

# Prevalence of *GJB2* gene mutations correlated to presence of clinical and environmental risk factors in the etiology of congenital sensorineural hearing loss of the Romanian population

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Received January 20, 2021; Accepted February 19, 2021

DOI: 10.3892/etm.2021.10044

**Abstract.** Although etiologically heterogeneous at least 50% of all early on-set hearing losses have a genetic cause and of these, the large majority, 75-80% are most probably autosomal recessive and 70% are non-syndromic. The rest of the congenital hearing losses are determined by clinical and environmental factors such as ototoxic medication, prematurity, and complications at birth. During the last decade it became clear that 50-80% of all such afflictions result from mutations in a single gene, *GJB2*, which encodes the protein Connexin 26. In order to, at least partially clarify this problem, especially in an emerging country such as Romania, where the problem is not studied adequately, we developed a comprehensive study of genetic, clinical and environmental risk factors for congenital hearing loss. The two most common variations of this gene, *35delG* and *W24X* in children with positive diagnosis of bilateral severe to profound sensorineural hearing loss were investigated. A cohort of 34 children (20 female and 14 male), ages between 2 and 10 (mean age 4.62 years), coming from 33 non-related families were evaluated. All cases were diagnosed with severe or profound bilateral congenital SNHL. A statistical comparison of genetic and environmental/clinical prevalence was also attempted since the presence of a genetic disorder cannot rule out the role of other documented risk factors in the etiology of SNHL. The results showed that,

29.4% of cases (10/34) were homozygotic for the *35delG* mutation *35delG/35delG*, also known as genotype  $\Delta/\Delta$ . 5.88% of cases (2/34) belong to the heterozygotic bi-genic group *35delG/W24X*. The clinical factors with high statistical significance for SNHL in a non-genetic group have no significance for genetic SNHL patients. Thus, the present study confirms the relatively high prevalence of the *35delG* and *W24X* mutations in cases of congenital non-syndromic severe or profound bilateral SNHL.

## Introduction

According to data reported by the World Health Organization, over 250 million individuals currently suffer from hearing loss of variable etiology, which represents 4.2% of the world's population (1-5). Congenital hearing loss is relatively frequent, with a prevalence reported by different sources in literature as varying between 1-3/1000 newborns (6) and 1/500 newborns (7).

Due to the etiological heterogeneity of congenital hearing loss, genetic, clinical and environmental risk factors often combine and provide a very complex picture that makes genetic evaluation and council extremely difficult, especially for very small children. Recent findings indicate the mutations of the *GJB2* and *GJB6* genes on the 13q11-q12 chromosome as responsible for more than 50% of all types of non-syndromic autosomal recessive congenital hearing loss in certain populations. This information facilitates, to some extent, the genetic diagnosis and gives families increased information regarding this genetic affliction. Although some hearing losses can be diagnosed with certainty as having a genetic cause, the presence of other risk factors such as ototoxic medication, prematurity, and complications at birth cannot be ruled out and the statistical significance of combined genetic and environmental factors represents an important but very little studied issue (5-7).

The *GJB2* and *GJB6* genes encode the Connexin 26 (Cx26) and Connexin 30 (Cx30) membrane proteins which form the

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**Key words:** Connexin Cx26, *GJB2*, sensorineural hearing loss, DNA mutational analysis, risk factors, environment, gene frequency

intercellular heteromeric channels known as gap-junctions. These structures have an important role in the cochlea homeostasis and ensure the influx of potassium ions from the internal and external hair cells to the cochlea supporting cells. The *GJB2* gene has over 100 alleles, especially for the autosomal recessive forms of deafness. The deletion of a single guanine, *35delG*, is responsible for over 50% of the non-syndromic sensorineural hearing loss (SNHL) in Europe, North America and Asia (8,9). The common deletion 342-Kb of the *GJB6* gene (GJB6-D13S1830) appears in up to 20% of all SNHL cases in the US and could be responsible of approximately 10% of all the DFNB1 alleles, since it has a very wide action range, based on ethnic origin and is frequently associated with the *35delG/GJB2* mutation (8,10,11).

Recent findings have shown that the *GJB6* mutations have no active role in the etiology of congenital non-syndromic SNHL and that the Connexin 30 protein would only modulate the action of the Connexin 26 protein. This would mean that in the case of an SNHL with a *GJB6* deletion, the deafness would be, in fact, the result of a *GJB2* deletion modulated by the presence of the *GJB6* deletion (12).

## Materials and methods

**Subjects.** A cohort of 34 children (20 female and 14 male), ages between 2 and 10 (mean age 4.62 years), coming from 33 non-related families was evaluated. All cases were diagnosed with severe or profound bilateral congenital SNHL, over a time span of 3 years. After establishing a complete family and personal history, the patients underwent a thorough ENT examination which excluded the syndromic forms of deafness and other associated diseases. A personal interview of the parents provided essential information regarding the pregnancy period (mother's health, pollution, use of ototoxic drugs, alcohol, smoking during pregnancy), birth (premature child, hypoxia, cranial trauma, extended ICU care after birth >5 days) and evolution of the SNHL (when and how it was discovered).

**Methods.** After excluding any middle ear pathology, the audiological diagnosis was established by using otoacoustic emissions (OAE), brainstem auditory evoked response (BAER) and auditory steady state response (ASSR) for children under 4 years of age (following the current international guidelines-Year 2007 Position Statement: Principles and Guidelines for Early Hearing Detection and Intervention Programs: Joint Committee on Infant Hearing) and Pure Tone Audiometry for children over 4 years of age. Syndromic or acquired hypoacusis were excluded from the study. Patients with a suggestive SNHL family history (deaf parents) were also excluded but patients with suggestive risk factors (ototoxic treatments, hypoxia at birth, and prematurity) were included, since it has been shown that the presence of such factors does not exclude a genetic etiology of the SNHL. A statistical correlation between the presence of these risk factors and a genetic SNHL was calculated and compared to another that only took into consideration the statistical significance of the clinical and environmental factors on a much larger cohort of newborns.

None of the patients benefited from neonatal screening even though some of them presented obvious risk factors for congenital SNHL. The mean age for diagnosis was 2.7 years.

**DNA and AS-PCR.** After obtaining the informed, written consent of the parents, a blood sample was taken for molecular testing and establishing a possible genetic cause for hearing loss. Peripheral blood (3 ml) was collected in EDTA tubes (ethylenediaminetetraacetic acid) from each participant by venous puncture.

DNA was extracted from peripheral blood leukocytes using the commercially available kit QIAmp DNA Minikit (Qiagen GmbH), using the following protocol: Electrophoresis 1.5% agarose gel in 1X TAE, 80-100 V; DNA control= $\lambda$  DNA standard; Samples=7  $\mu$ l DNA + 3  $\mu$ l LB (Luria broth); Control=5  $\mu$ l  $\lambda$ DNA + 3  $\mu$ l LB. The cultures were stopped with colchicine during the metaphase. The DreamTaq DNA polymerase from ThermoScientific DreamTaq Green PCR Master Mix (2X) was used. The reactants for the PCR reaction are presented in Table I. The molecular analysis was carried out using AS-PCR and multiplex-PCR techniques by directly amplifying the DNA with specific primers. Sequences of the forward and reverse primers ( $\rightarrow$ , direction) are: Forward primer 5'-TCTTTTCCAGAGCAAACCGC-3'; Reverse primer 5'-GCTGGTGGAGTGTGTTGTTACACCCGC-3'. For W24X mutation, 5'-AAGGTGACCAAGTTCATGCTCATGCTA AATGAAGAGGACGGTGAGCT-3' detected the mutations and 5'-CGCCCAGAGTAGAAGATGGATT-3' served as the common reverse primer.

After thawing the DreamTaq Green PCR Master Mix (2X), gently vortexing and briefly centrifuging, the amplification program was carried out as follows: Initial denaturation at 95°C for 3 min, followed by 30 cycles, each comprising denaturation at 94°C for 40 sec, annealing at 65°C for 60 sec, extension at 72°C for 60 sec and a final extension at 72°C for 7 min.

PCR products were submitted to a 2.5% agarose gel electrophoresis, stained with 1  $\mu$ l ethidium bromide and visualised under a UV light transilluminator. Weight marker 100 pb was calculated as 6  $\mu$ l Marker (GeneRuler 100 bp DNA ladder) + 2 LB (6X DNA Loading Dye).

The 89 bp resulted as PCR products of amplification were digested at 37°C with *Bst*NI restriction enzyme (New England Biolabs Inc.). In absence of deletion, the enzyme breaks occurred as 2 fragments, 69 and 20 bp. If deletion was present, the enzyme did not break the DNA sequence (89 bp).

**Statistical analysis.** After asserting the genetic etiology of the SNHL for all cases, the results were correlated to the presence of other risk factors in the patient's history. All data regarding the newborns (NBs) and the risk factors were centralized in a Microsoft Excel database and were attributed codes for easy statistical analysis. Statistical analysis was carried out using the Excel and SPSS ver. 15.00 programs. The data were synthesized as percentages, means, mean  $\pm$  standard deviations and quartiles. The statistical significance level used was  $P \leq 0.05$ . The data were analyzed in relation to the presence of environmental and clinical risk factors. For quantity variables, according to data distribution, parametric (Student's t-test) and non-parametric tests (Mann-Whitney) were applied whereas for quality variables, proportion equality tests (Fisher's exact and Likelihood Ratio) were applied.

In order to identify the significant risk factors, the univariate logistic regression was initially used, by calculating the odds

Table I. Reactants of the PCR reaction.

Substance	1X (X=number of samples)	34X
Master Mix 2X	12.5 $\mu$ l	425 $\mu$ l
Forward Primer-Oligo 1	2.5 $\mu$ l	85 $\mu$ l
Reverse Primer-Oligo 2	2.5 $\mu$ l	85 $\mu$ l
Water, nuclease free	6 $\mu$ l	204 $\mu$ l
Total	23.5 $\mu$ l	799 $\mu$ l

ratio (OR) together with the 95% confidence interval and then the multivariate logistic regression including all variables and using a Forward Stepwise (Wald) selection percentage of variables.

The results of this statistical study were then compared to those of a similar study performed on 854 patients that were screened for congenital SNHL but had no proven genetic etiologic involvement. The aim was to determine whether the known environmental risk factors have the same statistical significance in genetic cases.

## Results and Discussion

Since genetic testing is an expensive method and the financial means of the study were limited, a selection of patients was necessary. From the initially evaluated cohort of 58 cases with severe or profound bilateral SNHL, only 34 cases were selected: Children that had no other health issues (non-syndromic cases) and had no family history of SNHL (normal hearing parents). The 34 subjects underwent a genetic screening for the *35delG* and *W24X* mutations of the *GJB2* gene.

The results showed that, 29.4% of cases (10/34) were homozygotic for the *35delG* mutation (*35delG/35delG*), also known as genotype  $\Delta/\Delta$  (the Greek letter  $\Delta$  preceding the name of a gene, signifies that the gene has a chromosome deletion while the letter N represents the lack of a deletion). No case of heterozygosity *35delG/N* ( $\Delta/N$ ) was present for the *35delG* mutation while 5.88% of cases (2/34) belong to the heterozygotic bi-genic group *35delG/W24X*.

The *W24X* mutation was present in 5.88% of cases (2/34) as homozygotic genotype (*W24X/W24X*) and in 2.94% of cases (1/34) as heterozygotic genotype (*W24X/N*). Results are presented in Table II. The overall prevalence of the mutant allele was 32.35% for *35delG* and 10.29% for the *W24X*.

As far as environmental factors are concerned, the urban environment becomes statistically significant ( $P=0.027$ ) for SNHL with genetic etiology compared to the patients with non-genetic etiology but that could also be attributed to the small number of cases included in the study. Other known environmental risk factors such as prematurity ( $P=0.042$ ), age of the mother ( $P=0.048$ ), ototoxic medication during pregnancy ( $P=0.023$ ) also have great statistical significance for genetic SNHL (Table III).

The clinical factors with high statistical significance for SNHL in a non-genetic group have no significance for genetic SNHL patients (Table III). The statistical values included in this table were specifically calculated for genetically diagnosed patients (*35delG* and *W24X* mutations) with the

Table II. Results of genetic testing in the present study.

Genotype	No. of subjects/total no. of patients (%)
<i>35delG/35delG</i>	10/34 (29.4)
<i>35delG/N</i>	0/34 (0)
<i>35delG/W24X</i>	2/34 (5.88)
<i>W24X/W24X</i>	2/34 (5.88)
<i>W24X/N</i>	1/34 (2.94)
<i>N/N</i>	19/34 (55.88)

N, absence of mutation (*35delG* or *W24X*).

purpose of demonstrating that sometimes, the presence of a genetic mutation can be statistically correlated to the presence of known environmental factors that also generate SNHL (i.e. prematurity, urban environment, and ototoxic medication).

SNHL is one of the most frequent human disabilities and the genetic factor plays a central role in its research and diagnosis. Over 130 loci for human non-syndromic SNHL have been identified and it is estimated that more than 100 mutations of the *Cx26* encoding genes may be involved (13). This variety of mutation makes the genetic diagnosis extremely difficult and even more, the prevalence of certain genes varies for different population groups.

The *35delG* mutation of the *GJB2* gene remains, however, the main cause of genetic SNHL in the Caucasian population. The prevalence of mutation carriers varies from 1/35 for the south European population to 1/79 in Northern Europe (14,15). The maximum prevalence is recorded in countries around the Mediterranean (13). This mutation also has one of the highest pathogenic potential in humans, with a frequency of carriers similar to that of the deltaF508 mutation of the cystic fibrosis gene (*CFTR*) (14,16). It can be found both in homozygosis and heterozygosis state and it can also be associated to other mutations of the *GJB2* gene or the *GJB6* gene such as D13S1830, which is the second-ranking mutation involved in the etiology of SNHL in Europe. In certain population groups such as the Japanese, the Chinese, Ashkenazi Jews and the Roma populations, the *35delG* mutation is very rare and is replaced by other mutations such as 235delC, 167delT or *W24X* (17).

The present study evaluated the prevalence of the *35delG* and *W24X* mutations within the Romanian population suffering from congenital non-syndromic SNHL and attempted to associate the presence of the genetic mutation to other known risk factors for SNHL. Although the number of studied cases was relatively small and all diagnosed in the same clinic, the results showed that the screening for known mutations and a rigorous selection of cases can pinpoint the presence of the genetic factor in almost 50% of cases of severe or profound congenital SNHL.

The prevalence of the *35delG* mutation among the deaf patients was 29.4% in accordance with similar studies that reported 13.6% in Jordan, 14% in Palestine (13,18), 16% in Egypt (13,19), 5.66% in Iran (12,13) but lower than other studies that report 94% in Lebanon (13,20) and 60-80% in European populations (13,21) (Table IV). The relative frequency of the *35delG* mutant alleles was also in line with previously

Table III. Statistical comparison of environmental and clinical factors for genetic and non-genetic SNHL.

Risk factors	Cohort		P-value (test)
	Screening (N=854)	Genetic (N=34)	
Gender=Female	432/854 (50.6%)	20/34 (58.8%)	0.384943 (Fisher exact)
Environment=Urban	563/852 (66.1%)	16/34 (47.1%)	0.027233 (Fisher exact)
Prematurity=Yes	115/854 (13.5%)	9/34 (26.5%)	0.042056 (Fisher exact)
Gestational age	39 [38.00, 39.00]	38 [36.00, 40.00]	0.138 (Mann-Whitney)
Apgar score	8.38±0.8694	8.41±0.9883	0.826370 (Student T)
Mother's age	28.84±5.951	26.79±4.841	0.048529 (Student T)
Maternal pathologies=Yes	164/854 (19.2%)	9/34 (26.5%)	0.275933 (Fisher exact)
Chemicals, Radiation=Yes	1/854 (0.1%)	0/34 (0.0%)	1.000000 (Fisher exact)
Ototoxic medication for mother=Yes	88/854 (10.3%)	8/34 (23.5%)	0.023347 (Fisher exact)
Birth complications=Yes	50/854 (5.9%)	3/34 (8.8%)	0.449632 (Fisher exact)
Congenital infections=Yes	110/854 (12.9%)	3/34 (8.8%)	0.608554 (Fisher exact)
Periintraventricular hemorrhage=Yes	22/854 (2.6%)	1/34 (2.9%)	0.597264 (Fisher exact)
Respiratory distress=Yes	46/854 (5.4%)	3/34 (8.8%)	0.427561 (Fisher exact)
Perinatal hypoxia=Yes	98/854 (11.5%)	4/34 (11.8%)	1.000000 (Fisher exact)
Mechanical ventilation=Yes	81/854 (9.5%)	4/34 (11.8%)	0.558063 (Fisher exact)
Neonatal Intensive Care (NICU)=Yes	78/854 (9.1%)	4/34 (11.8%)	0.545578 (Fisher exact)
Neonatal hypotension=Yes	37/854 (4.3%)	0/34 (0.0%)	0.393117 (Fisher exact)
Hypoxic ischemic encephalopathy (HIEP),=Yes	3/853 (0.4%)	0/34 (0.0%)	1.000000 (Fisher exact)
Hyperbilirubinemia=Yes	135/854 (15.8%)	3/34 (0.0%)	0.341235 (Fisher exact)
Cranial trauma=Yes	56/854 (6.6%)	0/34 (0.0%)	0.265235 (Fisher exact)
Ototoxic medication for NB=Yes	64/854 (7.5%)	5/34 (14.7%)	0.176912 (Fisher exact)
Craniofacial abnormalities=Yes	1/854 (0.1%)	0/34 (0.0%)	1.000000 (Fisher exact)
Other abnormalities=Yes	91/853 (10.7%)	4/34 (11.8%)	0.777523 (Fisher exact)
Very Low Birth Weight (VLBW)	18/854 (2.1%)	0/34 (0.0%)	1.000000 (Fisher exact)
Extremely Low Birth Weight (ELBW)	8/854 (0.9%)	0/34 (0.0%)	1.000000 (Fisher exact)

published studies (Table V). Extensive research conducted in the Middle East determined that the frequent presence of the *35delG* mutation is directly linked to the high level of consanguinity of the studied populations, which does not apply to the European and therefore the Romanian population.

The frequency of the *35delG* mutation carriers varies in different studies from 1.1% in Jordan to 1.66% in Syria and up to 3.2% in Italy, the highest known frequency thus far (13).

Concerning the presence of the homozygotic genotype *35delG/35delG* in the Romanian population, the study is in accordance with those of other Romanian researchers from different areas such as: Bucharest (36%), Craiova (24%) and Transylvania (25.33%) (22–24) (Table VI). A particular feature of the results is the total absence of the heterozygotic genotype, *35delG/N*. This result had been reported only once before by Romanian researchers, in a 2010 study by Lazăr *et al* on 75 patients from Transylvania (22).

The results of the present study suggest that the high prevalence of the *35delG* mutation within the studied population may represent enough reasons to implement genetic diagnosis and council on a larger scale and also continue the research on this subject. However, the techniques used for detecting mutations are very expensive and occasionally prone to sequencing errors (14).

The *W24X* mutation is another relatively common cause for the non-syndromic SNHL, especially in populations from Northern and Southern India (13,25,26). A study on the Roma population of Slovakia reports a relatively large prevalence of *W24X* carriers (26.1%), probably in close correlation to the Indian origin of these populations. The present study found a 10.29% prevalence of the homozygotic genotype (*W24X/W24X*) and a 5.88% prevalence of the digenic heterozygotic genotype (*35delG/W24X*) for this mutation, similar to another Romanian study by Lazăr *et al* which reported a 1.33% prevalence for the *W24X/W24X* genotype and a 2.66% prevalence for the *35delG/W24X* genotype (22) (Table VI).

The diagnosis of SNHL in children is very complex and requires a number of specialized tests for establishing the etiology (27). However, testing for *GJB2* mutations is currently recommended as an initial test, followed by *GJB6* testing in the case of a negative result (28). Genetic testing has become a very useful tool for the ENT specialist in the attempt to explain the etiology of the SNHL. The main benefit of genetic testing is that it can explain deafness without any additional examinations (29). Additionally, information can be obtained regarding the manner in which the mutation was transmitted and the probability of appearance in other possible offspring (29,30).

Table IV. Prevalence of genotypes in the present study compared to other published studies.

Genotype	Present study (%)	Cordeiro-Silva <i>et al</i> 2010 (36) (%)	Pfeilsticker <i>et al</i> 2004 (37) (%)	Piatto <i>et al</i> 2004 (38) (%)	Battissoco <i>et al</i> 2009 (39) (%)	Nogueira <i>et al</i> 2011 (8) (%)	Al-Achkar <i>et al</i> 2011 (13) (%)	Khushvakova 2010 (5) (%)
35delG/35delG	10/34 (29.4)	3/77 (3.9)	2/75 (2.66)	5/33 (15)	22/300 (7.3)	4/95 (4.2)	15/50 (30)	146/374 (39)
35delG/N	0/34 (0)	5/77 (7.8)	2/75 (2.66)	3/33 (9)	12/300 (4)	8/95 (8.4)	9/50 (18)	57/374 (14)
35delG/Other mutation	2/34 (5.88)	1/77 (1.35)	N.A.	1/33 (3)	3/300 (1)	6/95 (6.3)	2/50 (4)	23/374 (6)

N, absence of mutation (35delG or W24X); N.A., not analyzed.

Table V. Prevalence of mutant alleles (35delG and W24X) in the present study compared to other published studies.

Geno-type	Present study (%)	Lazăr <i>et al</i> 2010 (22) (%)	Cordeiro-Silva <i>et al</i> 2010 (36) (%)	Pfeilsticker <i>et al</i> 2004 (37) (%)	Piatto <i>et al</i> 2004 (38) (%)	Battissoco <i>et al</i> 2009 (39) (%)	Nogueira <i>et al</i> 2011 (8) (%)	Al-Achkar <i>et al</i> 2011 (13) (%)	Khushvakova 2010 (5) (%)	Hamid <i>et al</i> 2009 (17) (%)	Frei <i>et al</i> 2002 (14) (%)
35delG	32.35	33.33%	7.8%	4%	21%	9.8%	40%	42%	53%	40.9%	77%
W24X	10.29	5.3%	N.A.	N.A.	N.A.	N.A.	2%	0%	N.A.	N.A.	N.A.

N.A., not analyzed.

Table VI. Prevalence of genotypes in the present study compared to other published studies from Romania.

Genotype	Present study (%)	Mocanu and Neagu 2013 (23)	Totolin <i>et al</i> 2011 (24)	Lazăr <i>et al</i> 2010 (22)
35delG/35delG	10/34 (29.4)	30/84 (36%)	6/26 (24%)	19/75 (25.33%)
35delG/N	0/34 (0)	15/84 (18%)	5/26 (19%)	0/75 (0%)
N/N	19/34 (55.88)	39/84 (46%)	15/26 (57%)	
W24X/W24X	2/34 (5.88)	N.A.	N.A.	1/75 (1.33%)
W24X/N	1/34 (2.94)	N.A.	N.A.	
35delG/W24X	2/34 (5.88)	N.A.	N.A.	2/75 (2.66%)

N.A., not analyzed.

Although genetic diagnosis is very expensive and is not routinely performed in developing countries, medical research can sometimes require a high degree of abstraction (31), and investigating the mutations of the *GJB2* gene is essential in clarifying the problem of congenital hypoacusis. This type of diagnosis leads to genetic council for family members and facilitates the rapid rehabilitation of the child. The neuro-linguistic development of a newborn requires auditory stimulation within the first two years of life and deficits during this period are almost impossible to recover. For this reason, early diagnosis of a congenital hearing loss is of outmost importance. If such a diagnosis is possible, the children will be able to develop normal language and will no longer require special education (32,33). The etiology of congenital SNHL entails environmental, clinical and genetic factors. Hearing disorders also were significantly associated with the presence of maternal pathologies but not with other prenatal or environmental factors such as radiation, or use of illegal drugs (34,35).

In summary, the genetic affliction of a child also brings social and psychological consequences on the family (concern for the child's wellbeing, stigmatization, guilt for passing the disease on to the child) which can affect the parent's capabilities of coping with the situation. After the birth of a deaf child, the parents do not usually know the etiology and most of them do not anticipate a genetic factor, especially without a suggestive family history.

The present study confirms the relatively high prevalence of the *35delG* and *W24X* mutations in cases of congenital non-syndromic severe or profound bilateral SNHL, in accordance with previously published studies. Two of the patients presented a double mutation *35delG/W24X* (bigenic heterozygosity). These results confirm the importance of genetic testing in clarifying the etiology and implementing a precocious recovery program for the patient. The purpose of such a program is optimal hearing and linguistic rehabilitation. The prevalence of the *35delG* mutation within the European population also suggests the necessity of genetic screening which would facilitate the early identification of mutation carriers and would lead to genetic council accordingly.

Although genetic etiology is confirmed, the involvement of other environmental and clinical risk factors cannot be always ruled out. A clinician must always bear in mind that the reverse situation is even more common and complicated; the patients with suggestive risk factors (ototoxic treatments,

hypoxia at birth, and prematurity) do not exclude a genetic etiology of the SNHL.

#### Acknowledgements

Not applicable.

#### Funding

No funding was received.

#### Availability of data and materials

Not applicable.

#### Authors' contributions

HM and AN contributed in all the stages of the article, they designed the article and revised the manuscript for important scientific content. AIM, AB and GC acquired the data, managed testing and evaluation and provided data analysis. HM and AIM also contributed to the conception of the work and revised the language. All authors read and approved the final manuscript.

#### Ethics approval and consent to participate

For this study, the agreement was obtained from the Research Ethics Committee of the Faculty of Medicine, Titu Maiorescu University (Romania). For any patients that required PCR testing written consent was obtained and benefited from anonymity by code designation.

#### Patient consent for publication

All patients provided informed consent and approved the publication of data.

#### Competing interests

The authors declare that they have no competing interests.

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