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Tissue-specific genotype-phenotype correlations among USH2A-related disorders in the RUSH2A study

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All authors contributed equally to the data collection, drafting, review, and finalization of manuscript. Robert Hufnagel takes responsibility for the data and analysis in the manuscript.

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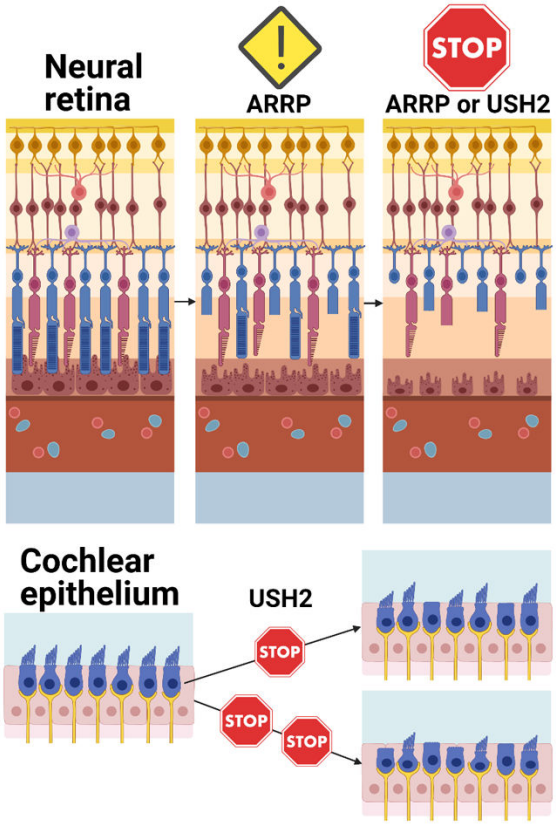
Abstract

We assessed genotype-phenotype correlations among the visual, auditory, and olfactory phenotypes of 127 participants with Usher syndrome (USH2) (n=80) or nonsyndromic autosomal recessive retinitis pigmentosa (ARRP) (n=47) due to *USH2A* variants, using clinical data and molecular diagnostics from the Rate of Progression in *USH2A* Related Retinal Degeneration (RUSH2A) study. *USH2A* truncating alleles were associated with USH2 and had a dose-dependent effect on hearing loss severity with no effect on visual loss severity within the USH2 subgroup. A group of missense alleles in an inter-fibronectin domain appeared to be hypomorphic in ARRP. These alleles were associated with later age of onset, larger visual field area, better sensitivity thresholds, and better electroretinographic responses. No effect of genotype on the severity of olfactory deficits was observed. This study unveils a unique, tissue-specific *USH2A* allelic hierarchy with important prognostic implications for patient counseling and treatment trial endpoints. These findings may inform clinical care or research approaches in others with allelic disorders or pleiotropic phenotypes.

Graphical Abstract



Keywords

USH2A ; hearing loss; photoreceptor degeneration; genotype; Usher syndrome; retinitis pigmentosa

INTRODUCTION

Retinitis pigmentosa (RP; MIM# 268000) is a form of retinal degeneration characterized by early loss of rod photoreceptor function, manifesting as nyctalopia, peripheral field loss, and diminished dark-adapted electroretinographic (ERG) recordings. The later stages include cone dysfunction, including constricted visual fields, loss of central vision, and reduced light-adapted ERG responses. RP has extreme locus heterogeneity, with >90 genes associated with the nonsyndromic form, and is associated with hundreds of syndromic disorders, including ciliopathies, peroxisomal disorders, and multiple (>500) malformation syndromes (Hartong et al., 2006; Schneider et al., 2021; Verbakel et al., 2018). Recently, an FDA-approved gene-directed therapy, the first in its class, has emerged for early-onset retinal degeneration caused by variants in the *RPE65* gene (MIM# 180069). However, there are no effective treatments for the vast majority of patients with RP. Defining genotype-phenotype correlations may allow for better selection of outcome measures for future clinical trials.

Usher syndrome (Usher syndrome, MIM# 276900) comprises a group of autosomal recessive disorders characterized by congenital, childhood-onset, or progressive post-lingual hearing loss and retinal degeneration. Genes associated with various forms of Usher syndrome encode proteins that localize mainly to the stereocilia and synaptic regions of inner ear hair cells and the connecting of cilium of retinal photoreceptors. Variants in the *USH2A* gene (MIM# 608400) are the leading cause of Usher syndrome type 2 (USH2) (USH2A; MIM# 276901). Notably, patients with USH2 have congenital hearing loss with progressive vision loss, providing a window of opportunity for intervention as the hearing loss is often diagnosed early in life and genetic testing often reveals the potential for subsequent retinal degeneration before vision loss actually begins. *USH2A* mutations can also cause nonsyndromic autosomal recessive RP (ARRP, isolated RP with normal hearing at birth) (RP39; MIM# 613809). In many populations, the most common pathogenic variants are located in exon 13 of the *USH2A* gene, in particular NM_206933.4:c.2299delG p.(Glu767Ser*fs*Ter21), which accounts for as high as ~16% of disease alleles (Lenassi et al., 2015; Pierrache et al., 2016). As such, *USH2A* exon 13 variants are the current targets for allele-directed therapy (NCT03780257).

Optimal design of gene therapy trials relies on natural history studies and deep clinical phenotyping to select reliable outcomes of treatment response. However, phenotypic correlates are poorly understood for many Mendelian conditions, and as a result the interplay between genotype and treatment response is largely overlooked. With over a thousand variants reported in the literature, *USH2A* offers a valuable opportunity for elucidating treatment-informing genotype-phenotype correlations.

Presumed truncating alleles, including nonsense, frameshift, and canonical splice variants, have been more frequently associated with hearing loss and, therefore, syndromic disease. Biallelic truncating variants are associated with more severe hearing loss (Hartel et al., 2016; Inaba et al., 2020; Meng et al., 2020; Pierrache et al., 2016). Notably, while earlier onset of visual impairment was noted in patients with USH2, the role of truncating variants has not been clearly established as a risk factor for severe visual impairment. Intriguingly, a subset of missense alleles is enriched in patients without hearing loss and ARRP (Lenassi et al., 2015; Molina-Ramirez et al., 2020). Overall, there appears to be a genotype-diagnosis correlation for *USH2A* truncating and specific missense variants for USH2 and ARRP, respectively.

The Rate of Progression in *USH2A*-related Retinal Degeneration (RUSH2A) natural history study includes 127 international participants with USH2 and ARRP related to variants in *USH2A*. Recently, RUSH2A baseline visual field data was reported, indicating that USH2 participants have more severe visual field loss than those with ARRP after adjusting for duration of disease and age of enrollment (Duncan et al., 2020).

Given the known association between diagnosis and genotype, we hypothesized that genotype influences audiometric and visual outcomes independent of the clinical diagnosis (USH2 versus ARRP). Here, we performed a deep analysis of *USH2A* genotypes to investigate whether the allelic hierarchy for hearing impairment applied to both severity of hearing loss and retinal degeneration. Through standardized variant classification and case-control analyses to ascertain pathogenic genotypes enriched in USH2 and ARRP subgroups, we ascertained genotype-phenotype correlations that are both tissue-specific and independent of clinical diagnosis. This work demonstrates the importance of genotype analysis in natural history studies and treatment trials for rare disorders.

PATIENTS AND METHODS

This multicenter, longitudinal, international natural history study enrolled participants with bi-allelic *USH2A* variants at 16 clinical sites in Canada, France, Germany, the Netherlands, the United Kingdom, and the United States (US). The protocol and informed consent process adhered to the tenets of the Declaration of Helsinki and were approved by the ethics boards associated with each participating site, including compliance with the associated federal regulations. Informed consent was obtained from all participants prior to enrollment. The RUSH2A protocol is listed on www.clinicaltrials.gov (NCT03146078), with registration completed prior to enrolling the first participant. Inclusion criteria stated that participants were required to have a clinical diagnosis of USH2 or ARRP and two pathogenic or likely pathogenic variants in *USH2A* from a certified testing lab obtained prior to study enrollment. Variants were demonstrated to be *in trans* for individuals with ARRP.

Variant analysis and interpretation

USH2A variant analysis was performed by two reviewers independently who used a five-tier classification system recommended by the 2015 American College of Medical Genetics and Genomics (ACMG) and Association for Molecular Pathology (AMP) guidelines and each variant was classified as benign, likely benign, variant of unknown

significance (VUS), likely pathogenic, or pathogenic.(Richards et al., 2015) Discordant results were resolved by an independent adjudicator. Variant analysis of the entire cohort was performed following the initial review, to standardize evidence used for recurrent variants. Healthy population frequency data were obtained from gnomAD (v2.1.1 accessed on Oct. 30, 2018, https://gnomad.broadinstitute.org/).(Karczewski et al., 2019) A consensus verdict for *in-silico* pathogenicity predictions for missense variants was acquired from Varsome (https://varsome.com/) and Franklin (https://franklin.genoox.com/clinical-db/home) webtools. Individual in silico predictions were acquired from Variant Effect Predictor (VEP; http://grch37.ensembl.org/Homo_sapiens/Tools/VEP) (Supp. Table S1).

Statistics

Statistical analysis was performed using the R system (v. 3.5.1) and SAS software (v. 9.4) for statistical computing. Statistical tests employed are listed in the text and figure legends. All t-tests assume two tails and unequal variance.

RESULTS

Of the 127 participants enrolled in RUSH2A, 80 were clinically diagnosed as USH2 and 47 as ARRP. Across the cohort, 140 unique variants comprising 128 single-nucleotide variants (SNVs) or small indels and 12 exonic deletions were determined to be disease-associated by variant analysis. Variants considered benign were excluded from analysis.

To assess genotype-phenotype correlation in the RUSH2A cohort, we first established disease-association of each variant by (i) standardized clinical variant interpretation using 2015 ACMG/AMP criteria (Supp. Table S1) and (ii) case-control comparison of *USH2A* allele frequencies (AF) in the RUSH2A cohort compared to a general subpopulation (gnomAD database v2.1.1).

USH2A variants in ClinVar and gnomAD

The *USH2A* canonical transcript, NM_206933.4, encodes for a large 6002 amino acid protein, Usherin. The *USH2A* transcript in the human population is highly variable, including many rare missense (gnomAD missense constraint Z-score = −2.5) and truncating variations (low probability of being loss-of-function [LoF] intolerant; gnomAD LoF score = 0). The variations observed in gnomAD appear to be randomly distributed throughout the coding region (Supp. Figure S1A). To determine whether disease-associated variants are distributed non-randomly, we then examined the distribution of *USH2A* coding variants present in the ClinVar database (Supp. Figure S1B). While ClinVar may have submission or population bias, we observed no apparent spatially restricted clusters of pathogenic or likely-pathogenic variants. However, exon 13 harbors the most frequently submitted variants, c.2276G>T p.(Cys759Phe) and c.2299delG. Among the pathogenic or likely-pathogenic variants in ClinVar, c.2276G>T p.(Cys759Phe) has the highest gnomAD AF of 0.0010. The c.2299delG p.(Glu767Ser*fs*Ter21) variant is the most frequent LoF variant (AF_{gnomAD} = 0.0007) in the *USH2A* gene in the gnomAD dataset. It is noteworthy that 94% of the LoF variants were classified as pathogenic or likely-pathogenic in ClinVar. However, only 12% of missense or in-frame-indel variants with gnomAD AF less than 0.001 were classified as

pathogenic or likely-pathogenic, and 68% such rare variants were classified as a VUS (Supp. Figure S1C). This represents a major challenge for definitive classification of rare missense variants as pathogenic or benign.

USH2A variant enrichment in the RUSH2A cohort

We next applied a similar analysis to the RUSH2A cohort. Similar to ClinVar, there is no hotspot for disease associated *USH2A* variation (Figure 1A). The c.2299delG, p.(Glu767Ser*fs*Ter21) (AF_{RUSH2A} = 0.138) and c.2276G>T p.(Cys759Phe) (AF_{RUSH2A} = 0.083) variants in exon 13 are the most frequent in this cohort (Figure 1A), and these variants demonstrate clear enrichment of AF_{RUSH2A} compared to AF_{gnomAD} (Fig. 1B–C). To establish which *USH2A* alleles are significantly associated with disease status, allele frequencies were compared between the RUSH2A and gnomAD cohorts. Among *USH2A* variants present in the RUSH2A cohort, 58% (74/128) SNVs or indels were also present in the general population (gnomAD) (Figure 1B). We applied Fisher's exact test to determine which variants in the RUSH2A cohort were enriched as compared to the gnomAD database (Figure 1C). A Bonferroni-corrected *P*-value of 0.00039 (=0.05/128 variants) was used as the cut-off to determine significant enrichment. Of the 128 variants, 23% (30/128) were statistically enriched in the RUSH2A cohort. An additional 9% (12/128) of *USH2A* variants were reclassified after application of the 2015 ACMG guidelines to determine pathogenicity level PS4, which is based on enrichment of variants in the affected population compared to controls (further description in Supplemental Methods and Results and Supp. Figure S2).

Association of clinical diagnosis and hearing loss severity with truncating variants

Following the establishment of individual variant disease-association, we sought to investigate phenotype associations using the power of this cohort. Typically, truncating alleles represent total loss of function and may be more likely to correlate with phenotypic severity. We grouped exonic deletions, nonsense, frameshift, canonical (+/−2) splicing site, and non-canonical splicing variants that were supported by RNA or minigene-based evidence as truncating variants. Consistent with previous studies, the predicted LoF variants or exonic deletions in the RUSH2A cohort were detected more frequently in participants with USH2 than ARRP (Figure 2A).(Iannaccone et al., 2021)

Next, we sought to determine if the number of truncating variants was associated with clinical diagnosis. In the RUSH2A cohort, the majority (50%) of participants had 1 truncating variant, followed by those with 2 truncating alleles (33%) and 0 truncating variants (17%). The number of truncating variants in each patient was significantly associated with the clinical diagnosis (χ^2 = 36.9, ***P*** <0.001) (Figure 2B). All 42 participants with two truncating variants were in the USH2 group and constituted 53% of all USH2 participants.

Given the association between truncating variants and clinical diagnosis of USH2, we hypothesized that the number of truncating variants also correlates with a greater degree of hearing loss.(Hartel et al., 2016) The number of truncating variants in each participant correlated positively with hearing sensitivity represented by a 4 frequency (.5/1/2/4 kHz) pure tone average in the entire cohort (Supp. Figure S3A) and the USH2 group (Figure

2C, Supp. Figure S3B). No such correlation was observed in the ARRP subgroup (data not shown). Notably, more severe hearing loss was associated with the presence of 2 truncating variants than 0 or 1, as shown by the Tukey multiple comparisons of means analysis (adjusted *P*-value for pair-wise comparisons < 0.03) (Figure 2C, Supp. Figure S3B).

Association of vision loss onset age and visual function with truncating variants

Participants with ARRP self-reported a later age of vision loss onset than those with USH2 (mean vision loss onset age in ARRP vs USH2: 31.8 vs 18.4, ***P*** < 0.001) (Supp. Figure S4A). While the presence of two truncating variants was associated with earlier vision loss onset across all study participants (Tukey multiple comparisons of means, 1–0, ***P*** = 0.39; 2–0, ***P*** = 0.001; 2–1, ***P*** = 0.004) (Supp. Figure S4B), there was no association between vision loss onset and the number of truncating variants within either the USH2 or ARRP subgroups (Supp. Figure S4C). In addition, USH2 participants had lower static perimetry full field hill of vision (mean V_{TOT} in ARRP vs USH2: 37.1 vs 22.7 decibel-steradian (dB-sr), ***P*** = 0.001) and lower kinetic perimetry V4e seeing area (mean in ARRP vs USH2: 9878 vs 6477 deg^2, ***P*** < 0.001) compared to ARRP participants (Supp. Figure S4D–E). We find similar results when adjusting for disease of duration and age (Supp. Table S2A). Similarly, these differences in hill of vision and kinetic perimetry characteristics were not associated with the number of truncating variants in either the entire cohort or the USH2 or ARRP subgroups when adjusting for disease duration and age (adjusted ***P*** = 0.67 and ***P*** = 0.26, respectively; Supp. Figure S4D–E; Supp. Table S2A–B). Therefore, unlike hearing loss, the earlier and more severe vision loss observed in USH2 compared to ARRP may not be dependent on the number of truncating variants, suggesting that a different genotype association determines variability among retinal phenotypes.

Missense alleles cluster in ARRP

To determine whether other variant classes determine clinical endpoints in the RUSH2A cohort and USH2 and ARRP subgroups, we compared the variant landscape between these clinical diagnoses. The most frequently observed variants in both groups were in exon 13, c.2299delG p.(Glu767Ser*fs*Ter21) and c.2276G>T p.(Cys759Phe). However, the AF of c.2276G>T was greater in the ARRP subgroup, while c.2299delG was greater in the USH2 group (Figure 3A–C and Supp. Table S3). Further, missense or in-frame-indel variants were more frequent in the ARRP group (Figure 2A, 3B–C). Previous studies indicated that specific *USH2A* missense variants are associated with a clinical diagnosis of ARRP. (Lenassi et al., 2015) Comparisons of allele frequencies of individual variants between the ARRP and USH2 groups revealed a group of missense alleles with enriched AF in the ARRP group (Figure 3C). Fisher's exact test showed five alleles statistically associated with the ARRP group (***P*** < 0.05): p.Cys759Phe (***P*** < 0.001), p.Cys3358Tyr (***P*** < 0.001), p.Cys3294Trp (***P*** = 0.02), p.Arg4192His (***P*** = 0.05), and *cis* variants p.Cys2040Gly (***P*** = 0.05) and p.Ser2492Leu (***P*** = 0.05) (Figure 3C, Table 1 and Supp. Table S2). Three of these variants, p.Cys759Phe, p.Cys3358Tyr, and p.Arg4192His, were previously reported to be enriched in patients with ARRP.(Lenassi et al., 2015) Thus, this comparison of allelic diagnoses confirms and expands the known hierarchy of missense variants in disorders.

ARRP-associated missense variants are hypomorphic

Because patients with ARRP have later vision loss onset and better retained visual function compared to USH2, we next sought to understand if these ARRP-associated missense variants have hypomorphic effects on retinal photoreceptors and, therefore, patient phenotypic outcomes, when compared to other missense variants. Since the diseases are inherited in an autosomal recessive manner, it has been challenging to perform in-depth genotype-phenotype association studies. We postulated this could be studied by examining the missense variants *in trans* to the truncating alleles among the 1-truncating variant group. Among these 62 participants, there were 63 missense variants (including 3 pairs of *cis*-variants) known or presumed to be *in trans* to a truncating variant in 60 participants (Figure 3D and Supp. Table S4). Of the five participants with known or predicted pairs of missense variants *in cis*, each had at least one pathogenic or likely pathogenic variant. Thus, we only included the likely pathogenic or pathogenic missense variant of these pairs for further analysis.

To compare clinical correlates with missense genotypes, we evaluated the subgroup of participants with one missense variant and one truncating variant. Of this subgroup, we postulated that ARRP-enriched missense variants would have milder retinal manifestations than USH2. As described above, 62 participants harbored 1 truncating variant and at least one pathogenic or likely pathogenic missense. By comparing the disease phenotypes to Usherin protein location of the missense variants, we noted that missense variants in the N-terminus including the laminin N-terminal domain and the C-terminus including the fibronectin type-III domain, appear to be associated with the USH2 in this 1-truncating group (Figure 3D), which was observed previously.(Pierrache et al., 2016)

The ARRP-enriched missense variants represented multiple times among those with 1-truncating variant were cysteine substitutions, p.Cys759Phe, p.Cys3294Trp, and p.Cys3358Tyr (Figure 3D and Supp. Table S4). These three variants, defined as “ARRP-enriched” in the subsequent analyses, had significantly higher AF in the ARRP group as compared to the USH2 group both in the whole RUSH2A cohort (Table 1 and Supp. Table S2) and in the 62 participants with compound heterozygous truncating and missense variants. We then evaluated clinical characteristics among patients harboring one of these ARRP-enriched missense variants. Patients with ARRP-enriched missense alleles in the 1-truncating subgroup had later vision loss onset regardless of clinical diagnosis (32.9+/−12.8 years ARRP-enriched vs 20.8+/−10.1 years Other; $P < 0.001$) (Figure 4A and Supp. Table S5). V_{TOT} and III4e isopter visual field areas were also increased in these participants (***P*** < 0.001 for both), indicating larger visual fields at their initial study visit (Figure 4B–C and Supp. Table S5). ERG measures including cone 30-Hz flicker response, which corresponds to the function of cone photoreceptors, were also increased in those with ARRP-enriched missense alleles (***P*** = 0.04) (Figure 4D and Supp. Table S5).

To further investigate functional vision mediated by photoreceptor subtypes, full-field stimulus testing (FST), which evaluates rod and cone-mediated function sensitivity responses, was examined using white, blue, and red wavelengths.(Birch et al., 2020) Notably, FST stimulus testing enables determination of the type of photoreceptor mediating

sensitivity; white FST thresholds < −30 dB indicate preserved rod photoreceptor function. (Birch et al., 2020) Patients with ARRP-enriched missense alleles had lower FST thresholds for white (−40.0+/−12.6dB ARRP-enriched vs −29.8+/−11.7dB Other; ***P*** = 0.007). The difference in sensitivity to blue relative to red is also an index of rod-mediated sensitivity. Patients with ARRP-enriched missense alleles had greater blue-red differences (−19.6 +/−7.8dB ARRP-enriched vs −9.3+/−9.0dB Other; ***P*** < 0.001), indicating better preserved rod function in those with ARRP-enriched missense variants (Figure 4E–F and Supp. Table S5). Thus, ARRP-enriched alleles appear hypomorphic on multimodal retinal assessments including psychometric and electrophysiologic measures.

To determine whether ARRP-enriched alleles exhibit hypomorphic properties independent of clinical diagnosis, we repeated this in only those with ARRP. Remarkably, all above measures (with the exception of vision loss onset age; ***P*** = 0.10) indicated better visual function in ARRP participants with ARRP-enriched missense variants in conjunction with a truncating allele (Supp. Figure S5A–F and Supp. Table S5). We also eliminated the possibility of younger age as a confounding variable, as participants with ARRP-enriched missense alleles were, on average, older in the 1-truncating group (47.9+/−15.1 years vs 38.9+/−12.29 years; ***P*** = 0.017) and of the same age in the ARRP subgroup (***P*** = 0.05). Additionally, ARRP-enriched missense alleles in the ARRP 1-truncating group appeared to have no effect on hearing among patients with Usher syndrome (***P*** = 0.61) and olfaction measures (***P*** = 0.23). These missense alleles have a milder effect on retinal dysfunction and degeneration, yet no effect on auditory or olfactory outcomes. This indicates a tissue-specific genotype-phenotype correlation, where retinopathy onset and progression are influenced by a subset of hypomorphic missense alleles, and hearing by the number of truncating alleles.

Variants in exon 13 are not significantly different from other regions

Finally, we investigated the effect of the most common individual variants, c.2299delG p.(Glu767Ser*fs*Ter21)and c.2276G>T p.(Cys759Phe) in exon 13, which is the target of a current gene therapy clinical trial (NCT03780257). We found no differences in measures of auditory or visual function with 0, 1, or 2 copies of c.2299delG p.(Glu767Ser*fs*Ter21) in the 2-truncating genotype subgroup (Supp. Figure S6 and **data not shown)**. We also observed no differences among patients with and without p.Cys759Phe in the 1-truncating subgroup, or among those with 0 or 1 copy of p.Cys759Phe in the 2-missense genotype subgroup (Supp. Figure S6 and **data not shown**). Therefore, the observations in the RUSH2A cohort of the influence of truncating variants on hearing loss endpoints, and missense variants for retinopathy endpoints, are not primarily driven by these commonly observed exon 13 variants.

DISCUSSION

RUSH2A is a natural history study of visual phenotypes and a cross sectional study of hearing and olfactory phenotypes among patients with *USH2A*-related disease, with the goal of identifying reliable clinical endpoints in the assessment of progression or therapeutic outcomes as well as identifying subpopulations most likely to benefit from treatment.(Birch et al., 2020; Duncan et al., 2020; Iannaccone et al., 2021) Here, we analyze the effect of

genotype on clinical measures to better understand whether genotype determines clinical diagnosis, and whether variant effects are global or tissue-specific.

First, we standardized clinical variant interpretation at the cohort level using a case:control analysis and reclassified 2.4% of VUSs as likely pathogenic or benign, and 7.8% of likely pathogenic variants as pathogenic. Such classifications are tantamount to standardizing clinical variant interpretations for gene therapy trials, and for public repositories such as ClinVar, LOVD, and ClinGen.(Richards et al., 2015) The advantage of this study cohort is the large number of cases (127) which allowed us to both calculate disease-specific allele frequencies as critical evidence for pathogenicity ascertainment and separately analyze the USH2 and ARRP subgroups to explore genotype effects independent of clinical diagnosis, which has not been achieved previously.

Next, we demonstrated several important genotype-phenotype correlations at the tissue- and diagnosis-levels. First, USH2 is associated with truncating alleles, where biallelic truncating alleles almost always cause USH2.(Lenassi et al., 2015; Pierrache et al., 2016) Second, in the RUSH2A cohort, hearing loss severity in USH2 is directly related to the number of truncating alleles, as similarly noted by Hartel et al. and Molina-Ramirez et al, as well as the RUSH2A study.(Hartel et al., 2016; Iannaccone et al., 2021; Molina-Ramirez et al., 2020) Third, truncating alleles are also associated with vision loss in USH2 patients, with earlier onset of and more severe retinal degeneration compared to ARRP.(Inaba et al., 2020; Meng et al., 2020; Pierrache et al., 2016) However, we found that the impact of truncating alleles on retinal degeneration may be dependent on clinical diagnosis, as we found no differences in visual symptom onset or severity in those with and without truncating variants in the USH2 and ARRP subgroups.

Furthermore, we confirmed and expanded the list of ARRP-associated missense alleles, adding p.Cys3294Trp and *cis* variants p.Cys2040Gly and p.Ser2492Leu through the RUSH2A study. Intriguingly, several of the hypomorphic missense alleles are located in the inter-fibronectin domain p.Cys3358Tyr, p.Cys3294Trp, and p.Glu3448Lys. Additionally, p.Arg4192His is in a fibronectin-3 repeat domain. Usherin interacts with fibronectin in retinal basement membranes, and is disrupted with certain mutations found in *USH2A*-related disorders.(Bhattacharya & Cosgrove, 2005) Further, human disease-associated variants in fibronectin-3 domains in usherin appear to be located within a “hotspot” for pathogenic missense variation.(Baux et al., 2014)

Analysis of both the entire cohort and the ARRP subgroup indicated that ARRP-enriched missense alleles among patients with 1-truncating allele have a later age of onset and better-preserved cone and rod photoreceptor function as measured by psychometric and electrophysiological testing. Thus, the effect of ARRP-specific missense alleles on visual phenotypes and truncating alleles on the auditory phenotype are independent of the phenotypic differences observed between USH2 and ARRP. Further, we did not observe differences in hearing loss in individuals with ARRP-enriched missense alleles, nor did we observe differences in vision loss with different numbers of truncating alleles in the USH2 or ARRP groups. This implies these variant classes may have mutually exclusive effects,

with less severe photoreceptor degeneration occurring with retinal-specific hypomorphic missense variants, and cochlear hair cells being more sensitive to truncating alleles.

Multiple studies from different countries have recognized an *USH2A* allelic hierarchy, where truncating alleles are associated with the clinical diagnosis of USH2 and hearing loss, and several missense alleles are associated with clinical diagnosis of ARRP.(Gao et al., 2021; Hartel et al., 2016; Inaba et al., 2020; Lenassi et al., 2015; Meng et al., 2020; Molina-Ramirez et al., 2020; Pierrache et al., 2016) The presence of specific missense alleles enriched in ARRP is associated with differences in age of onset and severity of retinal degeneration. Previously, Lenassi et al. described six variants, five missense and one intronic variant, that were found more frequently in ARRP than USH2, indicating that a different mutational spectrum exists between these two clinical diagnoses, which goes beyond the association of truncating variants with syndromic disease.(Lenassi et al., 2015) Here, we establish that the ARRP-enriched missense alleles are hypomorphic, in multiple tests of cone and rod photoreceptor function, and that these effects are independent of clinical diagnosis, even when adjusted for age of onset and disease duration.

Despite being the most expansive *USH2A* genotype-phenotype study to date, there are several limitations. First, we controlled for retinal dysfunction attributed to individual missense alleles by selecting patients with one truncating and one missense variant. As we and others have demonstrated, truncating variants predispose to Usher syndrome, which is an independent risk factor for more severe retinal degeneration. However, it is likely that the milder effects of ARRP-associated missense alleles are underestimated by this analysis design. Patients with homozygous or compound heterozygous missense alleles were not frequent in this population and would provide a better comparison.

Prospective longitudinal studies in cohorts such as these will be critical to determine if these effects indeed alter disease progression in addition to the onset and measures of phenotype severity performed here. Larger studies would also permit analysis of variant-specific effects. However, in our analysis, we did not find that the most common truncating variant c.2299delG p.(Glu767Ser*fs*Ter21) had different effects on visual and auditory endophenotypes from other truncating alleles, and patients with the most common missense variant c.2276G>T p.(Cys759Phe) did not have milder disease course than those with other missense alleles. This is likely because the other hypomorphic *USH2A* alleles were included in the control group of this analysis.

In conclusion, we demonstrated correlations of *USH2A* truncating variants with the presence and severity of hearing loss and of hypomorphic missense variants with the onset and severity of retinal degeneration (Supplemental Graphic). Importantly, these effects are independent of clinical diagnosis, and will allow for further subgrouping of patients to provide prognostic information and clinical endpoints for gene therapy trials. As such, these findings highlight the importance of considering the effect of genotype on outcome measures for clinical trials. A deep understanding of genotype-phenotype correlations is critical in this era of gene augmentation therapy. Understanding the mechanism of disease, improving clinical molecular diagnostics for eligibility, and providing prognostic

information for disease onset and progression are essential for determining the efficacy of new therapies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Conflict of Interests Statement

J. Duncan is a consultant for ConeSight Theraputics, DTx Pharma, Inc., Editas Theraputics, Eyevensys Theraputics, Nacuity, PYC Therapeutics, Spark Therapeutics, and Vedere Bio, Astellas; she receives financial support for clinical trials from Acucela, Abbvie/Allergan, AGTC Theraputics, Biogen/Nightstarx Theraputics, Inc., ProQR Therapeutics, Second Sight Medical Products, Inc and Neurotech USA, Inc., ;and she serves as a clinical advisory board member for SparingVision, Gyroscope Therapeutics, AGTC Therapeutics, Spark Therapeutics, ProQR Therapeutics, Nacuity, RD fund, and Foundation Fighting Blindness; Spouse: stock in RxSight.

E. Heon is consultant for Novartis, Janssen, Deep Genomics

M. Singh is a consultant/ advisor for Novartis, Janssen, Bayer, ReVision Therapeutics, and Acucela

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I. Audo is a consultant/advisor for Novartis, Sparing Vision, Janssen, Roche

C. Kay is a consultant for AGTC, Spark Therapeutics, Novartis, Astena Therapeutics; and receives clinical trial funding/investigator for AGTC, Foundation Fighting Blindness, Alkeus, Gyroscope, Regenx Bio, Nightstar Therapeutics/Biogen, Iveric Bio, ProQR Therapeutics, MeiraGTx/Janssen, and Kodiak; and receives equity from Astena Therapeutics

A. Iannaccone is a consultant for ClearView Healthcare Partners, Teladoc Health, GLG Group, Guidepoint, Astellas Institute for Regenerative Medicine, Roivant Pharma, Editas, Rhythm Pharmaceuticals, IQVIA, Gyroscope, Ocugen, and is a board member for Alia Therapeutics, and receives financial support from AGTC, Allergan, Acucela, ProQR, Retinagenix, 4D Molecular Therapeutics, BridgeBio Pharma/Retinagenix

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A deidentified database is available upon request through the public domain on the FFB/Jaeb public website.

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Web Resources:

ClinVar: https://www.ncbi.nlm.nih.gov/clinvar/

gnomAD: https://gnomad.broadinstitute.org/

Varsome: https://varsome.com/

Franklin: https://franklin.genoox.com/clinical-db/home

Variant Effect Predictor: http://grch37.ensembl.org/Homo_sapiens/Tools/VEP

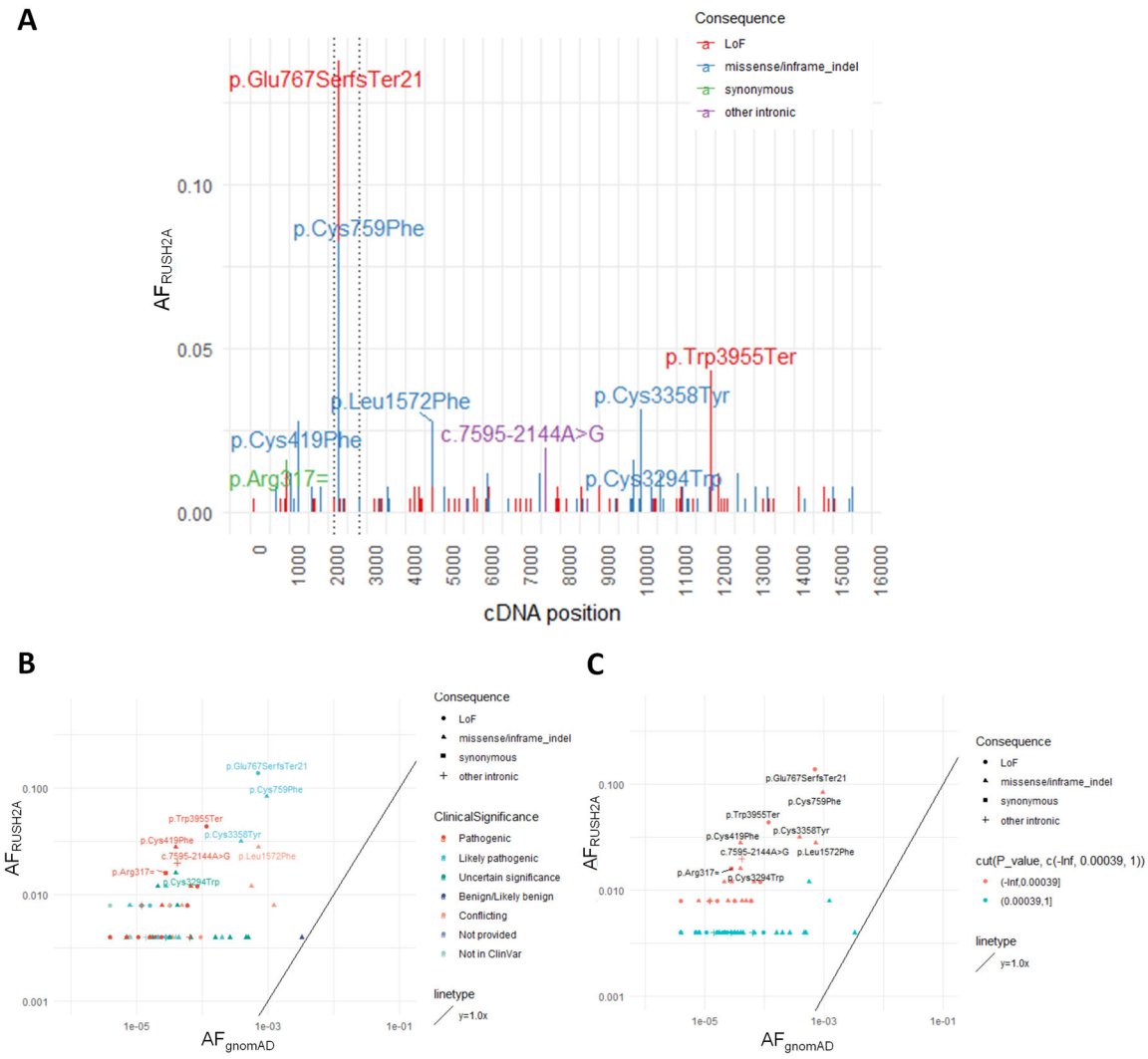

Figure 1.

Variant enrichment in the RUSH2A cohort. **A**. *USH2A* variant allele frequency in the RUSH2A cohort by cDNA position. **B-C**. *USH2A* variant allele frequency in the RUSH2A cohort vs allele frequency in gnomAD. Only variants present in both RUSH2A and gnomAD are shown. **B.** Clinical significance was obtained from ClinVar. **C.** Variants statistically (Fisher's exact test) enriched in the RUSH2A cohort as compared to gnomAD are shown in orange. Dotted lines in **A** represent exon 13 boundary; LoF, predicted loss of function variants; Variants labeled are those with allele frequency over 0.015.

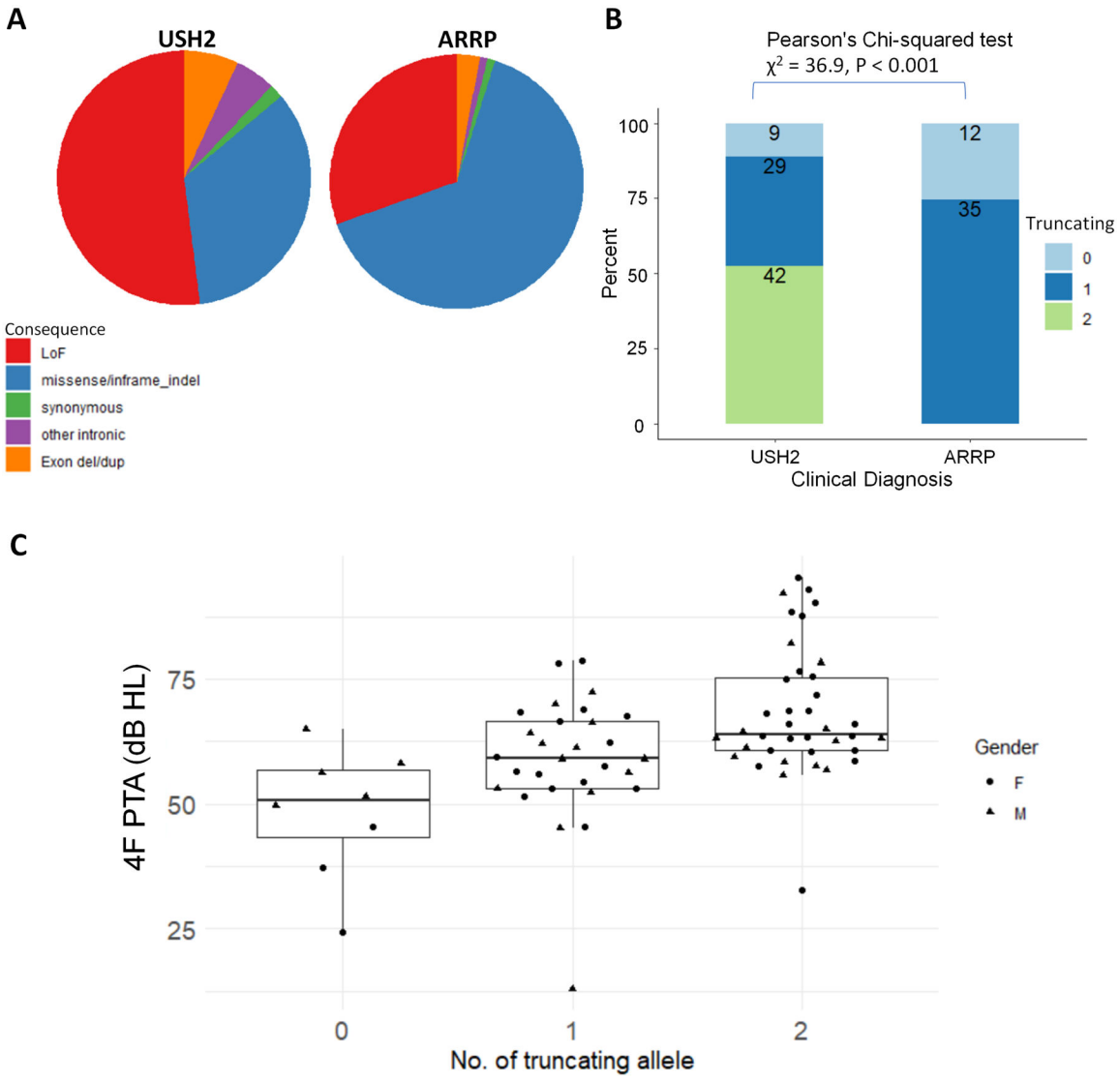

Figure 2.

Truncating alleles correlate with USH2 and degree of hearing loss. **A**. *USH2A* variant types in USH2 and ARRP. **B**. Bar chart showing patient diagnosis and number of truncating alleles. **C**. Box and dot plot showing 4 frequency (.5/1.2/4 kHz) pure tone average (4F PTA) in dB HLby number of truncating alleles in the USH2 group, adjusted for sex and age according to International Organization for Standardization (ISO) standards (ISO 7029: 2017; ANOVA, $\boldsymbol{P} = 0.0001$). Larger numbers mean worse hearing. Adjusted *P*-values in the Tukey multiple comparisons of means between truncating allele groups in **C**. 1–0, $\boldsymbol{P} = 0.10$; 2–0, $\boldsymbol{P} < 0.001$; 2–1, $\boldsymbol{P} = 0.01$.

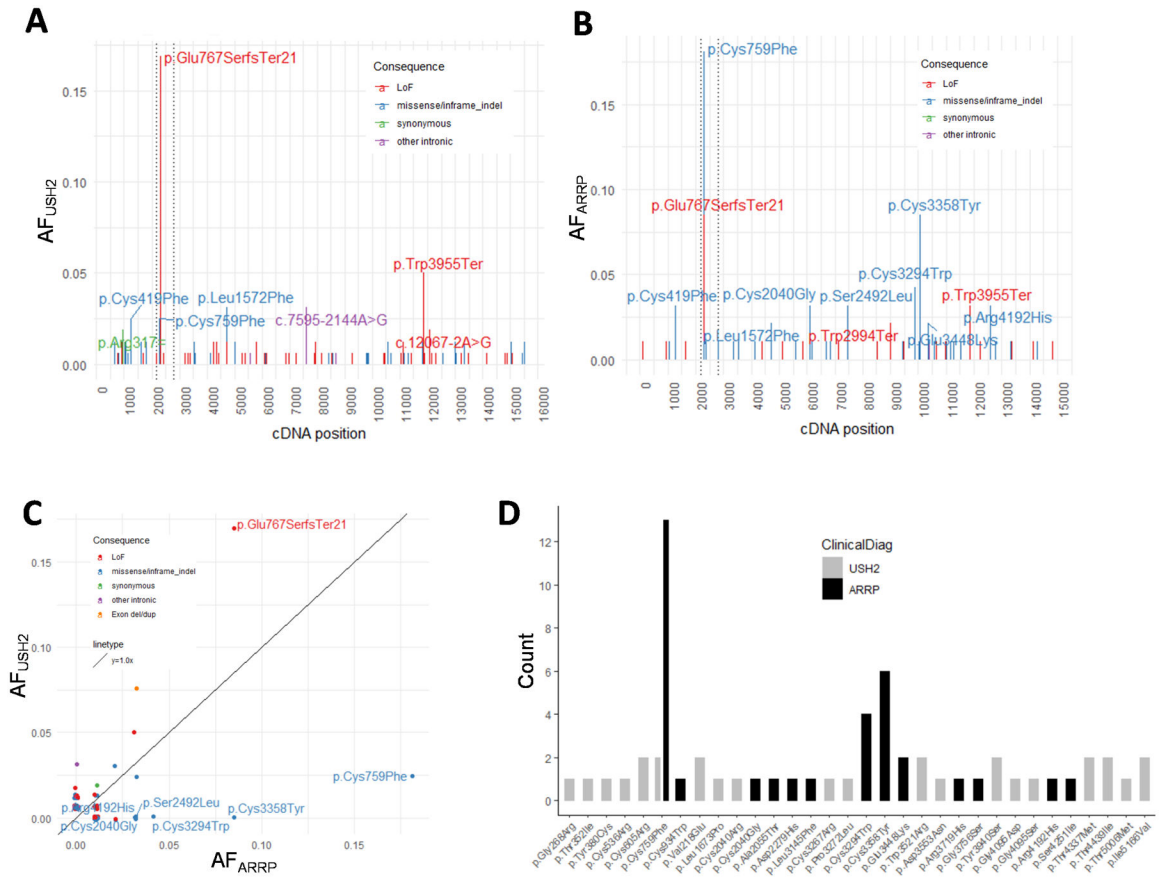

Figure 3.

USH2A variants enriched in patients with USH2 and ARRP. **A-B**. *USH2A* variant allele frequency in USH2 (**A**) or ARRP (**B**) by cDNA position. Variants labeled are those with allele frequency in patient subgroup over 0.015. Dotted lines, exon 13 boundary. **C**. *USH2A* variant allele frequency comparison by diagnosis. Variants labeled in **C** are those with *P*-value (Fisher's exact test) less than 0.05 (blue) or c.2299delG p.(Glu767Ser*fs*Ter21) (red, ***P*** = 0.09). LoF, predicted loss of function variants. **D**. Histogram of missense variants within the 1-truncating variant subgroup by protein position.

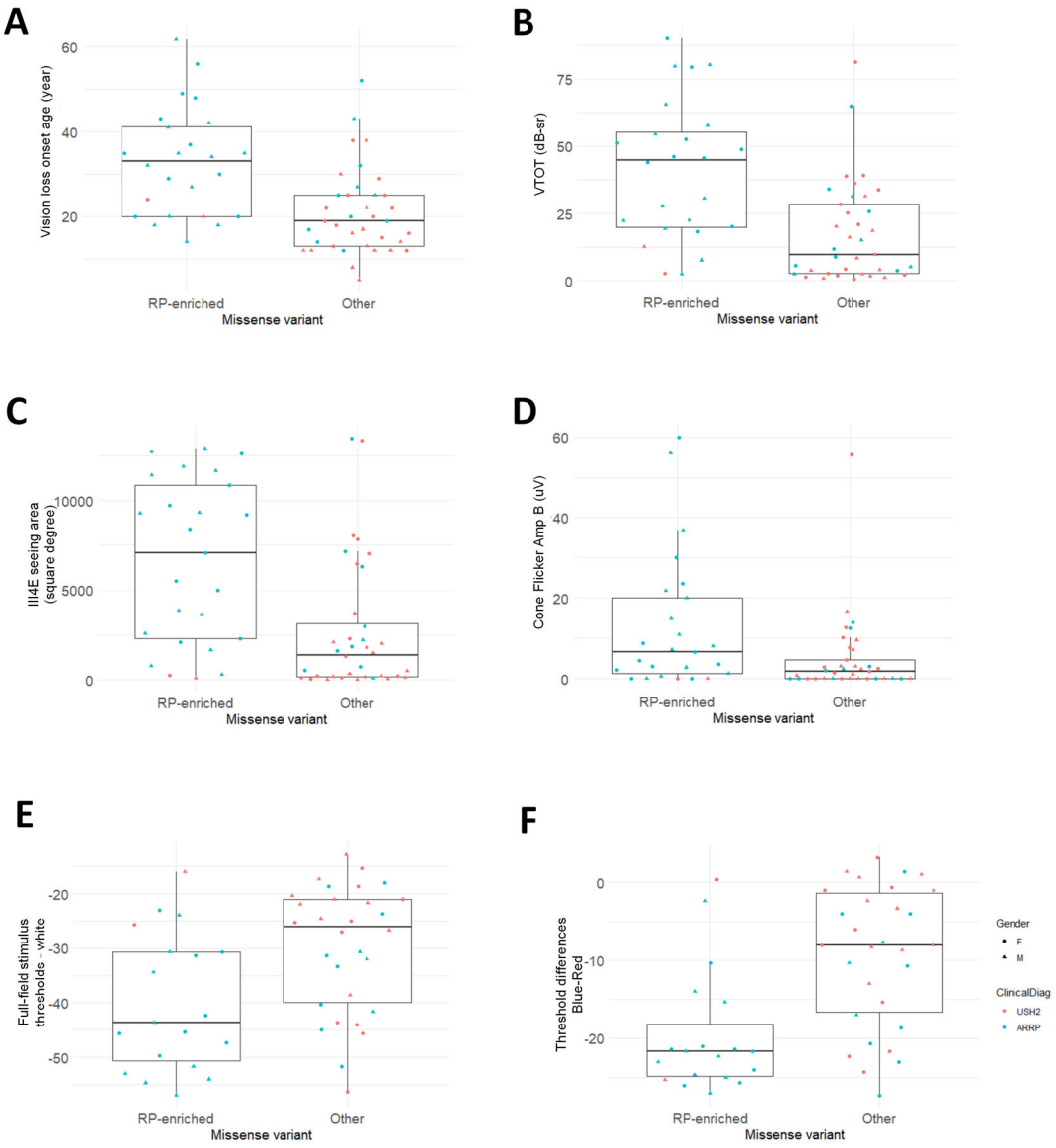

Figure 4.

Retinal phenotypic differences due to RP-enriched *USH2A* missense variants. **A**-**E**. Box and dot plot comparing RP-enriched and Other missense variants in the 1-truncating group, for age of vision loss onset (**A**; Welch's t-test; $\boldsymbol{P} < 0.001$), full-field hill of vision (**B**; $\boldsymbol{P} < 0.001$), iii4E seeing area (**C**; $\boldsymbol{P} < 0.001$), cone flicker amplitude (**D**; $\boldsymbol{P} = 0.04$), and full-field stimulus thresholds for White (**E**; $\boldsymbol{P} = 0.007$) and threshold differences Blue-Red (**F**; $\boldsymbol{P} < 0.001$). Circles = females, triangles = males, red = ARRP, blue = USH2. Full field hill of vision units as V_{TOT}, decibel-steradian (dB-sr).

Table 1.

USH2A variants enriched in patients with Usher syndrome type 2 (USH2) or nonsyndromic retinitis pigmentosa (ARRP).

cDNA	Protein	AF_ARRP	AF_USH2	Odds Ratio	95% Confidence Interval	*P*-value
c.2276G>T	p.Cys759Phe	0.181	0.025	8.54	2.66;36.04	<0.001
c.10073G>A	p.Cys3358Tyr	0.085	0	Inf	3.07;Inf	<0.001
c.9882C>G	p.Cys3294Trp	0.043	0	Inf	1.14;Inf	0.02
c.12575G>A	p.Arg4192His	0.032	0	Inf	0.71;Inf	0.05
c.6118T>G	p.Cys2040Gly	0.032	0	Inf	0.71;Inf	0.05
c.7475C>T	p.Ser2492Leu	0.032	0	Inf	0.71;Inf	0.05
c.2299del	p.Glu767SerfsTer21	0.085	0.169	0.46	0.17;1.1	0.09
c.7595–2144A>G	p.?	0	0.031	0	0;1.84	0.16
Exon deletion	p.?	0.032	0.075	0.41	0.07;1.57	0.18