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Bruijn, S.E. de; Smits, J.J.; Liu, C.; Lanting, C.P.; Beynon, A.J.; Blankevoort, J.; ... ; DOOFNL Consortium

Original research

A *RIPOR2* in-frame deletion is a frequent and highly penetrant cause of adult-onset hearing loss

Suzanne E de Bruijn ,[1,2] Jeroen J Smits ,[2,3] Chang Liu,[4] Cornelis P Lanting,[3] Andy J Beynon,[3] Joëlle Blankevoort,[1] Jaap Oostrik,[2,3] Wouter Koole,[1] Erik de Vrieze,[2,3] Cor W R J Cremers,[3] Frans P M Cremers,[1,2] Susanne Roosing,[1,2] Helger G Yntema,[1,2] Henricus P M Kunst,[3,5] Bo Zhao,[4] Ronald J E Pennings,[2,3] Hannie Kremer,[1,2,3] DOOFNL Consortium

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[1]Department of Human Genetics, Radboudumc, Nijmegen, The Netherlands
[2]Donders Institute for Brain, Cognition and Behaviour, Radboudumc, Nijmegen, The Netherlands
[3]Department of Otorhinolaryngology, Radboudumc, Nijmegen, The Netherlands
[4]Department of Otolaryngology-Head and Neck Surgery, Indiana University School of Medicine, Indianapolis, Indiana, USA
[5]Radboud Institute for Health Sciences, Radboudumc, Nijmegen, The Netherlands

Correspondence to
Professor Hannie Kremer, Department of Otorhinolaryngology and Human Genetics, Radboudumc, Nijmegen 6500 HB, Netherlands;
Hannie.Kremer@radboudumc.nl

SEdB and JJS contributed equally.
RJEP and HK contributed equally.

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ABSTRACT

Background Hearing loss is one of the most prevalent disabilities worldwide, and has a significant impact on quality of life. The adult-onset type of the condition is highly heritable but the genetic causes are largely unknown, which is in contrast to childhood-onset hearing loss.

Methods Family and cohort studies included exome sequencing and characterisation of the hearing phenotype. Ex vivo protein expression addressed the functional effect of a DNA variant.

Results An in-frame deletion of 12 nucleotides in *RIPOR2* was identified as a highly penetrant cause of adult-onset progressive hearing loss that segregated as an autosomal dominant trait in 12 families from the Netherlands. Hearing loss associated with the deletion in 63 subjects displayed variable audiometric characteristics and an average (SD) age of onset of 30.6 (14.9) years (range 0–70 years). A functional effect of the *RIPOR2* variant was demonstrated by aberrant localisation of the mutant RIPOR2 in the stereocilia of cochlear hair cells and failure to rescue morphological defects in RIPOR2-deficient hair cells, in contrast to the wild-type protein. Strikingly, the *RIPOR2* variant is present in 18 of 22 952 individuals not selected for hearing loss in the Southeast Netherlands.

Conclusion Collectively, the presented data demonstrate that an inherited form of adult-onset hearing loss is relatively common, with potentially thousands of individuals at risk in the Netherlands and beyond, which makes it an attractive target for developing a (genetic) therapy.

INTRODUCTION

Hearing loss (HL) is one of the most prevalent disabilities worldwide[1] and genetic factors importantly contribute to this condition. So far, 118 genes have been associated with non-syndromic forms of sensorineural HL and variants in these genes explain a significant part of subjects with an early onset of HL (ie, congenital or in childhood).[2–4] Our knowledge of the genetic architecture of adult-onset HL is limited despite a high heritability which is estimated to be 30–70%.[5–7] Differences in phenotypic parameters that are used and age ranges of study participants may well contribute to the variation in the reported heritability. As summarised by Lewis *et al*,[8] genome-wide association studies (GWAS) of hearing status in adults and genetic analyses of families with dominantly inherited post-lingual onset HL indicate that both common variants and rare variants contribute to adult-onset HL with a small and large effect size, respectively. Such variants may or may not affect genes that are already known to function in the auditory pathway.

Previously, we identified a 12.4 Mb locus for adult-onset HL on chromosome 6 (p24.1–22.3): DFNA21.[9 10] However, the underlying pathogenic variant in the studied family (W97-056) remained elusive. Here, we present the identification of an in-frame deletion (c.1696_1707del; NM_014722.3) in *RIPOR2* (RHO Family Interacting Cell Polarisation Regulator 2 (MIM: 611 410)) to underlie autosomal dominant non-syndromic HL (adNSHL) in this family and in 11 additional (large) families of Dutch origin. The allele frequency (AF) of this variant suggests that it potentially explains adult-onset HL in thousands of individuals in the Netherlands and northwest Europe. Our study expands the phenotypic spectrum associated with *RIPOR2* defects which had so far only been described to underlie early-onset recessively inherited HL.[11]

METHODS

Study approval

The study of human subjects was approved by the medical ethics committee of the Radboudumc (registration number: NL33648.091.10) and performed in accordance with the principles of the World Medical Association Declaration of Helsinki. Written informed consent was obtained from all participants or their legal representatives. All animal experiments were approved by the Institutional Animal Care and Use Committee of Indiana University School of Medicine (registration number 19075).

DNA sequencing

Next generation sequencing was performed for identification of DNA variants. Details of employed sequencing techniques are provided in online supplementary methods.

Variant interpretation

For exome sequencing and molecular inversion probe (MIP) datasets, annotated variants were filtered based on a population AF of ≤0.5% in the gnomAD database v.2.1, and our in-house exome database (~15 000 alleles). Variants in coding and splice site regions (−14/+14 nucleotides) were analysed. Interpretation of missense variants was performed using the in silico tools CADD-PHRED (≥15),[12] SIFT (≤0.05),[13] PolyPhen-2 (≥0.450)[14] and MutationTaster (deleterious)[15] to predict potential functional effects. Variants were considered if a pathogenic effect was predicted by at least two different tools. Potential effects on splicing of missense and synonymous variants were evaluated using the algorithms embedded in the AlamutVisual software (v.2.10, Interactive Biosoftware). A change of ≥5% in splice site scores predicted by at least two algorithms was considered significant. For candidate variants, segregation analysis was performed by Sanger sequencing. PCR conditions are available on request.

Clinical evaluation

Medical history was taken from all participants with special attention paid to acquired and noise-induced HL. Both affected and unaffected participants underwent general ear nose and throat (ENT) examinations, or this medical information was obtained from previous examinations. Age of onset of HL was reported by the subjects themselves. Only reports of a specific age of onset were used in the calculations. The audiometric data in this study are described according to GENDEAF guidelines.[16] Pure tone- and speech- audiometry and click-evoked auditory brainstem response (ABR) was performed in a sound-attenuated booth, according to current standards (International Organisation for Standardisation: ISO 8253-1:2010, ISO 389–1, ISO 389–5 and ISO 389–6).[17] Individuals were considered affected when pure tone thresholds for at least three individual frequencies were below the frequency-specific 95th percentile of age- and sex-specific thresholds (ISO7029:2017) for the best hearing ear. HL was considered asymmetric if pure tone audiometry showed a difference of more than 10 dB between both ears at two individual frequencies.[16] Longitudinal (individual) progression of HL was calculated if there was a follow-up duration of at least 10 years, after onset of HL. The progression rate is defined as the mean increase pure tone average (PTA) at 0.5–4 kHz ($PTA_{0.5\text{-}4kHz}$) in dB/year between first and last audiometry. For symmetric HL, the average of both ears was used to calculate progression; for asymmetric HL, the best-hearing ear at first audiometry was used. In case of profound HL at 0.5–4 kHz at the latest audiometry, the most recent audiometry at which all thresholds at 0.5–4 kHz could be measured was selected. Cross-sectional linear regression analysis was applied on pure tone thresholds to calculate an age-related typical audiogram (ARTA),[18] using Prism 6.0 software (GraphPad). A k-means clustering analysis was performed as described in online supplementary methods.

Injectoporation of *Ripor2*-constructs and immunostaining

The generation of *Ripor2*$^{LacZ/LacZ}$ mice has been described previously.[19] For *Ripor2* DNA construct generation, *Ripor2* cDNA (NM_029679.2, without exon 13) was amplified from a mouse-cochlear cDNA library and cloned into a pEGFP-N3-derived vector from which the EGFP (enhanced green fluorescent protein) coding sequence was deleted. Procedures for injectoporation and immunostaining have been described previously,[19] and are detailed in online supplementary methods.

Immunoprecipitations and western blots

Cell culture, immunoprecipitations and western blots were carried out as described.[19 20] Experiments were carried out at least three times. Antibodies used are listed in online supplementary methods.

Methods and materials for VNTR (variable number of tandem repeats) marker analysis, vestibular testing and allele-specific expression analysis are provided in online supplementary methods.

RESULTS

Exome sequencing revealed an in-frame deletion in *RIPOR2*

To identify the genetic defect underlying the HL in family W97-056 (figure 1), exome sequencing was performed in three affected family members (III:22, IV:20 and IV:25). After applying the variant filtering and prioritisation described above, two variants were shared between the three affected individuals (online supplementary table 1). A *SPATS1* (MIM: 613 948) variant (c.419G>A; p.(Gly140Glu); NM_145026.3) did not completely segregate with HL within the family as seven out of 23 affected subjects did not harbour the variant (online supplementary figure 1). Also, *SPATS1* expression was not detected in the mammalian cochlea[21 22] and SPATS1 function has only been related to spermatogenesis.[23] Therefore, this variant was deemed non-causative. The in-frame deletion was present in exon 14 of *RIPOR2* (c.1696_1707del; p.(Gln566_Lys569del); NM_014722.3; Chr6:g.24,843,303_24,843,314del; rs760676508). It affects a highly conserved protein region of RIPOR2 which is present in all RIPOR2 isoforms (online supplementary figure 2). *RIPOR2* has previously been associated with recessively inherited early-onset hearing loss and is positioned 0.9 Mb centromeric of the *DFNA21* locus.[10 11] No copy number variants were detected that were shared by all three subjects.

Segregation analysis identified the *RIPOR2* variant in 20 of 23 affected subjects of family W97-056 (figure 1). The variant was not found in subjects III:14, III:20 and III:21; a recombination event in subject III:14 previously delimited the centromeric border of the *DFNA21* locus.[10] The *RIPOR2* c.1696_1707del variant was also found in three unaffected family members (V:2, age 23 years; IV:26, age 40 years; and III:28, age 51 years). The strong association of the *RIPOR2* variant with HL in this family urged us to further address this and other variants in *RIPOR2* in families with (adult-onset) HL.

The *RIPOR2* variant c.1696_1707del associates with adNSHL in 11 additional families

An exome sequencing dataset of 1544 index cases with (presumed) hereditary HL was evaluated for rare *RIPOR2* variants. In these cases, (likely) pathogenic variants in known deafness genes were previously addressed in a clinical diagnostic setting. The c.1696_1707del variant was identified in 10 index cases, all diagnosed with adNSHL (figure 2). Analysis of a dataset obtained through MIP sequencing of 89 HL-associated genes in 64 index cases with (presumed) adNSHL revealed another subject (V:1, W08-1421; figure 2) with this variant. No other rare *RIPOR2* variants (AF ≤0.5%) that met the variant filtering criteria were identified.

For six of the 11 index cases with the c.1696_1707del *RIPOR2* variant, family members were included in the study and segregation analysis was performed (figure 2). The variant was detected in 39 of 40 affected subjects, but not in subject III:10 of family W04-262. As observed in family W97-056, the *RIPOR2* variant was also found in unaffected subjects, namely III:14 of family

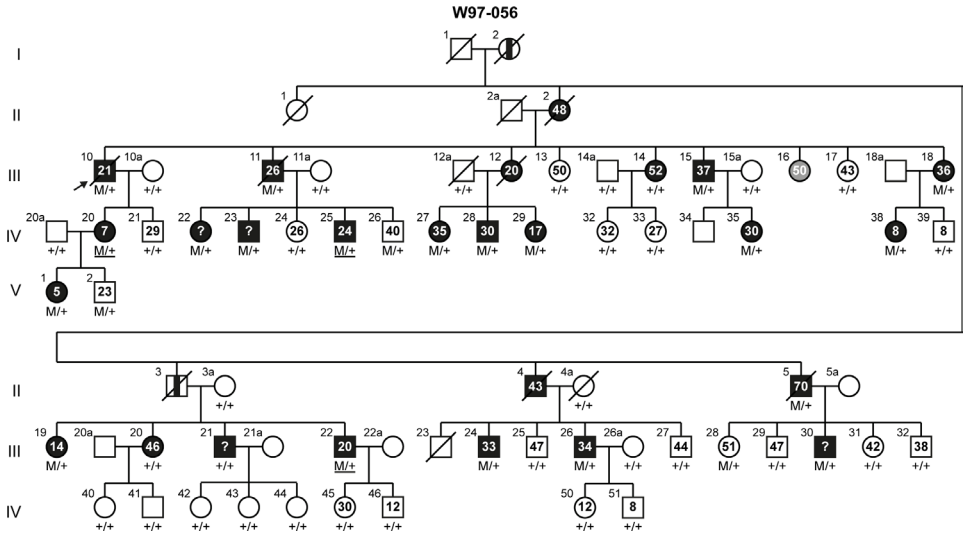

Figure 1 Pedigree of family W97-056 and segregation of *RIPOR2* variant c.1696_1707del for affected and unaffected family members. The age of onset of hearing loss or the age at the most recent audiometric evaluation are indicated in the pedigree symbols, respectively. Subjects who did not report an age of onset are indicated with a question mark. The index case is marked by an arrow. Exome sequencing was performed in subjects with an underlined genotype. Subjects determined to be affected by heteroanamnesis are indicated with a vertical black bar. The subject marked in grey is diagnosed with intellectual disability and excluded from further participation in this study. Subject identifiers correspond to those in de Brouwer *et al*.[10] . M, c.1696_1707del; +, wild-type.

W04-262 and III:4 of family W15-1495, aged 49 and 50 years, respectively.

For all 11 index cases, targeted re-analysis of sequencing data for known adNSHL-associated genes[4] was performed to reveal other (likely) pathogenic variants. No rare variants were identified that both segregated with HL in the family and were classified as (likely) pathogenic in ClinVar (online supplementary table 2).

The presence of an identical *RIPOR2* variant in 12 families of Dutch origin is suggestive for a common ancestor. Indeed, a shared haplotype of ~0.71 Mb, flanking the variant (D6S2439-D6S1281), was observed in the seven families and potentially the five single cases (online supplementary results, online supplementary figure 3).

Clinical evaluation of individuals with the c.1696_1707del variant

To characterise the HL associated with the c.1696_1707del *RIPOR2* variant, 200 affected and unaffected subjects from seven families and five single index cases were evaluated between 1997 and 2018. The *RIPOR2* variant was found to be present in 64 of the 200 subjects. Detailed clinical data per individual are provided in online supplementary table 3.

The mean (SD) reported age of onset is 30.6 (14.9) years with a wide range from congenital to 70 years (online supplementary figure 4). Evaluation of audiometric data showed that subjects with the *RIPOR2* variant have progressive sensorineural HL, ranging from mild to profound, with variable audiometric configurations (figure 3, online supplementary figures 5 and 6). In order to distinguish audiometric patterns, a k-means clustering algorithm, independent of subject age, was applied on the latest audiogram of each subject. This unbiased approach yielded four audiometric patterns, each with a distinct audiometric configuration (figure 4). Asymmetry of HL was seen in 16 cases. Inter-aural differences in progression of HL were also seen (figure 3C). For three subjects (III:30 of family W97-056; II:11 of family W04-262; and V:1 of W08-1421), an explanation for asymmetry was noted (online supplementary table 3).

Longitudinal analysis of HL in individual subjects revealed a large variation in progression of HL between subjects (online supplementary table 3). We could not identify a specific pattern, such as a certain progression (in dB/year (dB/y)) in certain decades. There was a median progression of 1.2 dB/y (range 0.5–2.7 dB/y) for the frequencies 0.5–4 kHz. Cross-sectional linear regression was applied to calculate ARTA (figure 3D). Progression ranged from 0.7 dB/y (0.25 kHz) to 1.3 dB/y (8 kHz). Progression of HL was significant for all frequencies (*F*-test, $p<0.0001$).

Speech reception thresholds were generally lower than, or comparable to, $PTA_{0.5\text{-}2kHz}$ (online supplementary table 3). This indicates absence of retrocochlear pathology and is in line with normal results of click-evoked ABR in four subjects (online supplementary table 3). CT and/or MRI of the bilateral temporal bones and cerebellopontine angle in six subjects revealed normal inner and middle ear anatomy (online supplementary table 3).

Vestibular testing, performed in 11 randomly selected subjects with the *RIPOR2* variant aged 29 to 71 years, led to the conclusion that c.1696_1707del *RIPOR2* is not associated with vestibular dysfunction (online supplementary table 4). Further details are provided in the online supplementary results.

Transcript levels of *RIPOR2* do not correlate with age of onset in affected subjects

We hypothesised that the variability in age of onset of HL associated with the c.1696_1707del *RIPOR2* variant might be explained by differences in expression levels of the wild-type allele. Alternatively, variants in cis-regulatory elements of the affected allele more distantly located from *RIPOR2* could influence expression levels of the mutant allele and might thereby modulate the age of onset. To test these hypotheses, allele-specific transcript levels of *RIPOR2* were determined in peripheral blood cells of 33 subjects using quantitative RT-PCR. Subjects were divided into three groups based on self-reported age of onset: <20 years (n=7),

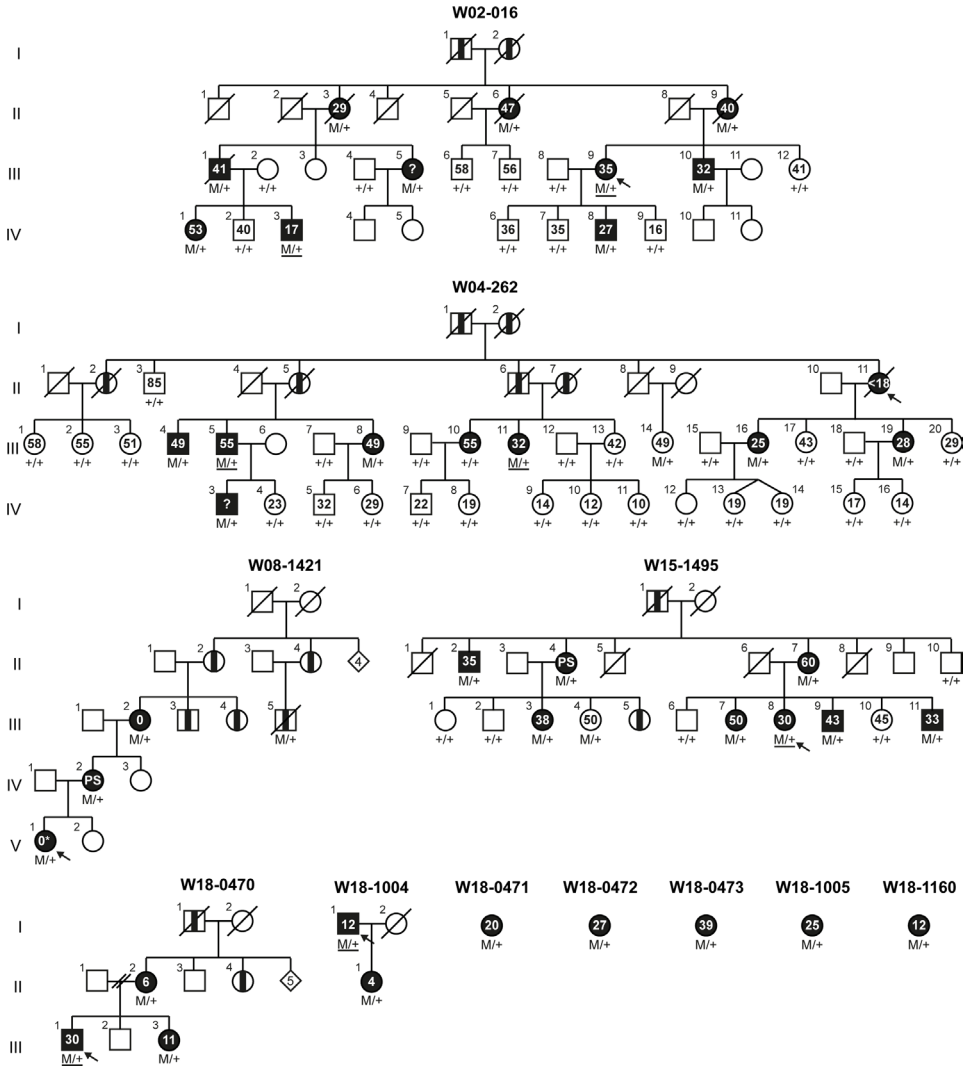

Figure 2 Family pedigrees and segregation of *RIPOR2* variant c.1696_1707del for affected and unaffected family members, the age of onset of hearing loss or the age at the most recent audiometric evaluation are indicated in the pedigree symbols, respectively. Subjects who did not report an age of onset are indicated with a question mark. Index cases are marked by arrows. Exome sequencing was performed in subjects with an underlined genotype. Subjects determined to be affected by heteroanamnesis are indicated with a vertical black bar. Based on the information provided in the questionnaires, an autosomal dominant inheritance pattern of hearing loss is likely for each of the single cases. M, c.1696_1707del; +, wild-type; PS, primary school.

20–39 years (n=15), and ≥40 years (n=6). No significant differences were observed between the different subject groups, neither for the wild-type or c.1696_1707del variant *RIPOR2* alleles nor for total *RIPOR2* transcript levels (online supplementary figure 7). Also, no difference was observed between the ratios of *RIPOR2* mutant to wild-type relative transcript levels. A small difference was observed in total *RIPOR2* transcript levels between subjects with an early onset of HL and controls (p=0.0241). This could suggest a trend between low expression levels and an early onset of HL; however, considering the overall variability in transcript levels it is more likely that other factors play a role. A larger sample size would be required to confirm or negate the observed trend.

The in-frame deletion in *Ripor2* prevents correct localisation of the protein in mouse cochlear hair cells

Previous studies have shown that RIPOR2 is specifically localised to the base of the stereocilia in mouse cochlear hair cells.[19] RIPOR2 is highly conserved between mouse and human (87% amino acid identity). To study whether the localisation of mouse RIPOR2 with a deletion of the orthologous four amino acid residues (p.584_587del) is altered, plasmids encoding wild-type or mutant RIPOR2 were injectoporated into cochlear outer hair cells of wild-type mice (P2). Interestingly, mutant-RIPOR2 was detected in the stereocilia but in none of the 12 evaluated cells was it retained at the stereocilia base where the wild-type protein was found to be localised in all 11 evaluated cells (figure 5A). Morphology of the stereocilia was not significantly affected 2 days after injectoporation of the mutant *Ripor2* construct, suggesting the mutant protein did not visibly affect the stereocilia structure in the short term.

Potentially, the variant affects interactions of RIPOR2 that are essential for its localisation. The four deleted amino acids are predicted to be part of a disorganised coiled coil structure (predicted using KMAD (knowledge-based multiple sequence alignment for intrinsically disordered proteins)[24]). Coiled coil regions are indicated to mediate protein–protein interactions, which supports the hypothesis that the variant affects RIPOR2 protein interactions.[25] Co-immunoprecipitation (Co-IP) assays demonstrated that both the dimerisation ability (online

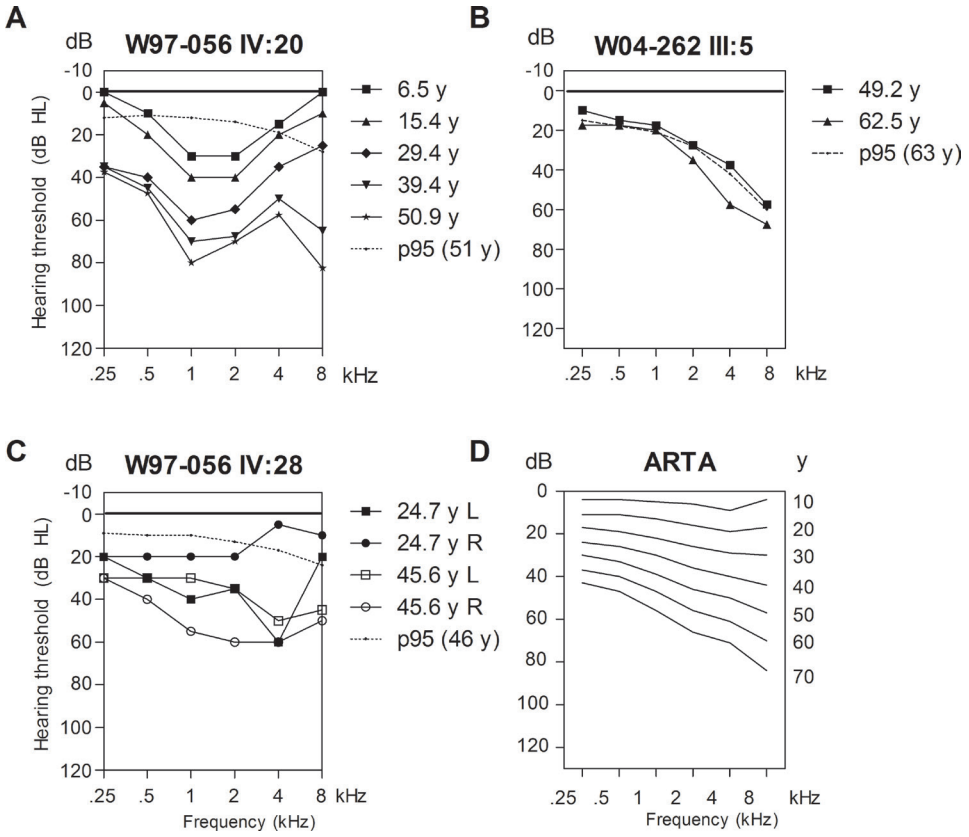

Figure 3 Selection of audiograms and the ARTA. (A–C) Air conduction thresholds of three selected individuals with the c.1696_1707del *RIPOR2* variant are depicted. For the individuals in panels A and B, hearing loss was symmetric and the average of left and right ear thresholds are depicted. For the individual in panel C, hearing loss was asymmetric and the thresholds for both right and left ears are depicted. The p95 values are matched to the individuals' sex and age at most recent audiometry, according to the iso 7029:2017 standard. (D) The age related typical audiogram (ARTA): cross-sectional linear regression analysis of last visit audiograms of affected subjects with the c.1696_1707del *RIPOR2* variant. dB HL, decibel hearing level; kHz; kilohertz; L, left; R, right; y, age in years; .

supplementary figure 8A) and the interaction with RHOC (Ras Homolog Gene Family Member C) (online supplementary figure 8B) of mutant RIPOR2 are intact.

Mutant RIPOR2 cannot rescue morphological defects in outer hair cells from *Ripor2* knockout mice

In *Ripor2* knockout mice, morphological defects were previously observed in hair cells, which included hair bundle polarity and cohesion and length of stereocilia.[19] After injectoporation of the *Ripor2* mutant construct into the outer hair cells of these mice, these defects could not be rescued in any of the 13 cells expressing mutant-RIPOR2. The typical V-shaped hair bundle was not formed, in contrast to the rescue effect observed in five out of six cells expressing wild-type RIPOR2 (figure 5B). This, together with the aberrant localisation of mutant-RIPOR2, confirms an effect of the 4-amino acid deletion on RIPOR2 function in outer hair cells.

DISCUSSION

This study identified an in-frame 12 nucleotide deletion in *RIPOR2* as a prevalent and highly penetrant genetic factor for adult-onset HL in the Netherlands and beyond. HL associated with the deletion is highly variable in age of onset and audiometric characteristics. Our study highlights that an increasing contribution of environmental factors and of low-penetrance genetic factors to hearing ability during life complicates the identification of highly penetrant genetic factors in adult-onset HL. This is best illustrated by family W97-056 in which the linkage interval was falsely delimited by a phenocopy.

The *RIPOR2* variant was significantly enriched in an in-house dataset, previously coined "SE-NL" (Southeast Netherlands) with exomes of 22 952 unrelated individuals with unknown hearing abilities.[26] Eighteen individuals were heterozygotes for the variant (AF 0.0392%), as compared with eight of 56 352 individuals (AF 0.0071%) and five of 32 287 individuals (AF 0.0077%) of non-Finnish European descent in the gnomAD exome database v.2.1.1 and gnomAD genome database v.3, respectively. As the variant was indicated to be inherited from a common ancestor, this individual might well be of Dutch origin or of neighbouring regions.

Several lines of evidence indicate the association of the c.1696_1707del *RIPOR2* variant with HL. First, the deletion affects four highly conserved amino acids of RIPOR2, which is known to have a crucial role in murine and zebrafish hair cell development, function and maintenance.[11 19 27] *Ripor2* knockout mice are already found to be deaf at 4 weeks of age due to impaired mechanotransduction.[19] Also, knockdown of *ripor2* in zebrafish induced loss of hair cells, and consequently profound hearing loss.[11] Second, aberrant localisation of the mutant RIPOR2 in early postnatal mouse hair cells, ex vivo, and failure to rescue the stereocilia defects of *Ripor2* knockout mice indicate a functional effect of the variant. Thirdly, neither other rare potentially causative variants in protein coding regions and splice sites of the shared haplotype region, nor structural

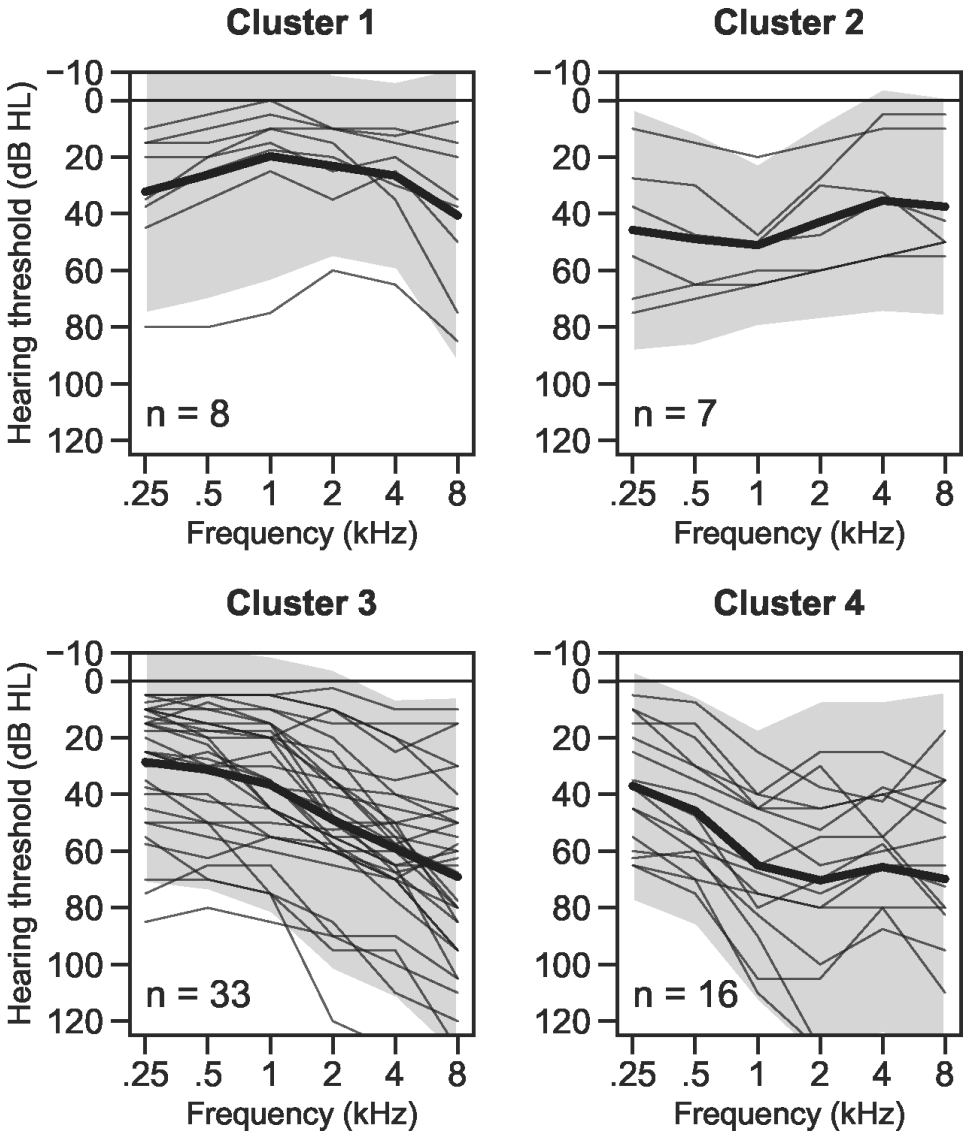

Figure 4 Four audiometric patterns of *RIPOR2*-associated hearing loss. Air conduction thresholds of all subjects were analysed with a k-means clustering protocol. The thick black lines depict the average of each cluster, the transparent grey areas represent the ±2 SD. Cluster 1: mild hearing loss (average ($PTA_{0.5\text{-}4\,kHz}$) 23 dB HL) with an inverse U-shape audiogram. Cluster 2: moderate hearing loss (average 48 dB HL), with relatively worse hearing in the lower frequencies. Cluster 3: moderate (average 39 dB HL) high-frequency hearing loss with a gently down sloping audiogram configuration (average of 28 dB HL difference between the mean of 0.5–1 and 4–8 kHz). Cluster 4: moderate (average 60 dB HL), mid-frequency hearing loss with a U-shape audiogram. Individual audiometry (online supplementary figure 5) shows relatively faster deterioration of higher frequencies later in life, for example W97-056 IV:20. dB HL, decibel hearing level; hl, kHz, kilohertz; PTA, pure tone average.

variants affecting this region, were revealed in exome or genome sequencing.

RIPOR2 is localised at the taper region of the mechanically sensitive stereocilia of murine hair cells[11 19 27] where it is organised in a ring-like fashion.[19] The latter is thought to be achieved by homo-oligomerisation in a head-to-head and tail-to-tail manner, regulated by RHOC.[19] The oligomerisation is essential for the structure of the taper region and for the morphology of the hair bundle as a whole, but the precise molecular mechanism is still elusive. The taper region is the specialised basal part of stereocilia that allows their deflection on mechanical stimulation.[28] CLIC5, PTPRQ, MYO6, TPRN, RDX, GRXCR2, and RIPOR2 are described to concentrate and co-function in the taper region and to be crucial for its structure and/or for hair bundle development and maintenance in mice.[19 29–34] Direct interactions of these proteins are indicated, for example, for CLIC5, RDX and TPRN, but not RIPOR2.[19 31] Also, interdependence for their concentration in the taper region has been observed.[19 30 31] In RIPOR2-deficient hair cells, for example, TPRN is no longer concentrated at the stereociliary base.[19 29] Depletion of TPRN in *Tprn* (MIM: 613354) knock-out mice leads to functional as well as (slowly) progressive morphological abnormalities of the stereocilia bundle.[29]

Based on the above described molecular structure of the stereociliary taper, we hypothesise that p.(Gln566_Lys569)del RIPOR2 affects this taper region and thereby the durability of the hair bundle, potentially via an effect on TPRN. Additionally or alternatively, the *RIPOR2* variant might affect the amount of the RIPOR2-interaction partner MYH9 in stereocilia, as well as the abundance of phosphorylated MYH9 and acetylated α-tubulin in the kinocilia, as these proteins are reduced in RIPOR2-deficient mice.[27] Interestingly, *MYH9* (MIM: 160775) defects in humans are also associated with progressive HL.[35]

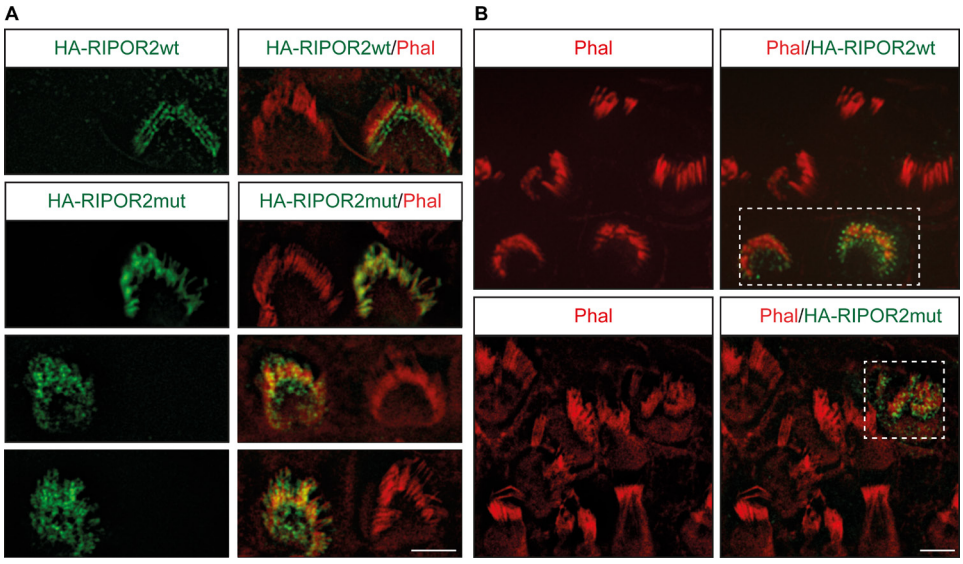

Figure 5 Functionality of mutant RIPOR2 is altered in mouse cochlear outer hair cells. (A) Mutant RIPOR2 differed in localisation from wild-type RIPOR2 in mouse cochlear outer hair cells. Outer hair cells of wild-type mice were injectoporated at P2 to express murine N-terminally HA-tagged wild-type RIPOR2 (RIPOR2wt) or mutant RIPOR2 (RIPOR2mut). Expression was evaluated after 2 days by immunohistochemistry and three representative images of cells expressing the mutant RIPOR2 are provided. Eleven cells expressing the wild-type construct and 12 cells expressing the mutant construct were evaluated. (B) Mutant RIPOR2 did not rescue stereocilia defects in RIPOR2-deficient hair cells. Cochlear explants of RIPOR2-deficient mice were prepared at P2 and injectoporated with constructs RIPOR2wt or RIPOR2mut. After culturing for 2 days, five out of six cells expressing the wild-type RIPOR2 construct demonstrated rescued hair bundle morphology but none of the 13 cells expressing the mutant RIPOR2 construct. Cells expressing the constructs are boxed. HA-tagged protein was stained in green, stereocilia were stained using phalloidin (phal) conjugated with Alexa Fluor 568 (red). HA, haemagglutinin. Scale bar represents 5 µm.

In light of developing therapeutic strategies, it is essential to determine whether the *RIPOR2* variant has a loss-of-function, a dominant negative or toxic gain-of-function effect. A haploinsufficiency effect of the variant seems to be the least plausible, as a loss-of-function *RIPOR2* variant in the heterozygous state was not indicated to be associated with HL.[11] Also, heterozygous *Ripor2* knockout mice displayed no significant hearing loss at 4 weeks[19] and 2 months of age (Zhao, 2019, unpublished data). A dominant-negative effect of the p.(Gln566_Lys569del) variant cannot be excluded as an interaction between the mutant and wild-type RIPOR2 was detected in Co-IP assays. However, a strong dominant negative effect would be expected to result in early-onset HL, comparable to that associated with the homozygous loss-of-function variant.[11] Therefore, we hypothesise that the variant has a toxic gain-of-function effect.

RIPOR2 is expressed in a wide range of tissues and cell types.[19] It is a known inhibitor of the small G-protein RHOA (Ras Homolog Family Member A) in neutrophils and T lymphocytes, where it regulates migration of these cells.[36] Additionally, *RIPOR2* is upregulated during muscle cell differentiation and induces the formation of filopodia.[37] We did not observe an effect of the four amino acid-deletion on filopodia formation (de Bruijn, 2019, unpublished data) which is in line with the fact that the deleted residues are not part of the RHOA interaction domain.[37] This might, at least in part, explain why the *RIPOR2* variant leads to HL only. The variant could affect a cochlear-specific protein interaction that determines RIPOR2 localisation in the hair bundle. Furthermore, in tissues other than the inner ear, loss of RIPOR2 function might be compensated by RIPOR1 and RIPOR3 which are described to have redundant functions.[38 39] Indeed, RNA levels of both *RIPOR1* and *RIPOR3* are low in hair cells (gene expression analysis resource).

The audiometric phenotype and age of onset of HL associated with c.1696_1707del *RIPOR2* displayed variation. Such intrafamilial phenotypic variation has also been reported for defects in several of the genes that can be associated with adult-onset adNSHL (eg, *EYA4* (MIM: 603 550), *MYO6* (MIM: 600 970) and *POU4F3* (MIM: 602 460)), and remains unexplained.[40–42] Non-penetrance is an extreme of phenotypic variability. In our study, five subjects with the c.1696_1707del *RIPOR2* variant had normal hearing—V:2, IV:26, III:28 (W97-056), III:14 (W04-262), and III:4 (W15-1495). They were aged 23, 40, 51, 49, and 50 years, respectively, at the latest audiometric evaluation. The average (SD) reported age of onset in the studied families is 30 (14.9) years and the highest reported onset age is 70 years. Therefore, unaffected subjects might develop HL in the future. However, incomplete penetrance of the variant cannot be excluded.

With a k-means cluster analysis, four distinct audiometric clusters could be distinguished. It is possible that subjects, due to increasing age, may go from one cluster to another cluster, which is not captured by the k-means clustering algorithm, since no longitudinal data are used. As no clear patterns of age of onset or audiometric configurations were observed within families or family branches with the *RIPOR2* variant, the phenotypic variability might well result from an interplay between environmental and genetic modifying factors. We have addressed differences in transcript levels of both wild-type and mutant *RIPOR2* alleles as potential modifiers of age of onset, but no clear correlations were observed. As the analysis was performed on RNA extracted from peripheral blood, we cannot exclude the possibility that *RIPOR2* mRNA levels determined by cochlear-specific cis or trans regulatory elements modify the onset of HL. Other candidate genetic modifiers are variants in the genes that encode proteins of the indicated complex of the stereocilia taper. As the taper region is thought to be essential for anchoring the mechanosensory stereocilia, noise exposure is an obvious candidate environmental modifying factor. Fourteen subjects with the

RIPOR2 variant reported noise exposure. However, we could not correlate onset or strong progression of HL with a preceding significant noise exposure.

Four subjects with HL who did not have the *RIPOR2* variant are considered to be phenocopies. In the light of the heterogeneity in the aetiology of HL, the occurrence of phenocopies is not unexpected. For individuals III:14 and III:20 (W97-056) a possible explanation for their HL is a Ménière-like disease (MIM: 156 000) and heavy smoking (COPD Gold III (MIM: 606 963)), respectively.[43 44] Subject III:10 (W04-262) might have inherited a cause of HL associated with vestibular problems from her mother, who married into the family. HL in subject III:21 of family W97-056 remains unexplained.

The c.1696_1707del *RIPOR2* variant was only reported in non-Finnish Europeans, with the exception of a single individual of African origin (gnomAD v3 genomes). Assuming that the AF of 0.0392% determined in the SE-NL cohort is comparable throughout the Netherlands, the c.1696_1707del *RIPOR2* variant is estimated to be present in more than 13 000 individuals who are therefore at risk of developing HL or have developed HL already due to this variant. About 30 000 additional individuals can be calculated to be at risk, based on the AF of 0.0096% of the variant in northwest Europe (gnomAD v2.1.1) with ~156 million inhabitants (United Nations Population Division estimates, 2019). This large number of individuals at risk of developing HL due to the c.1696_1707del *RIPOR2* variant illustrates the need to gain broader estimates of the penetrance of the variant, which was ~90% at the age of 50 years in the studied families. However, this calculated penetrance might be biased because these families were included based on index cases with HL. Further insight into the age-related penetrance of c.1696_1707del *RIPOR2* will pave the way for the identification of modifying factors which may convey measures for prevention.

In conclusion, we have demonstrated that an adult-onset type of HL (DFNA21) is relatively common and associated with a 'mild' variant in *RIPOR2*. Potentially thousands of individuals in the Netherlands and beyond are at risk of developing HL. Further such variants might well wait to be 'unmasked' as (population-specific) frequent and highly penetrant causes of adult-onset HL. Because of the large number of subjects estimated to be at risk for HL due to the c.1696_1707del *RIPOR2* variant, it is an attractive target for the development of a genetic therapy. The great progress that is being made in hearing disorders is promising.[45]

Twitter Suzanne E de Bruijn @SEdeBruijn and Susanne Roosing @suussuus84

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Contributors SEdB and JJS co-designed the study, conducted experiments, analysed the data, wrote the manuscript and JJS performed subject evaluation. CL performed the experiments in mouse cochlear explants and Co-IP experiments and revised the manuscript. CPL and AJB analysed the audiovestibular data and revised the manuscript. EdV discussed experimental design and critically read the manuscript. JB and JO conducted and analysed the genetic analyses. WK and HGY analysed the WES data and revised the manuscript. FPMC and SR discussed the experimental design and critically read the manuscript. HPMK performed clinical evaluations for members of family W97-056. BZ co-designed and supervised the studies in mouse cochlea and the Co-IP experiments, and revised the manuscript. RJEP clinically evaluated family members, RJEP and HK co-designed the study, supervised the project and revised the manuscript. All authors read and approved the final manuscript.

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ORCID iDs
Suzanne E de Bruijn http://orcid.org/0000-0003-2912-9265
Jeroen J Smits http://orcid.org/0000-0002-4241-0900

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