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Non-syndromic hearing loss: clinical and diagnostic challenges

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Abstract: Hereditary hearing loss is clinically and genetically heterogeneous. There are presently over 120 genes that have been associated with non-syndromic hearing loss and many more that are associated with syndromic forms. Despite an increasing number of genes that have been implemented into routine molecular genetic diagnostic testing, the diagnostic yield from European patient cohorts with hereditary hearing loss remains around the 50 % mark. This attests to the many gaps of knowledge the field is currently working toward resolving. It can be expected that many more genes await identification. However, it can also be expected, for example, that the mutational signatures of the known genes are still unclear, especially variants in non-coding or regulatory regions influencing gene expression. This review summarizes several challenges in the clinical and diagnostic setting for hereditary hearing loss with emphasis on syndromes that mimic non-syndromic forms of hearing loss in young children and other factors that heavily influence diagnostic rates. A molecular genetic diagnosis for patients with hearing loss opens several additional avenues, such as patient tailored selection of the best currently available treatment modalities, an understanding of the prognosis, and supporting family planning decisions. In the near future, a genetic diagnosis may enable patients to engage in preclinical trials for the development of therapeutics.

Keywords: clinical and genetic heterogeneity, hearing loss diagnostics, non-syndromic hearing loss, syndromic hearing loss

Introduction

With an incidence of 1–2 in 1000 newborns, hearing impairment is one of the most common congenital disorders in humans [1, 2]. In populations with high rates of consanguinity, the incidence increases to up to 3–4 in 1000 [3]. Because hearing is important for language and cognitive development, untreated hearing loss (HL) leads to impaired cognitive functions and social competencies. To prevent this, many countries, including Germany, require newborn hearing testing by measuring otoacoustic emissions or auditory brainstem responses, which ideally is done during the first three days to one month of life [4–6]. Clinically, hearing impairment can be further classified by virtue of the affected anatomical site(s), age of onset, severity, affected frequencies, progression, symmetry/laterality, and presence/absence of vestibular dysfunction (Figure 1). Hearing impairment may be acquired, most importantly, by intrauterine (e. g., cytomegalovirus) infections, as well as toxic compounds (e. g., aminoglycoside antibiotics) and noise exposure. In developed countries, up to 80 % of congenital cases have a genetic basis [7, 8]. Hereditary forms of HL can be syndromic (30 %) with other organ system abnormalities or non-syndromic (70 %) with isolated HL. To date, approximately 600 syndromes have been associated with HL [9], including Usher, Pendred, Stickler, branchio-oto-renal, Down, and many others. The absence of additional symptoms in newborns or infants does not necessarily exclude syndromic forms, as HL may appear as the first manifestation in a long list of syndromes.

Genetic heterogeneity of non-syndromic hearing loss (NSHL)

The auditory system is highly complex with many diverse and highly specialized cell types. Therefore, it is not unexpected that variants in a considerable number of genes interfere with normal hearing. To date, the Hereditary Hearing Loss Homepage (Van Camp G, Smith RJH, https://hereditaryhearingloss/org; last accessed November 2019) lists 46 autosomal dominant (DFNA), 76 autosomal recessive (DFNB), and 5 X-linked (DFNX) NSHL genes. There are

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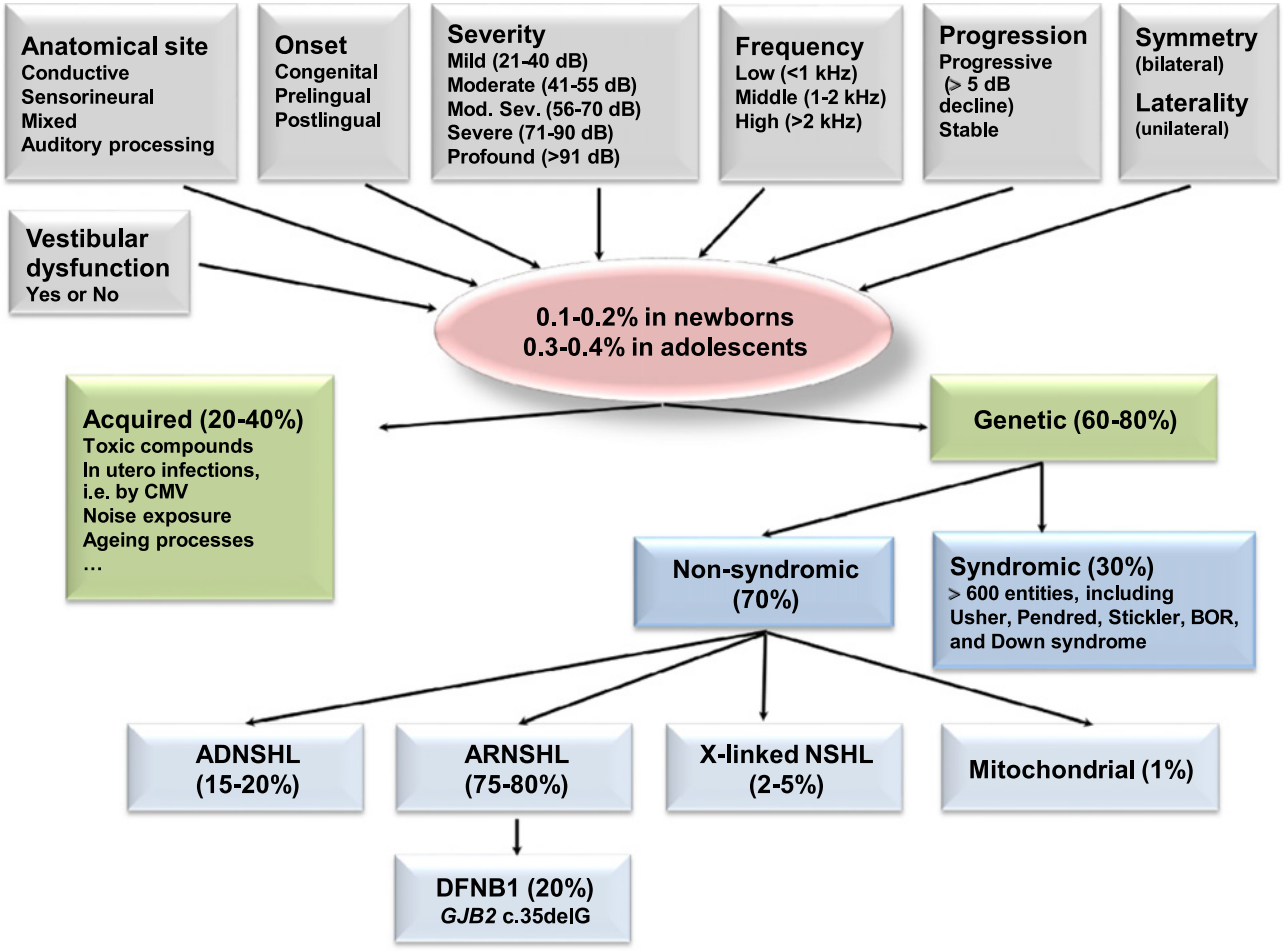

Figure 1: Characteristics and clinical classifications used to describe hearing loss.

Abbreviations: ADNSHL = autosomal dominant non-syndromic hearing loss, ARNSHL = autosomal recessive non-syndromic hearing loss.

new genes discovered almost every month. Although exceptions exist, autosomal dominant NSHL is commonly described as progressive with a postlingual (>5 years of age) onset, whereas autosomal recessive NSHL is usually non-progressive, severe to profound with a congenital or prelingual (until 5 years of age) onset. Despite unparalleled genetic heterogeneity, recessive alleles in *GJB2* (DFNB1A) predominate (Figure 1). It is noteworthy that one particular pathogenic variant, c.35delG, accounts for up to 80 % of recessive *GJB2* variants in European populations [10].

Different clinical outcomes can be evoked by variants in the same gene. For example, biallelic truncating variants in *GJB2* are generally associated with profound, compound heterozygous truncating and non-truncating variants with moderate, and biallelic non-truncating variants with mild hearing impairment [11]. Rare dominant variants in *GJB2* can also cause NSHL (DFNA3A), as well as several skin disorders (keratitis-ichthyosis-deafness syndrome, palmoplantar keratoma with deafness, hystrix-like ichthyosis with deafness, and Vohwinkel syndrome).

Next generation sequencing (NGS) has greatly transformed the molecular diagnostics of heterogeneous Mendelian disorders, allowing rapid screening of large gene panels or the entire exome. In a diagnostic setting, we currently use exome sequencing and apply targeted gene analysis of 129 genes (Table 1), 13 of which have been associated with both autosomal dominant and autosomal recessive inheritance. Forty-nine genes have been associated with both NSHL and other genetic entities (Online Mendelian Inheritance in Man; https://www.ncbi.nlm.nih.gov/omim/). Consistent with other studies [13–15], our solve rate in *GJB2*-mutation negative Caucasian patients, the majority of them being sporadic cases, is 20 %–30 % (using different panels over the years), whereas in consanguineous families from Iran and Pakistan with two or more affected individuals, it is over 50 % (Figure 2).

Table 1: Genes associated with non-syndromic hearing impairment.

Gene[1]	DFN locus/ inheritance[2]	Gene[1]	DFN locus / inheritance[2]	Gene[1]	DFN locus / inheritance[2]
ABCC1	AD	*GRAP*	DFNB114	*PDZD7**	DFNB57
*ACTG1**	DFNA20/26	*GRHL2**	DFNA28	*PJVK*	DFNB59
ADCY1	DFNB44	*GRXCR1*	DFNB25	*PLS1*	AD
*AIFM1**	DFNX5	*GRXCR2*	DFNB101	*PNPT1**	DFNB70
ATP2B2	Modifier; AD	*GSDME*	DFNA5	*POU3F4*	DFNX2
BDP1	DFNB112	*HGF*	DFNB39	*POU4F3*	DFNA15
*BSND**	DFNB73	*HOMER2*	DFNA68	*PPIP5K2*	DFNB100
CABP2	DFNB93	*IFNLR1*	DFNA2C	*PRPS1**	DFNX1
CCDC50	DFNA44	*ILDR1*	DFNB42	*PTPRQ*	DFNA73, DFNB84
CD164	DFNA66	*KARS1**	DFNB89	*RDX*	DFNB24
*CDC14A**	DFNB32	*KCNQ4*	DFNA2A	*REST**	DFNA27
*CDH23**	DFNB12	*KITLG**	DFNA69	*ROR1*	DFNB108
CEACAM16	DFNA4B, DFNB113	*LHFPL5*	DFNB66/67	*S1PR2*	DFNB68
CIB2	DFNB48	*LMX1A*	DFNA7	*SERPINB6*	DFNB91
*CLDN14**	DFNB29	*LOXHD1*	DFNB77	*SIX1**	DFNA23
CLDN9	AR	*LRP5**	AR	*SLC17A8*	DFNA25
CLIC5	DFNB103	*LRTOMT*	DFNB63	*SLC22A4*	DFNB60
COCH	DFNA9/DFNB110	*MARVELD2*	DFNB49	*SLC26A4**	DFNB4
COL4A6	DFNX6	*MASP1**	AR	*SLC26A5*	DFNB61 [12]
*COL9A1**	AR	*MCM2*	DFNA70	*SLC44A4*	DFNA72
*COL11A1**	DFNA37	*MET**	DFNB97	*SMPX*	DFNX4
*COL11A2**	DFNA13, DFNB53	*METTL13*	DFNM1, AD	*SPNS2*	DFNB115
CRYM	DFNA40	*MIR96*	DFNA50	*STRC**	DFNB16
*DCDC2**	DFNB66	*MPZL2*	DFNB111	*SYNE4*	DFNB76
DIABLO	DFNA64	*MSRB3*	DFNB74	*TBC1D24**	DFNA65, DFNB86
*DIAPH1**	DFNA1	*MT-RNR1**	Mitochondrial	*TECTA*	DFNA8/12, DFNB21
DIAPH3	AUNA1, AD	*MT-TS1**	Mitochondrial	*TJP2**	DFNA51
*DMXL2**	DFNA71	*MYH14**	DFNA4A	*TMC1*	DFNA36, DFNB7/11
ELMOD3	DFNB88, AD	*MYH9**	DFNA17	*TMEM132E*	DFNB99
EPS8	DFNB102	*MYO15A*	DFNB3	*TMIE*	DFNB6
EPS8L2	DFNB106	*MYO3A*	AD, DFNB30	*TMPRSS3*	DFNB8/10
*ESPN**	DFNB36	*MYO6**	DFNA22, DFNB37	*TMTC2*	AD
ESRP1	DFNB109	*MYO7A**	DFNA11, DFNB2	*TOP2B*	AD
ESRRB	DFNB35	*NARS2**	DFNB94	*TNC*	DFNA56
*EYA4**	DFNA10	*NLRP3**	DFNA34	*TPRN*	DFNB79
RIPOR2	DFNB104, AD	*OSBPL2*	DFNA67	*TRIOBP*	DFNB28
FOXF2	AR	*OTOA*	DFNB22	*TRRAP**	AD
GAB1	DFNB26	*OTOF*	DFNB9	*TSPEAR**	DFNB98
GIPC3	DFNB15/72/95	*OTOG*	DFNB18B	*USH1C**	DFNB18A
*GJB2**	DFNA3A, DFNB1A	*OTOGL*	DFNB84	*USH1G**	AR
*GJB3**	DFNA2B, AR [12]	*P2RX2*	DFNA41	*WBP2*	DFNB107
*GJB6**	DFNA3B, DFNB1B	*PCDH15**	DFNB23	*WFS1**	DFNA6/14/38
*GPSM2**	DFNB82	*PDE1C*	DFNA74	*WHRN**	DFNB31

[1] Genes indicated by an asterisk (*) are associated with different OMIM disease.

[2] Abbreviations: DFNA = autosomal dominant locus, DFNB = autosomal recessive locus, DFNX = X-chromosomal locus; DFNM = modifier locus, AUNA = auditory neuropathy locus, AD = autosomal dominant gene; AR = autosomal recessive gene.

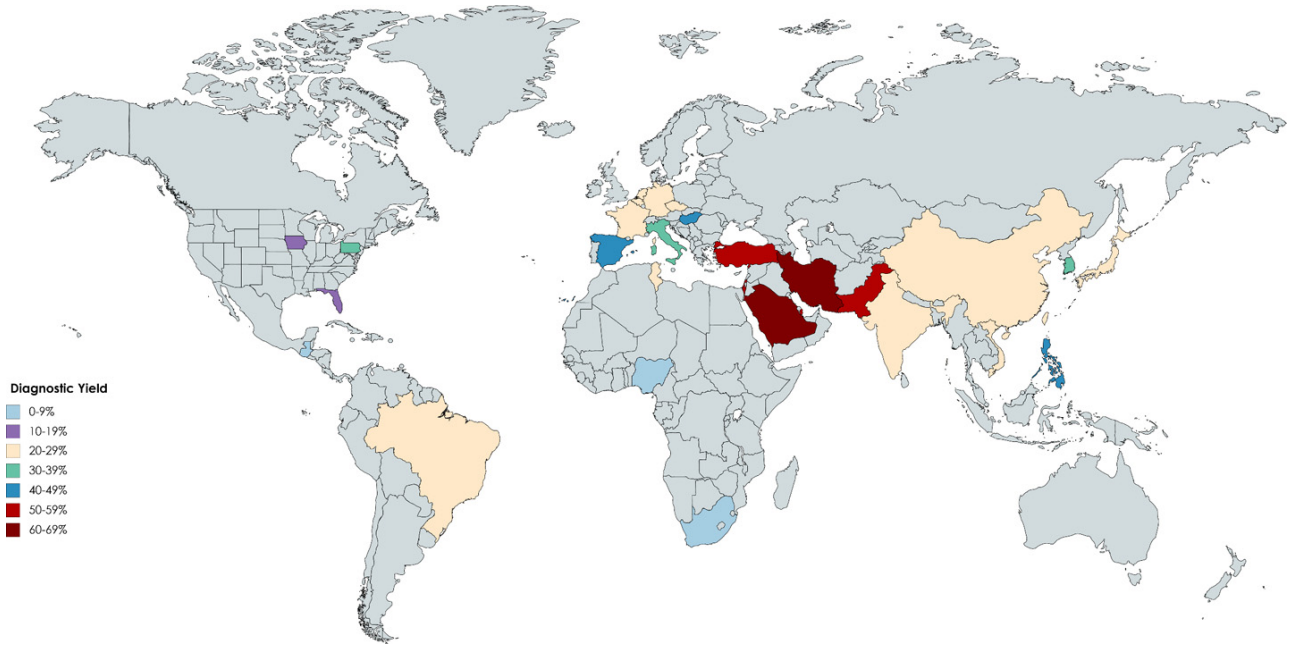

Figure 2: Worldwide diagnostic rates for HL that are attributed to NGS approaches, excluding *GJB2*. The unexplored genetic landscape for hereditary hearing loss is reflected by diagnostic yields less than 50 % for patients from European, African, North and South American, as well as a few Asian nations with data available. Some studies from the Middle East indicate diagnostic yields over 60 %. Created with mapchart.net [14, 16–30].

Syndromes that mimic NSHL

One important challenge in exome and panel diagnostics is that a considerable proportion of children and sometimes adults who are clinically diagnosed with NSHL present with variants in genes associated with syndromic forms of HL. This is one challenge that must be specifically addressed in pre-NGS diagnostic genetic counseling. One obvious explanation is that additional features of a given syndrome are not yet present at the time when NSHL is diagnosed. For example, the retinitis pigmentosa in a child diagnosed with Usher syndrome or the goiter in Pendred syndrome may manifest many years after onset of hearing impairment. Many bona fide NSHL genes also have been associated with syndromic phenotypes, including Bartter (*BSND*), branchiootic (*SIX1*), Stickler (*COL11A1* and *COL11A2*), Usher (*CDH23*, *MYO7A*, *PCDH15*, *USH1G*, and *WHRN*), and other syndromes (Tables 1, 2). When identifying new pathogenic variants in these genes, it is not always possible to predict the phenotypic outcome, i. e., whether a child with NSHL will develop retinitis pigmentosa or not. Diagnostic screens of apparently NSHL patients [18, 31] will inevitably identify a growing number of clinically relevant undiagnosed syndromes, which initially mimic NSHL (Table 2). For example, we identified a 45 kb homozygous in-frame deletion in the gene *COL9A1* in two affected brothers with moderate to severe high-frequency HL. The index patient, who was last clinically evaluated at the age of 28 years, did not have any additional features of autosomal recessive Stickler syndrome. Although it is difficult to exclude a very mild, subclinical form of Stickler syndrome in this family, more likely and similar to *COL11A1* and *COL11A2*, *COL9A1* may also qualify as a bona fide NSHL gene [32]. In a sporadic case of NSHL in a consanguineous family, we identified a homozygous nonsense mutation in *KCNQ1*, which has been associated with Jervell–Lange-Nielsen syndrome (Table 2). This molecular diagnosis in a patient with isolated HL has immediate consequences for genetic counseling and prevention of sudden cardiac death. In prepubertal children, we regularly find syndromic forms of HL, which are associated with male (due to biallelic *STRC-CATSPER2* deletions in deafness-infertility syndrome or *CDC14A* variants in hearing impairment infertile male syndrome) or female infertility (Perrault syndrome) later in life (Table 2). In our experience, up to 30 % of *GJB2*-mutation negative children who are clinically identified as having NSHL and receive a NGS diagnosis consistent with HL exhibit pathogenic variants in syndrome-associated genes.

Table 2: Syndromic genes which can mimic NSHL.

Gene[1]	Inheritance	OMIM[2]	
COL4A5	X-linked	# 301050	Alport 1
COL4A4	AR	# 203780	Alport 2
COL4A3	AR	# 203780	Alport 2
	AD	# 104200	Alport 3
*BSND**	AR	# 602522	Bartter 4A
*SIX1**	AD	# 608389	Branchiootic 3
*ACTG1**	AD	# 614583	Baraitser-Winter 2
CHD7	AD	# 214800	CHARGE
	AD	# 612370	Hypogonadotropic hypogonadism 5 with/without anosmia
FGF3	AR	# 610706	Congenital deafness with inner ear agenesis, microtia, and microdontia
PSIP1	AD	[4]	Deafness and optic neuropathy
SLITRK6	AR	# 221200	Deafness and myopia
FITM2	AR	# 618635	Siddiqi
GPRASP2	X-linked	[33]	Hearing loss with inner ear abnormalities and facial dysmorphism
SLC7A8	AR	[34]	Age-related hearing loss; damage of cochlear structures
*CDC14A**	AR	# 608653	DFNB32, with or without immotile sperm
PEX1	AR	# 234580	Heimler 1
		# 214100	Peroxisome biogenesis disorder 1A and 1B
		# 601539	
PEX6	AR/AD	# 616617	Heimler 2
		# 614862	Peroxisome biogenesis disorder 4A and 4B
		# 614863	
PEX26	AR	#614873	Peroxisome biogenesis disorder 7B
			Heimler
GATA3	AD	# 146255	Hypoparathyroidism, sensorineural deafness, and renal dysplasia
KCNQ1	AR	# 220400	Jervell–Lange-Nielsen
	AD	# 192500	Long QT 1
KCNE1	AR	# 612347	Jervell-Lange-Nielsen
	AD	# 613695	Long QT 5
KMT2D	AD	# 147920	Kabuki 1
PTPN11	AD	# 151100	LEOPARD 1
		# 153950	Noonan 1
FOXI1	AR	# 600791	Enlarged vestibular aqueduct (Pendred)
KCNJ10	AR	# 600791	Enlarged vestibular aqueduct, digenic (Pendred)
		# 612780	SESAME
SERPINF1	AR	# 613982	Osteogenesis imperfecta VI
HSD17B4	AR	# 233400	Perrault 1
		# 261515	D-bifunctional protein deficiency
HARS2	AR	# 614926	Perrault 2
CLPP	AR	# 614129	Perrault 3
LARS2	AR	# 615300	Perrault 4
TWNK	AR	# 616138	Perrault 5
ERAL1	AR	# 617565	Perrault 6
SGO2	AR	[35]	Perrault
PHYH	AR	# 266500	Refsum
RAI1	AD	# 182290	Smith Magenis
COL2A1	AD	# 108300	Stickler 1
		# 156550	Kniest dysplasia
		# 271700	Spondyloperipheral dysplasia
		# 132450	Epiphyseal dysplasia multiple with myopia and deafness
*COL11A1**	AD	# 604841	Stickler 2
	AR	# 228520	Fibrochondrogenesis 1
		# 154780	Marshall

Table 2: (continued)

Gene[1]	Inheritance	OMIM[2]	
*COL11A2**	AR/AD	# 614524	Fibrochondrogenesis 2
	AD	# 184840	Otospondylomegaepiphyseal dysplasia
	AR	# 215150	Otospondylomegaepiphyseal dysplasia
*COL9A1**	AR	# 614134	Stickler 4
	AD	# 614135	Epiphyseal dysplasia, multiple, 6
COL9A2	AR	# 614284	Stickler 5
	AD	# 600204	Epiphyseal dysplasia, multiple, 2
COL9A3	AD	# 600969	Epiphyseal dysplasia, multiple, 3, with or without myopathy
ABHD12	AR	# 612674	Polyneuropathy, hearing loss, ataxia, retinitis pigmentosa, and cataract
*MYO7A**	AR	# 276900	Usher 1B
*CDH23**	AR	# 601067	Usher 1D, 1D/F digenic
*PCDH15**	AR	# 602083	Usher 1F
	AR	# 601067	Usher 1D/F digenic
*USH1G**	AR	# 606943	Usher 1G
CIB2	AR	# 614869	Usher 1J, refuted by [36]
*WHRN**	AR	# 611383	Usher 2D
ADGRV1	AR	# 605472	Usher 2C
USH2A	AR	# 276901	Usher 2A
CLRN1	AR	# 276902	Usher 3A
HARS	AR	# 614504	Usher 3B, refuted by [37]
	AD	# 616625	Charcot–Marie–Tooth, axonal, 2W
ARSG	AR	# 618144	Usher-like-4
CEP78	AR	# 617236	Cone-rod dystrophy and hearing loss
CEP250	AR	# 618358	Cone-rod dystrophy and hearing loss 2
PAX3	AD	# 193500	Waardenburg 1
	AR/AD	# 148820	Waardenburg 3
	AD	# 122880	Craniofacial-deafness-hand
MITF	AD	# 193510	Waardenburg 2A
	AD	# 103500	Tietz-albinism-deafness
	AR	# 617306	COMMAD
	AD	# 103470	Waardenburg syndrome/ocular albinism, digenic
SNAI2	AR	# 608890	Waardenburg 2D
	AD	# 172800	Piebaldism
EDNRB	AR/AD	# 277580	Waardenburg 4A
	AR	# 600501	ABCD
EDN3	AR/AD	# 613265	Waardenburg 4B
SOX10	AD	# 613266	Waardenburg 4C
	AD	# 611584	Waardenburg 2E, with/without neurologic involvement
		# 609136	PCWH
CISD2	AR	# 604928	Wolfram 2

[1]Genes indicated by an asterisk (*) are associated with both NSHL and a syndromic phenotype(s).
[2]For gene-associated syndromic phenotypes without OMIM entries (#) a literature reference is given.

Genetic diagnostics: past and present

Diagnostic testing in the pre-NGS era

Genetic testing for HL has been performed since the late 1990s and was limited due to the low-throughput nature of Sanger sequencing or variant-specific screening using restriction fragment length polymorphism (RFLP) reactions [7]. Considering the extremely heterogeneous nature of NSHL, *GJB2* screening yielded an unexpectedly high diagnostic rate. Furthermore, the single-coding exon gene was simple to screen and could be easily analyzed using direct sequencing or RFLP testing. Genetic evaluation of *GJB2* in multiple large German hearing-impaired cohorts has shown diagnostic yields ranging from 17 % [38] to 31 % [39]. Two additional studies in German pa-

tients returned diagnostic yields of 21 % [11] and 22 % [40]. Following *GJB2* screening in the majority of undiagnosed patients, subsequent gene prioritization was difficult. Several groups tried to address this limitation in the pre-NGS era by developing more-inclusive resequencing arrays and single-primer extension microarrays [41, 42]. However, these methods were limited to previously identified pathogenic variants and did not provide an unbiased screening approach for all deafness genes [7]. Other groups developed Sanger sequencing assays for targeted sequencing of candidate genes based on clinical suspicion of a syndrome. This has been successfully performed in instances of Pendred syndrome in a series of German families with sensorineural HL and goiter [43], among other clinically distinct syndromes.

State-of-the-art next generation diagnostics

Because *GJB2* is a single-coding exon gene accounting for roughly 20 % of NSHL (DFNB1), Sanger sequencing and MLPA testing are still recommended as a first-line exclusionary diagnostic test prior to NGS testing [50]. In children with mild to moderate high-frequency HL, homozygous or compound heterozygous (together with a genetic point variant) deletions involving *STRC* (DFNB16) are a frequent cause of NSHL in 5 %–10 % of patients [51]. Because *STRC* and a pseudogene with 99.6 % identical coding sequence reside in a tandemly duplicated region, one cannot rely on NGS for copy number variations (CNVs) and sequence analysis. Therefore, we developed a Sanger sequencing method with pseudogene exclusion for targeted analysis of *STRC* [52]. In Caucasian patients, DFNB1 and DFNB16 should be excluded before NGS analysis [50].

Similar to other molecular genetic testing approaches, diagnostic testing for HL takes the form of gene panels that usually contain over 100 genes, or whole-exome or whole-genome sequencing with subsequent targeted data analysis. Using whole-exome sequencing and targeted gene analysis, 20 %–30 % of the *GJB2* and *STRC* mutation-negative patients in our diagnostic cohort received a molecular diagnosis. In a conceptually related larger screening study [14], variants in eight genes, including *GJB2* (9 % of cases), *STRC* (6 %), *SLC26A4* (3 %), *TECTA* (2 %), *MYO15A* (2 %), *MYO7A* (2 %), *USH2A* (2 %), and *CDH23* (2 %) accounted for 296 (27 %) of 1119 patients. The overall solve rate with a 66 and 89 deafness-associated gene panel identified pathogenic variants in 49 genes, accounting for a diagnostic rate of 39 %. When comparing the most frequently implicated genes in HL diagnoses across several different studies, it appears that the most frequently involved genes are recurrent in several populations (Table 3) and that roughly half of diagnoses can be explained by pathogenic variants in five to ten genes.

Because of the smooth transition between non-syndromic and syndromic HL, it is important to genetically counsel patients before initiating NGS diagnostics and to clarify whether defined in silico gene panels for the most frequent forms of autosomal dominant or recessive NSHL or larger panels, including all NSHL genes, syndromic genes mimicking NSHL, a larger number of candidate genes, and/or newly identified genes with limited clinical validity, should be analyzed and if so, how variants that may cause phenotypes other than NSHL should be addressed. Ideally, children and, if conspicuous, also their parents should be evaluated by a clinical geneticist or expert in dysmorphology before testing.

Factors that influence diagnostic rates in HL patients

Patient ethnicity

Diagnostic rates for NSHL are currently the highest for patients from Middle Eastern countries (Figure 2). This is largely attributed to high rates of consanguinity that not only enhance diagnostic screening in NSHL, a predominantly autosomal recessive disorder, but also make these populations appealing for research studies. Although molecular genetic studies in NSHL patients from African and South American countries are sparse in the literature, it appears that a diverse molecular landscape unlike what has been observed in other populations can be expected. For example, while pathogenic variants in the gene *GJB2* are common in most ethnic groups around the world, they appear to be rare in native African populations [53].

Hearing loss laterality

HL can manifest as bilateral, asymmetric, or unilateral. A genetic diagnosis is significantly more likely to be achieved in patients with bilateral forms of HL (44 %) compared to asymmetric (22 %) or unilateral (2 %) [14].

Age at hearing loss diagnosis

There is a negative correlation between the age of HL onset in patients in whom environmental factors have been excluded and the diagnostic yield from molecular diagnostic testing. In a study reporting on the diagnostic yield of 1119 hearing-impaired patients, the authors correlated the age

Table 3: Genes frequently mutated in hearing loss according to diagnostics in the respective populations.

Population	European						Asian		Middle Eastern		
Reference	Unpublished data[1]	[30]	[17]	[26]	[18][2]	[44]	[45]	[46]	[47]	[48]	[49]
Country	Germany	Netherlands	France	Belgium	Spain	Czech Republic	Japan	Korea	Iran	Pakistan	Turkey
Patients (*N*)	213	200	207	131	50	51	1025	32	302	321	29
GJB2	1st	1st	1st	1st	1st	1st	1st	1st		3rd	1st
STRC	2nd	3rd	2nd		3rd	2nd	5th				
MYO15A	4th	5th	4th	2nd		2nd			2nd		
MYO7A		4th	3rd	2nd					3rd	2nd	2nd
USH2A		2nd			5th	2nd					
OTOA			5th								
CDH23	5th[3]	4th					3rd		4th		
TECTA			4th		4th						
TMC1				2nd							3rd
SLC26A4	3rd						2nd	2nd	1st	1st	
KCNQ4							4th				
TMPRSS3						2nd					
PCDH15	5th[3]					2nd			5th		
LOXHD1	5th[3]					2nd					
POU3F4								3rd			
MYO6								4th			
COCH								4th			
CIB2										4th	
HGF										5th	
GJB6	5th[3]				1st						
ACTG1					2nd						

[1] Diagnostic cases (index patients) from Würzburg.
[2] Personal communication to clarify the lower than expected prevalence of *OTOF* in the Spanish population.
[3] Genes showing pathogenic mutations in 1 of 213 index patients.

of onset with diagnostic yield [14]. They observed a remarkable reduction in the diagnostic yield when comparing patients with a congenital onset (45 %), patients diagnosed with a childhood onset (30 %), and patients with an onset in adulthood (28 %). These findings were replicated in a Dutch cohort of 200 patients who were grouped according to congenital onset (50 %) or onset in the first (38 %) or second (20 %) decade of life [30].

Other considerations in HL diagnostics

With approximately one out of every two people by the age of 75 years affected, age-related HL (presbycusis) is one of the most common conditions interfering with the quality of life in elderly populations [54]. Although age-related HL is generally thought to be multifactorial, resulting from a complex interplay of genetic susceptibility and adverse environmental (e. g., noise) and systemic (e. g., diabetes) factors, it is plausible to assume that a proportion of cases represent mild forms of autosomal dominant NSHL with very late onset. Age-related HL shows a strong familial aggregation [55]. However, despite its high heritability, the genetic risk factors are largely unknown. A recent genome-wide association study for self-reported presbycusis phenotypes in 250,000 volunteers (between 40 and 69 years) identified 44 significant trait loci [56], including genes, e. g., *CLRN2*, which also has been associated with NSHL [57, 58]. Moreover, several genes, including *COL1A1* and *TGFB1*, have been associated with otosclerosis, one of the more common forms of adult-onset (conductive) HL [59]. The indigenous Filipino population has a 50 % prevalence of middle ear infections which can cause HL. Apart from environmental factors such as smoking and swimming, several gene variants, i. e., in *FUT2* and *A2ML1*, were shown to confer susceptibility to familial otitis media [60]. Similar to the overlap between syndromic and non-syndromic HL genes, there may be an overlap between the many mainly monogenic forms of early onset HL and multifactorial age-related HL. For example, genes associated with presbycusis may act as modifiers for NSHL, and vice versa, variants in NSHL genes may predispose to presbycusis.

Limitations of NGS

The biggest challenge in NGS diagnostics is the classification of numerous variants by various bioinformatic filtering strategies (e. g., by minor allele frequency) and prioritization of potentially pathogenic variants by manual interpretation. The Deafness Variation Database (DVD; http://deafnessvariationdatabase.org) provides information on >8000 pathogenic or likely pathogenic, >170,000 benign or likely benign, and approximately 700,000 variants of unclear significance (VUS) in 152 HL genes [61]. In diagnostics, new VUS are detected, even in well-studied genes. Moreover, >1000 variants have been re-classified in the past decade through expert variant curation according to the DVD. CNVs, most frequently in *STRC* (10 % of cases), *OTOA* (1 %), *GJB6* (0.5 %), *USH2A*, *MYH9*, *SLC26A4*, *TMC1*, and *TMPRSS3*, contribute to approximately 20 % of diagnoses [13]. Due to non-uniform sequence enrichment and low coverage, a substantial number of CNVs may remain undetected by exome analysis alone. Switching molecular diagnostics from whole-exome to whole-genome analysis may overcome this problem.

The many undiagnosed cases, the broad range of auditory pathophysiology, and systematic mouse screens [62] all suggest that a considerable number that is on the scale of several hundred HL genes remain to be elucidated. In addition, many pathogenic variants affecting known deafness genes may remain undetected using current diagnostic algorithms, because they reside in non-coding (intronic and regulatory) sequences or unannotated exons. Considering the many problems with classifying clinically relevant variants in coding and adjacent non-coding sequences, our understanding of the functional consequences of genetic changes outside the exome is even worse. Even if variants in these regions of genes are detected, our capacity for interpretation of these variants is extremely limited and requires experimental support. The integration of RNA sequencing in diagnostics may uncover the transcriptional consequences (reduced expression, aberrant splicing, mono-allelic expression) of genetic changes in genes with expression that is not limited to the inner ear, providing a potential avenue for improvement of variant prioritization and interpretation. Thus, the diagnostic yield should be improved by combined genomic and transcriptomic approaches, at least for disease genes and regulatory RNAs expressed in blood [63]. So far, DFNA50 is the only form of NSHL caused by variants in a regulatory RNA, *MIR96* [64].

The human inner ear may be the least accessible organ for gene expression analyses. The SHIELD database (https://shield.hms.harvard.edu) provides gene expression data for many cell types in the mouse inner ear, but typically for only two embryonic and four postnatal developmental time points [65]. This resource is particularly useful for the prioritization of new candidate genes, many of which are found in only a single patient or family.

At least in former times, assortative mating was common among hearing-impaired individuals. This may explain the enrichment of potentially pathogenic variants in different HL genes in individuals/families with NSHL [66]. Many current diagnostic reporting approaches only consider pathogenic or likely pathogenic dominant or biallelic recessive variants as disease-causing in a given patient and neglect the four or five variants (mainly recessive or VUS) in other HL-associated genes which are often also present. So far, there are few examples for digenic inheritance of HL, including *CDH23*/*PCDH15* [67]. However, one such example of digenism, *TMPRSS3*/*GJB2*, has recently been refuted in a study presenting three families with discordant segregation of pathogenic variants in each of the two genes [68]. This study highlighted how overinterpretation of NGS data in families of limited size may lead to erroneous associations. A modifier variant in *METTL3* suppresses DFNB26 due to biallelic variants in *GAB1* [69]. Since such complex disease etiologies are difficult to unravel, their role in HL may be largely underestimated, accounting for the highly variable expressivity and reduced penetrance of NSHL within families.

Do patients benefit from molecular diagnoses?

To date, comprehensive genetic testing for deafness leads to a diagnosis in approximately 50 % of patients. This removes uncertainty, e. g., whether hearing impairment is genetic or acquired by congenital infection. In many instances, the molecular diagnosis has important implications for treatment (e. g., hearing aids vs cochlear implants [CIs]), individual management (e. g., of additional symptoms of a syndromic disease), prognosis (progressive or stable HL), and family planning. Although cochlear implantation is the treatment of choice for children with severe to profound and/or progressive HL, the outcomes can vary considerably between different genetic forms. Recent research has suggested that patients with variants in *PCDH15* (DFNB23) and *PJVK* (DFNB57) have poor post-implantation CI outcomes, whereas patients with variants in *GJB2* (DFNB1) and *SLC26A4* (DFNB4) have good CI outcomes [70, 71]. Patients with inner ear malformations, e. g., due to variants in *POU3F4* (DFNX2), have an increased risk

for sudden loss of perilymph fluid (Gusher phenomenon) during cochlear implantation [72] or stapes surgery [73].

In murine models, a variety of gene therapy approaches using adeno-associated viral vectors for gene delivery, antisense oligonucleotides, or injection of genome editing agents into the cochlea have already yielded promising results for *Tmc1* [74, 75], *Otof* [76], *Slc17a8* [77], *Ush1c* [78], *Ush1g* [79], and *Whrn* [80]. According to the US National Library of Medicine (https://clinicaltrials.gov) several clinical trials using drug and gene therapies for treating HL are underway or in planning stages for 2020. Although initiating therapies in babies with HL already present at birth will be challenging, as there is already damage to the inner ear, it may be more realistic to initiate therapies for genes with a delayed disease onset and a greater therapeutic window to reduce progression of inner ear damage. In principle, NGS technologies enable comprehensive screening of newborns/children before manifestation or severe progression of sensorineural HL and, thus, prevention and management of HL by classical, and maybe in the future, gene-targeted therapies [8].

Conclusions

Unraveling the genetic diagnosis of HL is important to guide genetic counseling, to support prognostic outcomes and decisions with currently available treatment modalities (e. g., hearing aids versus CIs), and for patient eligibility in future therapy trials. A genetic diagnosis can provide tremendous value by diagnosing syndromes in patients who are pre-symptomatic, allowing for patients to be under the care of respective specialists before symptoms are present. Many challenges remain with respect to obtaining higher diagnostic yields, which may mean that variants in not yet discovered genes or those that reside in regions that are challenging to interpret need to be addressed by the field.

Bullet points for clinical practice

- Genetic testing for HL should be the next test to follow a clinical diagnosis of HL by audiometric testing.
- *GJB2* and *STRC* are highly valuable to pre-screen in patients of European ethnicity before NGS testing is initiated.
- Genetic testing can potentially uncover sub-clinical or pre-symptomatic syndromes in patients, which may be powerful for timely interventions with other medical specialists. This must be addressed in pre-NGS diagnostic counseling.
- The achievement of a diagnosis is heavily influenced by several aspects such as patient ethnicity, laterality of HL, and age at diagnosis.

Patients' rights and animal protection statement: The article does not contain any studies with human or animal subjects.

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