS) has become extensively used with the aim to identify variants responsible for the traits studied through the use of hundreds of thousands of molecular markers disposed at short intervals over the entire genome. In most cases all these studies identified a genetic variant in linkage disequilibrium with the functional variant.

In addition the data of the "1000 Genomes Project" (an international study started in 2008, that has described to date more than 37 million SNPs, 1,000,000 insertions and deletions and 20,000 structural variants (http://www.1000genomes.org)) can be used as a reference point to infer, with specific software, the variants that have not been genotyped on their samples: this allows us to directly test all variants on the genome.

In the study of complex traits it is also very useful to use "genetic isolates", i.e. populations founded from a small number of individuals ("founder effect"), or shaped by drastic environmental phenomena such as famines and wars ("population bottleneck"): the geographical and cultural isolation greatly restricts migration flows and a "genetic reserve" is created over generations. In recent years, many of the genes responsible for rare recessive Mendelian diseases have been identified through studies that used genetic isolates, such as the Finnish populations and Amish groups. In fact, the high degree of endogamy (inbreeding) produces a high frequency of recessive diseases and affected families show a homogenous phenotype due to the presence of the same environment that can be found only in that particular type of population (Peltonen et al., 2000). Moreover, the fact that individuals belonging to these populations share the same lifestyle, eating habits and natural environment, makes it easier to identify the environmental factors predisposing the onset of common diseases.

Therefore, assuming that specific SNPs located on taste genes can affect taste perception and BMI, we hope that the observations and the results of this work will give a contribution to the heterogeneous puzzle of obesity, thus the start of personalized and innovative nutritional treatments.

1.6 Aim and organization of thesis

The general aim of this thesis is to investigate how taste perception affects BMI and predisposes to complex diseases such as obesity (Figure 10).

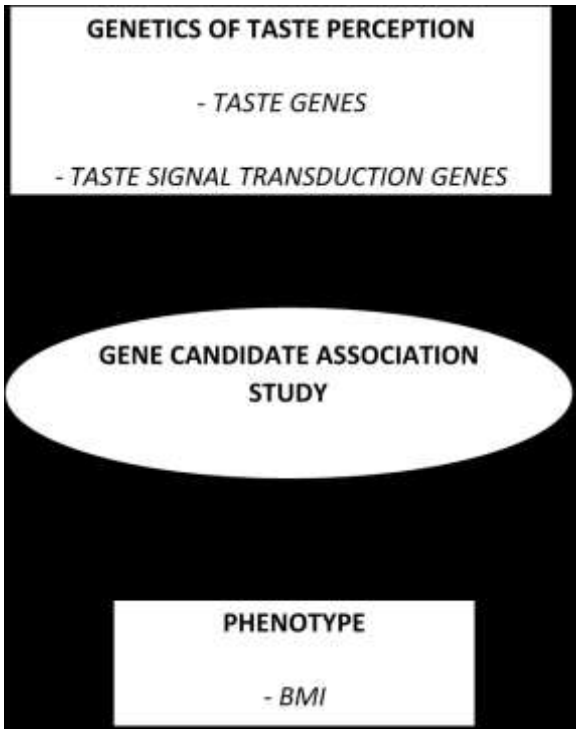

FIGURE 10. *Outline of aim of the thesis.*

We carried out a "candidate gene association study" and meta-analysis of data coming from 3 different Italian populations performing the following tasks:

1)- selection of genes directly involved in taste perception, such as the genes encoding the receptors for bitter, fat, salty, sour, sweet and umami taste, and other genes indirectly involved in taste perception because involved in the cascade of signal transduction;
2)- definition of the phenotypic trait to be analyzed, "phenotype analysis" and definition of covariates;
3)- "genotyping and imputation" of extracted DNA samples;
4)- "genotype analysis";
5)- "candidate gene association study" for each cohort;
6)- "meta-analysis" of results of association study of three cohorts.

CHAPTER 2

MATERIAL AND METHODS

2. MATERIAL AND METHODS

2.1 Participants

This research includes 5100 participants from three different projects within the Italian Network on Genetic Isolate (INGI) consortium:

- "Project Genetic Park Friuli-Venezia-Giulia" (INGI-FVG), which involved a total of 1600 participants from six villages in the North-East of Italy (San Martino del Carso, Erto & Casso, Illegio, Sauris, Resia, Clauzetto), all located in the Friuli-Venezia-Giulia region;
- "Project Carlantino" (INGI-CARL), which involved a total of 1500 participants from a small village in the South of Italy (Foggia) named Carlantino;
- "Project Val Borbera" (INGI-VAL), which involved a total of 2000 participants in the Northwest of Italy from eight villages of which Albera, Cabella, Cantalupo, Carrega, Rocchetta, Mongiardino, Roccaforte belong to "Alta Val Borbera" and Grondona belongs to "Valle Spinti".

These subjects belong to "genetic isolates", populations founded from a small number of individuals, isolated for a long time and with a high degree of endogamy, and therefore useful in identifying the environmental factors predisposing the onset of recessive diseases produced by shared lifestyle, eating habits and natural environment.

Each participant compiled a questionnaire on socio-demographic information, as well as data on clinical parameters, professional activity, lifestyle, eating habits and family history.

The Protocols of "Project Genetic Park Friuli-Venezia-Giulia" and "Project Carlantino" were approved by the Ethics Committee of IRCCS - Burlo Garofolo (Trieste) and the protocol of "Project Val Borbera" was approved by the Ethics Committee of Scientific Institute San Raffaele (Milano).

2.2 Gene selection

We selected the receptor genes for bitter, fat, salty, sour, sweet and umami taste, and the genes involved in the cascade of taste signal transduction through consultation of bibliographic database PubMed.

2.3 Phenotype analysis and definition of covariates

For each of the three populations that we considered, we chose to analyze, as a phenotype, the BMI as a continuous trait to maximize the power of the study, because by grouping subjects into the categories of normal weight, obese and overweight, would inevitably decrease the power of the study. Then, for each of the three populations that we studied, we performed the "analysis of the phenotype".

We performed the Shapiro-Wilk normality test: the BMI distribution was not normal in all populations (Figure 11, 12 and 13; Table 3).

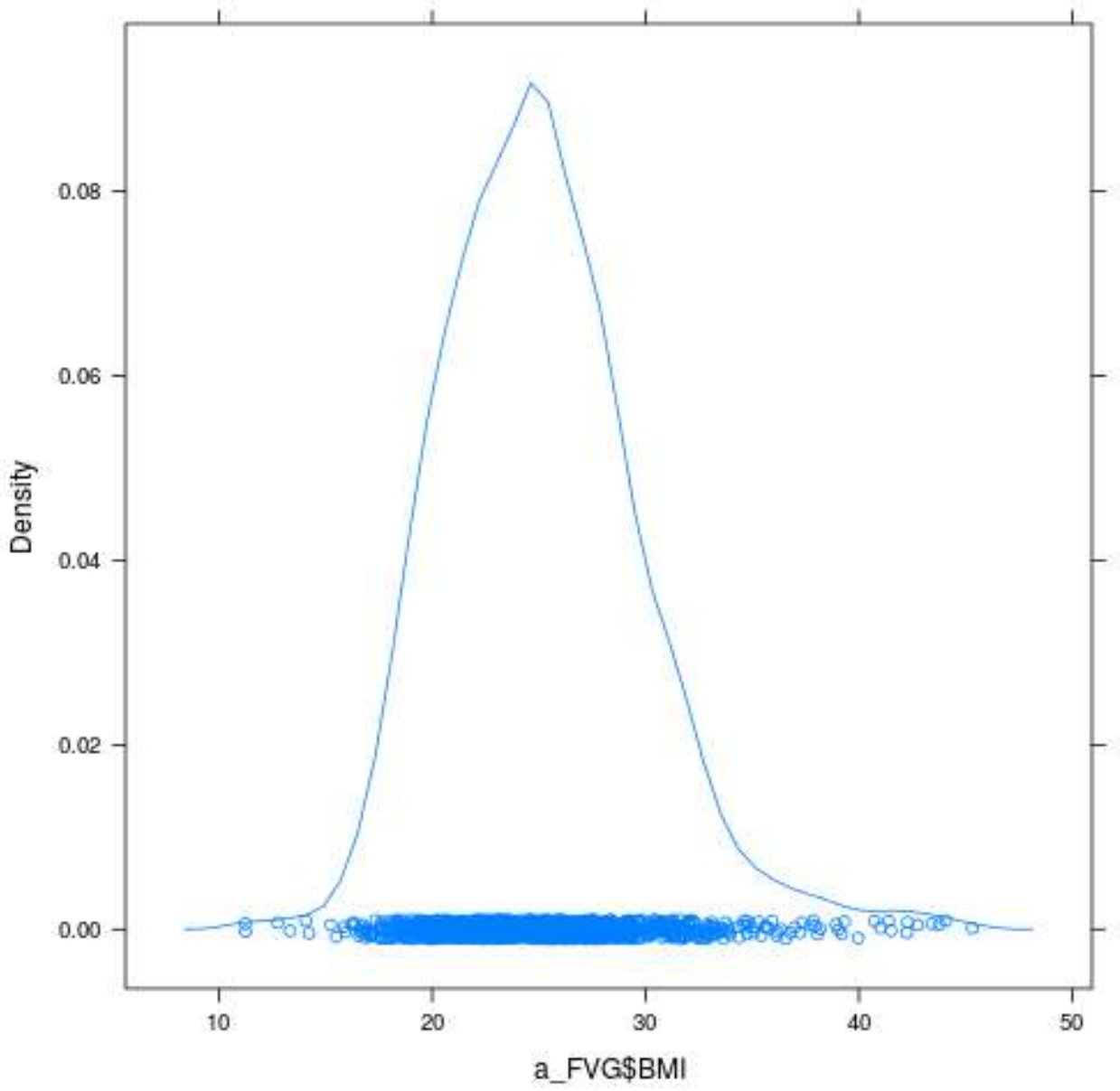

FIGURE 11. *Density plot of BMI raw data in FVG.*

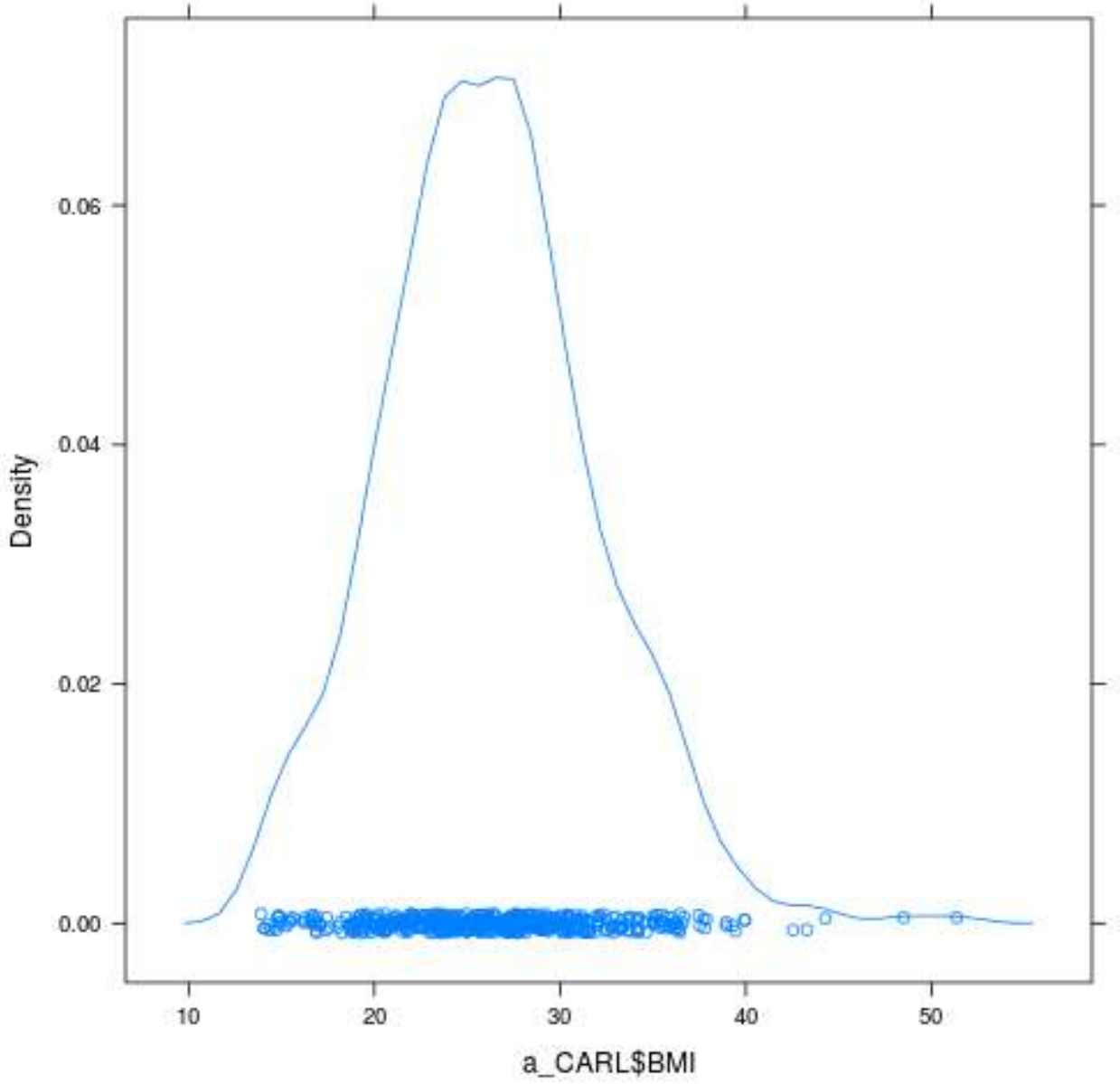

FIGURE 12. *Density plot of BMI raw data in CARL.*

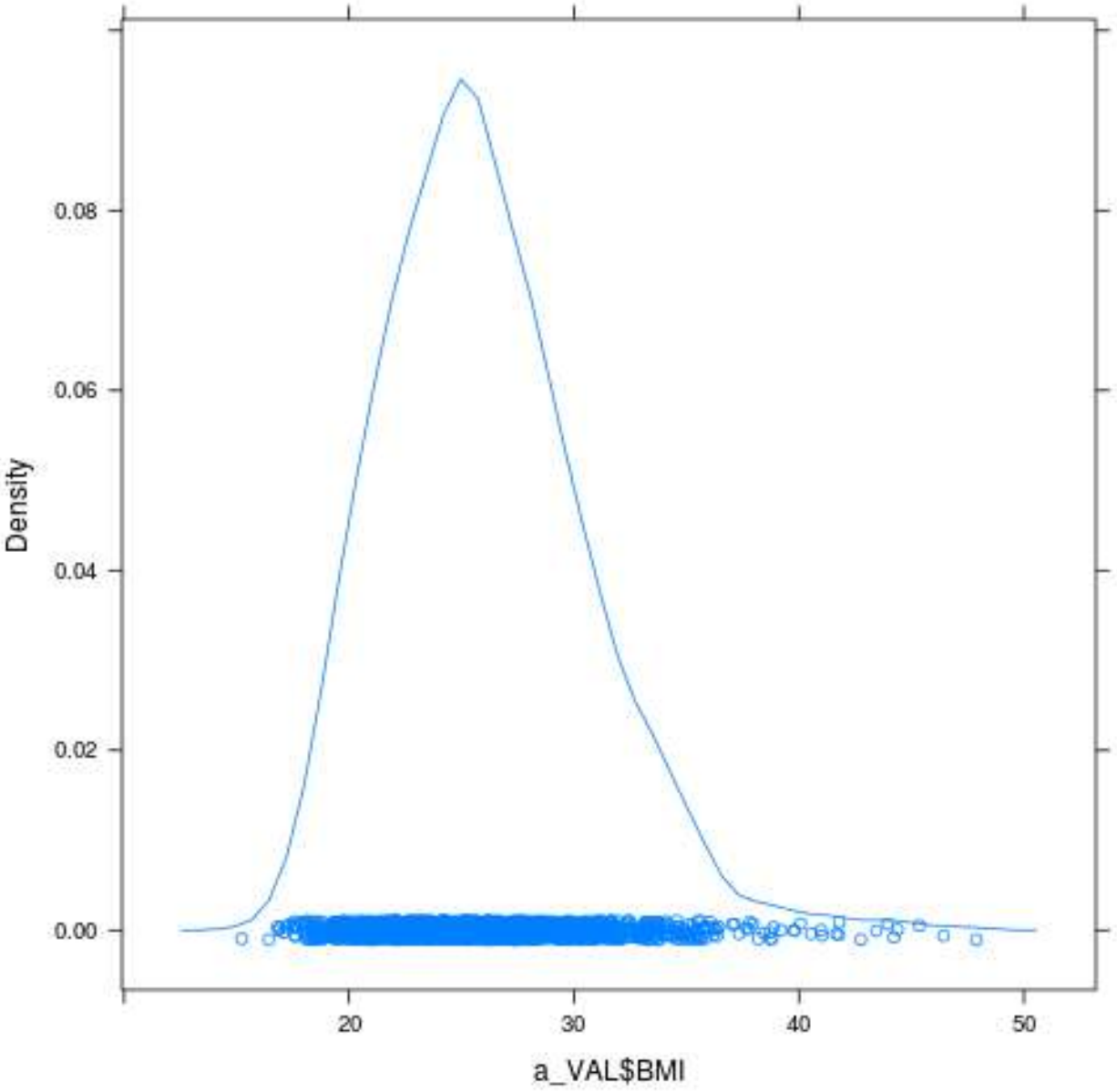

FIGURE 13. *Density plot of BMI raw data in VAL.*

	FVG	CARL	VAL
p-value	1.227e-14	7.017e-05	< 2.2e-16

TABLE 3. *Shapiro-Wilk normality test of BMI raw data of three populations.*

Then we adopted strategies to normalize the trait of BMI: we eliminated from the study subjects aged over 70 years and under 18 years and those with extreme values of BMI in both positive and negative sense. We also performed a log-transform of BMI adopting a method frequently used in association studies by big consortia such as GIANT (Genetic Investigation of Anthropometric Traits) (Simonson et al., 2014), avoiding the use of stronger methods of normalization such as the normal reverse (León-Mimila et al., 2013), which could have distorted our data too much.

In the following figures we report density plot of logBMI data after normalization and correction for covariates of age and gender in three populations (Figure 14, 15 and 16).

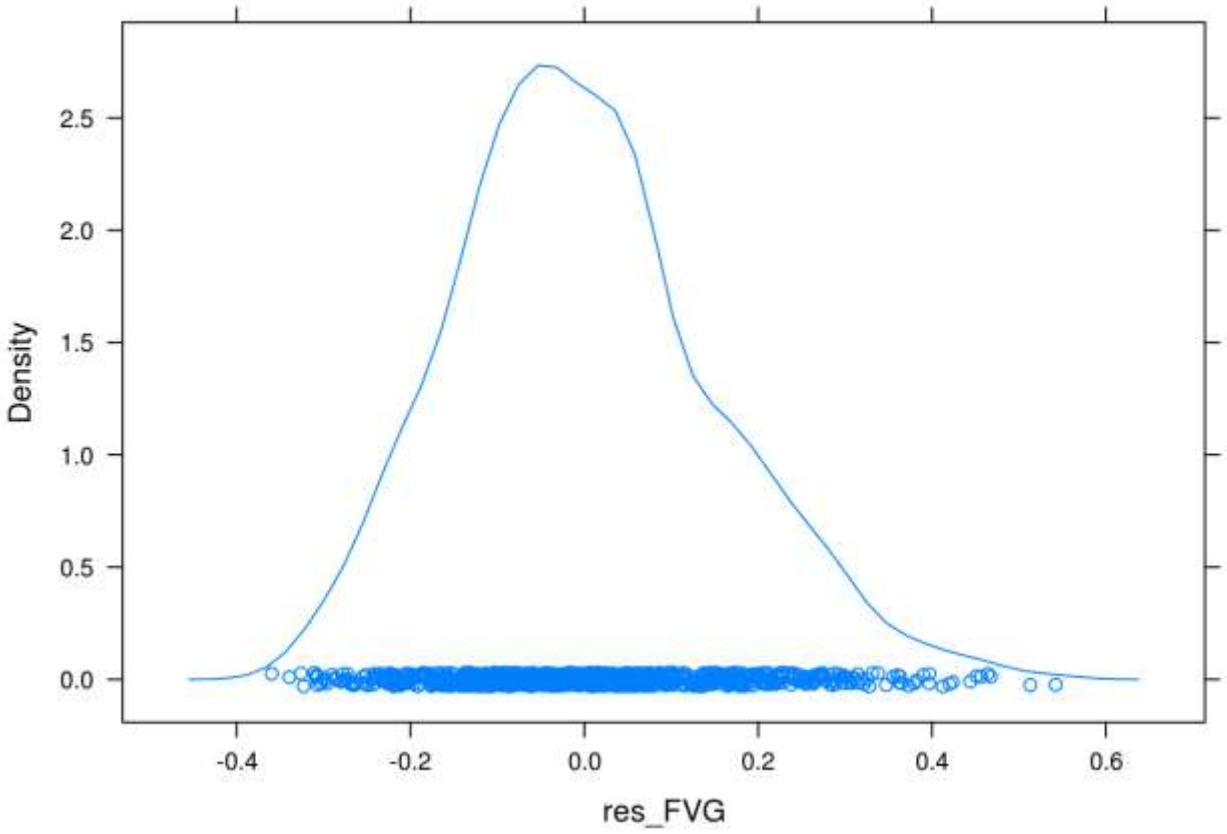

FIGURE 14. *Density plot of logBMI data after normalization and correction for covariates of age and gender in FVG.*

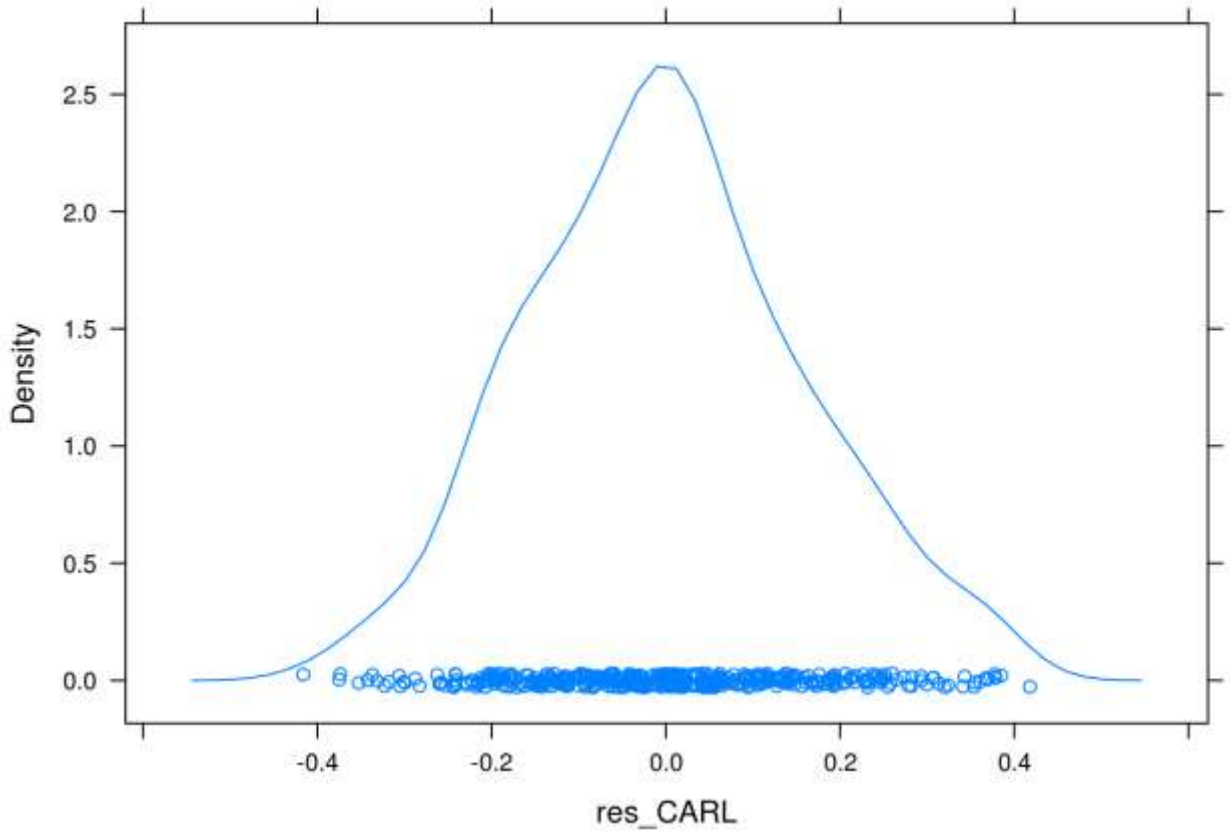

FIGURE 15. *Density plot of logBMI data after normalization and correction for covariates of age and gender in CARL.*

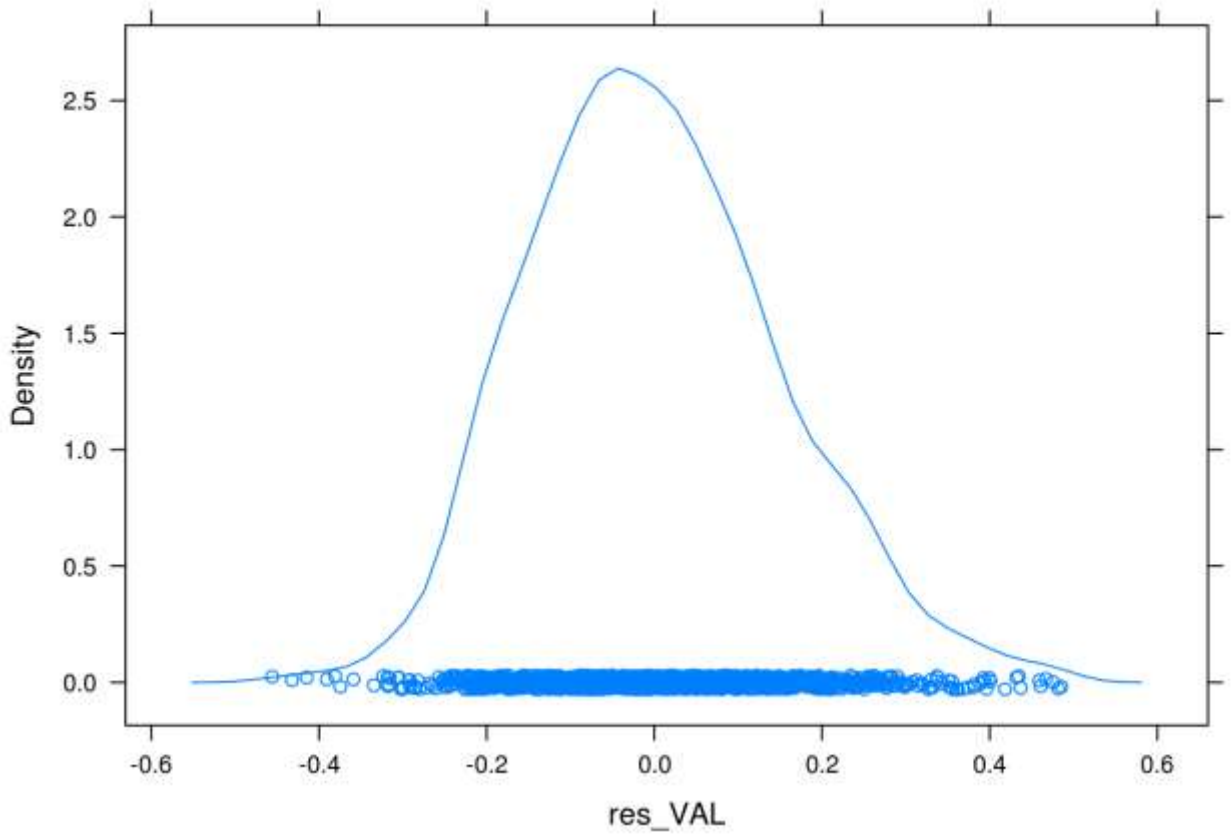

FIGURE 16. *Density plot of logBMI data after normalization and correction for covariates of age and gender in VAL.*

We chose to use gender and age as covariates, in order to correct possible phenotypic differences by gender and by age: they are covariates known and always used in literature.

2.4 Genotyping and imputation of extracted DNA samples

Genomic DNAs of subjects were extracted from blood samples.

Then, extracted DNAs were genotyped using Illumina 370k high-throughput SNP arrays, for a total of about 370.000 SNPs (mainly tagSNPs) per samples.

The tag-SNPs are a small number of characteristic SNPs "chosen as proxies for nearby SNPs, utilizing the local correlation structure of SNPs (or linkage disequilibrium) to find associations: many current association studies are performed using commercially available high-throughput genotyping products that define a set of tag SNPs", in order to maximize statistical power and to reduce greatly genotyping effort (Han et al., 2008).

In summary, the tag SNPs are used for the following reasons: they are highly polymorphic, as they

are not subject to selection; they are found throughout the genome, but especially in the non-coding regions and, if located in the latter, they also provide information on the coding regions because of haploblocks of haplotypes.

With the Human Genome Project (HPG), in fact, it was discovered that the human genome is characterized by block structures, named "haplotype blocks" or "haploblocks", structured in a smaller number of haplotypes (defined as "a sequence of alleles from the same chromosome" (Li et al., 2003) and consisting of tightly linked loci with high LD (linkage disequilibrium) and low recombination rate ("recombination coldspots")): all pairs of polymorphisms within a haploblock are hypothesized to be in strong linkage disequilibrium and haploblocks are hypothesized to be flanked by "recombination hotspots" ((Ge et al., 2010), (Cardon and Abecasis, 2003)).

In all three populations genotypes were then imputed using SHAPET2 software for the phasing step and IMPUTE software for the imputation by the reference panel of 1000 Genomes phase I v3 for a total of about 8 million SNPs (Howie et al., 2012).

2.5 Genotype analysis

Quality control was conducted independently in each population.

All SNPs with minor allele frequency (MAF)<0.05, Hardy-Weinberg equilibrium (HWE) deviation p-value<0.001 and missing genotype rate>0.05 were removed respectively by "maf", "hardy" and "geno" functions. All duplicate samples, all samples with reversed gender and with missing genotype rate>0.01 were removed respectively by "genome", "check-sex" and "mind" options.

Genotype analysis was performed using software *plink* version 1.9.

2.6 Gene candidate association analysis and meta-analysis

The association between SNPs of all selected genes and log10 of BMI was tested by fitting a linear model, where the log10 of BMI was considered as the dependent variable while SNPs as the regressors. Gender and age were used as covariates.

Association analysis was conducted using the GenABEL package for genotyped SNPs and PropABEL package for imputed SNPs.

The kinship matrix based on all available genotyped SNPs was used as the random effect in order to correct for relatedness population stratification.

In our study, the genomic kinship was calculated using data of whole genome genotyping with the "ibs" function in the GenABEL R package by using shared genotype counts as a measure of genetic distance between individuals.

For the association analysis an additive genetic model was assumed, because the polymorphisms that have an impact on quantitative and complex traits usually follow an additive model.

Association analysis was conducted separately for each cohort and, then, results were pooled together through meta-analysis. Meta-analysis was conducted using the inverse variance weighting method by GenABEL R package.

2.7 Data analysis

We performed all the statistical analyses using the software R version 3.0.2 (http://cran.r-project.org/).

CHAPTER 3

RESULTS

3. RESULTS

3.1 Characteristics of the populations

After phenotype and genotype analysis, 2827 subjects were analyzed overall.

We analyzed 1030 subjects belonging to the project INGI-FVG, 433 males (42%) and 597 (58%) females: BMI mean is 25.04; age mean is 47.62.

We analyzed 427 subjects belonging to the project INGI-CARL, 172 males (40%) and 255 (60%) females: BMI mean is 26.74.; age mean is 46.98.

We analyzed 1370 subjects belonging to the project INGI-VAL, 606 males (44%) and 764 (56%) females: BMI mean is 25.67; age mean is 48.34.

Table 4 shows descriptive characteristics of each population.

	FRIULI-VENEZIA-GIULIA (FVG)	**CARLANTINO (CARL)**	**VAL BORBERA (VAL)**
N	1030	427	1370
AGE (years)	47.62±13.40	46.98±14.94	48.34±14.41
GENDER			
M	42% (433)	40% (172)	44% (606)
F	58% (597)	60% (255)	56% (764)
BMI (kg/cm^2)	25.04±4.24	26.74±4.79	25.67±4.27

TABLE 4. *Description of the samples that were analyzed in the three populations: absolute numerousness of subjects, mean ± standard deviation of age and BMI, relative numerousness and absolute numerousness of gender.*

3.2 Gene selection

Through an extensive study of the literature, we selected the following genes involved in taste reception or in taste signal transduction cascade (Table 5):

GENE	FUNCTION
TAS2R1 (TRB7)	Receptor bitter taste
TAS1R2 (ht1r2, GPR71)	Receptor sweet taste
TAS1R3	Receptor sweet taste
TAS2R3	Receptor bitter/sweet taste
TAS2R4	Receptor bitter taste
TAS2R5	Receptor bitter taste
TAS2R7 (TRB4)	Receptor bitter taste
TAS2R8 (TRB5)	Receptor bitter taste
TAS2R9 (TRB6)	Receptor bitter taste
TAS2R10 (TRB2)	Receptor bitter taste
TAS2R13 (TRB3)	Receptor bitter taste
TAS2R14 (TRB1)	Receptor bitter taste
TAS2R16	Receptor bitter taste
TAS2R19 (TAS2R23, TA2R48)	Receptor bitter taste
TAS2R20 (TAS2R49, TAS2R56)	Receptor bitter taste

TAS2R38 (TAS2R61, PTC)	Receptor bitter taste
TAS2R39 (TAS2R57)	Receptor bitter taste
TAS2R40 (TAS2R58, GPR60)	Receptor bitter taste
TAS2R41 (TAS2R59)	Receptor bitter taste
TAS2R42 (TAS2R24, TAS2R55)	Receptor bitter taste
TAS2R43 (TAS2R52)	Receptor bitter taste
TAS2R44 (TAS2R31, TAS2R53)	Receptor bitter taste
TAS2R45 (ZG24P, GPR59)	Receptor bitter taste
TAS2R46 (TAS2R54)	Receptor bitter taste
TAS2R47 (TAS2R30)	Receptor bitter taste
TAS2R50 (TAS2R51)	Receptor bitter taste
TAS2R60	Receptor bitter taste
TRPV1 (VR1)	Receptor salt taste
SCNN1A	Receptor salt taste
SCNN1B	Receptor salt taste
SCNN1D	Receptor salt taste
SCNN1G	Receptor salt taste
PKD1L3	Receptor sour taste

PKD2L1	Receptor sour taste
TAS1R1 (GPR70)	Receptor umami taste
TAS1R3	Receptor umami taste
MGLUR1 (GRM1)	Receptor umami taste
MGLUR4 (GRM4)	Receptor umami taste
CD36	Receptor fat taste
GPR40 (FFAR1)	Receptor fat taste
GPR120 (FFAR4)	Receptor fat taste
GNAT3	Gene encoding a taste-selective G protein involved in the transduction of sweet, bitter and umami tastes
TRPM5	Gene encoding a $Ca(^{2+})$-activated non selective cation channel involved in the transduction of sweet, bitter and umami tastes
PLCB2	Phospholipase C beta 2 involved in the transduction of sweet, bitter and umami tastes
PDE1A	Phosphodiesterase 1A calmodulin-dependent involved in signal transduction
PDE1B	Phosphodiesterase 1B calmodulin-dependent involved in signal transduction
PDE4B	Phosphodiesterase 4B cAMP-specific involved in signal transduction

GLUT2	Facilitated glucose transporter member 2 involved in the transduction of sweet taste
AC= ASAH1	N-acylsphingosine amidohydrolase involved in the transduction of sweet taste
DRD2	Dopamine receptor D2 involved in sweet taste preference

TABLE 5. *List of selected candidate genes.*

3.3 Gene candidate association study

Meta-analysis identified some SNPs associated with the BMI by a direct or inverse correlation based respectively on the positive or negative Beta coefficient.

Table 6 shows the most significant results obtained using additive genetic model (p-value<0.01).

Gene	SNP	Chr	Position	A0	A1	No	Beta	p-value
SCNN1B	rs118132852	16	23372413	C	T	1457	-1.12	0.0021
SCNN1B	rs75061058	16	23376439	C	A	1457	-1.01	0.0034
GNAT3	rs10226853	7	80140402	A	C	2827	-0.31	0.0056
GNAT3	rs10230573	7	80141069	A	G	2827	-0.31	0.0055
GNAT3	rs10954212	7	80140217	A	G	2827	-0.32	0.0041
GNAT3	rs113783893	7	80104435	A	G	2827	0.58	0.0043
GNAT3	rs2030709	7	80140874	C	T	2827	-0.31	0.0049
GNAT3	rs7801018	7	80141418	A	G	2827	-0.33	0.0027
GNAT3	rs7805711	7	80142245	A	G	2827	-0.34	0.0023
GNAT3	rs6962693	7	80134032	T	G	2827	-0.44	0.0002
PDE4B	rs141755790	1	66352943	T	C	2827	0.39	0.0052
PDE4B	rs502958	1	66731182	A	T	2827	-0.30	0.0068
PDE4B	rs6700403	1	66735601	T	C	2827	-0.29	0.0089
PDE4B	rs6701329	1	66732529	A	T	2827	-0.29	0.0094

TABLE 6. *List of SNPs with p-value<0.01 associated to phenotypic trait of log of BMI. "Chr" indicates the chromosome. "A0" and "A1" indicate both alleles. "No" indicates the total number of individuals analyzed. "Beta" indicates the beta correlation coefficient between the independent variable - SNP and the dependent variable – log10 of BMI. "p-value" indicates the p-value.*

CHAPTER 4

DISCUSSION AND CONCLUSION

4. DISCUSSION AND CONCLUSION

In genetic association studies, which aim to identify the causal genes of human complex diseases, the sample size and the statistical power have a fundamental relevance.

In fact the "genome-wide association studies require a much larger sample size to achieve an adequate statistical power " (Hong and Park, 2012): there are numerous association studies conducted by large consortia on a big number of subjects.

So, we analyzed the samples of the largest Italian cohorts available, to date, with the information required for our study.

These samples under study come from genetic isolates of Friuli-Venezia-Giulia, Piemonte and Puglia. Generally, as previously described in the introduction, the use of genetic isolates provides considerable advantages for association studies. In addition, the genetic isolates of this work have very different characteristics from one another and this is an important element, because it can increase the statistical power to find genetic variants associated with the phenotype to study.

These samples were genotyped for ~300.000 SNPs, so we had to consider the "multiple-testing problem": "the multitude of comparisons made in a GWAS will result in both false positive (Type 1 errors) and, if the correction for multiple comparisons is overly conservative or power is inadequate, false negative (Type 2 errors) results" (Johnson et al., 2010).

Therefore, to avoid this problem, we performed, rather than a GWAS, an association study only with the SNPs of genes involved in taste perception.

However, the availability of DNA samples genotyped with many markers gave us the advantage of calculating the molecular kinship matrix of all individuals and performing a very precise association analysis of selected SNPs.

The originality and the relevance of our study is that it is the first candidate gene association study regarding all taste receptor genes and those involved in taste signal cascade transduction.

By this association study conducted on 50 selected genes some statistically significant interesting associations emerged: in particular our study showed that 2 SNPs of SCNN1B gene, 8 SNPs of GNAT3 gene and 4 SNPs of PDE4B gene were associated significantly with BMI with a p-value<0.01.

Precisely, for each SNP associated with BMI, according to the positive or negative Beta coefficient, subjects homozygous for A1 allele, which is the coding allele or reference allele, have or could have in the future a BMI respectively higher or lower than heterozygous or homozygous subjects for A0 allele, which is instead the non-coding allele.

These polymorphisms were not associated with BMI before.

The SCNN1B gene encode for one of four subunits of the sodium-specific amiloride-sensitive epithelial sodium channel (ENaC), which is involved in the perception of salty taste. The study of

Dias et al. (Dias et al., 2013) found that 2 SNPs of this gene, rs239345 and rs3785368, modify suprathreshold salt taste perception, indicating therefore the action of SCNN1B gene as EnaC-β subunit in salty perception. We found that two novel SNPs of this gene, rs118132852 and rs75061058, are associated with the phenotypic trait of BMI. Information about SCNN1B gene is still scarce, but to date, in support of our discovery, it is known that obese subjects prefer salty foods and, then, consume a diet rich of these foods ((Pasquet et al., 2007), (Keskitalo et al., 2008), (Cox et al., 1999)).
We also found four new SNPs of PDE4B gene, rs141755790, rs502958, rs6700403 and rs6701329, associated with BMI. The PDE4B gene is a putative taste involved in taste signal transduction: a human fungiform papillae cDNA library was created and in this library by PCR several genes were identified that are known taste-related and putative taste-related genes including also PDE4B gene (Rossier et al., 2004). Again, it's well known that taste tissue contains high levels of several types of phosphodiesterases (Ruiz-Avila et al., 1995b).
Finally, we found eight novel SNPs of GNAT3 gene that are significantly associated with the analyzed phenotype. GNAT3 gene is located on chromosome 7 and encodes the Gα-protein-specific taste, which is the α subunit protein Gustducin.
The Gustducin is a G-protein expressed in the taste buds of the tongue and it is involved in the signaling of sweet, bitter and umami taste. The binding of tastant to hT1R or hT2R causes the release of Gustducin.
In general, the involvement of Gustducin in the mechanism of signal transduction of sweet, bitter and umami taste by functional studies dates back to several years ago (the first study there was in 1992 (McLaughlin et al., 1992)), even before that there was evidence at the genetic level of involvement of GNAT3 gene.
In literature there are several works about GNAT3 gene and its role in the perception of sweet taste.
A study in 2010 showed that genetic variation in the sweet receptor subunit, TAS1R3, and in the second messenger gustducin encoded by GNAT3 gene affects people's ability to correctly rank ascending concentrations of sucrose, so variations in TAS1R3 and GNAT3 genes could have implications for diabetes and obesity; furthermore GNAT3 is close to another taste gene, CD36, and this association might be originated with linkage disequilibrium (Reed and Margolskee, 2010).
The study by Fushan et al. (Fushan et al., 2010) summarized recent advances in the understanding of the mechanisms of taste transduction in mammals and several signaling molecules identified including gustducin, polypeptides G-protein beta 3 and gamma 13, phospholipase C-β2, inositol triphosphate receptor and, more recently, the M5 channel transient receptor like-potential (TRPM5). Furthermore, this study stated that the gustducin is an essential component of the transduction pathway of the sweet taste in mammals; in fact, biochemical and histochemical studies suggested that the function of this protein is very similar to that of ortholog animals. Specifically, the association

analysis of this study revealed a significant correlation of scores AUC (area under curve) of sucrose with the genetic variation in GNAT3 gene ($p < 0.005$) regarding the following 11 SNPs: rs7792845 (that is in strong linkage disequilibrium with rs2012380); rs940541; rs1107660; rs1107657; rs1524600; rs6467217; rs6970109; rs6975345; rs10242727; rs6467192; rs6961082. In summary, the genetic variation of GNAT3 explains 13% of the variation in the perception of sucrose in worldwide human population.

Even the review by Bachmanov et al. (Bachmanov et al., 2011) on genetic preferences of sweet taste referred to the study by Fushan of 2010.

The report by Hayes et al. (Hayes et al., 2013) focused on the genetic receptors and on current knowledge on ingestive human behavior. The SNPs in coding regions, such as regions of promoter, may also have an effect on receptor function and there is evidence that, about the sweet taste, SNPs in the promoter region of GNAT3 are responsible for some variations in the perception of sweet, although this has not yet been confirmed. The variation in gene of gustducin, GNAT3, may also be associated with the variation in the intensity of sweet sensations. In addition to the variation in the perception of sweet explained by SNPs of TAS1R3, a variation can be explained by an additional 11 SNPs in GNAT3 (Fushan et al., 2010): the SNP rs7792845 is the most significant and, because it is located 10kb upstream of the coding region, presumably changes the promoter of the gene.

Furthermore, there are many functional studies on Gustducin protein.

Spielman showed that, in mice deficient for gustducin, there was the destruction of the perception of bitter and sweet taste, so gustducin is involved in the mechanism of signal transduction of both the bitter and sweet (Spielman, 1998).

In the work of Ruiz-Avila some control experiments were performed, where the expression of wild-type α-gusducin as transgene in mice null for α-gusducin restored responsiveness to bitter and sweet compounds, showing that deletion of α-gusducin gene produces deficit of taste in mice null. In contrast, the transgenic expression of the mutant G352P had not restored responsiveness of mice null to sweet or bitter compounds (Ruiz-Avila et al., 2001).

The work of Drayna referred to the previously mentioned work of Ruiz-Avila of 2001, even assuming a plausible evolutionary relationship of two sweet and bitter taste modes (Drayna, 2005).

In the study by Danilova (Danilova et al., 2006), comparing behavioral and electrophysiological responses to 11 different sweeteners by wild-type and KO α-gustducin mice, the integrated responses of the nerves of the chorda tympani and glossopharyngeal to all sweeteners, except neotame, were smaller in KO mice compared to wild-typet mice. These data indicate that the α-Gustducin participates in general in the transduction of sweet taste.

The work by Stone et al. (Stone et al., 2007) revealed that, in contrast to the lingual taste buds, the taste cells of the palate positive for T1R2 and T1R3 mostly coexpress the gustducin, suggesting that

transduction of sweet taste in the mouth is almost entirely dependent on gustducin.

Miura et al. (Miura et al., 2007), after studying the expression pattern of gustducin and IP3R3 in the taste buds of the soft palate and tongue papillae of rats, showed that the gustducin was expressed in almost all cells expressing IP3R3 (96.7%) in the taste buds of the soft palate, whereas cells positive for gustducin were 42.4% and 60.1% of cells expressing IP3R3 respectively in the fungiform and circumvallate papillae. So, gustducin is involved in signal transduction of sweet, umami and bitter taste in the soft palate, in contrast to its limited role in the tongue.

Clapp et al. (Clapp et al., 2008a) suggested an important role of α-gusducin in keeping cAMP levels tonically low to ensure proper signaling Ca^{2+}, so a key role for the α-gusducin in taste can be to maintain the taste buds in an active state for responding to the taste stimulus with strong signals of Ca^{2+}.

Garcia-Bailo referred to a work by Margolskee (Margolskee, 2002), in which α-gusducin is proposed as a pathway of signal transmission downstream, before the sweet taste receptor is activated by its ligand (Garcia-Bailo et al., 2009b).

In the work by Tomonari et al. (Tomonari et al., 2012) the expression of Gα-gustducin, Tas1rs and Tas2rs in fungiform taste buds and in the soft palate was examined and the impact of knockout Gα-gustducin (GKO) mice on neural responses to several bitter and sweet compounds in both the nerve of the chorda tympani and the greater superficial petrosal nerve was assessed. In summary, it has been shown that in the soft palate the Gα-gustducin plays a critical role in the transduction of both sweet and bitter; fungiform taste buds in its functional significance for the transduction of bitterness was only noticed for the cycloheximide compound among four analyzed bitter tastants.

Results of these studies support the data obtained in our work, suggesting that the functional mechanism by which variations in taste receptors may influence the BMI and, therefore, the risk of becoming overweight and obese is very complex and probably closely related to expression and function of the taste receptors.

Our work led to the identification of interesting associations helping to clarify this complex relationship and emphasizing that taste can be one of many factors influencing the risk of becoming overweight and obese.

Our results confirm that these genes are highly polymorphic and these polymorphisms are related to either protein receptor or protein involved in signaling cascade.

We hypothesize a functional characterization of gene products related to polymorphism in the attempt to correlate BMI categories (namely overweight/obese) with proper polymorphism and with a special emphasis to diet-related diseases (obesity, diabetes type II, etc.).

CHAPTER 5

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CHAPTER 6

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6. REFERENCES

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