

Review Article

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Applying Next Generation Sequencing to an Early Detection of Hearing Loss Program

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ABSTRACT

Current early hearing detection and intervention (EHDI) programs for children indicate that the diagnosis and treatment must be carried out within the first six months of life. Genetic testing can identify the causal variant of the hereditary hearing loss and is very useful for that. Implementing this testing would enable personalized medicine, avoiding other more costly and time consuming tests, and the negative effect of treating a child outside of the period of greatest hearing sensitivity.

Genetic tools are not part of EHDI programs. These programs are based on testing hearing. If genetic tools are used, the most likely gene is selected and analyzed via Sanger sequencing. The latest next-generation sequencing (NGS) can be applied to EHDI programs. From 100 to 200 genes associated to hearing loss can be analyzed through NGS in one blood sample or saliva set, bringing down the cost of analysis and enabling the causal diagnosis of hearing loss in a short time span.

This paper reviews the current state of early hearing detection and intervention programs in children, discusses the next generation gene sequencing tools applied to hearing loss in children, presents potential approaches to the EHDI programs and analyzes the key issues to personalize the treatment of hearing loss.

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Introduction

Hearing loss is a matter of public health due to its high prevalence. The WHO estimates that 360 million people live with hearing loss around the world. This entails some degree of disability, and in the case of children, beyond the prevalence, it impacts on the cognitive, emotional, educational and social development of the child. Additionally, it burdens healthcare systems with elevated costs (>€3,500/child). Under the current programs, there is an indication to diagnose and intervene within the first 6 months of life, as hearing and language are bound together. Otherwise, an extended hearing loss may have permanent, negative effect on the communicative, cognitive and social development of a child untreated or treated outside of the periods of greater hearing sensitivity [1, 2]. This is currently not possible for all hearing loss cases in children, due to the limiting factor of having detected the hearing loss without an accurate diagnosis.

In our countries, it is estimated that 40% of hearing loss cases are acquired (infections, hyperbilirubinemia, low weight <1500 gr,

ototoxicity, head injury, etc.) and 60% are caused by an alteration to one or several genes [3, 4]. This accounts for the usefulness of genetic testing to identify the causal variant of that congenital hearing loss for the purpose of diagnosing, predicting its nature and forecasting its evolution, and defining a personalized treatment. Besides, identifying the causal variants before 6 months of age would spare them more expensive and time-consuming testing in the future. This would save costs and benefit patients, as it avoids the negative effect on child development that treatment outside of the periods of greater hearing sensitivity entails.

Despite their potential, these genetic tools are not currently part of the universal early hearing detection programs in newborns. These programs still build their strategy on hearing tests, be it auditory evoked potentials or acoustic otoemissions [3]. We must strive for more, and the authors consider that a disruptive approach to this procedure, eliminating false positives and false negatives and learning more about the causes of the hearing loss by using next generation sequencing techniques (NGS) can be applied to early hearing detection and intervention programs in children. This paper reviews the new approach to managing hearing loss in children and the programs' detection strategies.

Present Status of Early Hearing Detection Programs Based on Hearing Screening

The incidence of permanent hearing loss in newborns is 1 for every 1000 births. This and other data, justify the creation of early hearing detection and intervention programs in newborns in order to treat them early and facilitate an optimal development of children affected by hearing loss [3, 5].

Even though there are many objective tests to detect hearing loss, the screening is basically done via hearing assessment methods, such as automated evoked potentials and acoustic otoemissions [3]. Both techniques are highly effective to diagnose hearing loss, they are quickly performed and easy to use, and they are particularly indicated to be used with small children, as they do not require their collaboration. According to the European Consensus Statement on Neonatal Hearing Screening, from 1998, these techniques can identify at least 80% of permanent hearing losses in children, where the false positive rate is between 2-3% of normal-hearing children. Late onset or progressive hearing losses are another limitation of current hearing detection programs in newborns.

Moreover, the American Academy of Pediatrics established that early hearing loss detection strategies must comply with the following features: study both ears in at least in 95% of all newborns; detect all cases (or at least 80%) of bilateral hearing losses greater than 40 dB, a false positive (FP) rate <3% and a false negative (FN) rate of 0%. The rate of referral to an audiological study and confirmation of the diagnosis must be <4% [6]. However, these criteria are not always met in practice. In fact, in one of the first papers published in Spain with Brainstem Auditory Evoked Potentials (BAEPs) with a risk-population, the FP rate is between 24.55% and 7.69%, in the first and second stage respectively [7]. In a more recent paper, also in Spain, these data improve to such an extent that the average referral period for diagnosis in a period of 7 years during the program was 5.69% (range 2.69-15.50%), and the total FN rate was 0.011% [8]. Between the first and the second stages of the program, there may be less children that resort to the necessary testing, which will directly compromise the potential need of receiving a necessary early treatment. The decrease in the number of children monitored was 26.82% for the first group of authors, and 4.50% for the second [7, 8]. Add to this that the onset of 50% of hearing losses in children may happen in the postnatal period [7].

In short, as explained in the document by the Commission for the Early Detection of Hearing Loss in Children (CODEPEH by its Spanish acronym) from 2017 regarding universal screening, these programs have limitations to detect mild and moderate hearing losses, as well as late onset and acquired hearing losses, and/or those that are progressive after birth, on top of the non-negligible number of patient drop outs and organizational, economic restrictions to massive implementation regardless of socio-economic environments. Therefore, CODEPEH, in its 2019 recommendations, points at how many genetic hearing losses cannot be detected with the current universal screening, and informs that they must be complemented with genetic testing, trying to screen for the most common mutations. This could bring up the number of hearing losses detected [3, 9]. However, as explained in the next section, there are next generation sequencing tools that would not only identify the most common mutations, but also the least frequent, those particular to each family. Therefore, implementing these tools could clearly contribute to improving the quality of current early hearing detection programs, besides other advantages brought about by the implementation of personalized medicine to treat hearing loss.

Material and Methods

Next Generation Gene Sequencing Tools for Hearing Loss in Children

Genetic hearing losses are discrete diseases in 75% of the cases. The remaining 25% of genetic hearing losses are associated to another disorder or part of a syndrome. At present, the London Dysmorphology Database (Oxford Medical Publishers) lists 396 syndromes where hearing loss plays a significant part. And the database of hereditary human diseases includes over 900 syndromes where hearing loss is caused by some 500 different genes (*Online Mendelian Inheritance in Man*, OMIM). One may deduce from this genetical heterogeneity and complexity that we must develop validated, universally useful and rational genomic tools for the genetic diagnosis of hearing loss. Over the last 15 years, the knowledge gained of the human genome has enabled research on the genetic bases of may frequent—and sometimes severe—traits, as is hearing loss. Over the last few years, dozens of genes have been identified. If altered, they may take to a certain degree or symptoms of hearing loss. The most frequent mutation in our environment and in the world is located in gene GJB2 (connexin 26); and OTOF (otoferlin) would be the second most frequent [10, 11, 12]. Other than GJB2, GJB6 and OTOF, there is a broad group of genes of variable frequency, depending on the papers reviewed, found in Caucasian Spaniards [3, 11]. One may highlight hearing losses associated to genes such as STRC (stereocilin), CDH23 (cadherin 23), *MYO7A* (myosin 7A), one of the genes that causes Usher syndrome, *MYO15* (myosin 15), *TMC1* (transmembrane channel-like protein 1), *TMRPSS3*, *MYO3A* (myosin 3A) and *SLC26A4* (pendrin), follow an autosomal recessive, hereditary pattern. Besides, hearing losses associated to genes such as USH2A (usherin), one of the causes of Usher syndrome, MITF and SOX1, which cause Waardenburg syndrome, follow an autosomal dominant inheritance pattern. Within the group of hearing losses with an X-linked inheritance pattern, one must highlight the gen POU3F4 and the group of collagen genes, which cause Alport syndrome and other connective tissue diseases that affect the inner ear [6, 13-20]. Within the group of mitochondrial inherited hearing loss, one must highlight homoplasmic mutations associated to gene RNRI-MT [21].

However, not even all these gene mutations can explain all the cases of hereditary, genetic hearing loss. There are still many patients without a final diagnosis. As it was just mentioned, bear in mind that there are some 500 known genes whose mutations can cause hearing loss, 200 of them are broadly supported by evidence.

Historically, the genetic diagnosis of hearing loss would be performed by selecting the gene with the highest likelihood of a causal mutation, based on the outcome of the hearing evaluation. The suspected gene would be analyzed through Sanger sequencing [11, 22]. An existing issue is that there may be different underlying gene alterations that genetically cause the same phenotype. Therefore, applying the Sanger method and ruling out suspected genes until a certain diagnosis is reached might become a long and costly endeavor, which often leaves individuals with hearing loss who are carriers of germline mutations in less common genes undiagnosed.

At present, Next Generation Sequencing (NGS) offers an opportunity to analyze the entire human genome (including the 25,000 known genes) in one single blood or saliva sample; but at a very high cost. Interestingly, more economical NGS tests can be designed to analyze sets of 100 or 200 genes. This could be applied to the 200 genes for which there is greater evidence of association to hereditary hearing loss, avoiding the full genome

analysis and even single-gene analysis. This would optimize the cost and time to diagnose tremendously, while offering other potential advantages, namely, earlier personalized therapeutical intervention for the patient [11, 12, 22]. Undoubtedly, the cost of NGS is high, compared with Sanger, when the object of analysis are 1 or 2 genes. But it is a lot more cost effective when we need to analyze more than 5-10 genes to reach a diagnosis.

The fact that we can analyze more genes in one single NGS assay allows for the identification of gene mutations accountable for a smaller percentage of hereditary hearing loss, and the diagnosis of syndromes that would otherwise remain unsolved, as they are associated to genes generally left out of the single gene analysis. Therefore, it is clear that a NGS assay that analyzes simultaneously the 100 or 200 genes with mutations accountable for a large percentage of hereditary hearing loss in the first 6 months of life would enhance diagnostic capabilities, providing a certain diagnosis more quickly and therefore, enabling early therapeutic intervention to support the cognitive and language development of the child. All this is vital in the age of personalized medicine.

Panel Design

The authors have designed a targeted panel NGS test to analyze 180 genes causally associated to hereditary hearing loss, both syndromic and non-syndromic, regardless of the type of inheritance. First, genomic DNA is extracted from a peripheral blood sample or saliva, then sheared into random fragments by ultrasonication, using the Adaptive Focused Acoustics (AFA) technology (Covaris). Then, the fragments are ligated with adaptors that will make the DNA molecules recognizable by the sequencing team MiSeq (Illumina). Before the sequencing, molecules that contain information about the genes or regions of interest are selected with the hybridization probe SureSelect XT (Agilent), specifically designed for the genomic regions of interest. This probe design and the methodology implemented (including the bioinformatic analysis) enable the identification of single Nucleotide Variants (SNVs), small insertions or deletions (indels), copy number variants (CNVs) and other structural variants (SVs) in the full coding region of 9 mitochondrial and 171 nuclear genes with mutations associated with hereditary hearing loss, including splicing alterations and some intronic or intergenic alterations [22].

Once identified with NGS, just as when they are identified with Sanger or any other molecular method, the clinical interpretation of variants is based on the American College of Medical Genetics and Genomics (ACMG) guidelines. Variants are classified in 5 categories: benign, likely benign, uncertain significance, likely pathogenic and pathogenic [23]. All the results obtained must be discussed in multidisciplinary sessions among clinicians and geneticists, in order to validate the phenotype-genotype correlation and discuss unresolved cases.

When using targeted panel-NGS, the analysis is limited to genes with alterations associated to the phenotype—the hearing loss in this case. NGS enables the full sequencing of the exome or genome (it is more expensive, and therefore less feasibly implemented in clinic). However, we must take into account that this option increases the risk of secondary findings, identifying variants of no pathological significance or unrelated to the reason that brought the minor to the doctor's office. This ethical angle of the analysis is relevant when applying NGS analysis to early hearing detection programs in children.

GHELP Project Development: New Outlook on Early Hearing Detection and Intervention Programs in Children

It has been mentioned that various papers have been published over the last few years, explaining how NGS techniques are applied to diagnosing hearing loss genetically [11]. However, there are few initiatives to apply this technology to early hearing detection programs. GHELP is a transnational collaboration project, engaging 8 institutions from 7 SUDOE regions, and funded by the EC's Interreg program. Our collective goal is to innovate in hearing loss detection and treatment. The goals of our project include: proving the usefulness of a 180-gene genetic panel, as these genes have been scientifically associated to hearing loss and analyzed with NGS technology, and then study the economic, social and healthcare-related feasibility of building this tool into current detection programs undertaken by Healthcare Systems (*the GHELP project is made public: first presentation in Pamplona – Interreg V Sudoe – Interreg Sudoe Program Sudoe [Internet]. [cited 2020 Jul 3]. Available from: <https://interreg-sudoe.eu/comunicacion/noticias-sudoe/215-el-proyecto-ghelp-se-da-a-conocer-primer-evento-de-presentacion-en-pamplona/categoria-3-Gestion-de-los-proyectos-aprobados>*)

What are the specific advantages of implementing a panel, as the one designed by GHELP, in an early hearing detection program? (Figure 1):

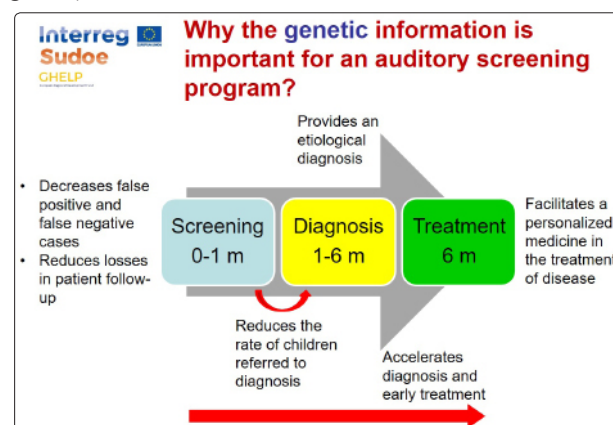


Figure 1: Advantages of building next generation sequencing techniques into an early hearing detection program in newborns

- Complementing the audiological tests of a hearing detection and intervention (EHDI) program with genetic testing may help reduce the percentages of FP and FN in a program, bringing it closer to meeting the quality criteria a hearing detection and intervention program in children should have [3, 6]. Reducing false positives is key to reduce the economic cost of testing during the detection, and especially during the diagnosis. Reducing the percentages of FN prevents a late diagnosis of hearing loss and the subsequent start-up of early treatments, which are essential to optimize the results obtained with them.

- Carrying out one single genetic test in the first days of life of a newborn child, following the delivery, when they are still hospitalized, would reduce the drop-out rate during the different stages of an EHDI. The drop-out rate fluctuates from one healthcare system to another within the 4.50%-50% range [8, 24]. High drop-out rates hinder the efficacy of the detection and the early treatment of hearing loss.

- Because 60% of neonatal hearing losses have a genetic etiology, applying NGS techniques would lead to an etiological diagnosis of hearing loss in a high percentage of the population. This is undoubtedly valuable; it enables the advantages listed and described in the following points.

- Obtaining a genetic diagnosis sheds light to optimize the genetic counsel to parents and patients regarding the nature of their disease: likelihood of developing hearing loss, risk of transmitting it, prevention measures, early diagnosis and available treatments.

- The etiological diagnosis of hearing loss optimizes and personalizes the diagnostic process, as it helps characterize other diseases associated to a syndrome, it provides insights about the evolution of the hearing loss and it could prevent certain triggers or worsening of the hearing loss.

- The insights gained through genetic testing can spare other (more expensive and time-consuming) testing, which cuts both the expense and inconvenience of unnecessary testing for patients. This is aligned with the CODEPEH recommendations about the pursuance of an etiological diagnosis [3]. The document establishes the sequence to investigate the cause of congenital hearing loss. Genetic analyses are recommended as a second step, following the examination and anamnesis, as from all the available, complementary tests, genetic analysis yields the best diagnostic performance and identifies a genetic cause in 44% of patients with bilateral, sensorineural hearing loss.

- Building NGS techniques into the analysis of hearing loss would shorten the time-to-diagnosis and reduce the anxiety of parents in the face of lack of information over a lengthy diagnostic process, which can often drag on for some years.

- An early diagnosis clearly enables early treatment, which is of paramount importance, as it is one of the main prognostic factors, and it adds to the cost-benefit equation of the treatments used [25].

- Having a genetic etiological diagnosis would enable personalized palliative or curative care, to be selected specifically based on the mutation diagnosed [23, 25]. The next chapter will discuss this concept in greater detail.

- Finally, having a specific genetic diagnosis allows for cheap and quick analysis of mutations within a family, creating value for the family's genetic counsel and informed reproductive decision making.

All this provides the public administrations charged with implementing EHDI programs and the centers that carry them out with a tool that yields a positive cost-benefit analysis now, and potentially better in the short term, as NGS technology is becoming widespread and therefore becoming exponentially cheaper. Let us not forget the valuable knowledge that this technology offers to the scientific community. That will undoubtedly help healthcare professionals establish a clear roadmap for the development of gene therapy for deafness now and in the future. Additionally, the genetic and clinical data discovered will help create new genomic tools with greater diagnostic performance and less costly, which will contribute to their universal implementation across EHDI programs.

In sum, the GHELP project is an attempt to improve current detection programs by implementing a genomic diagnostic tool to act early and develop personalized treatments.

Discussion

Future Perspectives: Personalized Medicine to Treat Hearing Loss

The future of medicine lies in personalization. Personalized therapies for each patient allow for treatments applied more

precisely and promptly, and therefore, with better results. Traditional medicine tends to adopt the "one for all" approach. However, the goal of personalized medicine is to apply an individualized treatment based on the genetic traits of a patient. It has already been mentioned that 60% of hearing losses are genetic. Hearing loss is caused by the alteration of one or a few genes. Therefore, genetic testing is very useful to diagnose and predict the nature and evolution of a disease, and to establish accurate, personalized treatments. Let us look at some examples:

- Detecting a mutation in OTOF, the gene encoding the protein otoferlin, gives way to a hearing loss clinically framed within the auditory neuropathies [26]. Within this group of diseases, genetic tests as part of the EHDI avoid false negatives when the program is basically built on the otoacoustic emissions test. Therefore, they are a key for the differential diagnosis of this disease, which would eventually lead to cochlear implantation.

The OTOF gene, locus DFNB9 (DF for deafness, B for recessive inheritance and locus number 9), encodes otoferlin, the protein that bonds to intracytosolic calcium and is anchored to the plasmatic membrane. It is part of the exocytosis of synaptic vesicles of hair cells in the cochlea [26]. OTOF gene mutations are accountable for a very homogenous non-syndromic, prelingual profound sensorineural hearing loss, without malformations associated with the inner ear. Besides, patients affected by a DFNB9-type of hearing loss present auditory neuropathy, which is a distinct clinical sign of these patients [27]. Therefore, one could expect a positive result with the CI's post-synaptic stimulation. There are many post-synaptic stimulation studies that prove that the CI yields clinically positive results in restoring auditory perception to patients with this genetic alteration [28, 29].

- A very complex issue when managing hearing loss in children is the information provided to parents. As part of the guidance given, it is key to convey clearly and confidently what is the prognosis to be expected from a given treatment. Therefore, when we have the diagnosis for one of the most frequent alterations in our environment, as is the mutation of the GJB2 gene, which tends to involve severe to profound hearing loss, we can predict an optimal result with the early use of a cochlear implant [30]. GJB2 encodes a gap junction protein called connexin 26 (Cx26), a transmembrane protein that is oligomerized with five other connexin molecules to create a connexon. Adjacent cells' connexons combine to produce the gap junctions, great "pores" that enable the cytoplasmatic exchange of electrolytes, second messengers and metabolites [31]. We now have evidence that proves that GJB2 gene mutations in Caucasian, European populations (DFNB1) account for up to 50% of recessive, non-syndromic hearing loss. Interestingly, GJB2 mutations are found in many apparently sporadic cases of deafness [20, 30, 31].

- One example of personalized, preventive treatment would be detecting mutations of the mitochondrial gene MT-RNR1 in people who are going to be treated with aminoglycoside antibiotics, such as gentamycin and streptomycin. These patients may develop sensorineural hearing loss [32]. In a study performed by a Spanish group, 55 patients were explored. There were 17 patients within the group who had been previously exposed to ototoxic drugs and had developed severe hearing loss in almost all cases. One of them was deaf mute. Most patients developed bilateral, symmetrical hearing loss that progressed slowly, particularly within the high frequencies. Carriers of mutation A1555G in mitochondrial DNA generally present mild to moderate bilateral, symmetrical, sensorineural hearing loss, particularly in the high frequencies, that

develops slowly and starts in the second decade. It is significantly worsened by the ototoxic effect of aminoglycosides [32].

The genetic diagnosis of hearing loss opens the door to curative treatment based on gene therapy. Gene therapy can act on a cell's genome and modify the genes that determine a disease. To this end, work has been undertaken with animal models, using a small-size virus—generally an adenovirus or an adeno-associated virus—to express the genetic material previously added to the virus artificially, in the genome of target cells.

Hearing loss is a disease that could benefit tremendously from these therapies. In fact, gene therapy can be pursued for genetic hearing loss in the realm of animal testing. However, research must develop further to achieve safety and efficacy in human beings, as demonstrated by the exponential increase in scientific publications on gene therapy for hearing loss over the last few years (Figure 2).

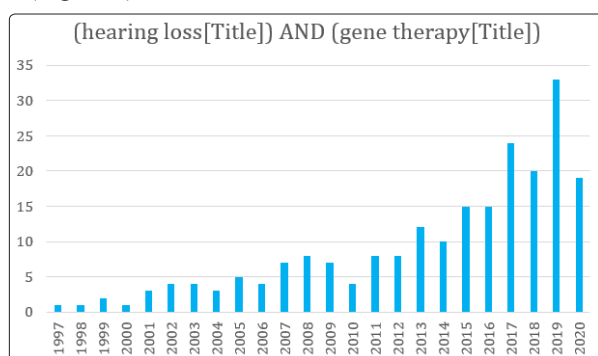


Figure 2: This graph shows the frequency of papers indexed by PubMed on gene therapy and hearing loss.

Technically, it is required to administer the viral preparations into the inner ear so that they would act directly on the cells affected by the specific mutation. For example, given the mutation of gene GJB2, the preparation should reach the support cells that make up the gap-junctions in the organ of Corti. Given the anatomical features of the ear, there is a discussion about the approach and the route to administer these drugs. Basically, three routes to administer the drugs have been described: through the round window, via cochleostomy or posterior canalostomy [33]. Figure 3, drawn from Ahmed et al, shows the 3 routes of administration [33].

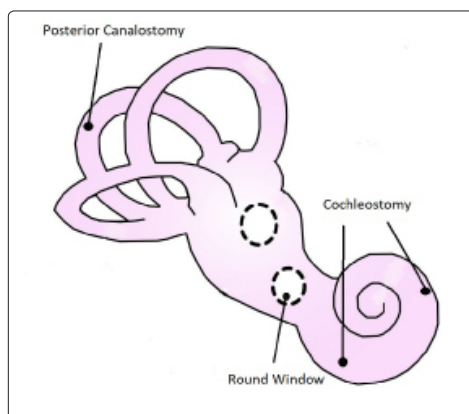


Figure 3: Illustration showing the 3 possible routes to inject the preparations with the vector to ensure their distribution to the target cells in a gene therapy procedure

The first satisfactory results published were presented in 2012 by Akil. The gene they modified was VGLUT3 [34]. At present,

there are several clinical trials underway or finalized to modify genes that cause hearing loss [34]. One may highlight the work of Pan et al 2017 with gene USH1C in an animal model, where they achieve the full restoration of hearing [35].

Curative treatment for hearing loss caused by a mutation of GJB2 would require restoring the expression of Cx26 to therapeutic levels in the affected cells within the cochlea. Lizuka et al 2015 and Crispino et al 2017 detail the full or partial restoration of hearing by using gene therapy for GJB2 gene mutations [36]. Regarding otoferlin, Akil et al and Al-Moyed et al present an improvement in hearing levels with gene therapy applied to the OTOF gene [37]. However, there are many other groups studying gene therapy for other less prevalent genes, such as *TMCI*, *WHRN*, *MYO7A*, *MSRB3*, *KCNQ1* and *SLC26A4* [33, 38, 39].

Clinical success with these lines of treatment requires reaching the right cells, efficiently and with high specificity. Technological developments in viral vectors may help achieve that. The two first gene therapy products with adenovirus (AAV) have been recently approved to treat single genes mutations (Luxturna™ and Zolgensma™, targeting the eye and central nervous system, respectively) [40].

Conclusions

This paper has reviewed the present status of early hearing detection and intervention programs in children, showing the next generation gene sequencing tools available for hearing loss in children, and describing how these tools might be applied to early hearing detection programs, thereby speeding up the diagnostic process and making it more affordable, and opening the door to the personalized treatment of hearing loss.

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