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Genes and lifestyle in normal hearing function and Age-Related Hearing Loss

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To my dearest

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ABSTRACT

The auditory system is a complex machinery, constituted by many molecules involving hair cells, cochlear neurons, the stria vascularis, and combinations thereof. The analysis of complex genetic traits/diseases such as normal hearing function and Age-Related Hearing Loss has long been an enigma of genetic biology, whether in the animal or in medical sciences. In particular, Age-Related Hearing Loss is the most prevalent sensory impairment in the elderly affecting 30% of people aged over 60. The disease is not directly life threatening but it contributes to loss of autonomy and is associated with anxiety, depression, and cognitive decline largely compromising the quality of life.

Until now, only few genes are known to contribute to variability of normal hearing function and Age-Related Hearing Loss. In both cases interactions between lifestyle and environmental determinants as well as several molecular and cellular basis and pathways should be taken into account.

The main aim of the thesis is the understanding of the molecular bases of variation of normal hearing function and Age-Related Hearing Loss using:

a) Genome Wide Association Studies to identify new genes/loci, b) immunohistochemistry to evaluate their expression in the mouse cochlea and c) epidemiological studies to identify environmental/lifestyle factors.

Genome-Wide Association studies and the following Meta-analysis have been carried out on 3815 people coming from isolated villages located in Italy, Croatia, Caucasus and central Asia recruited within the International Consortium G-EAR leading to the identification of 3 loci (rs614171 on chromosome 13, rs3786724 on chromosome 19, rs11711388 on chromosome 3) with p-value$\approx 1*10^{-8}$, 26 loci with p-value $\approx 1*10^{-7}$ and many others with high p-value.

23 genes have been then chosen for evaluation using expression studies in wildtype mice by immunohistochemistry and confocal microscopy. Five of them (*Arsg, Slc16a6, Dclk1, Gabrg3, Csmd1*) display strikingly specific expression in the cochlea and additional eight *(Ptprd, Grm8,*

Kiaa056/GlyBP, Evi5, Irg1, Rimbp2, Ank2, Cdh13) show expression in multiple cell types of the cochlea.

As regards environmental/lifestyle factors, the epidemiological analysis revealed that coffee consumption (coffee yes vs. no) and coffee intake (cups/day) displayed a significant association with better hearing function in four out of ten populations investigated. In particular, coffee drinking was associated at low and high frequencies (lowest p-value=0.006) while the intake only at high frequency (lowest p-value=0.003). Moreover, a statistical significant association between ARHL and education level was detected (lowest p-value=0.0003) confirming previously reported data.

INTRODUCTION

1.1 General overview of the auditory system

The auditory system is one of the most complex mechanisms of sensation ability in humans. The mammalian inner ear is an intricate structure functionally organized into auditory and vestibular components designed to transform mechanical energy into electrical stimuli, which will eventually be translated in the brain (Dror A. et al. 2010). The hearing system is characterized by three structures: a) the outer part, b) the middle ear, and c) the inner ear, that all play a role in hearing function. The hearing system is difficult to study through biochemical routes, due to the small amounts of tissue available for analysis and by key molecules that may be present in only a few tens of copies per cell, thus compounding the difficulty (Steel K.P. et al. 2001). The hair cells in the organ of Corti located in the inner ear, are composed of an inner row and three outer rows of hair cells. The apical side of the hair cells facing the scala media contains the stereocilia, actin-rich projections arranged in bundles at their upper surface, packed with actin filaments, and deflected by the vibrations of sound (Steel K.P. et al. 2001). This activity opens ion channels modulating potential within the cell, and releasing neurotransmitters to synaptic junctions between hair cells and neural fibers of the auditory nerve. The neural spike subsequently propagates in the auditory nerve fiber and impulses are perceived by the brain, mainly in the temporal lobe where they can be processed and assigned meaning.

1.2 Hearing loss: genes and phenotypes

Given the complexity of the hearing mechanism, it should come as no surprise that many genes are involved in hearing. So far, more than 140 loci associated with Non-Syndromic Hearing Impairment (NSHL) have been mapped, and approximately 80 genes identified in humans

(http://hereditaryhearingloss.org/). In mouse models more than 230 genes have been so far described (http://hearingimpairment.jax.org/index.html) to cause inner ear malformations or dysfunction. Despite the identification of these genes, the molecular basis of variation of normal hearing function is still largely unknown. Several molecules have been identified as having a role in auditory function and hair cell transduction because they are specifically expressed in or around the stereocilia and mutations in their genes lead to hearing impairments in either humans or mice models (Quint E. et al. 2003). These dysfunctional proteins are involved in impaired molecular-physiologic processes of potassium and calcium homeostasis, apoptotic signaling, stereocilia linkage, mechano-electric transduction, electromotility, and many other processes (Van Laer L. et al. 2003). Briefly, these molecules include myosins which represent one of the largest group of deafness associated molecules, adhesion protein such as cadherins, member of the ferlin family, components of the tectorial membrane, genes involved in ion homeostasis such as connexins (Martınez A.D. et al. 2009) and many others (Hilgert N. et al. 2009).

Hearing Loss (HL) can also be multifactorial or complex in causality such as Age-Related Hearing Loss (ARHL) (Reiss M. et al. 2009) and Noise-Induced Hearing Loss (NIHL) (Konings A. et al. 2009), reflecting the interaction of a number of genetic and environmental factors. ARHL or presbycusis is a growing problem that has been reported to reduce the quality of life (Ciorba A. et al. 2012). Many studies define heritability of ARHL being in the range between 25% and 75% in different analyzed cohorts (Wolber L.E. et al. 2012, Gates G.A. et al. 1999, Viljanen A. et al. 2007, Karlsson K.K. et al. 1997).

Patients usually show a high tone hearing loss, which has a major adverse effect on communication, particularly in noisy and/or reverberant listening situations. Later, it progresses in the 2–4 kHz range which means that the ability to detect, identify, and localize sounds is impacted (Huang Q. et al. 2010). Regarding the frequency of ARHL, the data are largely variable depending on the sample size, the ethnicity, and the use of

different classification systems (Roth T.N. et al. 2011). The disease is partially preventable, and can be only treated symptomatically with the provision of hearing aids or, in a minor proportion of cases, of a cochlear implant (Zahnert T. 2011). Many pathophysiological processes underlying age-related changes in the auditory mechanism have been described. Histologically, the aged cochlea shows degeneration of the stria vascularis, the hair cells, the primary afferent neurons and the central auditory pathways (Fetoni A.R. et al. 2011). Age-related hearing changes do not occur uniformly and more than one pathological process may be acting upon the auditory system. Classically, ARHL was categorized into different etiopathogenic categories: sensory with high frequency loss, hair cell loss but also non-sensory and supporting cells loss, neural with loss of word discrimination in the presence of stable pure-tone thresholds caused by a loss of neurons in the spiral ganglion, metabolic/strial with atrophy of the lateral wall and the stria vascularis of the cochlea and a flat or slightly descending pure-tone threshold audiometric pattern associated with excellent word discrimination scores (Schuknecht H.F. et al. 1993). Additional type has been described as mechanical where there is no cochlear abnormalities and is characterized by a linear descending pure-tone audiogram which is considered to be often coupled with degeneration in the spiral ligament along the cochlear lateral wall. Finally, ARHL could be mixed and indeterminate showing combinations of flat, gradually sloping, and abrupt high-tone hearing loss with observable light microscopy abnormalities of multiple cochlear elements. This multifactorial process can vary in severity from mild to severe and it could worsen by the contribution of a lifetime of insults to the auditory system. Environmental factors (such as diet, level of education, medical condition, exposures to environmental-ototoxic agents, trauma etc.) (Bovo R. et al., 2010) as well as risk factors (free radical, vascular insults, metabolic changes, hormones, immune system etc.) are essential components in the development of ARHL (Huang Q. et al. 2010). Despite some relevant efforts done to identify the molecular bases of these conditions, up to now,

only a few genes have been associated with either ARHL (Reiss M. et al. 2009, Newman D.L. et al. 2012) or NIHL (Konings A. et al. 2009).

1.3 Isolated populations

The use of isolated populations to reduce heterogeneity of complex and/or quantitative traits has already proven very useful in identifying DNA polymorphisms associated with these traits even if this argument is still an open issue (Shifman S. et al. 2001). In principle, the inbreeding, typical of small communities, reduces genetic heterogeneity, increases homozygosity and Linkage Disequilibrium (i.e. LD refers to correlations among neighbouring alleles, reflecting 'haplotypes' descended from single, ancestral chromosomes), and reduces environmental factors providing greater power for detection of susceptibility genes (Varilo T. et al. 2004, Lowe J.K. et al. 2009). Isolated populations are often either geographically isolated, for example on islands, such as the Sardinian, Icelandic and Orkney populations, or linguistically isolated, such as the Saami (Huyghe J.R. et al. 2011, Francalacci P. et al. 2003) or inhabitants from Resia, a village located in the Friuli Venezia Giulia (FVG) genetic park (Esko T. et al. 2012). Moreover, a population isolate can exist without physical or linguistic barriers, as is the case in Kuusamo and Southern Ostrobothnia (Jakkula E. et al., 2008, Hovatta I. et al., 1997). In these populations the enrichment of certain rare alleles and diseases could be very common as demonstrated for example with the high frequencies of multiple sclerosis in Sardinia, Southern Ostrobothnia and Orkney islands (Pugliatti M. et al. 2005; Tienari P.J. et al., 2004; Rothwell P.M. et al. 1998). As regards hearing impairment, our isolated populations/communities coming from Italy, Caucasus, Central Asia and Tajikistan showed many differences. In particular, the proportion of affected in people aged < 40 increased from Caucasus region to Tajikistan while in those aged ≥ 40, Caucasus region showed the highest percentage (Girotto G. et al. 2011 **A**).

Considering that for most disorders only few common variants, detected

with Genome Wide Association Studies (GWAS) are implicated and the associated SNPs explain only a small fraction of the genetic risk, isolated populations could be particularly useful to detect rare variants, those with less than 1% allele frequencies in the population and can have a stronger effect compared to the common ones. In fact, rare variants, may even be private mutations that only appear in a few individuals/families or be very widespread in isolated population as a consequence of a founder effect. Moreover, rare variants, despite being seen in a small fraction of those who are affected, are expected to raise the risk in carriers at a higher rate and to have larger effect sizes than common variants (Wray N.R. et al. 2001).

1.4 Genotyping and Imputation

The effect of genome variation might be investigated using high-density single nucleotide polymorphism (SNP) array. Current genotyping platforms use single array that contains up to 5 million SNPs to genotype up to twelve samples, while in the past less dense arrays were used. In order to increase the number of available markers for analysis, additional information to predict missing values in a sample might be imputed. Imputation is a method where genotypes of markers that are not genotyped are computationally estimated based on the LD information from variation in a reference population (Marchini J. et al. 2007, Servin B. et al. 2007). In this light, appropriate software that exploited linkage disequilibrium among SNPs and accurately imputed large numbers of "missing" genotypes (Cantor R.M. et al. 2010) has been created. Tools such as the 2.5M HapMap CEU SNP set v22 and the 1000 Genomes Project (1000GP et al. 2010) now provide more dense coverage of the genome at increasingly affordable costs. These developments have resulted in an explosion of positive GWAS and the identification of many new genes for common diseases (Cantor R.M. et al. 2010).

Since in medical genetics, the ultimate objective is to identify causal functional variants and elucidate the mechanisms through which they

exert their effects (Stranger B.E. et al. 2011), new high density SNP arrays aimed at detecting functional variants have been developed. These arrays contain approximately 900.000 optimized common tag SNPs and novel functional exonic variants taken from over 12,000 sequenced exomes.

1.5 Association studies

Association analyses have become increasingly popular for mapping genes involved in complex traits and diseases. In a population association having a specific allele (e.g. A) could make you more or less susceptible to a particular disease (e.g. D) compared with the frequencies reported in a reference population. Usually, a general population contains several genetically distinct subsets caused by differences in allele frequencies (population stratification) and both the disease and allele A could be particularly frequent in one population rather than in another. In association studies, a given number of markers (in some cases specific of one or more candidate genes or regions) are genotyped and the statistical dependence between the genotypes and the phenotype is statistically measured testing any association between those markers and a disease. Moreover, SNPs are often selected based on LD, to tag as much regional genetic variation as possible with as few markers, or polymorphisms, as possible. Association is considered significant if the likelihood of falsely rejecting the null hypothesis is less than 5% (p-value < 0.05) but the raw p-value needs a correction for the number of tests performed.

1.5.1 Association studies vs. Genome wide association studies

Thanks to the development of high-throughput sequencing and genotyping technologies, one of the new uses of the association method is to conduct a Genome wide association studies (GWAS) which are an excellent complement to Quantitative Traits Loci (QTL) mapping (Miles C.M. et al. 2008). GWAS is a genetic association study design in which a sample is genotyped for a large number of genetic markers (usually SNPs). The main aim of GWAS design is to capture all common genetic variation

across the genome and to relate this variation to disease risk. These studies have become the preferred experimental design in exploring the genetic etiology of complex human traits and diseases (Wang X. et al. 2012). GWAS require three essential items: 1) a large number of samples accurately phenotyped from populations that effectively provide genetic information regarding the research question, 2) polymorphic alleles that can be inexpensively and efficiently genotyped and cover the whole genome adequately, and 3) analytic methods that are statistically powerful and can be employed to identify the genetic associations in an unbiased fashion.

In particular, as regard the first item, a large use of Meta-analyses studies which combine results across different studies and populations have been applied. Therefore, by combining association studies the sample size grows, which in turn increases the statistical power to detect association signals. Several Meta-analysis methods exist, that differ, for example, in the way they weigh the contribution of each population and they can generally account for population stratification (Nelis M. et al., 2009, Consortium WTCC, 2007, Devlin B. et al., 1999, Pritchard J.K. et al., 2000).

Regarding the second item, as already described, many different arrays are available to detect variation in the genome.

Concerning the third item, many different approaches could be used based on the analyzed trait. Analysis of continuous variables requires linear model (LM) when data are normal distributed or generalized linear model (GLM) for non-normal distributed data and discrete variables. In addition, Mixed Models (both linear LMM, and generalized mixed model GLMM) are more accurate for representing clustered, and therefore dependent, data, arising for example from a large group of individuals; these models are largely applied in GWAS to account for population stratification and in statistical epidemiological studies (Bolker B.M. et al. 2009). These methods can model population structure, family structure and cryptic relatedness being the main used approach for the analysis of

inbred populations. In particular, in a GWAS this method could be applied to model phenotypes using a mixture of fixed and random effects. Fixed effects include the candidate SNP and optional covariates such as gender or age, while random effects are based on a genotypic covariance matrix (Price A.L. et al. 2010).

1.5.2 Experimental bias

All steps in the process of conducting GWAS (e.g. clinical ascertainment, DNA sampling and processing, DNA quantification, GWAS genotyping, genotype calling and statistical analysis) have the potential for introducing bias. In GWAS there are half a million or more opportunities to find a SNP that is correlated with disease either by chance or though experimental bias. Therefore, by themselves, even very small p-values do not necessarily imply a true association has been found (Neale B.M. et al. 2008).

1.5.3 How to overcome these biases? Multiple testing

With the number of markers collected in genetic association studies ever growing, it is important to correct for random events that falsely appear significant (Noble W.S. 2009). Since multiple testing increases the risk of false positive findings, or by chance observations, of a p-value less than 0.05, the level of significance needs to be readjusted (Risch N. et al. 1996). Methods for correcting for multiple testing in genetic studies include, for example, Bonferroni adjustment that considers the number of tests, permutation methods for empirical p-value, and the Nyholt method that takes into account the LD between the markers (Nyholt D.R. 2004). The limit for a significant uncorrected p-value has been calculated to be $5*10^{-8}$ in a GWAS, assuming 1,000,000 independent association tests and 95% probability of no false positives (Risch N. et al. 1996).

1.6 Immunohistochemistry and confocal microscopy

Immunohistochemistry or IHC refers to the process of detecting antigens (e.g., proteins) in cells of a tissue section by exploiting the principle of antibodies binding specifically to antigens in biological tissues. It uses cells that are broken by the sectioning process and could be used for example in the mouse cochlea to detect gene expression and distribution in the inner ear. Another approach is to combine Immunofluorescence (IMF) with confocal microscopy that use whole-mounts of cochlear tissues retaining much of the structure of the tissue but can lead to artifacts due to variable access of the antibody to different cells or parts of cells. The high resolution provided by confocal microscopy enabled the detailed study of the expression of single cells. Therefore, the combination of these two approaches provide enough information on the clear and real expression of the genes studied.

1.7 Epidemiological studies: environmental and lifestyle factors

Several environmental risk factors such as noise, aging, ototoxic drugs, viral and bacterial infections, and interactions between these factors might affect hearing function. In particular, it has been suggested the role of tobacco, noise exposure, frequent infections, trauma, ototoxic substances, alcohol, leisure activities like hunting and shooting, imbalanced diet (low fruit and vegetable intake), level of education, etc. in the development of non-Mendelian forms of hearing loss, such as ARHL and NIHL (Gaur K. et al. 2012, Bovo R. et al. 2011, Huang Q. et al 2010, Zhan W. et al. 2011, Van Eyken E. et al. 2007). In epidemiological studies, statistical analysis might rely on different models such as GLMM. In this case fixed effects (e.g. covariates) are evaluated in addition to random effects such as population, medical center, operator, repeated-measures etc.

2. MATERIALS & METHODS

2.1 Subjects

Thanks to the International consortium called G-EAR, subjects coming from several isolated villages have been recruited: Carlantino located in South Eastern Italy (Bedin E. et al. 2009), Friuli Venezia Giulia Genetic Park, characterized by 6 villages located in North Eastern Italy (Nelis M. et al. 2009), Korcula, an island in the Adriatic sea (Croatia) (Polasek O. et al. 2009), Campora and Cardile two isolated villages located in the Cilento National's park characterized by 2 different isolated villages in South Western Italy (Bedin E. et al. 2009), and, Talana, Seulo, Urzulei some isolated villages from Ogliastra Genetic Park in central part of Sardinia, Italy (Fraumene C. et al. 2006), plus Split an outbred not isolated population located on the Dalmatian coast (Croatia). Finally, additional samples were coming from Silk Road isolated villages/communities (3 communities from Ukraine, 10 from Caucasus (Armenia, Georgia and Azerbaijan) and 16 from Central Asia (Tajikistan, Uzbekistan, Kyrgyzstan and Kazakhstan) (Girotto G. et al. 2011 **A**). An overall number of 3815 (aged from 18 to 95 years old) were available for the genetic analysis while 4401 (aged from 4 to 95 years old) for epidemiological studies. Among all these samples, during my thesis, I was directly involved in collecting saliva and performing audiometric evaluation of all Silk Road samples (874) and part of those coming from Friuli Venezia Giulia Genetic Park (252). All tests were performed using standard portable audiometers designed for field studies (Madsen Micromate 304, GN Otometrics, Denmark). Subjects underwent pure-tone audiometry obtained after any acoustically obstructing wax had been removed. The analysis of hearing function was done calculating the pure-tone average of air-conduction (PTA at the lower 0.25, 0.5 and 1 kHz, medium 0.5,1 and 2 kHz, and high frequencies 4,8 kHz) (Girotto G. et al. 2011 **B**).

A questionnaire to obtain socio-demographic information, as well as data on physical activity (i.e. job, sport, etc.), lifestyle (e.g. smoking, alcohol consumption, coffee intake, diet including taste and food preferences, etc.), clinical examinations (psychological, neurological, cardiological, etc.), clinical chemistry including blood count, more than 20 parameters related to drugs intake, diseases presence and other information regarding the health status (BMI, bone density, blood pressure, etc.) have been collected for each subject. Clear familial forms of severe hearing loss have been excluded from the study.

A problem that is often encountered in both population-based and family-based GWAS is that of identifying cryptic relatedness and population stratification because it is well known that failure to appropriately account for both pedigree and population structure can lead to spurious association. A number of methods have been proposed for identifying relatives in samples from homogeneous populations, such as isolated ones. One of them, is based on the use of the kinship coefficients which can be written as a function of the IBD-sharing probabilities. When pedigrees are known, software programs are available for calculating IBD-sharing probabilities and kinship coefficients. When pedigrees are partially or completely unknown, genome-screen data can be used for estimating measures of relatedness. In our case, before running GWAS analyses we calculated the genomic kinship by using shared genotype counts as a measure of genetic distance between individuals.

2.2 Phenotypes

For both the analyses we used only the threshold from the better ear, defined as the ear with lowest value of hearing loss for each individual and the following quantitative traits have been tested:

- Seven different thresholds (125Hz, 250 Hz, 500 Hz, 1 kHz, 2 kHz, 4 kHz, and 8 kHz)

- Pure Tone Average at low, medium and high frequencies: PTAL are

defined as the mean between 250Hz, 500Hz, 1000Hz, PTAM as the mean between 500Hz, 1000Hz, 2000Hz while PTAH as the mean between 4000Hz and 8000Hz. In the case of PTAs, when calculating the right and the left PTA, we chose the lowest.

- The first 3 Principal Components (PC1, PC2, PC3) were estimated on all frequencies. All these components were adjusted by sex and age. Each component describes a different pattern in the data (Van Laer L. et al. 2010): PC1 is a "size variable" that represents an overall measure of a subject's hearing ability, PC2 and PC3 are instead "shape variables". In particular PC2 shows the ratio of hearing between the high and the low frequencies and is a measure of the slope of the audiogram, PC3 contrasts the middle frequencies with the lower and higher frequencies and can be considered a measure of the concavity of an audiogram.

2.3 DNA sampling, genotyping and imputation

All studies had appropriate ethical approval by the Institutional Review Board of IRCCS-Burlo Garofolo, (Trieste-Italy) and by the other involved members. Consent forms for clinical and genetic studies have been signed by all the participants in the study. Blood samples were collected and DNA extracted according to standard protocols. After measuring quantity and quality of DNA, 3927 samples were genotyped with Illumina 370k platform (Carlantino, FVG Genetic Park, Cilento, Korcula and Split), Affymetrix 500K (Talana) or Illumina 700K (Silk Road). Genotype quality control and data cleaning were conducted independently by each study group resulting in a final number of 3815 samples. As regards our group, genotypes were obtained through our Genomics core facility, while imputation to the 2.5M HapMap CEU SNP set v22 was carried out by the biostatisticians belonging to the same core facility.

2.4 Statistical analysis: quantitative GWAS 1

In this first analysis, each trait was first linearly regressed against age. Being residuals from this regression bell-shaped, data were cleaned from outliers following these standard criteria of exclusion (mean +/- 6 SD) and normalized with rank normal transformation. I then performed association analysis using sex as a covariate and through a mixed model linear regression where the random effect was given by the genomic kinship. The analysis was implemented in GenABEL (Aulchenko Y.S. et al. 2007) package for genotyped SNPs and ProbABEL (Aulchenko Y.S. et al. 2010) for imputed data. Meta-analysis was conducted using the inverse variance model as implemented in the MetABEL (Aulchenko Y.S. et al. 2007) R library. For PC traits fixed effects meta-analysis was conducted where Z-scores were estimated from p-value and weighted on the sample-size as implemented in METAL (Willer C.J., et al. 2010). SNPs with imputation quality (Rsq in MACH) less than 0.3 or with less than 30 copies of an allele in each population, were excluded. After quality control, 3409 subjects and approximately 2.2 million SNPs were used for this first meta-analysis (Girotto G. et al. 2011 **B**).

2.4.1 Statistical analysis: GWAS2

I carried out a second analysis on a larger number of 3815 individuals with a slight modification of the protocol. As described in Girotto G. et al. 2011 **A**, after observing 1) a non-linear relationship between the hearing trait and age and 2) age-related audiometric profile between men and women, several adjustments procedures were performed. In this light, to better adjust for age, a more precise function based on double linear regression was applied while to overcome the sex differences, men and women datasets were firstly separated, then traits were corrected for age and finally merged for both sexes.

As regards PCs, in order to be more accurate, this correction was applied to raw data before computing them (Van Laer L. et al. 2010).

2.4.2 Statistical analysis: quantitative GWAS3

Finally, in a sex-separated analysis, as already described above, I firstly separated men and women adjusting for age, then performed the analysis in the two different datasets (males and females).

2.4.3 Statistical analysis: qualitative analysis GWAS4

This association analysis was performed using the same software as the quantitative one (see above, section 2.4). Moreover, PTAs already calculated in the quantitative analysis have been used to define cases and controls as follow:

Cases were people aged equal or more than 50 years old with PTAH≥ 40dB while controls those aged equal or more than 50 years old with PTAH≤25dB. Before running the analyses, the proportion of cases and controls was approximately 1:1 (within the sample size of 1622). Because people aged more than 50 years old show generally more hearing problems, in order to avoid confounding effects related to the trait and the age, we decided to adjust the trait for age.

2.5 Expression studies

2.5.1 Immunohistochemistry

A list of 23 candidate genes (*DCLK1, GRM8, PTPRD, CMIP, RIMBP2, DFFB, KIAA0562/GLYBP, C1orf174, FZD6, GABRG3, CDH13, FOS, FAM69A, EVI5, RPL5, SNORA66, SNORD21, ANK2, CSMD1, AMZ2, ARSG, SLC16A6, and IRG1*) has been created combining data arising from the different GWAS. Wild-type mice at postnatal day P4 and P5 stage from the albino C57BL/6J-Tyr^{c-Brd} or pigmented C3HeB/FeJ inbred strains were used for all experiments. The heads of all samples were dissected in PBS before fixation for two days in 10% formalin at 4°C, washing, dehydrating and embedding in paraffin wax. Embedded samples were cut into 8μm thick sections along the sagittal plane. Immunohistochemistry was then carried out on slides using the Ventana

Discovery machines with the manufacturer's reagents (CC1 (cat.no 950-124), EZPrep (cat.no 950-100), LCS (cat.no 650-010), RiboWash (cat.no 760-105), Reaction Buffer (cat.no 95-300), and RiboCC (cat.no 760-107) and according to the manufacturer's instructions. The DABMap™ Kit (Ventana; cat.no 760-124) with hematoxylin counterstain (cat.no 760-2021) and bluing reagent (760-2037) was used. All antibodies were diluted in 'Antibody staining solution': 10% fetal calf serum, 0.1% Triton, 2% BSA and 0.5% sodium azide in PBS. The primary antibodies of genes with positive staining were: anti-Dclk1 (Abnova, PAB2050, 1:50), anti-Arsg (Sigma Aldrich, HPA023245, 1:50), anti-Evi5 (Abgent, AP9168c, 1:10), anti-Ptprd (Abcam, ab103013, 1:200), anti-Slc16a6 (Santa Cruz, sc-51325, 1:20), anti-GlyBP (Abcam, ab28773, 1:100), anti-Grm8 (Abcam, ab53094, 1:50), anti-Rimbp2 (Santa Cruz, sc-169182, 1:25), anti-Gabr3 (Santa Cruz, sc-7371, 1:50), anti-Cadh3 (Abcam, ab36905, 1:200), anti-Ank2 (Santa Cruz, sc-28560, 1:50) anti-Csmd1 (Santa Cruz, sc-68280, 1:50) and anti-Irg1 (Santa Cruz, sc-84189, 1:100). Secondary antibodies used were Jackson ImmunoResearch biotin-conjugated donkey anti-rabbit (711-065-152, 1:100) and Jackson ImmunoResearch biotin-conjugated donkey anti-goat (705-065-147, 1:100). For each gene, slides covering the entire inner ear for at least three different mouse samples at P5 or P4 were stained, and the observed expression patterns were only considered reliable if present in all three samples. Stained slides were examined and images obtained using an AxioCam HRc camera mounted on a Zeiss microscope. Images were then processed in Photoshop CS5 extended.

2.5.2 Whole mount immunofluorescence

Heads from 5 days old mice (P5) were bisected and inner ears plus bone were removed from the skull and then fixed in 4% paraformaldehyde for 2 hours at room temperature. Subsequently specimens were fine dissected in PBS, then washed and permeabilized in 1% PBS/Triton-X-100 (PBT) and blocked with 10% sheep serum. Then, they were incubated with the primary antibody, goat polyclonal against Csmd1 (N-20), Santa Cruz

Biotechnology, cat. no. sc-68280, dilution 1:100) overnight at 4°C. After washes with PBT, samples were incubated with anti-goat Alexa Fluor 488 secondary antibody (Invitrogen, anti-rabbit, diluted 1:300) and rhodamine/phalloidin (Invitrogen, diluted 1:100). Samples were mounted in Prolong Gold Antifading reagent (Invitrogen). Images were acquired on a LSM 510 Meta confocal microscope (Zeiss, Welwyn Garden City). Post-acquisition image analyses were performed using Adobe Photoshop CS2.

2.6 Epidemiological studies

Epidemiological analyses for hearing function have been carried out on 4401 healthy individuals ranging from 4 to 95 years of age. The following variables: smoking, consumption of chocolate, coffee, tea, wine, beer, dairy products and spirits were taken into account and used as possible covariates for the normal hearing function, besides sex and age. All these lifestyle/environmental factors have been both analysed as binomial (yes/no) and quantitative (e.g. intake) traits. In particular, intake of coffee was calculated from 1 to 5 or more cups/day.

A simple linear regression analysis between hearing function (PTAL, PTAM and PTAH) and each of the six lifestyle-variables (smoke, coffee, wine, beer, spirits and dairy products) was carried out including age and sex as potential confounding variables. In order to find the best fitting model and to detect any possible interaction between hearing functions and the above mentioned variables, an automated step-wise selection process was then used. This is well achieved with the R software ("step" function, http://www.r-project.org/). This function fits all the possible models by adding and dropping covariates in a given linear or general linear model. Moreover, in order to verify if sex and age were interacting with the other covariates in the model, we used the Wilcoxon test to exclude those that showed significant associations. The significance level was set at 5%.

As regards ARHL the analysis was performed on 2767 cases and controls equally divided, ranging from 50 to 95 years of age. The level of education was assessed in five different categories comprehending 1) no education, 2) elementary school, 3) secondary school, 4) high school and 5) university. As far as occupation is regarded, the different jobs were clustered in four groups: unskilled manual, skilled manual, skilled non-manual and, professional work. An additional group consists of retired and unemployed people.

In this case, the main statistical analysis relied on the use of a mixed-effect model that is more accurate to analyze large group of individuals belonging to different populations (see section 1.5.1). Covariates sex, age and age-squared were added to the fixed effects of the model, to avoid confounding effects due to the strong correlation between the hearing trait and these variables. Furthermore population was considered as a random effect, in order to account for geographical and cultural differences.

Correlation between the categorical variables analysed was computed using the polychoric correlation technique, implemented in the R software ("hector" function). This method is used for estimating the correlation coefficient ϕ between two ordinal variables, in our case the five education levels and the professional categories of work. The significance level was set at 5%.

3. RESULTS

3.1 Genetic factors

Meta-analyses have succeeded in identifying some GWAS loci associated with hearing traits and ARHL, plus several additional ones strongly suggestive. As reported in Table 1A in the first quantitative analysis (GWAS1) 4 loci were strongly associated with the analyzed traits (max p-value=$3.18*10^{-7}$), 10 with p≈10^{-6} or 10^{-5} and many others suggestive with higher p-values (not shown). In the second quantitative analysis (GWAS2), one locus with p-value=$7.69E^{-08}$, 6 loci with p-value≈10^{-7} (Table1B) plus 77 with p-value≈10^{-6} (not shown) have been identified. Finally, in the quantitative study sex separated (GWAS3; Table 1C), 2 loci with p-value=$4.32*10^{-8}$ and $7.03*10^{-8}$, 16 with p-value≈10^{-7} and 150 with p-value≈10^{-6} (not shown) were identified. As regards the qualitative analysis, GWAS4, (i.e. ARHL; Table 1D) 7 loci with p-value≈10^{-6} were identified as well as others 64 showing a p-value≈10^{-5} (not shown).

In all cases, when the most significant hint was located outside of a given gene, we selected the closest gene/s among those located in a region of 200Kb upstream and downstream the most significant SNP.

Going into details, in GWAS1, the highest hit (p=$3.18*10^{-07}$) was found at 4kHz with a SNP in LD with *DCLK1* gene, a member of the doublecortin family expressed in the ear according to NCBI data (http://www.ncbi.nlm.nih.gov/gene/). Other suggestive hits were: rs2687481, rs10815873 and rs898967. The first one was detected with the lowest p-value ($3.22*10^{-7}$) at PC1, really close to *GRM8* gene, a glutamate receptor that inhibits adenylyl cyclase decreasing the formation of cAMP (Scherer S.W. et al. 1997). The second one was rs10815873 located within *PTPRD* gene; data available from the literature show that another member of this gene family plays an important role in the hearing system (Goodyear R.J. et al. 2003). The third SNP is located within *CMIP* gene, reported from NCBI (http://www.ncbi.nlm.nih.gov/gene/) as

expressed in the ear. Additional interesting associations were detected with the following genes: *RIMBP2, KIAA056/GLYBP, DFFB, C1orf174, FZD6, GABRG3, CDH13, FOS, EVI5, SNORA66, RPL5, SNORD21, FAM69A, ANK2, CSMD1, ARSG, AMZ2, SLC16A6*.

After running the first analysis, in GWAS2, a more accurate age correction (see section 2.4.1) has been performed, and the analysis carried out on a larger sample size as compared to that of GWAS1. The top SNP/gene (p-value of $7.69*10^{-08}$) was within Immunoresponsive 1 homolog (*IRG1*).

As regards GWAS3 (Table 1C), the analysis led to the identification of very interesting associations separately in males and females; some examples are: in the males cohort we detected *SMARCA4* gene ($p=4.3*10^{-8}$), a member of the SWI/SNF family of proteins while in the females cohort we found *MMP8* gene together with others members of the matrix metalloproteinase protein family (p-value=$1.24*10^{-07}$).

Regarding qualitative analysis (GWAS4), the study has been performed in a selected cohort (1622 cases and controls equally divided) leading to the identification of 7 SNPs with p-value thresholds of $\approx 10^{-6}$ (Table 1D). One possible interesting gene, among those marked by positive hints, is *EDN3* a member of the endothelin family, whose members have been already associated with hearing impairment or are expressed in inner ears (Uchida Y. et al. 2009, Ida-Eto M. 2001).

A

GWAS1

SNP	chr	position	p-value	gene	Trait	NSNP	closest genes (+/- 200 Kb)
rs9574464	13	36314793	3.18E-07	NA	4k	5	NBEA; DCLK1
rs2687481	7	125869122	3.22E-07	NA	PC1	9	GRM8
rs10815873	9	8436194	3.35E-07	PTPRD	125	2	
rs898967	16	81566780	9.64E-07	CMIP	1k	9	
rs10848114	12	130979355	4.75E-06	RIMBP2	PC1	4	
rs6673959	1	4123117	7.97E-06	NA	PTAH	8	DFFB;KIAA0562/GLYBP C1orf174
rs1110115	8	104098620	9.54E-06	NA	2k	5	FZD6
rs7182802	15	27347206	9.94E-06	GABRG3	PC1	6	
rs12758887	1	93394055	8.68E-06	FAM69A	PTAH	3	EVI5;RPL5 SNORD21;SNORA66
rs17195859	16	83318996	2.07E-05	CDH13	500	12	
rs11159133	14	75759172	2.98E-05	NA	PCA3	0	FOS
rs17045059	4	113775047	7.81E-05	ANK2	125	2	
rs10091102	8	3060466	8.75E-05	CSMD1	PC1	2	
rs8077384	17	66163231	9.66E-05	NA	500	0	AMZ2;SLC16A6 ARSG

B

GWAS2

SNP	chr	position	p-value	gene	Trait	NSNP	closest genes (+/- 200 Kb)
rs614171	13	77516752	7.69E-08	NA	PC1	5	IRG1
rs1328967	6	47810090	2.54E-07	NA	PTAM	12	OPN5;PTCHD4
rs13022609	2	36269425	3.21E-07	NA	PC1	4	NA
rs1420134	7	29530473	5.91E-07	CHN2	X2K	5	
rs2170756	4	10968348	6.83E-07	NA	X8K	1	NA
rs11731909	4	76421029	7.72E-07	RCHY1	PC3	0	
rs7617144	3	43848450	8.19E-07	NA	X8K	9	ABHD5;ANO10

C

GWAS3

SNP	chr	position	p-value	gene	trait	NSNP	closest genes (+/- 200 Kb)
rs3786724	19	11166791	4.32E-08	*SMARCA4*	500_M	0	
rs11711388	3	194759980	7.03E-08	*NA*	6K_M	0	*XXYLT1; ACAP2*
rs6590986	11	102598859	1.24E-07	*MMP8*	PTAL_F	51	
rs2717795	7	148195053	1.47E-07	*NA*	PTAH_F	7	*CNTNAP2;C7orf33*
rs3002330	9	92149146	2.32E-07	*NA*	PC2_F	5	*SEMA4D; GADD45G*
rs12868445	13	105831211	2.45E-07	*NA*	6K_F	22	*NA*
rs9390779	6	102209081	4.30E-07	*GRIK2*	PC2_F	43	
rs7844675	8	70961514	4.31E-07	*PRDM14*	250_M	1	
rs3769702	2	99195095	4.87E-07	*INPP4A*	PTAL_F	2	
rs260406	5	1890233	5.72E-07	*IRX4*	2K_M	0	
rs10171479	2	161596138	5.83E-07	*NA*	8K_M	72	*RBMS1*
rs9319217	13	25973403	5.99E-07	*ATP8A2*	4K_M	5	
rs2314305	5	154449947	6.76E-07	*NA*	8K_F	0	*KIF4B*
rs2710832	4	169409002	7.31E-07	*DDX60L*	PC1_F	4	
rs6903872	6	37728547	7.40E-07	*NA*	PTAL_F	3	*MDGA1; ZFAND3*
rs701554	13	103607804	8.25E-07	*NA*	PTAH_F	7	*METTL21EP; SLC10A2*
rs4356243	11	59596197	8.44E-07	*GIF*	8K_F	13	
rs10917398	1	23927205	9.76E-07	*MDS2*	6K_F	5	

D

GWAS4

SNP	chr	position	p-value	gene	trait	NSNP	closest genes (+/- 200 Kb)
rs156248	20	58013270	2.69E-06	*NA*	ARHL	0	*EDN3; PIEZO1P1*
rs9596486	13	51837299	3.02E-06	*FAM124A*	ARHL	5	
rs12648011	4	25956191	3.38E-06	*NA*	ARHL	2	*C4orf52*
rs16977831	15	58189489	4.58E-06	*NA*	ARHL	0	*ALDH1A2*
rs4974419	3	127124096	4.61E-06	*NA*	ARHL	4	*C3orf56; TPRA1*
rs7341233	6	108940280	7.84E-06	*FOXO3 SUMO2P8*	ARHL	3	
rs7236648	18	56761318	8.43E-06	*NA*	ARHL	0	*SEC11C*

Table 1. List of genes/loci selected from the four studies with details of lowest p-values. 1.A, 1.B, 1.C and 1.D show candidates genes from GWAS1, GWAS2, GWAS3 and GWAS4, respectively. In Table 1C the column trait distinguishes between top hints obtained in females (F) and those in males (M). Genes in bold belong to the list of the 23 candidates: in red are those expressed in the cochlea while in black those with negative staining.

SNP: referral SNP with the lowest p-value, Chr: chromosome position, position: genomic position, p-value: p-value, gene: gene related to the referral SNP, trait: trait in which the association is found, NSNP: overall number of SNPs showing an association (p-value equal or lower than $5*10^{-5}$) within the locus marked by the referral SNP, closest gene: closest gene/genes in a region of ±200 Kb upstream and downstream the referral SNP

3.2 Expression studies

GWAS data arising from the meta-analyses were used to define a first list of 23 candidate genes to be included in an expression analysis by immunohistochemistry. In particular, combining results coming from GWAS1 and GWAS4 we generated a list of 11 genes detected in both studies (*AMZ2, ARSG, DFFB, C1orf174, EVI5, FAM69A, KIAA0562/GLYBP, RPL5, SLC16A6, SNORA66, SNORD21*). Additional, 11 candidates have been selected among the top hints in the GWAS1 (*DCLK1, GRM8, PTPRD, CMIP, RIMBP2, GABRG3, CDH13, ANK2, CSMD1, FOS, FZD6*). Finally, the last gene corresponds to the most significant hint in GWAS2 (*IRG1*).

Results from these studies demonstrated specific cochlear expression for 13 of them, while in the remaining 10 cases results were not conclusive showing either no detectable labeling (*Amz2, Rpl5* and *Cmip*), or widespread labeling that appears non-specific (*Dffb, Fzd6* and *Fos*). Two different antibodies were tested for *Dffb, Amz2* and *Cmip*, but no specific pattern of labeling was seen with either. In addition, the RNA genes *Snora66* and *Snord21* were tested by in situ hybridisation using custom-designed Locked Nucleic Acid probes, but no labeling was seen in the ear. Finally, for two of them (*C1orf174* and *FAM69A*), antibodies were not available. All genes under investigation gave the same levels of staining from the apex to the base of the cochlea and for most of them data regarding the expression in the vestibular system have been also obtained. A summary of the distribution of labeling is given in Table 2 and according to the inner ear topography we could divide the pattern of expression into three different categories.

1) Expression in marginal cells of the stria vascularis:

Doublecortin-like kinase 1 (*Dclk1*) displays a strong pattern of expression in the marginal cells including projections towards the basal cells in the stria vascularis (Fig.1 A,B). In the vestibular system, staining of *Dclk1* could be seen in the dark cells adjacent to the crista (Fig.6 A,B).

2) Expression in the hair cells of the organ of Corti:

Arylsulfatase G (*Arsg*) shows striking specific expression at the top of sensory hair cells in the organ of Corti (Fig.1 C,D). No staining in the vestibular system has been detected.

Solute carrier family 16, member 6 (*Slc16a6*) shows expression at P4, at the top of the outer hair cells in the organ of Corti (Fig.1 E, F), but weak staining is also present in the hair cells and supporting cells of the maculae and cristae of the vestibular system (Fig.6 C, D). The staining of this gene is variable between mice of different genetic backgrounds: it is very clear in outer hair cells of C3HeB/FeJ mice (n=4), while a more faint and less specific pattern of expression was detected in C57BL/6J-*Tyr*^{c-Brd} mice (n=4).

Csmd1, a CUB and Sushi multiple domains 1, expression could be noted in the outer and inner hair cells in the organ of Corti (Fig.2 A, B). To better understand the precise localization of this expression, a confocal experiment has been also performed. The expression is strongly localized in the stereocilia of the inner hair cells but a faint staining is also present in the stereocilia of the outer hair cells (Fig.2 E). Heavy expression was also noted in the hair cells in the maculae and cristae of the vestibular system (Fig.2 C, D).

Gabrg3 is a member of the GABA receptor gene family, a group of proteins involved in the GABAergic neurotransmission of the mammalian central nervous system. It shows a striking specific expression in the outer and inner hair cells. In particular, the outer hair cells have the strongest staining (Fig.2 F,G).

3) Expression in multiple cell types in the cochlea

Protein tyrosine phosphatase, receptor type, D (*Ptprd*) is expressed in the outer as well in the inner hair cells, in the stria vascularis, in the cells of Kölliker's organ and in the spiral ganglion (Fig.3 A, B, C). Expression was also noted in the hair cells and supporting cells in the cristae and maculae of the vestibular system (Fig.6 E, F). Faint staining is also present in the neural dendrites.

Ankyrin 2, a member of the ankyrin family, group of molecules that link the integral membrane proteins to the underlying spectrin-actin cytoskeleton. *Ank2* expression could be noted in the Hensen's cells, Deiters' cells and Pillar cells and in the Reissner's membrane (Fig.3 G, H).

Cdh13 is a member of the cadherin superfamily. Cdh13 is expressed in cells of Claudius and Hensen cells, outer and inner hair cells, Deiters' cells and pillar cells, cells of Kölliker's organ, interdental cells and spiral limbus. Staining was also noted in the marginal cells of the stria vascularis, in the Reissner's membrane, spiral prominence and external sulcus cells (Fig 3 I, L).

Glutamate receptor, metabotropic 8 (*Grm8*) gave positive signals in the outer and inner cells in the organ of Corti, cells of Claudius, Hensen's cells as well as in the cells of Kölliker's organ (Fig.4 A, B, C, D). Expression could be seen in the spiral ganglion neurons. Staining of this protein was also noted in the root cells, in the spiral prominence and in the stria vascularis. Heavy expression was present in the hair cells and supporting cells in the maculae and cristae of the vestibular system (Fig. 7 A, B). As in the cochlea, neurons in the vestibular system showed strong expression of *Grm8*.

RIMS binding protein 2 (*Rimbp2*) could be noted in the outer and inner hair cells in the organ of Corti, in the lateral edge of the tectorial membrane, root cells, spiral prominence, spiral ganglion and in the marginal and intermediate cells of the stria vascularis (Fig.4 E, F, G, H). Heavy expression was also noted in the hair cells and supporting cells in the maculae, and cristae in the neural dendrites of the vestibular system. (Fig.7 C, D).

Centrosomal protein 104kDa (*GlyBP/KIAA0562*) expression could be seen in the outer and inner hair cells and in the Hensens' cells. Strong expression could also be seen in the spiral prominence, in the tectorial membrane and in the basilar membrane (Fig.5 A, B, C, D). In addition, discreet patches of *GlyBP* expression could be seen in the hair cells and

supporting cells in the maculae and cristae and in supporting cells adjacent to the sensory patches of the vestibular system (Fig.7 E, F).

The expression of *Evi5* could be noted in the outer and inner cells in the organ of Corti, in Deiters' and pillar cells, cells of Kölliker's organ, spiral ganglion (neuronal dendrites), root cell processes, in the spiral prominence and in the stria vascularis. A weak staining could be also seen in the cells of Claudius and Hensen cells (Fig.5 E, F, G). Heavy expression was also noted in the hair cells and in the supporting cells in the maculae and in the cristae of the vestibular system. Strong staining is also present in the neural dendrites (Fig.7 G, H).

Finally, *Irg1* expression could be noted in the nuclei of the outer and inner cells in the organ of Corti, in the root cells and in the spiral prominence (Fig.3 D, E, F). Expression was also noted in the hair cells and supporting cells in the maculae and cristae and in the neural dendrites of the vestibular system (Fig.6 G, H).

Gene	ihc	ohc	rc	sp	tm	bm	sv	pc	Dc	cC	Hc	Ko	sg
Dclk1							+						
Grm8	+	+	+	+			+			+	+	+	+
Ptprd	+	+					+					+	+
Rimbp2	+	+	+	+	+		+						+
GlyBP	+	+		+	+	+					+		
Gabrg3	+	+											
Cdh13	+	+		+			+	+	+	+	+	+	
Evi5	+	+	+	+			+	+	+	+	+	+	+
Ank2								+	+		+		
Csmd1	+	+											
Arsg	+	+											
Slc16a6		+											
Irg1	+	+	+	+									

Table 2. List of genes with specific labeling patterns in the cochlea with a summary of expression results.

ihc, Inner hair cells; **ohc**, outer hair cells; **rc**, root cells; **sp**, spiral prominence; **tm**, tectorial membrane; **bm**, basilar membrane; **sv**, stria vascularis; **pc**, pillar cells; **Dc**, Deiter cells; **cC**, cells of Claudius; **Hc**, Hensen cells; **Ko**, Kölliker's organ; **sg**, spiral ganglion, **+** indicates expression is detected.
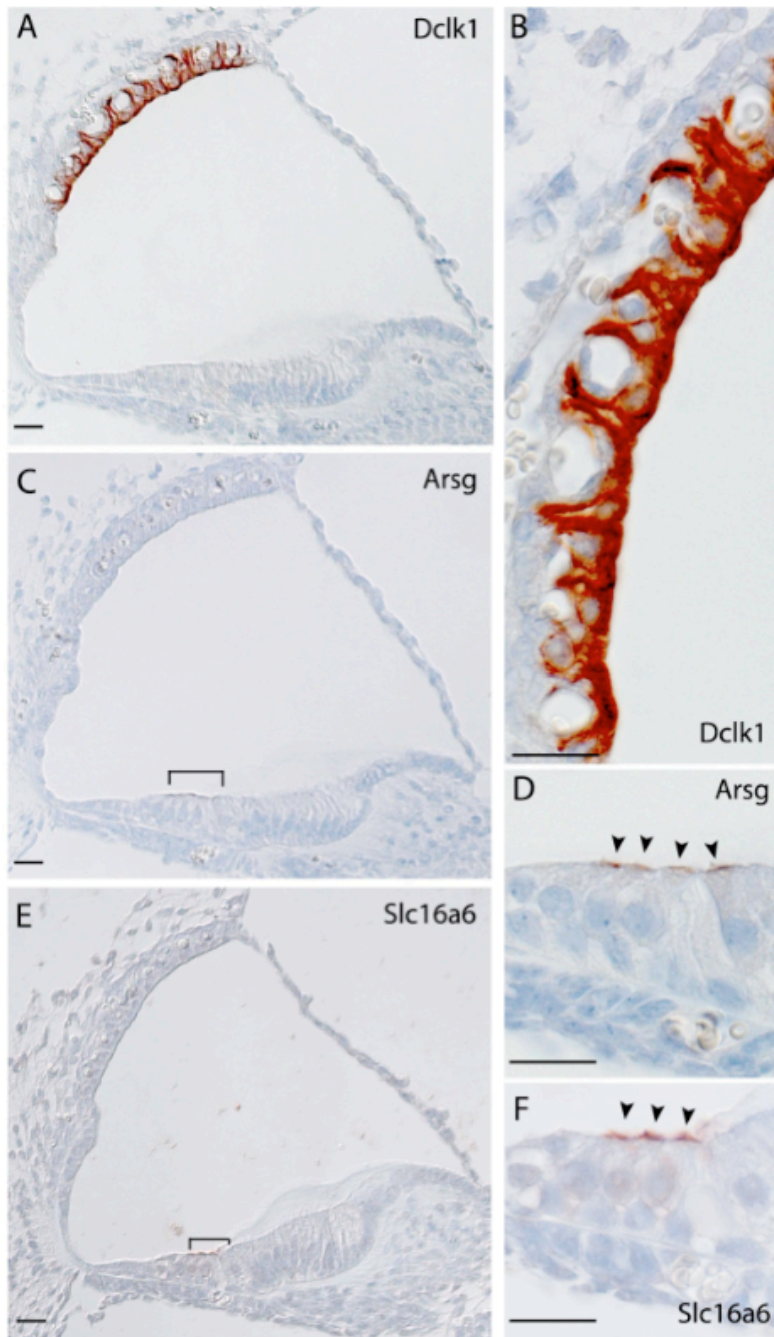
Figure 1. Immunohistochemistry in the mouse cochlea at P5 (P4 for *Slc16a6* gene)
Brown signals indicate positive staining. A, B) Expression of *Dclk1*, showing intense staining in the marginal cells of the stria vascularis; C, D) Expression of *Arsg* is localized at the top of sensory hair cells in the organ of Corti (bracket in C, arrowheads in D). E, F) Hair cells at P4, show staining in outer hair cells of *Slc16a6* (bracket in E, arrowheads in F). Note that these samples are from the C3HeB/FeJ strain, which is pigmented. Scale bar indicates 20μM.
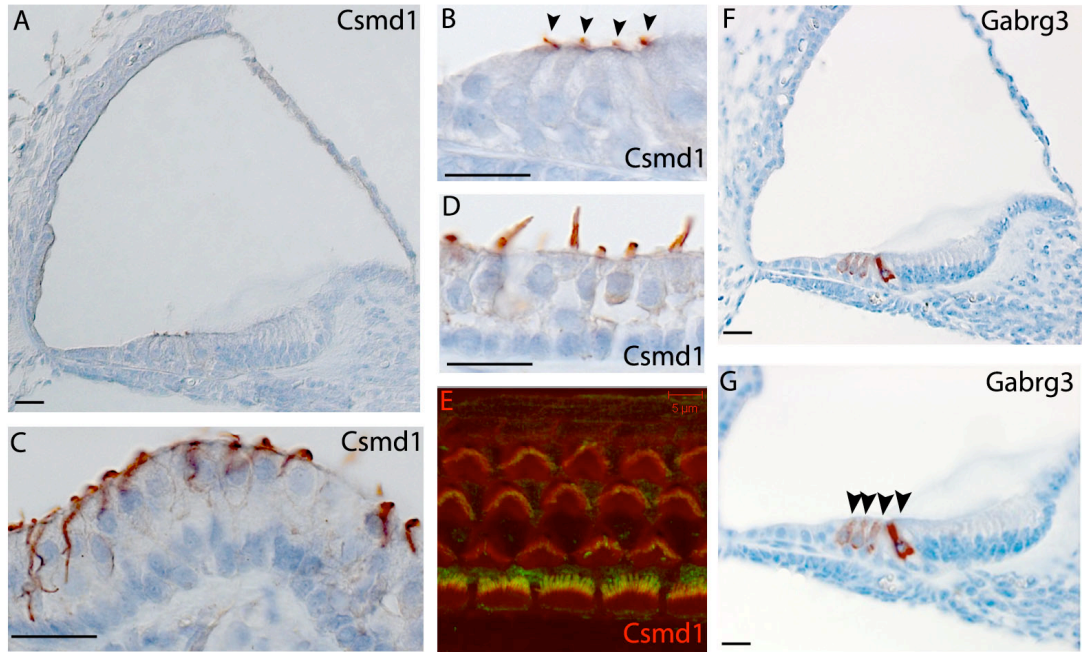
Figure 2. Immunohistochemistry and confocal microscopy in the mouse cochlea at P5

Brown signals indicate positive staining. A, B) Expression of *Csmd1* is localized at the top of sensory hair cells in the organ of Corti; C, D) Heavy expression of *Csmd1* was also noted in the hair cells in the cristae (C) and in the maculae (D) of the vestibular system. E) confocal expression shows a strong staining localized in the stereocilia of the inner hair cells and a faint staining is also present in the stereocilia of the outer hair cells. The red labels indicate the expression of rodhamin phalloidin, which marks the actin filaments in the stereocilia. The green labels indicate the expression of the *Csmd1* protein and the yellow labels indicate the merge between *Csmd1* and phalloidin, and therefore their actual co-localisation in the stereocilia bundles (E). F), G) *Gabrg3* shows a striking specific expression in the outer and inner hair cells (arrowheads in G). In particular, the inner hair cells have the strongest staining. Scale bar indicates 20μM.
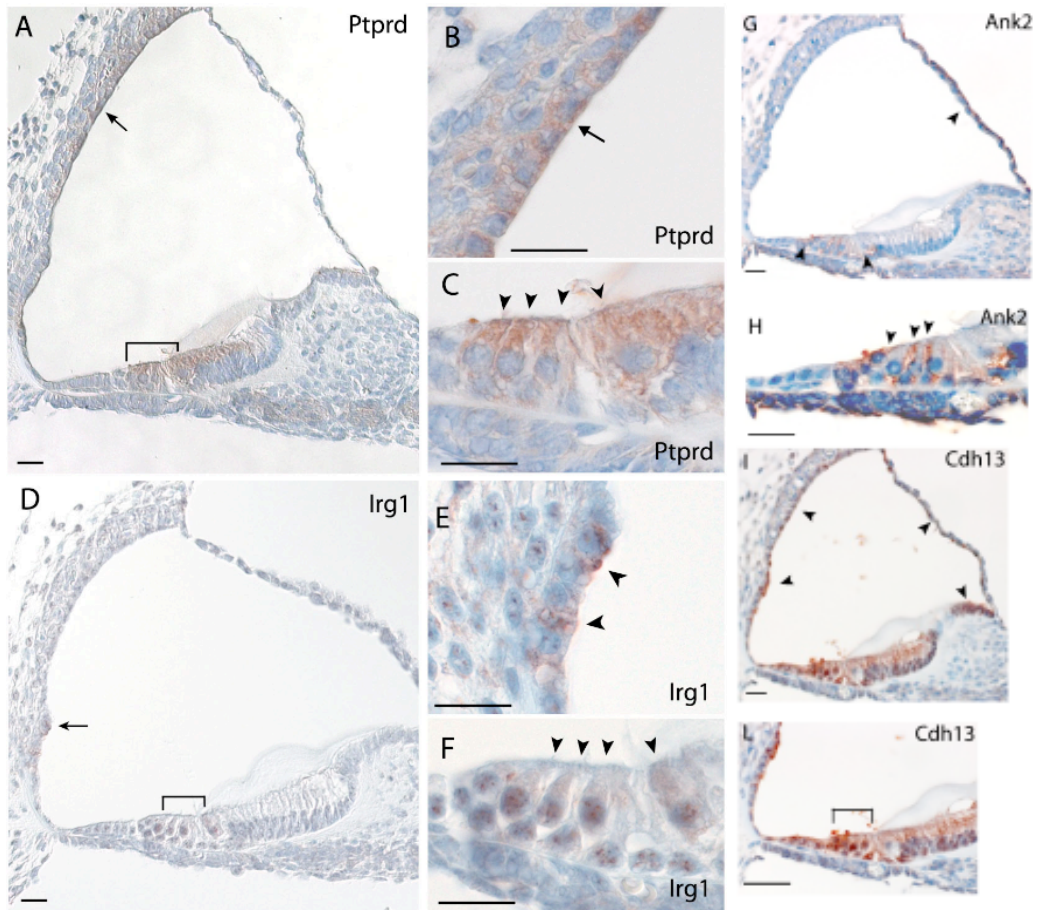
Figure 3. Immunohistochemistry in the mouse cochlea at P5

Brown signals indicate positive staining. A, B, C) *Ptprd* is localized in hair cells of the organ of Corti (bracket in A, arrowheads in C), in the marginal cells of the stria vascularis (arrow in A, and arrowheads in B), in the spiral ganglion and in the supporting cells of the Kölliker's organ; D, E, F) Expression of *Irg1* showing staining in nuclei of hair cells (bracket in D, arrowheads in F), root cells and spiral prominence (indicated by arrow in D and arrowheads in E). G) H) *Ank2* could be noted in the Hensen's cells, Deiters' cell and pillar cells and in the Reissner's membrane (arrowheads in G). I) L) *Cdh13* is expressed in cells of Claudius and Hensen cells, outer and inner hair cells, Deiters' cells and pillar cells, cells of Kolliker's organ, interdental cells and spiral limbus. Staining was also noted in Reissner's membrane, stria vascularis, spiral prominence and external sulcus cells (arrowheads in I, bracket in L). Scale bar indicates 20μM.
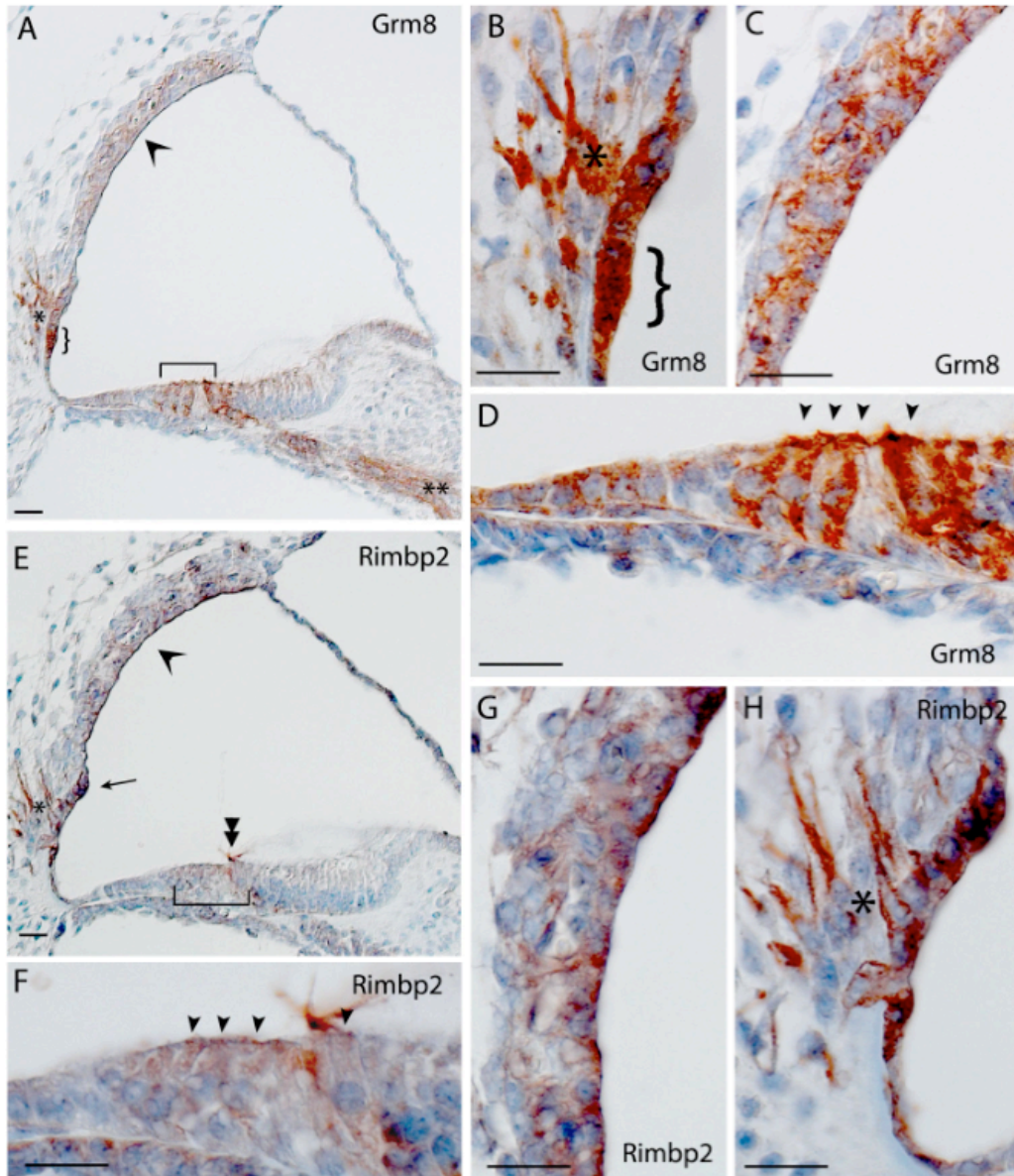
Figure 4. Immunohistochemistry in the mouse cochlea at P5

Brown signals indicate positive staining. A, B, C, D) Staining of *Grm8* showing expression throughout the cochlea, most notably the root cells (curly brackets in A and B) and root cell processes (asterisks in A and B), the stria vascularis (open arrowhead in A), the hair cells (brackets in A, arrowheads in D) and also in the spiral ganglion neurons (double asterisks in A); E, F, G, H) Expression of *Rimbp2* showing staining in hair cells (bracket in E, arrowheads in F), tectorial membrane (double arrowhead in E), the stria vascularis (open arrowhead in E) and in the spiral prominence (arrow in E) and root cell processes (asterisks in E and H). Scale bar indicates 20μM.
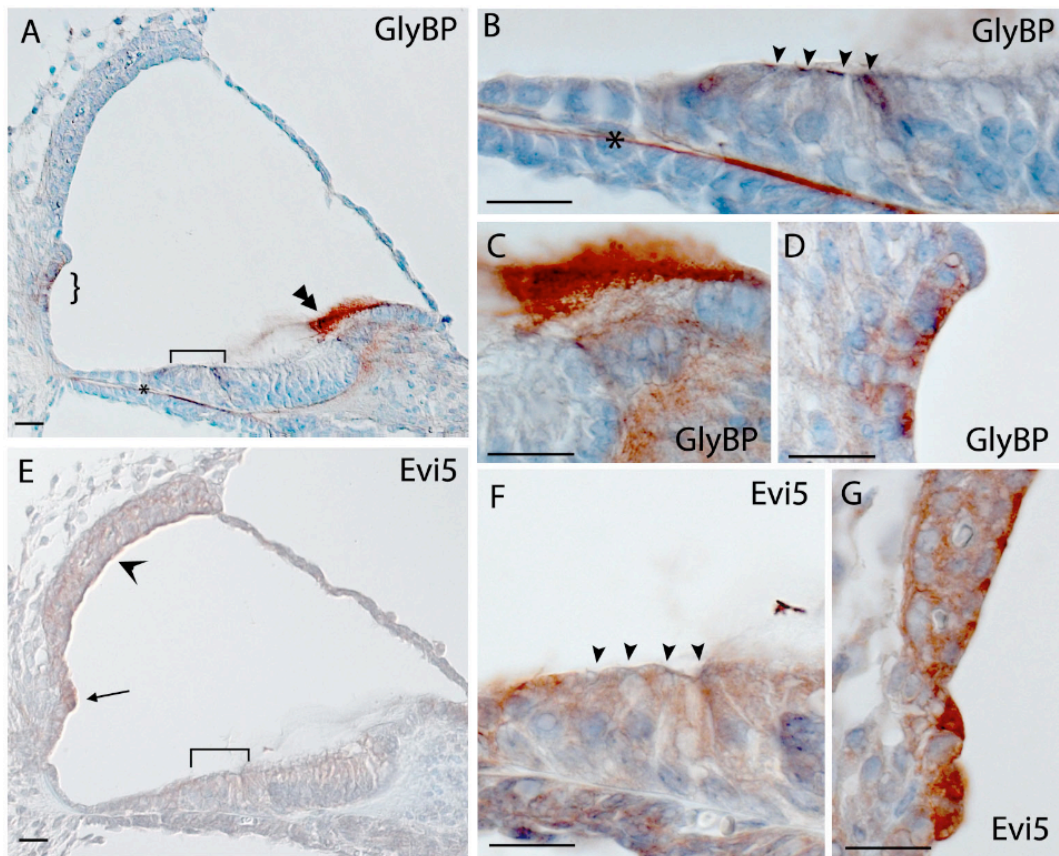
Figure 5. Immunohistochemistry in the mouse cochlea at P5

Brown signals indicate positive staining. A), B, C, D) Expression of *GlyBP* showing staining in hair cells (bracket in A, arrowheads in B), tectorial membrane (double arrowhead in A), root cells (curly brackets in A) and basilar membrane (asterisk in A and B). E, F, G) Staining of *Evi5* localized throughout the cochlea, including hair cells (bracket in E, arrowheads in F) and spiral prominence and stria vascularis (arrow and open arrowhead in A respectively, G). Scale bar indicates 20*µ*M.
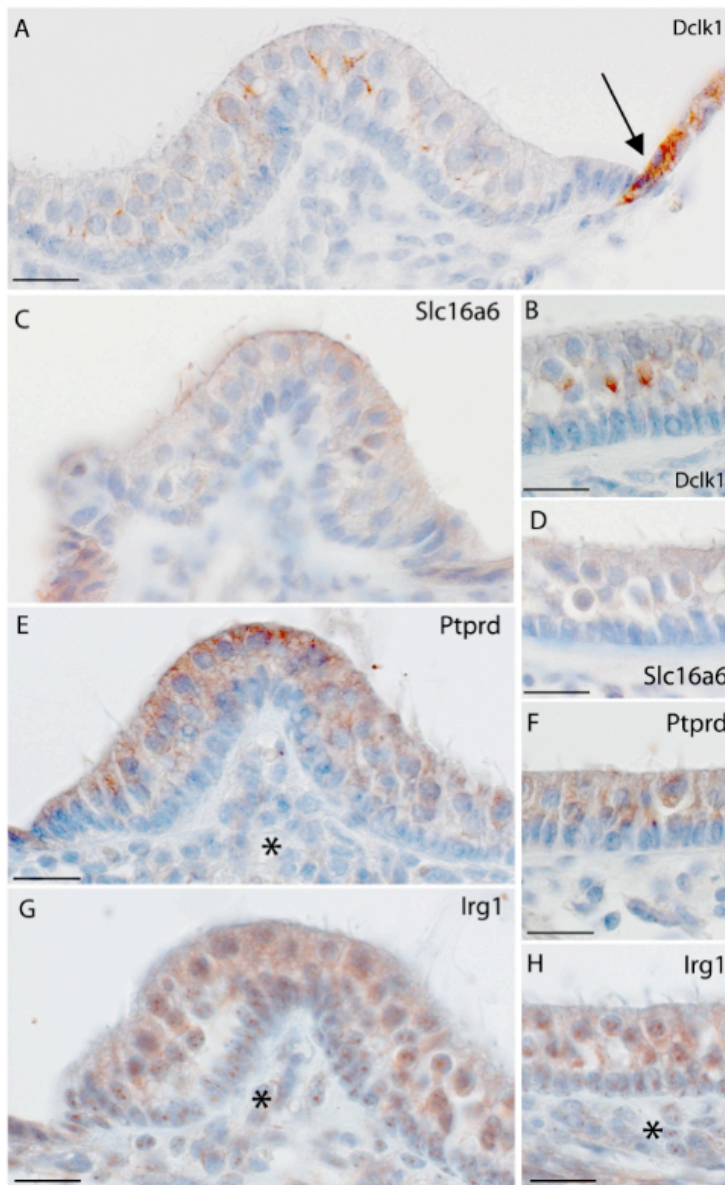
Figure 6. Immunohistochemistry in the mouse vestibular system at P5

Brown signals indicate positive staining. A, B) Expression of *Dclk1*, showing staining in the dark cells of the crista (arrow, A) and in supporting cells of the macula (B); C, D) *Slc16a6* showing a weak staining in the hair cells and supporting cells of the cristae (C) and maculae (D) of the vestibular system. E), F) Staining of *Ptprd* in the hair cells and in the supporting cells of the cristae (E) and the maculae (F) of the vestibular system and a faint staining in the neural dendrites (asterisk, E); G), H) Expression for *Irg1* is clear in the hair cells and in the supporting cells in the cristae (G) and maculae (H) and in the neural dendrites of the vestibular system (asterisks in G and H). Scale bar indicates 20μM.
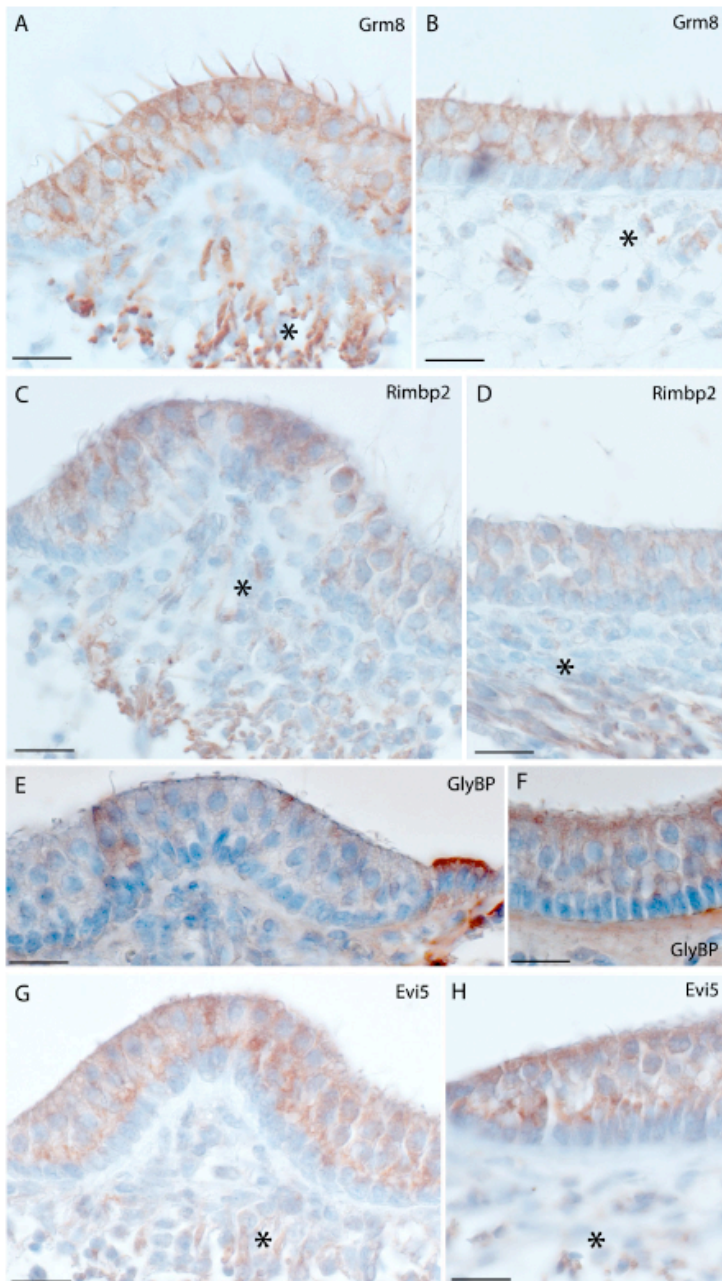
Figure 7. Immunohistochemistry in the mouse vestibular system at P5

Brown signals indicate positive staining. A, B) Strong staining of *Grm8* is present in the hair cells and in the supporting cells in the cristae (A) and in the maculae (B) of the vestibular system as well as in the neural dendrites (asterisks in A and B); C, D) Expression of *Rimbp2* showing staining in the hair cells and in the supporting cells in the cristae (C) and in the maculae (D) as well as in the neural dendrites of the vestibular system (asterisks in C and D); E, F) *GlyBP* expression could be seen in the hair cells, in the dark cells and in the supporting cells in the cristae (E) and in the maculae (F) of the vestibular system. G, H) Staining of *Evi5* is present in the hair cells and in the supporting cells in the cristae (G) and in the maculae (H) of the vestibular system and in the neural dendrites (asterisks in G and H). Scale bar indicates 20*µ*M.

3.3 Environmental/lifestyle factors

Among a series of analyzed variables such as smoking, drinking of spirits (e.g. yes/no), chocolate, tea, wine, beer, dairy products, coffee, and coffee intake (2-3 cups per day) only the last two showed a significant association with better hearing function in four out of ten populations investigated. In particular, coffee drinking was associated with better hearing at low and high frequencies (Figure 8; P=0.006 in Southern Italy, P=0.017 in Azerbaijan, P=0.016 in Tajikistan and P=0.038 in Sardinia).
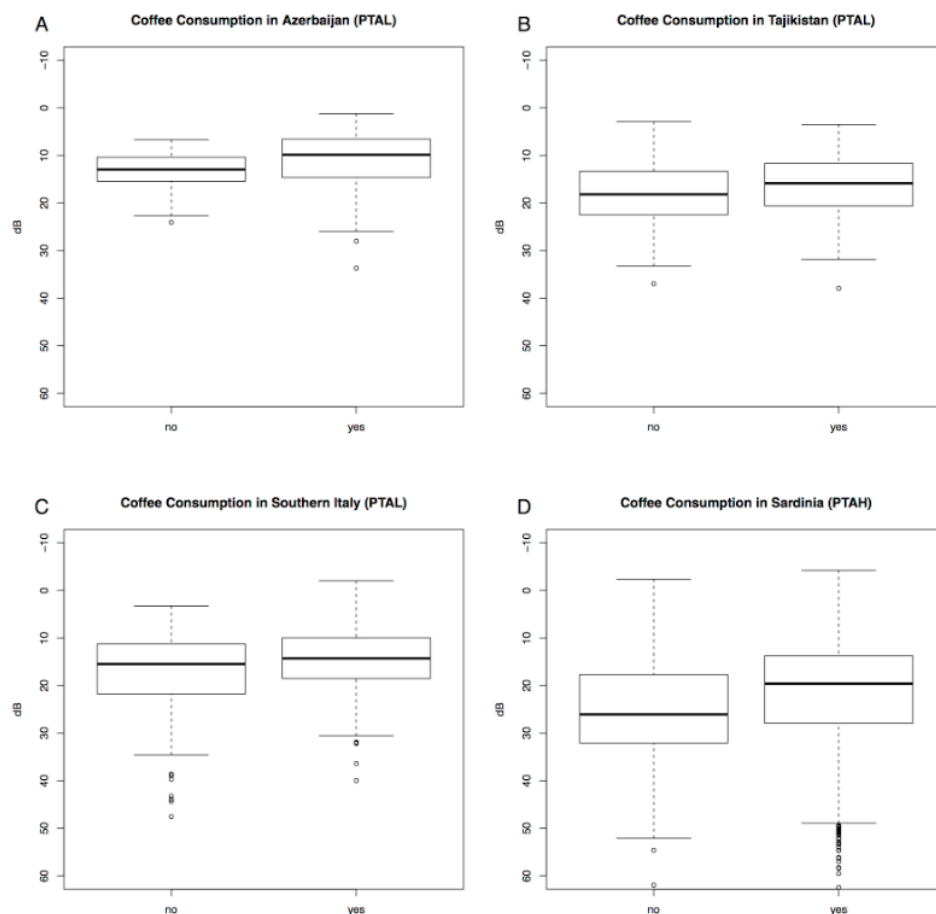
Figure 8. Coffee drinking and hearing trait

Boxplots represent hearing trait (PTAL and PTAH adjusted for sex and age), expressed in decibels (dB) along the y-axes referred to coffee-drinkers (labelled "yes") and coffee non-drinkers (labelled "no") on the x-axis. The difference in hearing ability at PTAL between coffee-consumers and non-drinkers is reported in four communities: Azerbaijan (A), Tajikistan (B), Southern Italy (C) and for PTAH in Sardinia (D). Notice that lower values correspond to better hearing.

As regards the intake of coffee (number of cups/day) we found an association between an intake of 2/3 cups/day (P=0.01, P=0.003, respectively) and better hearing at high frequencies (Figure 9). No association was found in the other populations, neither with other substances.
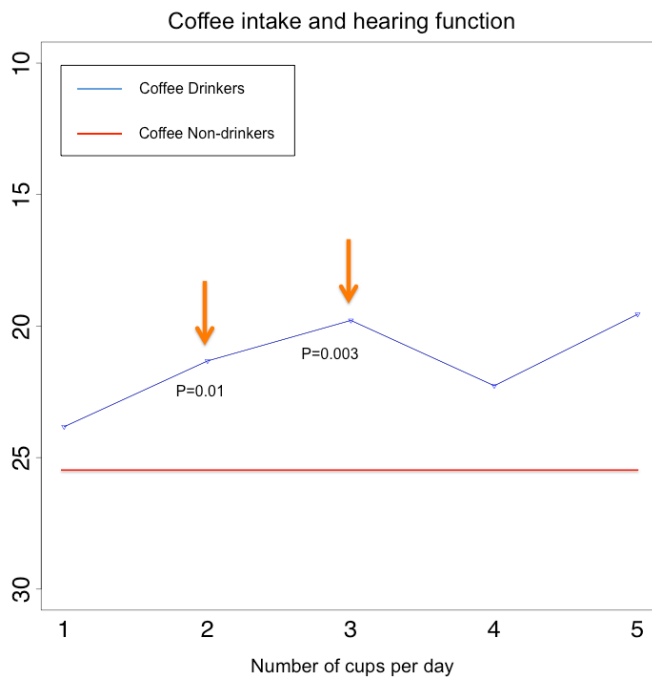
Figure 9. **Coffee intake and hearing function**

Blue line indicates the average of the hearing profile at PTAH for coffee consumers in the four populations where the association was detected. Red line shows the average of PTAH in non-consumers group.

On ARHL trait, educational/occupational factors have been tested. An association between education level and ARHL was found. Moreover, after detecting a suggestive disproportion in the distribution of cases across education levels, the association was tested using a mixed logistic regression model. Thanks to this method, we were able to overcome the complex structure of the data and to increase the statistical power. After excluding people with missing values, the overall number of people included in each categories was: 44 with no education, 918 with primary school, 863 with secondary school, 556 with high school and 185 with

university. The distribution of cases and controls is represented in Figure 10. The higher percentage of cases mainly belong to lower levels of education. For this reason we decided to better investigate any association between ARHL and the level of education. In fact, a statistical significant association between ARHL and education was detected (lowest P=0.0003). Moreover, people with no education showed a higher risk of developing the disease as compared with people with a higher education (the difference between the levels has P<0.001). Several explanations could be taken into account including individual jobs (classified as unskilled manual, skilled manual, skilled non-manual and, professional work); in fact a strong correlation (ϕ>0.60) between a noise-exposed job and level of education was also found.
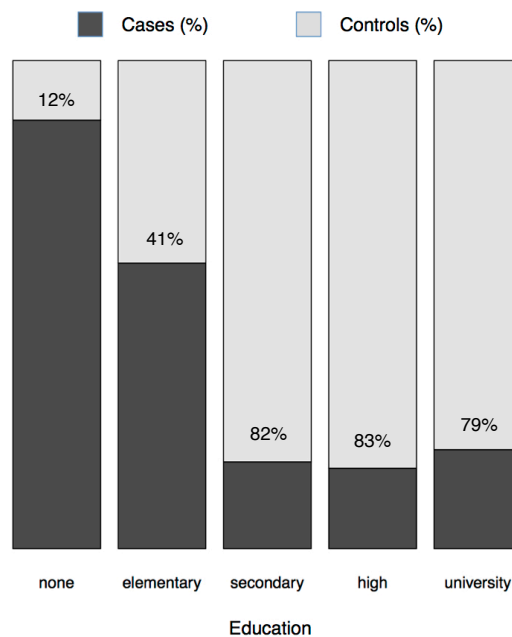

Figure 10. ARHL and level of education

The barplot shows the percentage of cases (dark grey) and controls (light grey) for each level of education considered. Cases are prevalent among lower education, while controls belong mostly to higher education levels.

4. DISCUSSION

4.1 Discussion genetics and expression studies

GWAS became the tool of choice for the identification of genes for quantitative and qualitative traits, since they are able to analyze large amounts of data. By this way it is possible to identify common variants resulting from very old mutations, each one adding to the risk of the trait in an individual. Moreover, the use of genetic isolates largely increases the possibility of detecting rare variants.

Despite recent progress, almost nothing is known about the molecular bases of variation of normal hearing or ARHL, apart from genes identified as being directly involved in HHL and one gene recently described for ARHL in human (Friedman R.A. et al. 2009). Here, four different kinds of GWAS have been performed: three on quantitative trait (one sex separated) and one on qualitative trait (ARHL). This multi-analyses strategy was based on the following considerations: 1) enlarge the sample size in order to increase the statistical power of the analysis (from GWAS1 to GWAS2), 2) define a more precise age correction based on our experience (from GWAS1 to GWAS2), 3) check for the possible difference in hearing ability between male and females (GWAS3), and finally 4) focus on ARHL (GWAS4).

In order to further support our GWAS data, a first list of 23 candidate genes has been defined to be included in the expression studies in the ear by immunohistochemistry. Results showed that 13 of them are clearly expressed in the mouse cochlea.

These findings suggest that integrating gene expression in specific cell types within the cochlea of a series of genes identified by GWAS may be an effective filtering and discovery approach, allowing one to uncover novel weakly associated genetic variants.

Going into details, among the most relevant genes identified for hearing function, the encoded protein of DCLK1 is involved in several different

cellular processes, including neuronal migration in the developing brain and in maturation of nervous system (Lin P.T. et al. 2000). All of these mechanisms could be interesting on hypothesizing an important role of this gene in the development of sensitive neurons that we know essential for the hearing function. The expression pattern observed for this gene is clearly localized into the marginal cells of the stria vascularis into the cochlea. Because the stria vascularis is essential for the secretion of (K^+) into the endolymph and for maintaining its associated endocochlear potential (i.e. cochlear power supply), this gene could control the expression of ion channels essential for endolymph production. For this reason, variants in this gene might alter the endolymph homeostasis, a feature that enhances the electrochemical gradient across the top of hair cells, making them more sensitive.

Another interesting gene is *PTPRD*, a member of the protein tyrosine phosphatase (PTP) family. PTPs are known to be signaling molecules that regulate a variety of cellular processes including cell growth, differentiation, mitotic cycle, and oncogenic transformation. Moreover, studies of homologous genes in others species suggest the role of PTPs family in promoting neurite growth and regulating neurons axon guidance, both mechanisms important for neuronal development (Burgoyne A.M. et al. 2009). PTPRD belongs to the same family of proteins that includes PTPRQ that, if mutated, causes autosomal recessive non-syndromic hearing impairment (Shahin H. et al. 2010). Moreover, *Ptprq* is expressed throughout the lifetime of most hair cells in hair bundles in the mouse (Nayak G. et al. 2011) and is required for formation of the shaft connectors of the hair bundle, the normal maturation of cochlear hair bundles, and the long-term survival of high-frequency auditory hair cells (Goodyear R.J. et al. 2003). A similar role might be hypothesized on the basis of the present results for the *PTPRD* gene.

Another interesting gene is *GRM8* a member, together with *GRM7*, of the group III of metabotropic glutamate receptors (GRMs) family that are neurotransmitter receptors (Cartmell J. et al. 2000). This family has been

divided into three groups on the basis of sequence homology, putative signal transduction mechanisms, and pharmacologic properties (Friedman R.A. et al. 2009, Scherer S.W. et al. 1997). GRM8 and GRM7 proteins show 87% of homology and 76% of identity using BLAST analysis (Friedman R.A. et al. 2009) and *GRM7* has been recently reported to be a candidate for involvement in ARHL (Friedman R.A. et al. 2009; Schulz H.L. et al. 2002). Consequently the strong expression of *Grm8* in the mouse cochlea but also in the spiral ganglion, supports a role for this gene. *Grm8* is located close to the recessive deafness loci DFNB14 and DFNB17 on chromosome 7, but falls outside of the reported loci (Mustapha M. et al. 1998, Greinwald J.H. et al. 1998).

Additional strong biological candidates are: *RIMBP2, GABRG3* and *CSMD1.* RIMBP2 is a member of a family of proteins that act as binding partners of the presynaptic active zone proteins RIMs (Mittelstaedt T. et al. 2007) as well as for voltage-gated Ca^{2+}-channels, such as CACNA1B and CACNA1D, the latter already known to be involved in deafness (Baig S.M. et al. 2011). In this light, the strong staining of this protein in the organ of Corti as well as in root cells, root cell processes and in the spiral prominence and the possible interaction with CACNA1D could consequently indicate an important role in hearing function. RIM-binding proteins (RIM-BPs) were suggested to form a functional link between the synaptic-vesicle fusion apparatus and Ca^{2+} channels (Mittelstaedt T. et al. 2007). Because the influx of (Ca^{2+}) ions is fundamental to depolarize the hair cell and to stimulate the release of neurotransmitter at the basal pole of the hair cells (Sziklai I. et al. 1996), any mutations in RIMBP2 could interfere with synaptic activity. Interestingly, RIMBP2 maps within the DFNA41 locus associated with dominant-inherited progressive hearing loss, very close to the marker showing the highest lod score (Blanton S.H. et al. 2002).

GABRG3 is a gamma-aminobutyric acid (GABA) A receptor, gamma 3. Very interestingly, expression studies already demonstrated a reduction of Gabrg3 expression levels in middle aged, old mild and severe presbycusic

mice when compared to young adult mice (Friedman R.A. et al. 2009). In addition, other members of this family have already been associated with hearing loss (Maison S.F. et al. 2009, Dong S. et al. 2010). Moreover, GABAergic component of the olivocochlear system contributes to the long-term maintenance of hair cells and neurons in the inner ear (Maison S.F. et al. 2006). The expression pattern of this gene very well localized in the inner and outer hair cells of the cochlea and all the literature findings reported above, further increase the importance of this gene as potentially involved in ARHL.

Also found with the lowest p-value at PC1 was *CSMD1*, a CUB and Sushi multiple domains 1. Analysis of Csmd1 mRNA expression by in situ hybridization and immunolabeling of neurons indicates that the primary sites of synthesis are the developing CNS and epithelial tissues (Kraus D.M. et al. 2006). Despite this, the immunohistochemistry analysis of this gene in the ear showed a clear staining localized in the stereocilia of the hair cells in the organ of Corti. The stereocilia bundles are sensory hairs linked each other with filamentous connections that led to the opening and the closing of the channels near the top of the hair cells. Because many molecules expressed in the stereocilia have already been related with hearing loss, we hypothesize that this gene, if mutated, could be associated with hearing impairment. Very interestingly a linkage study carried out on HHL identified a pedigree with non-syndromic deafness due to a homoplasmic mitochondrial mutation whose phenotype was modified by a locus defined by a marker which is within *CSMD1* gene (Bykhovskaya Y. et al. 1998). In support of this, it is very well known that mitochondrial DNA (mtDNA) mutations have been implicated in various age-related diseases such as ARHL. In fact, experimental evidence suggests that mitochondrial dysfunction associated with reactive oxygen species (ROS) plays a central role in the aging process of cochlear cells (Someya S. et al. 2010.).

Little is known about *GlyBP/KIAA0562*, a gene primarily associated with PTAH. It is expressed in the hair cells and in the border cells of the organ

of Corti, as well as in the basilar membrane and in the tectorial membrane. The inner ear is responsible for transforming the mechanical energy of the sound waves into electrical stimuli, and its function relies critically on the integrity of the extracellular matrix of the tectorial membrane and the basilar membrane in order to achieve proper mechanical stimulation of the cochlear sensory cells. For this reason, variants/mutations in this gene might affect this function (Dror A. et al. 2010).

Found to be principally associated at low frequencies were *ARSG* and *SLC16A6* that are located in the same locus. *ARSG* is a gene involved in hormone biosynthesis, modulation of cell signaling, and degradation of macromolecules. This protein is strictly localized to the apical surface of the outer and inner hair cells. Because the cochlear hair cells translate the mechanical forces evoked by sound into an electrical signal, any variant in this gene could alter the sensitivity of hearing. *SLC16A6*, belongs to the solute carrier (Slc) family, which includes several members encoding anion transporters and related proteins. Several members of this family have already been associated with different forms of deafness (Liu X.Z. et al. 2003). GWAS findings, coupled with the restricted expression of Slc16a6 at the top of outer hair cells in a specific mouse background, further support a relevant role of this gene in hearing function. Because the cochlear amplifier relies on an active process located in the outer hair cells, variants of this gene might cause a change in the amount of amplification provided by these hair cells.

Two additional genes detected at low frequencies (125 Hz and 500Hz) are *ANK2* and *CDH13*, both clearly expressed in specific cells type in the cochlea. Unfortunately, no data are provided about hearing function/loss in K/O mice models, apparently (Scotland P. et al. 1998, Hebbard L.W. et al. 2008). In particular, members of the Ankyrin's family link many different proteins like the TRPN proteins and a member of the TRPN family (TRPA1 channel) which has 17 ankyrin repeats, is expressed in mammalian inner ear hair cells and seems to be a candidate for the

mechanosensitive channel responsible for hearing (Gaudet R. et al. 2008). As regards cadherins, they are calcium dependent cell adhesion proteins and they preferentially interact with themselves in a homophilic manner in connecting cells (El-Amraoui A. et al. 2010). They have already been associated with hearing loss, most likely playing a fundamental role in the tip-link between the hair bundle of the hair cells (Sotomayor M. et al. 2010).

EVI5 gene, found to be associated with the 2kHz threshold, encodes a protein recently reported (Faitar S.L. et. 2005) as a novel centrosomal protein that binds to alpha and gamma tubulin, essential components of microtubules. Moreover, tubulin isoforms are enhanced in sensory hair cells and five different supporting cells (inner and outer pillar cells, Deiters cells, cells of Kölliker's organ, and cells of the tympanic covering layer) (Tannenbaum J. et al. 1997). Thus, the expression throughout the cochlea and in particular in the hair cells, might indicate EVI5 protein as an important player in translating mechanical forces evoked by sound into an electrical signal.

Finally, we should consider *IRG1*, whose expression is strictly localized in the nuclei of the cells of the organ of Corti and in the root cells. This specific pattern of expression might suggest an important role in regulating hearing function.

Interestingly, among genes identified to be associated with hearing in males we should mention *SMARCA4*, which belongs to a family of proteins with helicase and ATPase activities. On the contrary, in females *MMP8* was detected. It belongs to the matrix metalloproteinases family, whose members are very well known to play a significant role in inner ear structure and function (Kundu S. et al. 2012). For example, a polymorphism in *MMP1* has been also demonstrated to increase risk of sudden deafness in Korean population (Nam S.I. et al. 2011). Moreover, Hu et al. also demonstrated that in rats, MMPs and their related genes participate in the regulation of cochlear responses to acoustic overstimulation and that the modulation of MMP activity can serve as a

novel therapeutic target for the reduction of noise-induced cochlear damage (Hu B.H. et al. 2012). The most significant hints mark different genes in females as compared to males further supporting the possible role of specific gender factors such as hormones, jobs, etc.

As regards qualitative analysis, considering the p-value, the sample size and the lack of immunohistochemistry data, I consider them as preliminary ones. They need to be further replicated or obtained in even more large cohorts. Despite this, *EDN3* gene is worth to be mentioned as well as the absence of genes known to involved in aging processes.

In conclusion, our approach clearly confirms the usefulness of combining candidate gene data from GWAS with expression studies to further support the role of novel genetic associations including those with weak effects, which cannot be distinguished from spurious associations (due to low power) using a standard GWAS approach alone. In this light, the specific expression patterns of many GWAS candidate genes suggests that the standard correction for multiple testing may lead to cautious calls of significance. Even when SNPs show only suggestive association rather than significant association with the trait, consideration of expression data can be used in a Bayesian approach to assessing the significance of the observations.

4.2 Discussion Environmental/lifestyle factors

An association between coffee drinking/intake and a better hearing function in four different populations/communities has been identified and explanations could be many. Recently, experiments in animal models have shown that trigonelline, one of the main active compound of coffee extract (Allred K.F. et al. 2009), or other coffee compounds can potentially facilitate recovery from pyridoxine-induced auditory neuropathy (Hong B.N. et al. 2009). Another possible mechanism of action could be related to coffee's high content of various polyphenols which play a role against several oxidative stress related diseases including hearing loss and presbycusis (Ewert D.L. et al. 2012, Cascella V. et al. 2012, Sergi B. et al.

2004). Although the coffee antioxidant properties could depend on many different factors including the bean type and the roasting process, a recent study reported that coffee contains higher antioxidant concentration as compared to red wine, herbal teas, cocoa etc. (Krell J. et al. 2012). In this light, it is possible to hypothesize that antioxidants contained in the coffee interacts with the specific environment/genetic background of these four populations influencing in some way the hearing function. Moreover, the beneficial effect of coffee on hearing functions might go beyond anti-oxidation, thus involving mechanisms not yet known as suggested by recent data underlying the controversial role of antioxidants in ARHL (Sha S.H. et al. 2012, Gopinath B. et al. 2011). As far as coffee intake is concerned, our findings suggest that not only the consumption but also an optimal quantity (2-3 cups per day) of coffee is related to better auditory function. In this light, results could suggest that the consumption of more than 3 cups per day does not improve the audiometric profile. Interestingly, in the four communities there is a different way of preparing and drinking coffee. In particular, Italians drink mostly espresso while in Caucasus and Central Asia people mainly drink Turkish or instant soluble coffee. Thus, the positive effect is probably mediated by coffee compounds regardless of the different ways of preparing it.

As regards ARHL and the level of education, to date, it has been proven that the socio economic status and in particular the level of education can be considered as an indirect marker for many risk factors (that can be difficult to investigate one by one), and become good predictors for the risk of developing ARHL. Sixt E. et al. 1997 have already found evidence of correlation between low social class, low education level and ARHL in a single outbred cohort of Swedish people. Our data clearly confirm these findings in cohorts coming from different parts of Europe, Caucasus and Central Asia indicating that a low education level represents a risk factor for ARHL. Many possible explanations could explain this finding. First of all, as expected, there is a very high correlation between education and occupation; quite often people with a low education perform a noise-

exposed job. Furthermore, in principle, they might be also more exposed to ear infection and pay less attention to sense organ stimulation and preservation. In fact many different studies have proven that poor social conditions associated with deprived neighbourhood constitute one of the most relevant environmental risk factors for public health. Moreover, low education and insufficient employment opportunities may affect individual's self-esteem, social status, and consequently health in a net of concurrent causes hard to break down individually (Poortinga W. et al. 2012). On the other hand, people attending university courses are usually dedicated to professional activities (teaching and similar) that stimulate their sense of hearing and pay more attention to their hearing health status. In this light, education can reflect the socioeconomic status of the family of origin and hence influence personal habits for a subject, such as health care, hygiene, prevention and awareness of medical conditions.

4.3 Conclusion and future prospects

During these three years of PhD, results led to:

1. the identification of a series of new genes for quantitative and qualitative traits using GWAS and meta-analysis approaches

2. the support of the role of some of them using expression studies in the mouse inner ear

3. the detection of environmental/lifestyle factors involved in hearing function and ARHL

Preliminary results prove the useful combination of GWAS and expression studies providing new insights into the molecular basis of hearing function and ARHL. Moreover, these findings, together with the understanding of lifestyle/environmental factors involved in these complex traits, contribute to define new targets for hearing impairment treatment and prevention.

The next plan is:

1. to increase our population cohorts recruiting other samples

2. to replicate our candidate genes in other cohorts

3. to perform whole genome sequencing in a subset of individuals

4. to identify causative functional variants and their role in ARHL disease pathogenesis.

5. to further improve our methods of statistical analysis (i.e using new algorithms, software and tools)

In particular, in order to increase the sample size, a new sample collection is in progress in some isolated Sardinian villages (Urzulei and Seulo). Moreover, we recently imputed all of our samples up to 1000 genome in order to have accurate haplotype information on all forms of human DNA polymorphism in the hope of increase the power of our analysis (1000GP et al. 2010 et al. 2010). In fact, thanks to this improvement, a new GWAS will be performed. In addition to the additive model which displays most of the common genetic variants, future analyses will include dominant, over-dominant and recessive model that could be the alternative options providing in some cases (presence of certain rare alleles) strongest associations (Salanti G. et al. 2009). As regards replication of the data, it is already in progress in an outbred population (1958 British birth cohort in collaboration with Sally Dawson, UCL-UK).

Moreover, considering that: 1) replication of data obtained from isolated populations may be tricky; 2) rare variants, will require sophisticated analysis methods, and different functional studies; 3) common variants explain only a modest fraction of the genetic components of human common diseases, and 4) a large part of the heritability is still missing, Whole Genome Sequencing is now in progress in a subset of individuals as well as genotyping of 250.000 functional variants to increase genetic information.

Websources

http://hereditaryhearingloss.org/

http://hearingimpairment.jax.org/index.html

http://www.r-project.org/

http://www.ncbi.nlm.nih.gov/gene/

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