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ESTUDO GENÉTICO-CLINICO DE PACIENTES COM O ESPECTRO DA NEUROPATIA AUDITIVA

GENETIC-CLINICAL EVALUATION OF PATIENTS WITH AUDITORY NEUROPATHY SPECTRUM

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ORIENTADOR: Profa. Dra. Edil Lúcia Sartorato

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"Tudo é aliado do homem que sabe querer."

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"Saber interpor-se constantemente entre si próprio e as coisas é o mais alto grau de sabedoria e prudência."

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"Tudo aquilo que o homem ignora, não existe pra ele. Por isso o universo de cada um, se resume no tamanho de seu saber."

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RESUMO

A surdez é a doença sensorial mais prevalente em humanos, tendo etiologia genética e ambiental. A perda auditiva não-sindrômica (PANS) representa até 70% dos casos hereditários, sendo as formas autossômicas recessivas (AR) as mais frequentes. A heterogeneidade da PANS AR é extremamente elevada, e 71 *loci* e 40 genes já foram identificados. A neuropatia auditiva (NA) é um tipo de perda auditiva neurossensorial que consiste na alteração da condução do estímulo auditivo por acometimento das células ciliadas internas, nervo auditivo ou das sinapses entre eles. É caracterizada pela ausência ou alteração das ondas no exame de potenciais evocados auditivos de tronco encefálico, com emissões otoacústicas presentes e/ou identificação e presença do microfonismo coclear. Até o momento, foram mapeados 4 *loci* responsáveis pela NA não-sindrômica: DFNB9 (gene *OTOF*) e DFNB59 (gene *PJVK*), associados ao padrão de herança AR; AUNA1 (gene *DIAPH3*), autossômico dominante; e AUNX1, ligado ao cromossomo X. Além disso, mutações no gene *GJB2* já foram relacionadas à afecção. Mutações no *OTOF* desempenham papel significativo na NA. Mais de 80 mutações diferentes já foram identificadas em indivíduos com PANS AR, em populações de origens variadas, especialmente a mutação *p.Q829X* identificada em 3% dos casos de surdez em espanhóis. Achados genéticos responsáveis pela NA são um dos desafios que contribui para a compreensão dos diferentes fenótipos da perda auditiva. Além disso, a utilização de ferramentas moleculares para um diagnóstico mais rápido e eficaz tem grande interesse. Dessa forma, esses fatores são de extrema importância para um diagnóstico diferencial, desenvolvimento de tratamentos específicos e aconselhamento genético preciso. O objetivo do estudo é pesquisar alterações moleculares no gene da otoferlina (*OTOF*) em pacientes com diagnóstico de NA, pesquisar outras alterações genéticas/moleculares que

possam estar relacionadas com a NA, descrever os dados clínicos dos pacientes com diagnóstico clínico de NA seguidos na Otorrinolaringologia-Unicamp e detalhar o subgrupo de pacientes submetidos a tratamento com implante coclear (IC). A mutação do gene *GJB2* está presente em 7,5% dos pacientes estudados (p>.05) e nenhuma mutação do gene *OTOF* foi identificada. Quase 80% dos pacientes apresentaram início dos sintomas no primeiro ano de vida, e 53% apresentavam surdez severa/profunda. O IC teve excelente resultados nesses pacientes, com bom desenvolvimento de fala, aquisição de linguagem, além dos pacientes e suas famílias ficarem satisfeitos com tal tratamento. O tratamento deve focar na adequada interpretação da audição e comunicação dos pacientes. O treinamento auditivo e a estimulação sonora são fundamentais. O IC já apresenta evidências científicas que suportam esse tratamento e deve ser considerado quando há pouco desenvolvimento na aquisição/desenvolvimento de fala, mesmo com terapias adequadas. A NA ainda é um grande desafio diagnóstico e terapêutico, tendo sua etiologia genética e ambiental ainda incertas. Acredita-se que seja um conjunto de situações onde a avaliação eletrofisiológica auditiva e genética possam ser grandes ferramentas no seguimento desses pacientes. A NA é uma situação complexa e deve ser abordada multidisciplinarmente.

Palavras-Chave (DeCS): Transtornos da Percepção Auditiva. Perda Auditiva. Implantes Cocleares. Neuropatia Hereditária Motora e Sensorial. Surdez.

ABSTRACT

Deafness is the most prevalent sensory disease in humans, with genetic and environmental causes. Non-syndromic hearing loss (PANS) reaches for over 70% of hereditary cases, being more frequent as recessive autosomal (AR) forms. The heterogeneity of PANS AR is extremely high, and 71 loci and 40 genes have been identified. Auditory Neuropathy (NA) is a type of sensorineural hearing loss that consists of a change in the conduction of the auditory stimulus due to involvement of the inner hair cells, auditory nerve or synapses between them. It is characterized by absence or alteration of the waves of brainstem auditory evoked potentials, with positive otoacoustic emissions and/or identification of cochlear microphone. At the moment, 4 loci responsible for non-syndromic NA were mapped: DFNB9 (*OTOF* gene) and DFNB59 (*PJVK* gene), associated with the AR form; AUNA1 (*DIAPH3* gene), autosomal dominant; And AUNX1, linked to the X chromosome. Furthermore, mutations in the *GJB2* gene were related to NA. Non-*OTOF* mutations play a significant role in NA. More than 80 different mutations were identified in individuals with PANS AR in populations of varied origins, especially a *p.Q829X* mutation identified in 3% of Spanish. The genetic findings are the challenges that contribute to the understanding of the different phenomena of hearing loss in NA. In addition, the use of molecular tools for a faster and more effective diagnosis is of great interest. Thus, these factors are extremely important for differential diagnosis, development of specific treatments and accurate genetic counseling. The goal of this study is to search for *OTOF* mutations in patients diagnosed with NA, to investigate other genetic / molecular changes that are in a relationship with NA, to describe the clinical data of patients with clinical diagnosis of NA followed in

Otorhinolaryngology-Unicamp and to detail the subgroup of patients undergoing treatment with cochlear implant (IC). The mutation of the *GJB2* gene is present in 7.5% of the patients studied (p> .05) and no mutation of the *OTOF* gene was identified. Almost 80% of the patients had onset of symptoms in the first year of life, and 53% had severe / profound deafness. The IC patients had excellent results, with good speech development, language acquisition, as well as patients and their families were well satisfied. The treatment should focus on development of means where the patient can get interpret their hearing and have good communication. Auditory training and sound stimulation are essential. The IC presents scientific evidences that support this treatment and should be an option when there is poor development in the acquisition/development of speech, even with adequate therapies. NA is still a major medical and therapeutic challenge, with its genetic and environmental etiology still uncertain. We believe that NA is a set of diseases where an electrophysiological auditory and genetic evaluation are great tools for follow-up those patients. NA is a complex situation and must be approached multidisciplinarily.

Key-Words (DeCS): Auditory Perceptual Disorders . Hearing Loss . Cochlear Implants . Hereditary Sensory and Motor Neuropathy. Deafness.

LISTA DE ABREVIATURAS E SIGLAS

AASI – Aparelho auditivo de Amplificação Sonora Individual/ Próteses auditivas

AR – Autossômico Recessivo

BERA/PEATE – Potencial Evocado Auditivo de Tronco Encefálico

IC – Implante Coclear

HC – Hospital das Clínicas

MC – Microfonismo Coclear

MALDI-TOF MS - *Matrix-Assisted Laser Desorption Ionization Time-Of-Flight Mass Spectrometry*

NA – Neuropatia auditiva

OEA – Otoemissões Acústicas

OMS - Organização Mundial da Saúde

OTOF - Otoferlina

PANS – Perda Auditiva Não Sindrômica

UNICAMP- Universidade Estadual de Campinas

SUMÁRIO

1. INTRODUÇÃO

Perda auditiva

De acordo com a Organização Mundial da Saúde (OMS), a surdez refere-se à perda total da capacidade auditiva em uma ou nas duas orelhas. Já a deficiência ou perda auditiva, refere-se à perda parcial ou completa da capacidade de ouvir (OMS, 2009).

Os termos surdez, perda auditiva e deficiência auditiva neste trabalho serão adotados para se referir a qualquer comprometimento do sistema auditivo significante, independentemente do tipo, etiologia, grau e frequência. Essa mesma terminologia pode não se aplicar para outros profissionais, como por exemplo, otorrinolaringologistas e fonoaudiólogos.

Entretanto, em publicações científicas sobre genética, esses termos são os mais empregados, já que muitas vezes perdas genéticas com a mesma etiologia exibem gravidade de manifestação muito variável.

As deficiências auditivas podem ser classificadas de diferentes formas, como por exemplo, baseada na região afetada da orelha, podendo ser do tipo condutiva, neurossensorial ou mista. A perda condutiva é causada por problemas na orelha média e/ou externa; a neurossensorial é causada por problemas na orelha interna e/ou nervo coclear; e a mista é causada por uma combinação de ambos componentes condutivos e neurossensoriais (Ito *et al*., 2010).

De acordo com o início da perda auditiva, esta pode ser classificada como congênita, adquirida ou tardia. Se estiver presente ao nascimento ou antes da aquisição da linguagem, a perda auditiva pode ser pré-lingual e,

quando se manifesta após a aquisição da linguagem, pós-lingual (Ito *et al*., 2010).

A surdez pode ainda ser definida de acordo com o seu grau, como leve (de 26 a 40 dB), moderada (de 41 a 70 dB), severa (de 71 a 90 dB) ou profunda (acima de 90 dB) (Davis & Silvermann, 1970). A avaliação é feita através dos limiares tonais nas frequências que variam de 0,5 a 4 kHz, que são essenciais para o entendimento da fala.

Outro critério é quanto à lateralidade, sendo chamada de bilateral quando as duas orelhas são afetadas, e de unilateral quando a deficiência auditiva está presente em apenas um dos lados (Finsterer & Fellinger, 2005).

Quando os casos de surdez são acompanhados por outras manifestações clínicas, além dos sintomas auditivo-cocleares, são caracterizados como sindrômicos, ao contrário da perda auditiva não-sindrômica, onde a surdez é o único fenótipo **(**Dror & Avraham, 2009**).**

A surdez é a doença sensorial mais prevalente em humanos, causada por uma série de fatores genéticos e ambientais, que podem estar relacionados entre si. Os fatores ambientais incluem a exposição frequente à alta intensidade do som, trauma acústico, infecções, drogas ototóxicas, entre outros, enquanto os fatores genéticos são causados por mutações em diferentes genes ou em elementos regulatórios que estão envolvidos no desenvolvimento adequado, na estrutura e na função da orelha **(**Dror & Avraham, 2009**).**

Nos países desenvolvidos, mais de 60% de todos os casos de deficiência auditiva resultam de causas genéticas (Rodríguez-Ballesteros *et al*.,

2008). Entretanto, no Brasil, os fatores ambientais superam os de origem genética (Pupo et al., 2008; Calháu *et al*., 2011). Acreditamos que implantadas melhorias no setor de saúde, e com o avanço e aprofundamento nos estudos genéticos relacionados às perdas auditivas, a proporção de causas genéticas tende a aumentar.

Dados estatísticos internacionais mostram que 1 em cada 1.000 recém-nascidos apresenta perda auditiva (Tekin *et al*., 2001; Schrijver, 2004). No Brasil, a frequência é estimada em 4 a cada 1.000 nascimentos (Piatto & Maniglia, 2001). No entanto essa frequência varia, dependendo da amostra e região estudada, podendo a surdez estar presente em 2 a 7 para cada 1.000 recém-nascidos (Simões & Maciel-Guerra, 1992; Russo, 2000).

Nos últimos anos houve avanços significativos em pesquisas sobre as bases moleculares do sistema auditivo, permitindo a identificação e caracterização de vários genes e proteínas relacionados à audição. O conhecimento cada vez maior sobre esses genes contribui não só para uma melhor compreensão dos mecanismos da audição, mas também das bases moleculares da perda auditiva. Pesquisas nesse campo são pré-requisitos para o desenvolvimento do diagnósticos e terapias específicas para a surdez (Stover & Diensthuber, 2011).

Um dos maiores empecilhos na localização de genes envolvidos na deficiência auditiva é a dificuldade de acesso à cóclea e às demais estruturas da orelha interna. A construção de um banco de cDNA de material coclear fetal possibilitou o endereçamento de genes candidatos por uma abordagem tecido-específica (Robertson *et al.,* 1994; Wilcox & Fex, 1992). O grande número de

genes expressos na cóclea reflete a complexidade dos mecanismos moleculares envolvidos neste órgão de intrincada natureza (Heller *et al*., 1998).

Os diferentes *loci* ou regiões candidatas a apresentarem genes associados à surdez não-sindrômica recebem o prefixo DFN (do inglês *deafness*), seguido de um número que indica a ordem em que foram descobertos. As mutações genéticas conhecidas que levam à surdez podem ser de herança autossômica recessiva (DFNB), autossômica dominante (DFNA), **l**igada ao X (DFNX) ou mitocondrial **(**Dror & Avraham, 2009**).** A surdez não-sindrômica é a mais comum e ocorre em até 70%, de hereditários (Hilgert *et al*., 2009b).

Em relação aos mecanismos de herança, estima-se que aproximadamente 75 a 80% dos casos de surdez genética não-sindrômica sejam de herança autossômica recessiva, 20 a 25% autossômica dominante e cerca de 1 a 2% ligada ao cromossomo X. Além disso, a frequência da herança mitocondrial é estimada em 1% (Kokotas *et al.*, 2007; Hilgert *et al*., 2009b; Smith *et al*., 2010**).**

A heterogeneidade da deficiência auditiva não-sindrômica autossômica recessiva é extremamente elevada, para a qual 71 *loci* foram descritos e 40 genes foram identificados até o momento (Van Camp & Smith. Hereditary Hearing Loss Homepage. Disponível em <http://hereditaryhearingloss.org>).

O diagnóstico molecular é complicado devido a grande heterogeneidade genética. Mutações nos genes *GJB2* e *GJB6*, que codificam as conexinas 26 e 30, respectivamente, no *locus* DFNB1 são responsáveis por mais de 50% de todos os casos de perda auditiva autossômica recessiva (Del Castilho *et al*.,

2003; Hilgert *et al*., 2009a). No entanto, a contribuição de mutações em outros genes ainda está sendo investigada, e o estudo é complicado pelo fato da epidemiologia genética da perda auditiva não-sindrômica ser amplamente variável entre as populações (Petit *et al.,* 2001; Friedman & Griffith, 2003; Petersen & Willems, 2006).

Neuropatia auditiva

A neuropatia auditiva (NA) é um tipo de perda auditiva não-sindrômica que consiste na possível alteração da condução do estímulo auditivo por acometimento das células ciliadas internas, do nervo auditivo ou das sinapses entre eles. É caracterizada pela ausência ou alteração das ondas no exame de potenciais evocados auditivos de tronco encefálico com a presença de emissões otoacústicas (OEA) e/ou microfonismo coclear(MC) (Starr *et al*., 1996).

Ela é responsável por 7-10% dos casos de surdez em crianças (Sininger, 2002; Rance, 2005), podendo ser causada por uma série de fatores genéticos e ambientais, como hiperbilirrubinemia, prematuridade, anóxia, exposição a drogas ototóxicas, infecções, entre outros. Quanto à etiologia, estima-se que aproximadamente 42% dos casos sejam hereditários, 10% associados a fatores tóxicos, metabólicos, imunológicos e infecciosos (drogas ototóxicas, anóxia, hiperbilirrubinemia, desmielinização, infecções virais), e 48% idiopáticos (Starr *et al.,* 2000).

A neuropatia auditiva, que como descrito acima acontece na maioria das vezes como um sinal clínico isolado (de forma não sindrômica), pode em poucas situações estar associada a outros distúrbios, fazendo parte dos sinais clínicos de doenças sindrômicas e/ou neurodegenerativas sistêmicas, como a

Síndrome de Charcot-Marie-Tooth, Ataxia de Friedreich, Neuropatia de Guillan Barrè e doenças mitocondriais (Rodríguez-Ballesteros *et al*., 2008).

Diante das características peculiares dos pacientes com neuropatia auditiva, o tratamento desses indivíduos é um desafio. Há grande necessidade em conhecer melhor a fisiopatologia dessa afecção, e os estudos voltados para avaliação genética e molecular podem oferecer importante colaboração.

Na última década, a identificação de genes responsáveis pela neuropatia auditiva teve grande contribuição para o seu diagnóstico e melhor compreensão dos seus mecanismos.

O gene *OTOF*, que codifica a otoferlina, é um dos 40 genes associados à perda auditiva não-sindrômica autossômica recessiva (Yasunaga *et al*., 1999). Ele está localizado no *locus* DFNB9, na região cromossômica 2p22-23 (Chaib *et al*., 1996). O gene contém 48 éxons e codifica múltiplas isoformas de proteínas, longas e curtas, por *splicing* alternativo combinado com o uso de vários sítios de iniciação transcricional (Yasunaga *et al*., 2000). A otoferlina é expressa na cóclea, vestíbulo e cérebro (Yasunaga *et al.,* 1999; 2000), e está envolvida na exocitose das vesículas sinápticas das células ciliadas internas (Roux *et al*., 2006).

A otoferlina é uma proteína citosólica, que se encontra normalmente na membrana celular. Como já citado, aparentemente tem uma função da comunicação intercelular, nas sinapses e na transdução de sinal celular. Acredita-se que uma falha nesses processo, tanto na parte periférica (células ciliadas, sistema vestibular) ou na parte central pode causar alterações na sincronia auditiva por disfunção na comunicação sináptica (Jimenez *et al*.,

2007; Schug *et al*., 2006; Roux *et al*., 2006; Yasunaga *et al.,* 1999; Yasunaga *et al.,* 2000).

Outra informação que também contribui para a relevância do *OTOF* é que as mutações desse gene estão relacionadas com perda auditiva não sindrômica em 3,5% na população espanhola e até em 2,3% em países orientais, o que pode sugerir que este é o terceiro gene mais comum relacionado a surdez genética não sindrômica depois das mutações dos genes *GJB2* e *GJB6* (Rodríguez-Ballesteros *et al.,* 2003; Choi *et al*., 2009.)

Mutações no gene *OTOF* são responsáveis por um fenótipo bastante homogêneo de perda auditiva profunda pré-lingual, sem a associação de malformações na orelha interna, nos quais os indivíduos afetados apresentam neuropatia auditiva (Rodríguez-Ballesteros *et al*., 2003). Até o momento, foram descritas 43 mutações patogênicas diferentes no gene *OTOF* em indivíduos com surdez não-sindrômica autossômica recessiva, em populações de origens variadas (Rodríguez-Ballesteros *et al.,* 2008; Romanos *et al.,* 2009).

A maioria dessas mutações é particular, cada uma sendo relatada em apenas uma família. Entretanto, a mutação p.Q829X (c.2485C>T) é mais frequente identificada no gene *OTOF* e a terceira causa mais comum de perda auditiva não-sindrômica autossômica recessiva, na população espanhola (Migliosi *et al.,* 2002; Rodríguez-Ballesteros *et al*., 2003; 2008), sendo também identificada em 2 franceses, 1 mexicano, 2 argentinos e em 1 paciente inglês (Reynoso *et al.,* 2004; Rouillon *et al.,* 2006; Varga *et al.,* 2006). Porém, ela não é uma causa frequente de surdez na população brasileira (Oliveira *et al*., 2007; Romanos *et al*., 2009).

Em 2004, foi mapeado em uma família americana mais um *locus*, AUNA1, na região cromossômica 13q21-q24, associado à neuropatia auditiva pós-lingual de herança autossômica dominante (Kim *et al*., 2004), sendo o gene correspondente, *DIAPH3*, identificado posteriormente (Schoen *et al.,* 2010).

Wang e col., em 2006, mapearam AUNX1, um novo *locus* relacionado à neuropatia auditiva, porém dessa vez de herança ligada ao cromossomo X, na região Xq23-q27.3, em uma família chinesa (Wang *et al.,* 2006). O gene correspondente ainda não foi identificado.

Mais recentemente, foi identificado outro gene associados à NA de herança autossômica recessiva. O gene *PJVK* (DFNB59), localizado na região cromossômica 2q31.1-q31.3, codifica a *pejvaquina*, uma proteína da via auditiva aferente envolvida na sinalização de células ciliadas e neurônios (Delmaghani *et al.,* 2006). Mutações nesse gene não são uma causa frequente de neuropatia auditiva no Brasil, assim como as mutações no gene *OTOF* revelaram ser (Romanos *et al*., 2009).

Além disso, mutações no gene da conexina 26 (*GJB2*) também já foram relacionadas à neuropatia auditiva. Recentemente, dois estudos diferentes identificaram casos com neuropatia auditiva em indivíduos com perda auditiva que apresentavam mutações no gene *GJB2* (Cheng *et al*., 2005; Santarelli *et al.,* 2008).

O primeiro trabalho analisou 777 indivíduos que frequentavam escolas para surdos ou que recebiam atendimento para perda auditiva moderada a profunda. Aproximadamente 10% dos casos apresentavam emissões

otoacústicas presentes, sugerindo diagnóstico de neuropatia auditiva. Em cinco desses indivíduos foram identificadas mutações no gene da conexina 26, tanto em homozigose (c.35delG/c.35delG ou p.W77X/p.W77X) quanto em heterozigose composta (c.35delG/c.360delGAG, c.35delG/p.V95M e p.M34T/p.V84M) (Cheng *et al*., 2005).

No segundo estudo, foram analisadas 3 crianças com perda auditiva maior do que 30 dB e emissões otoacústica presentes. Duas delas eram homozigotas para a mutação c.35delG e a terceira heterozigota para p.M34T. Este último caso não foi considerado, já que não foi encontrada uma segunda mutação que pudesse explicar a causa da perda auditiva. Investigações posteriores descartaram o diagnóstico de neuropatia auditiva em uma das crianças com a mutação c.35delG em homozigose e, na outra, sugeriram lesão envolvendo células ciliadas internas, com ausência de potenciais evocados auditivos de tronco encefálico (BERA/PEATE) e presença de emissões otoacústicas e microfonismo coclear (Santarelli *et al*., 2008).

Atualmente sabe-se que alterações na conexina 26 interferem na homeostase iônica da orelha interna, levando ao acúmulo extracelular de potássio, que resulta em morte celular. Dessa forma, é bem provável que as emissões otoacústicas observadas nos pacientes citados nos estudos acima representem atividade residual de poucas células ciliadas externas que permaneceram vivas na parte apical da cóclea (del Castillo & del Castillo, 2012).

No entanto, acredita-se que algumas mutações no gene *GJB2* podem causar alterações nas células ciliadas internas e terminações nervosas das

células ciliadas (Matsunaga, 2009). Assim, são necessários estudos adicionais para esclarecer a relação entre neuropatia auditiva e mutações no gene *GJB2*.

Genotipagem utilizando espectrometria de massa

Há várias maneiras de se detectar alterações e/ou mutações no genoma, e recentemente as tecnologias de alto rendimento *(high-throughput)* têm surgido como uma alternativa interessante. A genotipagem utilizando o *MassARRAY® System* da *Sequenom*, Inc. (San Diego, CA), por meio da técnica *MALDI-TOF MS* (*Matrix-Assisted Laser Desorption Ionization Time-Of-Flight Mass Spectrometry*) e denominada *iPLEX® Gold Assay*, pode ser considerada uma ferramenta poderosa e com abordagem atrativa. Esse método de genotipagem é considerado de médio rendimento e permite a genotipagem de até 40 SNPs (do inglês *single nucleotide polymorphisms*), simultaneamente, utilizando de 96 a 3.840 amostras, de forma rápida e a um baixo custo.

A discriminação alélica é obtida utilizando *MALDI-TOF MS* (*Matrix-Assisted Laser Desorption Ionization Time-Of-Flight Mass Spectrometry*), a qual tem sido utilizada vastamente para analisar os produtos *multiplex* e os diversos genótipos com base em diferenças no peso molecular dos nucleotídeos.

Portanto, a espectrometria de massa atualmente representa um poderoso e versátil método analítico que fornece informações valiosas sobre a composição e estrutura de moléculas e também ajuda a esclarecer sobre a quantidade de analitos específicos em misturas (Oberacher, 2008). Esse método fornece uma solução atrativa para a genotipagem de SNPs, principalmente porque permite a medição direta e rápida de produtos do DNA, ao invés de detectar apenas uma marcação (fluorescente ou radioativa), sendo

que os resultados podem ser facilmente analisados por um *software* automatizado (Gut, 2002).

MALDI foi iniciado em 1988 por Karas e Hillenkamp como um método revolucionário para ionização e análise de massa de muitas biomoléculas. Esses pesquisadores descobriram que a irradiação de cristais formados por pequenas moléculas orgânicas adequadas (chamadas de *matrix*) com um curto pulso de laser causou uma transferência de energia e processo de desorção, produzindo íons da *matrix* em fase de gás. De maior importância ainda, eles descobriram que se uma baixa concentração de um analito não-absorbante, como uma proteína ou molécula de ácido nucleico, for adicionada à *matrix* em solução e embutida nos cristais sólidos da *matrix* formados por secagem da mistura, as moléculas intactas não-absorbantes do analito também seriam levadas à fase de gás e ionizadas a laser, facilitando sua análise de massa (Griffin & Smith, 2002).

Os componentes básicos de um espectrômetro de massa consistem de uma fonte de ionização (laser UV), um analisador e um detector. Para a análise, a amostra biológica (mistura de ácidos nucleicos) é misturada com um material (*matrix*), geralmente um ácido orgânico de baixo peso molecular com forte absorção no comprimento de onda do laser (Gut, 2002), em blocos de uma superfície de silicone do *chip*. O processo *MALDI-TOF* é então iniciado por uma desorção a laser da mistura analito-*matrix* (Irwin, 2008). Os subsequentes processos físicos resultam na predominante formação de íons carregados positivamente ou de íons carregados negativamente. Esses íons são extraídos com um campo elétrico e separados em função de suas massas moleculares e de suas cargas (Gut, 2002). As massas dos compostos de ácidos nucleicos

são calculadas através do “tempo de voo” (TOF), que reflete o tempo que o composto laser-ionizado e acelerado requer para ser levado através do tubo (1 a 2 m de comprimento) do analisador TOF e alcançar o detector do instrumento. No detector, os compostos ionizados geram um sinal elétrico que fica gravado por um sistema de dados e é finalmente convertido em um espectro de massa (Irwin, 2008). A resolução da atual geração de espectrômetros de massa *MALDI* permite a fácil distinção da substituição de nucleobases com variação de massa de 1 a 7kDa, o que corresponde ao tamanho de DNA de 3 a 25 nucleobases (Gut, 2002).

JUSTIFICATIVA

A capacidade de se ter elementos clínicos que auxiliem na orientação para o diagnóstico genético e etiológico de pacientes com neuropatia auditiva pode trazer benefícios na identificação correta desses pacientes, caracterizando-os de forma mais adequada e estabelecendo um tratamento mais adequado.

A identificação de alterações genéticas responsáveis pela neuropatia auditiva é um dos desafios que contribui para a compreensão das bases moleculares dos diferentes fenótipos da perda auditiva. Além disso, a utilização de novas ferramentas moleculares que permitem um diagnóstico mais rápido e eficaz é de grande interesse para esses pacientes. Dessa forma, esses fatores são de extrema importância para um diagnóstico diferencial, desenvolvimento de tratamentos mais específicos e aconselhamento genético mais preciso.

2. OBJETIVOS

2.1. Objetivo geral

O objetivo do presente estudo é pesquisar alterações moleculares no gene da otoferlina (*OTOF*) e outras alterações moleculares (c.35delG, gene *GJB2*, das deleções del(*GJB6*-D13S1830) e del(*GJB6*-D13S1854) no gene *GJB6*, e da mutação mitocondrial *m.1555A>G* no gene *MTRNR1* e correlacionar os achados com as características clínicas desse grupo de pacientes portadores de neuropatia auditiva.

2.2. Objetivos específicos

Pesquisar alterações moleculares no gene da otoferlina (*OTOF*) em pacientes com diagnóstico de neuropatia auditiva, e desenvolver um chip de DNA para o diagnóstico molecular da neuropatia auditiva, utilizando espectrometria de massa para a genotipagem.

- Investigar a presença da mutação c.35delG e outras alterações no gene *GJB2*, das deleções del(*GJB6*-D13S1830) e del(*GJB6*-D13S1854) no gene *GJB6*, e da mutação mitocondrial m.1555A>G no gene *MTRNR1*;
- Determinar a frequência da mutação p.Q829X no éxon 22 do gene *OTOF* em indivíduos brasileiros com neuropatia auditiva;
- Verificar a ocorrência de outras alterações genéticas através do sequenciamento completo do gene *OTOF*;
- Padronizar o método de genotipagem utilizando o sistema *MassARRAY®, da Sequenom*, para o diagnóstico molecular da neuropatia auditiva, com as principais mutações encontradas na população brasileira e na população mundial.

2.3. OBJETIVOS DO PRIMEIRO ARTIGO:

Investigar as alterações moleculares no gene *OTOF* em pacientes com neuropatia auditiva, e desenvolver um chip de DNA para o diagnóstico molecular de neuropatia auditiva usando espectrometria de massa para genotipagem.

Investigar a presença da mutação, c.35delG e outras alterações no gene *GJB2*, supressões del(*GJB6-D13S1830*) e del(*GJB6-D13S1854*) no gene *GJB6*, e o m.1555A mutação mitocondrial> G no gene *MTRNR1*.

Determinar a frequência da mutação p.Q829X no éxon 22 do gene *OTOF* em pacientes brasileiros com NA.

Verificar a ocorrência de outras alterações genéticas por sequenciamento do gene *OTOF* completo;

Padronizar o método de genotipagem utilizando o sistema *MassARRAY®, Sequenom* para o diagnóstico molecular de NA, com as mutações que ocorrem predominantemente em populações brasileiras e mundiais.

2.4. OBJETIVOS DO SEGUNDO ARTIGO:

Analisar o padrão de relação clínica entre os pacientes com diagnóstico clínico com o espectro da neuropatia auditiva e correlacionar com mutações do gene *GJB2*.

2.5. OBJETIVOS DO TERCEIRO ARTIGO:

Descrever informações clínico, epidemiológicas e audiológicas de pacientes com diagnóstico de NA, acompanhados no serviço de saúde auditiva de um hospital universitário de referência terciária/quaternária.

2.6. OBJETIVOS DO QUARTO ARTIGO:

Demonstrar o desempenho e os resultados de pacientes com neuropatia auditiva submetidos a tratamento com o implante coclear.

2.7. OBJETIVOS DO QUINTO ARTIGO:

Avaliar a satisfação de pacientes com neuropatia auditiva que foram submetidos a tratamento com implante coclear.

2.8. OBJETIVOS DO SEXTO ARTIGO:

Discutir os aspectos diagnósticos, clínicos e terapêuticos que são desafiadores na condução e avaliação de pacientes com neuropatia auditiva.

3. CAPÍTULO 1

MOLECULAR STUDY OF PATIENTS WITH AUDITORY NEUROPATHY

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Molecular study of patients with auditory neuropathy

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Abstract. Auditory neuropathy is a type of hearing loss that constitutes a change in the conduct of the auditory stimulus by the involvement of inner hair cells or auditory nerve synapses. It is characterized by the absence or alteration of waves in the examination of brainstem auditory evoked potentials, with otoacoustic and/or cochlear microphonic issues. At present, four loci associated with non-syndromic auditory neuropathy have been mapped: Autosomal recessive deafness-9 [DFNB9; the otoferlin (*OTOF*) gene] and autosomal recessive deafness-59 [DFNB59; the pejvakin (*PJVK*) gene], associated with autosomal recessive inheritance; the autosomal dominant auditory neuropathy gene [AUNA1; the diaphanous-3 (*DIAPH3*) gene]; and AUNX1, linked to chromosome X. Furthermore, mutations of connexin 26 [the gap junction β2 (*GJB2*) gene] have also been associated with the disease. *OTOF* gene mutations exert a significant role in auditory neuropathy. In excess of 80 pathogenic mutations have been identified in individuals with non-syndromic deafness in populations of different origins, with an emphasis on the p.Q829X mutation, which was found in ~3% of cases of deafness in the Spanish population. The identification of genetic alterations responsible for auditory neuropathy is one of the challenges contributing to understand the molecular bases of the different phenotypes of hearing loss. Thus, the present study aimed to investigate molecular changes in the *OTOF* gene in patients with auditory neuropathy, and to develop a DNA chip for the molecular diagnosis of auditory neuropathy using mass spectrometry for genotyping. Genetic alterations were investigated in 47 patients with hearing loss and clinical diagnosis of auditory neuropathy, and the c.35delG mutation in the *GJB2* gene was identified in three homozygous patients, and the heterozygous parents of one of these cases. Additionally, *OTOF* gene mutations were tracked by complete sequencing of 48 exons, although these results are still preliminary. Studying the genetic basis of auditory neuropathy is of utmost importance for obtaining a differential diagnosis, developing more specific treatments and more accurate genetic counseling.

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Key words: auditory neuropathy spectrum disorder, evoked auditory brainstem response, otoacoustic emissions, *GJB2*, hearing loss

Introduction

Hearing loss is the most prevalent sensory disease in humans; it is caused by a variety of genetic and environmental factors. Whereas environmental factors include exposure to frequent high-intensity sound, acoustic trauma, infections and ototoxic drugs, among others, genetic factors are caused by mutations in different genes or regulatory elements involved in the proper development, structure and function of the ear (1).

In developed countries, >60% of all cases of hearing impairment result from genetic causes (2). However, in Brazil, environmental factors outweigh those of genetic origin (3,4). Considering that improvements are being implemented in the health sector, and with the advancements being made in genetic studies associated with hearing loss, the proportion of genetic causes tends to increase.

International statistics reveal that one in every 1,000 newborns has hearing loss (5,6). In Brazil, the frequency is estimated at four per 1,000 births (7). However, this often varies: Depending on the sample and study area, it may be present in 2-7 per 1,000 newborns (8).

Recent years have witnessed significant advances in research on the molecular basis of the auditory system, enabling the identification and characterization of several genes and proteins associated with hearing. The increasing knowledge about these genes contributes not only to an improved understanding of the mechanisms of hearing, but also to the molecular basis of hearing impairment. This basic research is a prerequisite for the development of molecular diagnostics and novel therapies for deafness (9).

One of the biggest obstacles in the search for genes involved in hearing loss is the difficult access to the cochlea and other inner ear structures. The construction of a cDNA library of fetal cochlear equipment allowed the issue of candidate genes to be addressed using a tissue-specific approach (10,11). The

large number of genes expressed in the cochlea reflects the complexity of the molecular mechanisms involved in this organ of intricate nature (12).

Most cases of hereditary hearing loss (~70%) are non-syndromic. Different loci or regions to submit candidate genes associated with non-syndromic deafness receive the prefix DFN (from deafness), followed by a number indicating the order in which they were discovered. The known genetic mutations that lead to deafness may be autosomal recessive (DFNB), autosomal dominant (DFNA), X-linked (X DFN) or mitochondrial (1,13-15).

Regarding the mechanisms of inheritance, it is estimated that 75-80% of cases of non-syndromic genetic deafness are autosomal recessive, 20-25% are autosomal dominant, and 1-2% are linked to the X chromosome. In addition, the frequency of mitochondrial inheritance is estimated at 1% (13-17).

The heterogeneity of non-syndromic autosomal recessive hearing loss is high, for which 71 loci have been described, and 40 genes have been identified to date (18).

Auditory neuropathy (AN) is a type of sensorineural hearing loss, consisting of auditory stimulus alteration as a result of the involvement of inner hair cells or auditory nerve synapses. It is characterized by the absence or alteration of waves in the examination of the brainstem auditory potential response in the presence of otoacoustic emissions and/or cochlear microphonism (19).

It is responsible for 7-10% of the cases of hearing loss in children, and may be caused by a number of genetic and environmental factors, including hyperbilirubinemia, prematurity, anoxia, exposure to ototoxic drugs and infections, among others. Regarding etiology, it is estimated that ~42% of cases are hereditary, 10% are associated with toxic, metabolic, immunological and infectious factors (e.g. ototoxic drugs, anoxia, hyperbilirubinemia, demyelination and viral infections), and 48% are idiopathic (20-22).

The AN may be associated with other disorders (syndromic) as part of the clinical signs of systemic neurodegenerative diseases, including Charcot-Marie-Tooth disease, Friedreich's ataxia, Guillain-Barré neuropathy and mitochondrial diseases, or there may be (non-syndromic) isolated clinical signs (2).

Given the peculiar characteristics of patients with AN, the treatment of these individuals is a challenge. There is a need to gain an improved understanding of the pathophysiology of this disease, and studies on genetic and molecular assessment can provide important collaboration. Over the course of the last decade, the identification of genes responsible for AN have contributed greatly to the diagnosis and improved understanding of the mechanisms involved in the disease.

The otoferlin (*OTOF*) gene is one of the 40 genes associated with autosomal recessive non-syndromic hearing loss. It is located in the locus DFNB9, chromosomal region 2p22-23. The gene contains 48 exons and encodes multiple isoforms of proteins generated via long and short alternative splicing events, combined by using different transcriptional initiation sites. OTOF is expressed in the cochlea, vestibule and brain, and is involved in the exocytosis of synaptic vesicles from inner hair cells (23-26).

Mutations in the *OTOF* gene are responsible for a prelingual, fairly homogenous phenotype of profound hearing loss, without associated defects in the inner ear. However, numerous affected individuals have AN. Over 80 pathogenic mutations have been identified in individuals with non-syndromic deafness, in populations of different origins (27-29).

In 2004, another locus, *AUNA1*, was mapped in an American family, in the chromosomal region 13q21-q24, associated with autosomal dominant postlingual AN, and the corresponding gene, diaphanous-3 (*DIAPH3*), was subsequently identified (30,31).

The *AUNX1* gene was mapped in 2006, a novel locus associated with AN, although this time linked to the X chromosome in the Xq23-q27.3 region, in a Chinese family inheritance. The corresponding gene has not been identified (32).

More recently, another neuropathy associated with the autosomal recessive gene was identified. The gene *PJVK* (DFNB59), located in chromosome region 2q31.1-q31.3, encodes pejvakin (PJVK), a protein of the afferent auditory pathway involved in signaling from hair cells and neurons. However, mutations in this gene are not a frequent cause of AN in Brazil, as are mutations in the *OTOF* gene (33,34).

Furthermore, mutations in connexin 26 [the gap junction β2 (*GJB2*) gene] have also been associated with AN. Two different studies have identified cases with AN among individuals with hearing loss, who had mutations in the *GJB2* gene (35,36).

Currently, it is known that changes in connexin 26 interfere with the ionic homeostasis of the inner ear, leading to the accumulation of extracellular potassium, which results in cell death. Thus, it is likely that otoacoustic emissions observed in patients in the studies cited above represent residual activity of a few outer hair cells that remained alive in the apical part of the cochlea. However, it is considered that certain mutations in the *GJB2* gene may cause changes in the inner hair cells and nerve endings of the hair cells (37,38).

Therefore, further studies are required to clarify the association between AN and *GJB2* gene mutations (37,38). A previous study has also investigated connexins and gap junctions, such as one involving the gap junction β4 (*GJB4*) gene (39), which may be associated with non-syndromic hearing loss.

There are several ways to detect changes and/or mutations in the genome and, recently, high-throughput technologies have emerged as an interesting alternative. Genotyping using the MassARRAY® system (Sequenom, Inc., San Diego, CA, USA) with the matrix-assisted laser desorption ionization time-of-flight mass spectrometric (MALDI-TOF MS) technique and iPLEX, termed Gold Assay®, may be considered a powerful tool with an attractive approach. Genotyping is considered a method for obtaining an average yield, and allows the genotyping of up to 40 single nucleotide polymorphisms (SNPs) simultaneously, using 96-3,840 samples, quickly and at low cost (40,41).

Allelic discrimination is achieved using MALDI-TOF MS, which has been widely used for analyzing multiplex products and different genotypes, based on differences in the molecular weights of nucleotides (40,41).

Therefore, MS currently represents a powerful and versatile analytical method that provides valuable information about the composition and structure of molecules, and also sheds light on the quantity of specific analytes in mixtures. This method provides an attractive solution for the genotyping

of SNPs, particularly since it allows direct and rapid DNA measurement, rather than only detecting a mark (radioactive or fluorescent), and the results may be easily analyzed by automated software (40,41).

MALDI-TOF MS was first employed in 1988 by Karas and Hillenkamp (42) as a revolutionary method for the ionization and mass analysis of numerous biomolecules. These researchers demonstrated that irradiation of crystals formed by suitable small organic molecules (called matrix) with a short-pulse laser caused an energy transfer and desorption process, producing the matrix ions in the gas phase. Of even greater importance, they determined that, if a low concentration of a non-absorbent analyte, such as a protein or nucleic acid molecule, is added to the matrix solution and embedded in the solid matrix of crystals formed by drying the mixture, non-absorbent intact molecules of the analyte also would be sent to the gas phase and ionized laser, facilitating its mass analysis (42,43).

The basic components of a mass spectrometer consist of an ionization source (UV laser), an analyzer and a detector. For analysis, the biological sample (nucleic acid mixture) is added to a material (matrix), usually a low-molecular-weight organic acid with a strong absorption at the laser wavelength in blocks of one silicon chip surface. The MALDI-TOF process is then initiated by laser desorption of an analyte-matrix mixture. Subsequent physical processes result in the predominant formation of either positively charged or negatively charged ions. These ions are extracted using an electric field, and separated according to their molecular masses and their charges (40,42,43).

The masses of nucleic acid compounds are calculated using TOF, which reflects the time that the laser-ionized compound requires to be carried through the flight tube (1 to 2 m length) TOF analyzer and reach the instrument detector. At the detector, the ionized compounds generate an electrical signal that is recorded by a data system and is finally converted into a mass spectrum. The resolution of the current generation of MALDI mass spectrometers enables easy distinction of nucleobase replacements, with a mass variation of 1,000 to 7,000 Da, which corresponds to a DNA size from 3 to 25 nucleobases (40,44,45).

The identification of genetic alterations responsible for AN is one of the challenges that contribute to the understanding of the molecular basis of the different serotypes of hearing loss phenotypes. Furthermore, the use of novel molecular tools that enable a more rapid and effective diagnosis is of great interest for these patients. Thus, these factors are important for a differential diagnosis, as well as for developing more specific treatments and a more accurate genetic counseling.

The present study aimed to investigate molecular changes of the *OTOF* gene in patients with AN, and to develop a DNA chip for the molecular diagnosis of AN, using MS for genotyping, specifically: i) To investigate the presence of the mutation, c.35delG, and other changes in the *GJB2* gene, the deletions del(*GJB6-D13S1830*) and del(*GJB6-D13S1854*) in the *GJB6* gene, and the mitochondrial mutation m.1555A>G in the *MTRNR1* gene; ii) to determine the frequency of the p.Q829X mutation in exon 22 of the *OTOF* gene in Brazilian patients with AN; iii) to check the occurrence of other genetic alterations by sequencing the complete *OTOF* gene; and iv) to standardize the method of genotyping using the MassARRAY®, Sequenom system for molecular diagnosis of AN, with the predominantly occurring mutations in Brazilian and world populations.

Materials and methods

Clinical subjects. This study comprised 47 patients from a tertiary care center diagnosed with hearing loss and AN. All subjects underwent audiological evaluation, including pure tone audiometry, speech audiometry, tympanometry, otoacoustic emissions and brainstem auditory evoked potential.

Patients who presented an absence of, or alterations in, the waves in the examination of the potential auditory brainstem response in the presence of otoacoustic and/or cochlear microphonic issues were clinically diagnosed with AN.

Initially, patients underwent a clinical evaluation performed by ear, nose and throat doctors. Subsequently, blood samples were collected and forwarded directly to the Human Molecular Genetics Laboratory, Center of Molecular Biology and Genetic Engineering (CBMEG)-UNICAMP, where genetic tests were performed.

All patients in this sample had previously authorized their participation by signing the informed consent statement, having received clarification on the study to be performed. This project was approved by the Research Ethics Committee of the Faculty of Medical Sciences, UNICAMP (form number 96/2006).

Genomic DNA extraction from peripheral blood. The genomic DNA extraction was performed from leukocytes obtained from 10-15 ml of peripheral blood collected in EDTA Vacutainer® tubes (BD Biosciences, Franklin Lakes, NJ, USA). The method of extraction with phenol and chloroform was used (46), standardized in the Human Molecular Genetics Laboratory of CBMEG.

Analysis of mutations in the GJB2 gene, deletions del(GJB6-D13S1830) and del(GJB6-D13S1854) in GJB6, and the mitochondrial mutation m.1555A>G in the MTRNR1 gene. The mutation c.35delG in the connexin 26 (GJB2) gene was screened by allele-specific polymerase chain reaction (PCR) using Amplification Refractory Mutations System ('ARMS') primers for the detection of point mutations (44). The normal (NOR) and mutant (MUT) primers were used in different reactions to amplify alleles with or without mutation, respectively. The common primer (COM) was used as the reverse primer (47). These two reactions (NOR and MUT) may be identified as being normal homozygote, heterozygote and mutant homozygote to mutation c.35delG in each individual. Primers A and B were used as internal controls for amplification reactions (47). This technique was developed in the Human Molecular Genetics Laboratory of CBMEG (patent no. P10005340-6, test method for deafness of genetic origin-UNICAMP, 2002).

Screening for mutations in the GJB2 gene by sequencing. The coding exon of the *GJB2* gene, with 681 bp, was divided for amplification by technical PCR, according to a previously described protocol (48,49).

Purification of the PCR products. First, the fragments to be amplified by PCR were purified using the Wizard® SV Gel and PCR Clean-Up system kit (Promega Corp., Madison, WI, USA). The purification, quantity and purity of the DNA sample were determined by optical density in a spectrophotometer (NanoDrop® ND-8000; Thermo Fisher Scientific, Waltham, MA, USA).

Sequencing reaction. Sequencing reactions were performed in the automatic sequencer ABI PRISM® 3700 DNA Analyzer, using the BigDye™ Terminator v3.1 Cycle Sequencing kit (Applied Biosystems Life Technologies, Foster City, CA, USA), according to the manufacturer's protocol. The reactions consisted of 40-80 ng DNA, 1 µl mix BigDye® and 1 µl primer (5 pmol), adding deionized water to a final volume of 10 µl. The amplification conditions were: 96˚C (1 min), followed by 30 cycles of 96˚C (10 sec), 57˚C (5 sec) and 72˚C (30 sec), completing the cycle at 72˚C (5 min).

Analysis of the obtained sequences. The obtained sequences were smoothed and compared with normal gene sequences, using the programs Chromas Lite®, Gene Runner® v. 3:01 and CLC Sequence Viewer 6.1 (CLC bio; Aarhus, Denmark) [see Technelysium 2012: http://www.technelysium.com.au/chromas_lite.html; and Generunner 2013: http://www.generunner.net].

Identification of the deletions, del(GJB6-D13S1830) and del(GJB6-D13S1854), in the GJB6 gene. The tracking deletions, del(*GJB6-D13S1830*) and del(*GJB6-D13S1854*), were performed according to protocols previously described by Del Castillo *et al* (50,51). The investigation of mutations was performed using a multiplex PCR protocol, searching for the presence of the two deletions in one reaction. Fragments resulted from the amplification of DNA containing the breakpoints of the two deletions, as well as a segment of exon 1 from the *GJB6* gene, which was used as a control to check the efficiency of the reaction and to distinguish between heterozygous and homozygous alleles for either of the two deletions.

Screening of the mitochondrial mutation m.1555A>G in the MTRNR1 gene. The conditions previously described by Friedman and Griffith (52) and Iwasaki *et al* (53) were used for tracking the m.1555A>G mutation (15,52,53). Following amplification, fragments of mitochondrial DNA of 2060 bp were generated, and subsequently subjected to restriction analysis using the restriction endonuclease *Bsm*AI (New England BioLabs, Inc., Ipswich, MA, USA) for 2 h at 55˚C. Among individuals who were not carriers of the mutation m.1555A>G, three fragments were generated of size 1,100, 516 and 444 bp, whereas in individuals with the mutation, one of the region restrictions was abolished, generating only two fragments, of 1,616 and 444 bp.

Detection of mutations in the OTOF gene. For mutation screening, detection of the mutation p.Q829X (c.2485C>T) in exon 22 of the *OTOF* gene was accomplished using the PCR- fragment length polymorphism technique [primers: 22F (forward): 5'-TGACACCCCCTCCTTCGC-3' and 22R (reverse): 5'-CCCGACCCCTTGGGCGC-3']. Following amplification, the fragment of 157 bp was digested with the enzyme *Bfa*I (New England BioLabs, Inc.). In the presence of the mutation, the product was digested into two fragments of size 98 and 59 bp. The fragments were observed on a 3% agarose gel (Invitrogen; Thermo Fisher Scientific, Inc.) run at 100 V for 2 h, following a protocol described previously (54).

Complete screening of mutations in the OTOF gene. Mutations in the *OTOF* gene were tracked through the entire sequencing of exon 48, according to the protocol previously described by Migliosi *et al* (54). The sequencing steps were performed as described above for the *GJB2* gene.

Genotyping using MS. Genotyping was performed using the MassARRAY® system of Sequenom, Inc. by MALDI-TOF MS, and the iPLEX® Gold Assay system.

Test definition. Capture oligonucleotides (amplification primers) and single-base extensions were drawn from selected mutations with and/or SNP sequences. The tests were performed using MassARRAY Assay Design® software (version 3.1; Sequenom, Inc.). This program also generates groups of SNPs (multiplex) to be evaluated together. As this is a platform for high level, there is the possibility of evaluating up to 40 SNPs, or changes simultaneously in a single reaction for a given sample.

Amplification products containing SNPs and/or mutations. After evaluating the mutations or SNPs, defined capture primers were used in amplification products ranging from 100 to 400 bp, encompassing the region with the polymorphic site. Amplifications were performed in a GeneAmp® PCR system 9700 (Applied Biosystems) thermocycler with two blocks of 384 sample plates, following the protocol described by Sequenom® (iPLEX Gold Application Guide).

At this stage, the fragments containing the changes are captured. Amplification reactions were performed in a final volume of 5 µl containing 10 ng DNA template, 10X buffer, 500 µM each deoxynucleotide triphosphate (dNTP), 25 mM $MgCl_2$, 500 nM each primer and 5 units HotStar *Taq* DNA polymerase.

Treatment with SAP. After PCR, the amplification products have undergone a treatment for neutralization of unincorporated dNTPs, using the shrimp alkaline phosphatase (SAP) enzyme. The SAP inactivates dNTPs that have not been incorporated during the amplification reaction, converting them into non-phosphorylated nucleotides, and making them unviable for future dNTP reactions. To each sample, 2 µl of the SAP reaction was added, and the plate was incubated in a thermocycler at 37˚C for 45 min for enzyme action.

Reaction extension-iPLEX®. Aliquots of 2 µl of a cocktail of extension (iPLEX Gold reaction), composed of primer extension enzyme (iPLEX enzyme), buffer (10X iPLEX Plus Buffer) and nucleotides modified with masses (iPLEX Terminator Mix; Sequenom, Inc.), were added to the treated amplification products.

This reaction also occurred with the aid of the thermal cycler mentioned above. During the reaction, the primer

was exactly of the correct length adjacent to the SNP site, extending only one base (single extended background process). The extended iPLEX Gold Reaction generates products of different masses, depending on the nucleotide that has been added, or depending on the allelic form present in this sample.

Cleanliness of reactions and MS. Prior to MS, the iPLEX reaction products underwent cleaning using a resin (Clean Resin; Sequenom, Inc.), which removes excess ions that can interfere with the reading laser. A total of 6 mg resin was added to each of the 384-well plates, and 16 μl water was added to the total volume to make a final volume of 25 μl in each sample.

Reactions were transferred from the 384 plates to SpectroCHIP, with the aid of a MassARRAY Nanodispenser (Sequenom, Inc.). The SpectroCHIP was analyzed from the MassARRAY Compact Analyzer (Sequenom, Inc.) using the technique of MALDI-TOF MS.

The MALDI-TOF process was initiated by laser desorption of the analyte-matrix mixture and the analyte that the amplification product generated and selected during the iPLEX reaction. The subsequent physical processes result in the predominant formation of positively charged or negatively charged ions. These ions were extracted with an electric field, and separated according to their molecular masses and their charges.

The mass of the nucleic acid compounds was calculated using TOF, which reflects the time that the laser-ionized and accelerated compound requires to be taken through the tube (1-2 m length) of the analyzer and reach the detector of the instrument. At the detector, the ionized compounds generate an electrical signal that is recorded by a data system and is finally converted into a mass spectrum (45).

The MassARRAY TyperAnalyzer 4.0.5 software package was used to assimilate the information generated during the process described above, and reports were provided describing all the results of the analyses of each of the samples: Genotypes and frequencies are the predominant information derived from this system. The peaks were used to calculate the frequencies of SNP alleles.

Results

Of the 47 patients with AN analyzed, 33 were men (70.6%) and 14 were women (29.4%). The subjects' ages ranged from 2-61 years. The hearing loss, congenital in a total of 27 cases (79.4%), began during childhood in three (8.8%), and during adolescence in four (11.8%), of the cases.

Molecular changes were tracked in all 34 patients who were involved in this study and three parents, and the results are shown in Table I.

The c.35delG mutation in the connexin 26 (*GJB2*) gene was found in the homozygous form in three patients, and in the heterozygous form in one of the parents of these cases. No other changes were identified in the *GJB2* gene. Deletions in the *GJB6* gene [del(GJB6-D13S1830) and del(GJB6-D13S1854)] were not identified in any of the subjects, and neither was the mutation m.1555A>G in mitochondrial gene, *MTRNR1*, detected in any case studied.

A subsequent project is track the other mutations in the *OTOF* gene through the complete sequencing of the 48 exons. This process is already under development; however, thus far only four exons (exons 2, 3, 5 and 7) from certain patients have been analyzed. The partial results obtained are presented in Tables II and III. A total of 11 different genetic alterations were identified: Three exonic variations (Table II) and 8 intronic changes (Table III), all of which have been previously described in the literature.

Among the changes that were exonic were two silent variants, p.D43D (c.129C>T) in exon 2 and p.T124T (c.372A>G) in exon 5, and a missense mutation, p.A53V (c.158C>T), in exon 3. The p.A53V and p.T124T variants are considered to be non-pathogenic polymorphisms, although p.D43D is likely not to be pathogenic, since it is a silent change (29,54).

Discussion

Deafness is an etiologically heterogeneous trait with numerous known genetic and environmental causes, with genetic factors accounting for at least half of all cases of profound congenital deafness (29,54). A study previously reported that the fraction of AN cases in a sample of individuals with non-syndromic hearing loss that are likely to be genetic is ~70% in Brazil (34).

Genetic deafness can be classified by the mode of inheritance (dominant or recessive) and the presence or absence of characteristic clinical features that may be associated and allow the diagnosis of a specific form of syndromic deafness. Currently, the identification of >120 independent genes for deafness has provided profound novel insights into the pathophysiology of hearing, with recessive mutations at a single locus (the gene *GJB2*, or connexin 26), accounting for more than half of all genetic cases in certain specific populations, such as in the example of familial non-syndromic hearing loss, and even 30% of sporadic cases (55,56).

Knowing the mutation may allow the physician to make predictions regarding the progression of hearing loss or other abnormalities, although more efficient methods of genetic testing are required. These methods may allow the screening of all genetic mutations simultaneously. Therefore, the study of the genetic basis of AN is most important for a differential diagnosis, as well as for developing more specific treatments and more accurate genetic counseling.

Among the intronic changes identified were three polymorphisms considered non-pathogenic, IVS2+62C>T, IVS3+55C>T39IVS5 and A>T, and likely to be a non-pathogenic variant, IVS510A>G. The other intronic variants, IVS2+28T>G, IVS2+75G>A, IVS5-59T>C and IVS7-39C>T, are of unknown clinical significance; however, they are likely to have no relation to the clinical picture, since they are located, relatively, at a further distance from splicing sites.

Although, at present, >80 pathogenic mutations have been identified in the *OTOF* gene in individuals with non-syndromic deafness in populations of different origins, there is no 'hot-spot' in this gene (28). However, all reported mutations are gathered in two regions, exon 13 to 30 and exon 35 to 48, which may explain the absence of pathogenic mutations in the present study, since only certain of the initial exons of the gene have been analyzed thus far (28).

In a Brazilian study, >60% of cases with AN had at least one pathogenic mutation in the *OTOF* gene (34). Since muta-

Table I. Molecular test results in patients and their parents. The genetic changes found are highlighted.

	GJB2 gene			*GJB6* gene		*MTRNR1* gene	*OTOF* gene
Subject	AS-PCR c.35delG	IVS1+1G>A	Sequencing allele 1/allele 2	del (*GJB6*-D13S1830)	del (*GJB6*-D13S1854)	m.1555A>G	p.Q829X
1	WT	WT	WT	WT	WT	WT	WT
2	WT	WT	WT	WT	WT	WT	WT
3	35delG/35delG	WT	35delG/35delG	WT	WT	WT	WT
4	WT	WT	WT	WT	WT	WT	WT
5	WT	WT	WT	WT	WT	WT	WT
6	WT	WT	WT	WT	WT	WT	WT
7	WT	WT	WT	WT	WT	WT	WT
7 (b)	WT	WT	WT	WT	WT	WT	WT
8	WT	WT	WT	WT	WT	WT	WT
9	WT	WT	WT	WT	WT	WT	WT
10	WT	WT	WT	WT	WT	WT	WT
11	WT	WT	WT	WT	WT	WT	WT
12	WT	WT	WT	WT	WT	WT	WT
13	WT	WT	WT	WT	WT	WT	WT
14	WT	WT	WT	WT	WT	WT	WT
15	WT	WT	WT	WT	WT	WT	WT
16	WT	WT	WT	WT	WT	WT	WT
17	WT	WT	WT	WT	WT	WT	WT
18	WT	WT	WT	WT	WT	WT	WT
19	WT	WT	WT	WT	WT	WT	WT
20	WT	WT	WT	WT	WT	WT	WT
21	WT	WT	WT	WT	WT	WT	WT
22	WT	WT	WT	WT	WT	WT	WT
23	WT	WT	WT	WT	WT	WT	WT
24	WT	WT	WT	WT	WT	WT	WT
25	WT	WT	WT	WT	WT	WT	WT
26	WT	WT	WT	WT	WT	WT	WT
27	WT	WT	WT	WT	WT	WT	WT
28	WT	WT	WT	WT	WT	WT	WT
29	WT	WT	WT	WT	WT	WT	WT
30	35delG/35delG	WT	35delG/35delG	WT	WT	WT	WT
31	WT	WT	WT	WT	WT	WT	WT
32	WT	WT	WT	WT	WT	WT	WT
33	WT	IVS1+1G>A/WT	WT	WT	WT	WT	WT
34	WT	WT	V153I/WT	WT	WT	WT	WT
35	WT	WT	WT	WT	WT	WT	WT
36	WT	WT	WT	WT	WT	WT	WT
37	WT	WT	WT	WT	WT	WT	WT
38	WT	WT	WT	WT	WT	WT	WT
39	WT	WT	WT	WT	WT	WT	WT
40	WT	WT	WT	WT	WT	WT	WT
41	WT	WT	WT	WT	WT	WT	WT
42	WT	WT	WT	WT	WT	WT	WT
43	WT	WT	WT	WT	WT	WT	WT
44	WT	WT	WT	WT	WT	WT	WT
45	35delG/35delG	WT	35delG/35delG	WT	WT	WT	WT
45 (f)	35delG/WT	WT	35delG/WT	WT	WT	WT	WT
45 (m)	35delG/WT	WT	35delG/WT	WT	WT	WT	WT

Table I. Continued.

Subject	*GJB2* gene			*GJB6* gene		*MTRNR1* gene	*OTOF* gene
	AS-PCR c.35delG	IVS1+1G>A	Sequencing allele 1/allele 2	del (*GJB6*-D13S1830)	del (*GJB6*-D13S1854)	m.1555A>G	p.Q829X
45 (b)	35delG/WT	WT	35delG/WT	WT	WT	WT	WT
46	WT	WT	WT	WT	WT	WT	WT
47	WT	WT	WT	WT	WT	WT	WT

AS-PCR, allele-specific polymerase chain reaction; WT, wild-type; b, brother; f, father; m, mother.

Table II. Summary of exonic changes identified to date in the *OTOF* gene.

Exon	Finding	Frequency	Genotype	Clinical outcomes
2	p.D43D (c.129C>T)	1/21	Heterozygosity	Probably non-pathogenic
3	p.A53 V (c.158C>T)	2/15	Heterozygosity	Non-pathogenic
5	p.T124T (c.372A>G)	5/20	Heterozygosity and homozygous	Nonpathogenic

Table III. Summary of intronic changes identified to date in the *OTOF* gene.

Intron	Finding	Frequency	Genotype	Clinical outcomes
2	IVS2+28T>G	1/21	Heterozygosity	Unknown
	IVS2+62C>T	1/21	Heterozygosity	Non-pathogenic
	IVS2+75G>A	1/21	Heterozygosity	Unknown
3	IVS3+55C>T	14/15	Heterozygosity and homozygous	Non-pathogenic
4	IVS5-59T>C	1/20	Heterozygosity	Unknown
5	IVS5+10A>G	1/20	Heterozygosity	Probably non-pathogenic
	IVS5+39A>T	13/20	Heterozygosity and homozygous	Non-pathogenic
6	IVS7-39C>T	3/5	Heterozygosity	Unknown

tions in this gene are a frequent cause of AN in this population, a large number of individuals with a genetic etiology would be expected to be found in our sample resulting from mutations especially in the *OTOF* gene.

AN is characterized by absent or abnormal auditory brainstem responses and preserved otoacoustic emissions and/or cochlear microphonics (57). It is estimated that the prevalence of AN ranges between 0.23 and 15% in individuals with hearing loss, although it may vary according to the different criteria of patient inclusion and/or methodology. A previous study in Brazil identified AN in 1.2% of the patients with sensorineural hearing loss (n=2.292) in an auditory health care service (58).

Various studies have shown that patients with AN are heterogeneous in their underlying etiology, age and clinical manifestation, and patients may range from newborns to adults (57). AN may be caused by a variety of environmental and genetic factors. Approximately 40% of AN cases may have a genetic etiology (20,22).

Another study demonstrated that AN was associated with hereditary neurological disorders in 42% of the patients; in 10% of the patients, it was associated with toxic, metabolic, immunological and infectious causes; while the cause was unknown in 48% of patients (20-22). Most cases with AN are sporadic, although researchers have identified a number of familial cases with two or more affected members. Mutations may occur, for example, substitutions, deletions or base-pair insertions, although familial cases suggest that AN is inherited in certain genetic inheritance patterns (59).

To date, four loci associated with non-syndromic AN have been mapped: DFNB9 (the *OTOF* gene) and DFNB59 (the *PJVK* gene), which are responsible for the autosomal recessive pattern; AUNA1 (*DIAPH3* gene) for autosomal dominant; and AUNX1 for X-linked (23,24,26,32,33).

AUNA1 was first cited by Kim *et al* (30), when the first gene found in a four generation family was reported to be responsible for non-syndromic, autosomal dominant AN. The linkage analysis revealed an association with a novel section

of DNA on chromosome 13q14-21, between the D13S153 and D13S1317 markers (30). In addition, mutations in the connexin 26 (*GJB2*) and mitochondrial 12S rRNA genes were also reported in subjects with AN (37,60).

Molecular diagnosis is complicated by the extensive genetic heterogeneity. The *GJB2* gene, and *GJB6* mutations encoding connexins 26 and 30, in the DFNB1 locus are responsible for >50% of all cases of autosomal recessive hearing loss. However, the contribution of other gene mutations continues to be investigated, and this research is complicated by the evidence that the genetic epidemiology of non-syndromic hearing loss is highly variable among populations (13,14,40,52,61,62).

The *OTOF* gene is located on chromosomal region 2p22-23 (2,23,26). This gene encodes OTOF, a membrane calcium-binding protein involved in vesicle membrane fusion that serves a role in the exocytosis of synaptic vesicles at the auditory inner hair cell ribbon synapse (23,26). OTOF is expressed in the cochlea, vestibule and brain (23,26,34). The currently available data suggest that mutations in *OTOF* are a major cause of AN in a number of populations, with >100 identified pathogenic mutations, as revealed in the Human Gene Mutation Database (29,63-65).

Genetic research has shown that mutations in the *OTOF* gene are associated with non-syndromic autosomal recessive AN (57). Most *OTOF* mutations are exclusive, each being reported in only one family. One notable exception is p.Q829X (c.2485C>T) and in the present study, the p.Q829X mutation was investigated in the *OTOF* gene, although it had not been identified previously in any of the patients (2,27). The p.Q829X mutation is the most frequent mutation of the *OTOF* gene, and the third most common cause of non-syndromic autosomal recessive hearing loss in the Spanish population (29,52), the second in French and Argentine populations (2,66), and the first in Mexican and English populations (2,26). However, it is not a common cause of deafness in the Brazilian population (34,67-69).

The *PJVK* gene is mapped to chromosome 2q31.1-q31.3, and encodes PJVK, a 352-residue protein of unknown function, which is possibly associated with the activity of neurons or hair cells. PJVK is expressed in the body cells of all spiral ganglion neurons. Little is known about its contribution to the total of AN cases with genetic origin (33,66).

Researchers consider that PJVK is crucial for auditory nerve signaling. A missense mutation in DFNB59 would result in the production of a protein other than PJVK, resulting in AN due to a disruption in neuronal signaling along the auditory pathway (57).

In 2004, the *AUNA1* locus was mapped in an American pedigree, on chromosomal region 13q14-21, associated with post-lingual AN. The corresponding gene, *DIAPH3*, was later identified. No information is currently available about precise expression patterns of the *DIAPH3* gene or the localization of DIAPH3 within the inner ear. The function of DIAPH3 in the cochlea remains uncertain (30,31).

Wang *et al* (64) mapped the *AUNX1* locus on chromosomal region Xq23-q27.3 in a Chinese pedigree, although the corresponding gene has yet to be identified (64,69).

There are only two studies that have reported *GJB2* mutations in patients with AN. This gene is expressed in cochlear non-sensory supporting cells, and encodes the connexin 26 protein, which is associated with cell communication (gap junctions), forming channels that mediate the passage of small ions and molecules across cell membranes (35,36).

Connexin 26 deficiency disrupts the inner ear ion homeostasis, which leads to a local extracellular accumulation of potassium and cell death. Conversely, it is hypothesized that it may cause impairment of inner hair cells and nerve endings under the hair cells, and may be responsible for non-syndromic recessive AN. Therefore, it has not been established whether pathogenic variants in connexin 26 may be involved with AN, or whether the otoacoustic emissions that were recorded in the patients only represent the residual activity of a few outer hair cells that remain alive in the apical part of the cochlea. However, it is considered that certain mutations in the *GJB2* gene may cause changes in the inner hair cells and nerve endings of hair cells. Further investigation is needed to clarify the link between *GJB2* mutations and AN (37,38,70).

It has also been reported that mutations in the *GJB6* and *GJB3* genes contribute to autosomal recessive and autosomal dominant hearing defects in a number of populations. Mutations within the connexin *GJB3* gene family are considered to be the next most frequent cause of non-syndromic hearing defects, associated with non-syndromic autosomal dominant hearing impairment (38,54).

Mitochondrial mutations are associated with aminoglycoside-induced hearing loss, and also with maternally inherited non-syndromic hearing loss without exposure to aminoglycosides. One of these mutations, m.1095T>C in the mitochondrial 12S rRNA gene, was originally identified in two Italian families, and later in several Chinese families. However, the phenotypic differences observed among the subjects suggest that the mutation may be not actually responsible for the clinical signs. The identification and the clinical and molecular characterization of novel cases may elucidate its association with AN (70-72).

In addition, AN associated with mitochondrial disease may also be associated with hereditary syndromes, including Charcot-Marie-Tooth disease, Leber's hereditary optic neuropathy, autosomal dominant optic atrophy, autosomal recessive optic atrophy, Fredreich's ataxia, Mohr-Tranebjaerg syndrome and Refsum's disease with different inheritance types: Autosomal recessive, autosomal dominant, X-linked recessive and mitochondrial. Peripheral neuropathies may also be associated with neuronal nitric oxide synthase gene deficiency (57,73).

Ongoing research aims to identify other genes that are associated with AN. With this knowledge, an improved understanding of the underlying pathophysiological basis may be acquired, and specific treatments can be developed since, once a gene responsible has been identified, the protein it encodes and the target of treatment may be determined. This is a possible explanation of the heterogeneity of the disease and the different responses of patients to an identical treatment (56).

AN is a challenging condition, as numerous factors concerned with its etiology and pathogenesis remain poorly understood. Additionally, studies are needed to provide an improved understanding and clarification of AN. Further studies, particularly in the fields of molecular and genetic research, are required, in addition to the oncology research field (74). The study of the genetic basis of AN is therefore

important to improve the diagnosis, management, therapy and genetic counseling of the affected subjects.

In conclusion, in the present study, three homozygous c.35delG deletions were detected in patients with AN. In the *OTOF* gene, 11 mutations were identified, , although they are likely to be non-pathogenic, and the majority have a heterozygous genotype. However, the associations between these mutations and their correlation with AN have yet to be fully elucidated, and further studies are required to improve on the understanding of the pathophysiology of AN.

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4. CAPÍTULO 2

RELATIONSHIP BETWEEN PATIENTS WITH CLINICAL AUDITORY NEUROPATHY SPECTRUM DISORDER AND MUTATIONS IN GJB2 GENE

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Relationship Between Patients with Clinical Auditory Neuropathy Spectrum Disorder and Mutations in Gjb2 Gene

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Abstract:

The auditory neuropathy is a condition which there is a dyssynchrony in the nerve conduction of the auditory nerve fibers. There is no evidence about the relationship between patients with clinical auditory neuropathy spectrum disorder and mutations in GJB2 gene. There are only two studies about this topic in the medical literature. Connexin 26 (GJB2 gene) mutations are common causes of genetic deafness in many populations and we also being reported in subjects with auditory neuropathy.

Objective:

To analyze the pattern of clinical relationship between patients with clinical diagnosis with auditory neuropathy spectrum disorder and GJB2 gene.

Patients and Methods:

Study Design - Retrospective analysis and genetic evaluation. Setting - Tertiary referral center. Subjects - 40 patients with Auditory Neuropathy Spectrum Disorder. Intervention - Clinical information and genetic evaluation (GJB2 gene) were analyzed.

Results:

Biallelic mutations that accounted for hearing loss (HL) were found in three patients, both with c.35delG mutation in homozygous state. The splice site mutation IVS1+1G>A was detected in heterozygous state in one individual. However, since the second mutant allele was not identified, it was not possible to establish its correlation with the phenotype.

Conclusions:

Mutations in GJB2 gene mutations were found in 7.5% of the patients with ANSD. We found no relationship between patients with clinical auditory neuropathy spectrum disorder and mutations in GJB2 gene ($p>0.05$).

Keywords: Auditory neuropathy spectrum disorder, evoked auditory brainstem response, otoacoustic emissions, GJB2, hearing loss.

INTRODUCTION

Auditory neuropathy spectrum disorder (ANSD) is the synchrony loss related to the auditory pathway, resulting in a deficitary nerve conduction in the auditory nerve fibers, probably secondary to a demyelination of the 8th cranial nerve. ANSD can affect individuals of any age, from newborns to adults, and can be caused by a variety of environmental or others clinical conditions (such as anoxia and hyperbilirubinemia) and genetic factors. The prevalence of ANSD in

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children with severe or profound hearing loss is estimated at 13.4% [1 - 3].

ANSD is characterized by outer hair cells (OHCs) function preservation associated with lack of response in the brainstem evoked response audiometry (ABR) test. The outer hair cells functionality is demonstrated by the positive response of otoacoustic emissions (OAEs) and/or cochlear microphonic (CM) detection, while no synchronous neural activity is detected in the ABR test [4, 5].

It is believed that there are several topographical transmission failure sites in the auditory action potential, such as damage at the tectorial membrane and internal hair cell; changes in neurotransmitter release in the synapses between the inner hair cell and spiral ganglion neurons fibers; changes in electrical transmission in cochlear nerve fibers or axons or problems related to cochlear nerve myelination [1, 2, 6].

Clinically, the patients with ANSD can present with many degrees of unilateral or bilateral hearing loss (symmetric or not). The speech discrimination is low and incompatible with audiometric thresholds. Patients with ANSD may have fluctuating hearing levels; sometimes, the individual appears to have normal hearing and its behavior resembles an individual with auditory disabilities [7].

The presence of OAEs with absent or abnormal ABR supports the clinical diagnosis. The CM may be present and the acoustic reflexes may be absent. There may be some correlation with pathologies that occur with peripheral neuropathy such as *Guillain-Barré* syndrome and *Charcot-Marie-Tooth* [8, 9]. The therapeutic intervention in patients with auditory neuropathy needs to be made by hearing stimulation and speech therapy, and the cochlear implants or hearign aids could be a great assistance and must be performed as soon as possible [10].

In the last decade, the genes identification responsible for ANSD had great contribution for the diagnosis and better understanding of the mechanisms involved in this disorder [5, 6, 11].

Four loci responsible for nonsyndromic ANSD were mapped to date: DFNB9 (*OTOF* gene) and DFNB59 (*PJVK* gene) for autosomal recessive ANSD; AUNA1 (*DIAPH3* gene) for autosomal dominant ANSD; and AUNX1 for X-linked ANSD. In addition, mutations in *GJB2* gene and mitochondrial 12S rRNA gene have been also related to this disorder [4, 7 - 10, 12].

Most genetic studies on the etiology of ANSD exclude cases that may have originated in *GJB2* gene, environmental or host factors, in order to be more effective in the search for new genes or genetic factors that may explain this pathology.

There are only two studies that reported the relationship of the *GJB2* gene with ANSD. The first one described five individuals with preserved OAEs, among 777 unrelated subjects attending schools for the deaf or receiving special services for their moderate to profound HL, carrying mutations in the *GJB2* gene, either in homozygous state (c.35del G/c.35delG or p.W77X/p.W77X) or in compound heterozygous state (c.35delG/c.360delGAG, c.35delG/p.V95M and p.M34T/p.V84M) [13].

However, the clinical diagnosis of ANSD was confirmed only in one case. So, this Asian study highlighted that 6.5 % of patients with clinical diagnostic of ANSD (OEA + / ABR -) feature homozigose compound heterozygous for the GJB2 (c.35delG/c.35delG or p.W77X/p.W77X / c.35delG/c.360 delGAG , c.35delG/p.V95M and p.M34T/p.V84M) [13].

The second paper, a case series, about *GJB2* gene and ANSD reported two children homozygous for c.35delG and one heterozygous for p.M34T, showing moderate to profound hearing loss and preservation of OAEs. However, further investigation revealed that only one child homozygous for c.35delG could have ANSD [14].

Despite mutations in *GJB2* gene are clearly related to unilateral non syndromic hearing loss and is the most common genetic finding in hearing loss diseases, the relationship with Auditory Neuropathy Spectrum Disorder (ANSD) is still unclear [13 - 16].

In this paper we conducted a literature review on the mutations in *GJB2* gene in patients with ANSD, and studied this relationship in our population. Only few articles studied this relationship and more data about this topic can help another studies and protocols of the investigation and development of knowledge of ANSD.

This research article is important because it is the first Brazilian and Latin American review on this topic, it shows the most frequent causes ANSD, and the peculiarities of ANSD and this relationship with mutations in *GJB2* gene.

We believe that these findings are of great importance to all professionals who work with patients with auditory

neuropathy, such as pediatrician, otolaryngologists, audiologists, genetics and researches teams.

Table 1. Patients' distribution according to gender, age of onset, audiological evaluation and genetic background.

ANSD	Sex	Age	OAE	ABR	CM	GJB2 Genotype	Past History
1	F	Childhood	+	No response	-	wt/wt	None
2	M	Congenital	+	No response	+	wt/wt	Preterm baby (35wks) / neonatal unity care for 10 days
3	M	Congenital	+	No response	-	wt/wt	None
4	M	Adolescent	+	No response	-	wt/wt	None
5	M	Congenital	+	No response	-	wt/wt	None
6	F	Congenital	+	No response	+	wt/wt	Preterm baby (36 wks)
7	M	Adult	+	No response	-	IVS1+G>A/wt	None
8	F	Congenital	+	No response	-	wt/wt	None
9	F	Childhood	+	No response	-	wt/wt	None
10	M	Congenital	+	No response	-	wt/wt	None
11	M	Congenital	+	No response	+	wt/wt	Brother with SNHL
12	M	Congenital	+	No response	-	wt/wt	Kernicterus
13	F	Congenital	+	No response	-	wt/wt	Preterm baby (29 wks), neonatal unity care for 35 days, neonatal CRA
14	M	Adult	+	No response	-	wt/wt	None
15	F	Congenital	+	No response	-	wt/wt	Neonatal jaundice, preterm baby (36 wks), neonatal unity care for 26 days
16	F	Congenital	+	No response	-	wt/wt	Axial ascending muscular atrophy
17	M	Adolescent	+	No response	-	wt/wt	None
18	M	Congenital	+	No response	-	c.35delG/c.35delG	None
19	M	Congenital	+	No response	-	c.35delG/c.35delG	None
20	F	Congenital	-	No response	+	wt/wt	None
21	M	Congenital	-	No response	+	wt/wt	None
22	F	Congenital	-	No response	+	wt/wt	None
23	M	Congenital	-	No response	+	wt/wt	None
24	M	Congenital	-	No response	+	wt/wt	Charge syndrome
25	M	Congenital	-	No response	+	wt/wt	None
26	M	Congenital	-	No response	+	wt/wt	Preterm baby (27 wks), neonatal unity care for 30 days
27	M	Congenital	-	No response	+	wt/wt	Preterm baby (28 wks), neonatal unity care for 42 days, meningitis
28	M	Congenital	-	No response	+	wt/wt	Preterm baby (32 wks), neonatal unity care for 14 days
29	M	Congenital	-	No response	+	wt/wt	Neonatal unity care for 10 days
30	M	Congenital	-	No response	+	wt/wt	None
31	F	Congenital	-	No response	+	wt/wt	Gestational toxoplasmosis
32	F	Congenital	-	No response	+	wt/wt	aspiration pneumonia 3 months old
33	M	Congenital	-	No response	+	c.35delG/c.35delG	Preterm baby (35 wks), neonatal unity care for 05 days
34	M	Congenital	-	No response	+	wt/wt	Preterm baby (35 wks), neonatal unity care for 19 days
35	F	Adolescent	-	No response	+	wt/wt	Systemic arterial hypertension, diabetes mellitus
36	M	Congenital	-	No response	+	wt/wt	Brother with SNHL
37	F	Adolescent	-	No response	+	wt/wt	None
38	M	Congenital	-	No response	+	wt/wt	Preterm baby (35 wks), neonatal unity care for 16 days
39	F	Congenital	-	No response	+	wt/wt	None
40	M	Childhood	-	No response	+	wt/wt	None

Ps.: M: male; F: female; +: positive; -: negative; N/A: not available; wt: wild type.

The underlying causes of ANSD are highly questionable and uncertain; therefore the study of GJB2 involvement in patients with ANSD can help to guide new studies, new discoveries and the interface of these genetic alterations with different clinical phenotypes of deafness.

This paper aims to study the relationship between the clinical diagnosis of auditory neuropathy spectrum disorder and mutations in the *GJB2* gene.

METHODS

We conducted a retrospective observational study through analysis of medical records of patients diagnosed with bilateral hearing loss, with suspected ANSD, accompanied in the auditory health care service of a tertiary care university hospital.

The analyzed variables were age, gender, age of onset of the hearing loss (congenital, childhood, adolescent or adult), past history, previous pregnancies, perinatal and genetic data and electrophysiological test results (ABR,

transient and distortion product otoacoustic emissions (OAE), and search of cochlear microphonics (CMs)).

The age group was defined as reported in the medical records of the early onset of symptoms, as follows: congenital (up to 1 year old), childhood (between 1 and 10 years old), adolescent (between 11 and 18 years of life) or adult (over 18 years of life).

The clinical diagnosis of ANSD was established as follows: absent or abnormal ABR with preservation of otoacoustic emissions and / or CMs. All patients had no acoustic reflex and the presence of the cochlear nerve was assessed by nuclear magnetic resonance (MRI) and computed tomography (CT).

Molecular Study

Genomic DNA was extracted from patient peripheral venous blood according to standard protocols. *GJB2* mutations were screened by direct sequencing of the gene coding region and the exon 1 and flanking splice donor site [17 - 19].

Inclusion Criteria

Inclusion criteria were: sensorineural hearing loss, normal otoscopy, absence of middle ear disease, absence of acoustic reflex, presence of transient and / or distortion product OAEs, absence or abnormality in ABR waves and imaging (MRI / CT) showing the presence of the cochlear nerve and excluding retrocochlear disturbances.

All patients who did not complete these criteria were excluded from the study.

Audiological Evaluation

Audiological tests were performed including impedanciometry, speech and pure tone audiometry. The tests were performed using an audiometer AC30-SD25, calibrated according to ISO 389 standards / 64.

For ABR, which were repeated at least two times, we used the device AT-235 (Interacoustics).

Table 2. Patients details with pathogenic variants in the connexin 26 gene.

ANSD	Case 18	Case 19	Case 33	Case 7 *
Gender	Male	Male	Male	Male
Age (at diagnosis)	Congenital	Congenital	Congenital	Adult
OAE	+	+	-	+
ABR	absent	absent	absent	absent
CM	-	-	+	-
GJB2 Genotype	c.35delG/c.35delG	c.35delG/c.35delG	c.35delG/c.35delG	IVS1+G>A/wt
Pure Tone Thresholds	50-70 dB	60-80 dB	80-100 dB	40-70 dB
IRF	68%	NA	50%	72%
LRF	65 dB	70 dB	60 dB	40 dB
Past History	None	None	Preterm baby (35 wks) NICU (5 d)	None
CT/MNR	Normal	Normal	Normal	Normal
Treatment	CI +HA + speech therapy	CI + HA + speech therapy	CI + HA + speech therapy	HA + speech therapy
Ps.		detects plate and barrel in behavioral audiometry with hearing aids and / a / & / u /		Understanding speech difficulty; PTA oscillations; Some audiogram similar to conductive HL

Ps.: M: male; F: female; +: positive; -: negative; N/A: not available; wt: wild type; CI: cochlear implant; HA: hearing aids. PTA: pure tone audiometry; HL: hearing loss. NICU: neonatal unity care unity.

Statistical Analysis

The data were analyzed using descriptive analysis, with production of means, medians and standard deviation tabs.

Chi-Square was used to compare the groups of our sample. Because of the small size of some of the variables analyzed Fisher's Exact test was also used to check the correlation between the groups.

The confidence Interval was of 95%, and *p-value* <0.05 was considered significant.

Ethical Considerations

This study was previously approved by the Research Ethics Committee of the Faculty of Medical Sciences of the University of Campinas (Report number 396/2006).

RESULTS

According to the criteria established in the methodology, 40 patients with ANSD were selected after the review of the medical records.

Most patients, 75%, had onset of symptoms in the first year of life (30 congenital; 3 child; 4 adolescence; 2 adult) and 65% were male (26 male; 14 female). In 23 subjects, 57.5% of the cases, no genetic or environmental factor that could be related to the etiology of the disease were observed.

We observed that ANSD could be associated with hyperbilirubinemia in only two cases, *i.e*., a neonatal jaundice with phototherapy and kernicterus. Three cases had family history of sensorineural deafness. In 25% of cases the patients were premature (mean of 32,8 weeks (range 28 to 36 days) – median of 28 weeks), and in 25% of cases. (Mean of 20,7 days (range 5 to 42 days) – median of 27,5 days) they were hospitalized in the neonatal intensive care unit (NICU)

Regarding infectious diseases, only one case of meningitis during the neonatal ICU was reported, one case of aspiration pneumonia at three months of life with need of hospital treatment and one case of gestational toxoplasmosis (case with also bilateral enlarged vestibular aqueduct in radiologic evaluation).

Syndromic features were observed in only one case (Charge syndrome). Regarding systemic neuromuscular diseases, axial ascending muscular atrophy was observed in only one subject. No others known causes of peripheral neuropathies were present. One of the patients (adult) had arterial hypertension and diabetes without other relevant risk factors for hearing loss.

ABR was abnormal or absent in all cases. The classic situation in which OAEs are present with absent ABR occurred in only 47.5% of cases; the presence of CM was also noted in three of these patients (OEA + ABR – CM +). The remaining patients (52.5%) showed absent OAE and ABR with the presence of CMs.

Radiologic abnormalities were identified in three cases, including enlargement of vestibular aqueduct bilaterally (OAE-, ABR-, CM + and gestational toxoplasmosis), a cyst arachnoid in left cerebellar point angle (OAE-, ABR-, CM +) and signs of involvement of kernicterus (OAE +, ABR -, CM-).

Genetic Testing

Sequence analysis of *GJB2* gene was performed in all 40 patients with clinical diagnosis of ANSD. Pathogenic variants in the connexin 26 gene were identified in four subjects. Biallelic mutations that accounted for hearing loss (HL) were found in three patients, all of them with c.35delG mutation in homozygous state. The splice site mutation IVS1+1G>A was detected in heterozygous state in one individual. However, since the second mutant allele was not identified, it was not possible to establish its correlation with the phenotype.

All the variables with *GJB2* gene' homozygous were matched (Chi-Square and Fischer-t-Test) and we did not found any relevant correlation.

We found correlation between the mutation of *GJB2* gene' homozygous only with moderate (p=0,03) and severe (p=0,02) hearing loss associated with the presence of CM, however the Fischer-t-Test did not confirm this relationship.

The variables analyzed about the relationship with *GJB2* gene were: neonatal care unit, past history, gender, prematurity, jaundice, other peripheral neuropathies, infectious disease, radiologic abnormalities, syndromic comorbidities, OEA+ABR-, OEA+ABR-CM+, age, presence and absence of OEA, presence and absence CM, hearing loss (mild to profound).

The relationships between hearing loss and the presence of CM and hearing loss with OEA+ABR- or OEA-ABR-CM+ were also evaluated, with no significant findings.

DISCUSSION

ANSD, unknown until recently, is a condition that has been more studied in the last decade, and electrophysiological tests for hearing are essential tools for proper diagnosis and knowledge of its pathophysiology. The diagnosis is primarily based on changes in ABR associated with normal OAEs [2].

OAEs are used to evaluate the function of outer hair cells and represent preneural phenomenon related to mechanical processes in the cochlea. The presence of OAE depends on an intact auditory system. They check the organ of Corti functioning and the efferent auditory system (OHCs) [1, 20].

CM is another way to verify the cochlear integrity and functioning. It is an alternating current potential that occurs during the sound stimulus and reflects the movement of the basilar membrane [20]. CM is a preneural electrical activity, *i.e*., it occurs before hair cell synapses in the auditory nerve, and thus appears before the wave I in the ABR record [21].

Cochlear microphonic was present in 60% (24 cases) of the patients, which denotes some degree of cochlear integrity and inner hair cells (IHCs) functioning in these individuals. However, it should be noted that 52,5% (21 cases) of patients had absent transient OAEs, which does not favor the diagnosis. In our study, a large portion of the subjects included had hearing loss onset at congenital age (75%).

ABR was absent or abnormal in all cases. ANSD classically presents with impaired speech discrimination. In individuals with congenital hearing loss, obviously that deficit would result in an impaired speech development [2, 20]. The study showed that 51% of the patients had poor speech development, with great difficulty understanding speech, other 26.7% had speech deemed modified (dislalia), although intelligible (reasonable) and 22.3% had good speech without compromising the understanding.

In our cohort, 42.5% (17 cases) of patients had environmental factors or other clinical conditions that may be related to ANSD, such as neonatal ICU, prematurity, family history of deafness, among others. Many recent studies have aimed to identify the possible mechanisms responsible for auditory neuropathy, particularly the involvement of genetic factors [1, 2, 22].

Three out of the 40 patients with clinical diagnosis of ANSD were homozygous for c.35delG mutation. Mutations in the connexin 26 gene are frequent in many populations, accounting for up to 50% of all ANSD cases. Interestingly, two different studies also reported the finding of ANSD in individuals with HL carrying *GJB2* mutations [13, 14].

There is no scientific evidence to support the real relationship between ANSD and mutations in the *GJB2* gene. This gene is expressed in the cochlea non sensory cells (support cells) and encodes the connexin 26 protein, which is associated with the cellular communication (gap junctions), forming channels that mediate passage of small ions and molecules across cell membranes, allowing for example, the recycling of potassium ions in the cochlear fluids [13, 14, 23, 24].

Mutations in this gene can alter the function of connexin 26 to cause deficiency in the flow of potassium ions, which can lead to cell death and consequently deafness since high potassium levels may affect the function and survival of cells necessary to support hearing [13, 14, 23, 24].

It is possible that OAE observed in patients with ANSD represents residual activity of a few outer hair cells remained alive in the apical part of the cochlea, and thus the hearing loss observed in these cases would not fit the diagnosis of ANSD [24].

It is currently known that the connexin 26 deficiency disrupts the inner ear ion homeostasis, which leads to a local extracellular accumulation of potassium and cell death [25].

Table 3. Audiometric thresholds of all subjects [27].

PTA	Total (n)	OEA + BERA -	OEA - BERA - MC +	OEA + BERA - MC+	MC +
Low	5	2	3	0	3
Moderated	15	9	6	0	6
Severe	11	3	8	1	9
Profound	9	5	4	2	6
Total (n)	40	19	21	3	24

Conversely, Matsunaga's recent reports also highlights that mutations in connexin 26 gene may cause impairment of inner hair cells and nerve endings beneath the hair cells and be responsible for nonsyndromic recessive ANSD [26]. Thereby, further studies are necessary to clarify the link between *GJB2* mutations and ANSD [20].

The identification of genetic alterations responsible for ANSD may contribute for a better understanding of the molecular basis and pathophysiological mechanisms involved in different hearing loss phenotypes. The use of new molecular tools allowing a faster and more effective diagnosis is of great importance for the prognosis and also for the patients' treatment.

Genetic testing, combined with clinical and audiological exams, allows an accurate diagnosis, as well as the development of more specific treatments and genetic counseling of patients and/or families [16].

There is no scientific evidence to support the real relationship between ANSD and mutations in the GJB2 gene. This

gene is expressed in the cochlea non sensory cells (support cells) and encodes the connexin 26 protein, which is associated with the cellular communication (gap junctions), forming channels that mediate passage of small ions and molecules across cell membranes, allowing for example, the recycling of potassium ions in the cochlear fluids [13 - 16].

In this paper we conducted a literature review on the mutations in GJB2 gene in patients with ANSD, and studied this relationship in our population. Only few articles studied this relationship and more data about this topic can help another studies and protocols of the investigation and development of knowledge of ANSD.

This research article is important because it is the first Brazilian and Latin American review on this topic, it shows the most frequent causes ANSD, and the peculiarities of ANSD and this relationship with mutations in GJB2 gene.

We believe that these findings are of great importance to all professionals who work with patients with auditory neuropathy, such as pediatrician, otolaryngologists, audiologists, genetics and researches teams.

The underlying causes of ANSD are highly questionable and uncertain; therefore the study of GJB2 involvement in patients with ANSD can help guide new studies, new discoveries and the interface of these genetic alterations with different clinical phenotypes of deafness.

The diagnosis of ANSD should be considered in patients with bilateral hearing loss initiated until adolescence. Studies should be performed to determine the role of perinatal and genetic conditions in the origin of the disease.

The knowledge of clinical features of ANSD, as specified in the above data, allows us to presume that individuals diagnosed with hearing loss were more likely to present ANSD. The clinical skills improvement, combined with additional tests (genetic and audiological), allows large knowledge of ANSD's etiologies that are still being studied.

CONCLUSION

We found, a 7.5% ratio of GJB2 gene mutations in patients with ANSD.

Evidences are lacking to show the actual relationship between ANSD and GJB2 gene.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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UNICAMP Approval. The study, including the protocols for subject recruitment and assessment, the informed consent for participants, and the overall analysis plan were reviewed and approved by UNICAMP boards for the Faculdade de Ciências Médicas da Universidade Estadual de Campinas e do Centro de Biologia e Engenharia Molecular (CBMEG), São Paulo Brasil, through their doctoral programs and their research ethics committee.

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5. CAPÍTULO 3

AUDITORY NEUROPATHY: CLINICAL EVALUATION AND DIAGNOSTIC APPROACH

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Neuropatia Auditiva: Avaliação Clínica e Abordagem Diagnóstica

Auditory Neuropathy: Clinical Evaluation and Diagnostic Approach

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RESUMO

Introdução: A neuropatia auditiva é uma condição na qual há alteração na condução neuronal do estímulo sonoro. Este trabalho pretende descrever e caracterizar a casuística de doentes com neuropatia auditiva.

Material e Métodos: Realizámos um estudo transversal, retrospetivo, com descrição de uma série de casos consecutivos. O diagnóstico da neuropatia auditiva foi definido nas seguintes situações: Presença de otoemissões acústicas com potenciais auditivos de tronco encefálico ausente ou anormal e presença do microfonismo coclear independentemente da presença de otoemissões acústicas.

Resultados: Foram avaliados 34 doentes com perda auditiva bilateral, 67% deles do sexo masculino. O aparecimento dos sintomas foi congênito em 80% dos casos. Na pesquisa das otoemissões acústicas, a resposta foi ausente em 67% dos doentes. O microfonismo coclear foi detetado em 79% dos doentes. Antecedentes gestacionais, perinatais ou ambientais relevantes estiveram presentes em 35,3% dos casos.

Discussão: A literatura médica ainda apresenta grande variabilidade nos achados relacionados com a neuropatia auditiva, tanto na sua etiologia quanto nos dados epidemiológicos.

Conclusão: A neuropatia auditiva apresenta um amplo espectro de alterações que podem resultar em disfunções leves a severas no funcionamento da via auditiva. Na nossa amostra, observámos que 80% das neuropatias auditivas terão tido origem congênita e/ou apresenta microfonismo coclear, 91% dos doentes apresenta défice auditivo significativo e 53% sofrem de surdez severa ou profunda.

Palavras-chave: Células Ciliadas Auditivas; Células Ciliadas Auditivas Externas; Células Ciliadas Auditivas Internas; Potenciais Evocados Auditivos do Tronco Encefálico; Neuropatia Auditiva.

ABSTRACT

Introduction: Auditory neuropathy is a condition in which there is a change in the neuronal transmission of the auditory stimuli. Our objective was to describe the patients' series within the clinical spectrum of auditory neuropathy.

Material and Methods: We designed a transversal, retrospective study, with a description of a consecutive case series. Auditory neuropathy was defined by the presence of acoustic otoemissions plus absent/abnormal auditory brainstem responses with cochlear microphonism.

Results: 34 patients with bilateral hearing loss, 23 males and 11 females, were included in the study. Eighty percent of the cases had congenital onset of hearing loss. Acoustic otoemissions were absent in 67% of them. Cochlear microfonism was present in 79% of all cases. Prenatal, perinatal or ambiental factors were present in 35.2% of the cases.

Discussion: Medical literature shows great variability in findings related to auditory neuropathy, both in its etiology and epidemiological data.

Conclusion: Auditory neuropathy presents a broad spectrum of changes that may result from mild to severe changes in the functioning of the auditory pathway, and in our sample we observed that 80% of Auditory neuropathy have congenital onset of hearing loss and/or with cochlear microphonism identified. 91% of patients experience significant hearing impairment and 53% suffer from severe or profound deafness.

Keywords: Auditory Neuropathy; Evoked Potentials, Auditory, Brain Stem; Hair Cells, Auditory; Hair Cells, Auditory, Inner; Hair Cells, Auditory, Outer; Mononeuropathies.

INTRODUÇÃO

A neuropatia auditiva (NA) ou dissincronia auditiva (DA) é um distúrbio auditivo onde a função das células ciliadas externas da cóclea é normal, mas há uma alteração na função das células ciliadas internas e/ou acometimento das fibras do nervo auditivo com dissincronia na condução nervosa do mesmo.[1,2]

Esta patologia auditiva pode acometer indivíduos de qualquer faixa etária, com prevalência variando entre 0,23 e 2% das crianças, sendo um fator de risco para deficiência auditiva. Acredita-se que por volta de 8% dos novos casos anuais de perda auditiva diagnosticada em crianças sejam relacionados com a NA.[3]

No Brasil, estão disponíveis poucos dados sobre prevalência e incidência dessa patologia na população geral. Num estudo recente, que avaliou cerca de 2 292 indivíduos com perda auditiva, foi encontrada uma prevalência de 1,2% de doentes com patologia no espectro da neuropatia auditiva.[4]

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A fisiopatologia da doença é ainda pouco conhecida. Provavelmente a NA não é uma doença única, mas um espectro de patologias que afetam a via auditiva. Em recém-nascidos, diversos fatores como prematuridade, hiperbilirrubinemia, hipercolesterolemia, hipóxia, imaturidade do sistema nervoso central, baixo peso ao nascer, condições idiopáticas, fatores genéticos e ainda outros foram propostos como contribuintes, isoladamente ou em combinação, para o desenvolvimento de NA.[3-6]

Estão propostas diversas teorias no que diz respeito aos locais de lesão auditiva que cursam com NA, tais como a membrana tectorial, as células ciliadas internas (CCI), as células ciliadas externas (CCE), alterações da liberação de neurotransmissores nas sinapses entre as células ciliadas internas e as fibras dos neurónios do gânglio espiral, alterações na transmissão elétrica das fibras do nervo coclear, problemas axonais diversos ou mesmo relacionados à mielinização do nervo coclear.[7]

A prevalência descrita da NA em doentes pediátricos com hipoacúsia severa ou profunda é de cerca de 13,4%.[8] De entre os fatores genéticos, muito estudados, estão estabelecidas algumas mutações em genes classicamente descritos e relacionados com a NA, como o OTOF e o PJVAK.[4] Acredita-se que tais genes possam ser responsáveis por disfunções relacionadas com as CCI.[9]

Estão já mapeados quatro *loci* considerados na literatura médica como responsáveis pela NA em doentes não-sindrómicos, de entre os quais: DFNB9 (*OTOF*) e DFNB59 (*PJVK*) para casos autossómicos recessivos; AUNA1 (*DIAPH3*) em casos autossómicos dominantes; 1 AUNX1 em casos ligados ao X. De acordo com alguns autores, mutações no gene GJB2 e mutações mitocondriais (12S rRNA) podem também estar relacionadas com a NA.[10-15] Apesar de estarem descritos casos clássicos de NA em doentes com o diagnóstico genético molecular de homozigotia da mutação 35delG no gene GJB2, a associação entre mutações de GJB2 e NA foi abordada ainda em poucos trabalhos, não estando estabelecida uma correlação.[4,16]

Na sua evolução, a perda auditiva pode ocorrer em graus variados, frequentemente com alterações na inteligibilidade da fala associadas a alterações nos exames eletrofisiológicos auditivos.

As otoemissões acústicas (OEA) apresentam-se normais com potencial evocado auditivo do tronco encefálico (PEATE)/BERA) normal ou ausente, sendo essa situação considerada como NA clássica. O microfonismo coclear (MC) pode estar presente e os reflexos estapédicos estão normalmente ausentes.[17,18]

Em algumas situações, há outras neuropatias periféricas concomitantes, sendo as mais descritas a síndrome de Guillain-Barré e a doença de Charcot-Marie-Tooth.[14,15]

A reabilitação dos doentes com o diagnóstico de NA, seja com terapia da fala isolada, implante coclear ou com aparelhos auditivos de amplificação sonora individual, deve ser realizada o mais precocemente possível, para uma otimização dos resultados.[18]

O presente estudo tem como objetivo descrever os dados epidemiológicos e audiológicos dos doentes pediátricos com diagnóstico de NA, acompanhados no serviço de saúde auditiva de um hospital universitário de referência terciária, evidenciando as características clínicas desses doentes.

MATERIAL E MÉTODOS

Realizámos um estudo transversal, retrospetivo, com a descrição de uma série de casos consecutivos, através da análise do processo clínico de doentes com diagnóstico de perda auditiva bilateral, com suspeita de patologia no espectro da neuropatia auditiva, acompanhados no serviço de saúde auditiva de um hospital universitário.

As variáveis analisadas foram idade, sexo, data de início da perda auditiva (congénita, na infância, na adolescência ou em adulto), antecedentes gestacionais, perinatais e genéticos, e resultados dos exames eletrofisiológicos: PEATE, emissões otoacústicas transientes e por produto de distorção (OEA), e pesquisa de microfonismo coclear (MC).

Como antecedentes perinatais e gestacionais foram consideradas quaisquer situações descritas nos antecedentes desses doentes como: prematuridade, icterícia, *kernicterus*, internamento em unidade de cuidados intensivos neonatal, meningite, infeções neonatais graves (como sépsis e pneumonia), síndromes genéticas, doenças neurológicas concomitantes, doenças infeciosas (como rubéola e toxoplasmose), comorbilidades sistémicas clínicas (como hipertensão arterial, diabetes mellitus). Os antecedentes familiares de surdez também foram pesquisados e identificados.

O grupo etário foi definido conforme o início do aparecimento dos sintomas, sendo: congénito (até um ano de vida), infância (entre o primeiro e o 10º ano de vida), adolescente (entre o 11º e o 18º ano de vida) ou adulto (mais do que 18 anos de vida).

O diagnóstico clínico da NA foi estabelecido da seguinte forma: OEA normal e/ou MC com PEATE com respostas ausentes ou anormais, com exames de imagem que excluíssem qualquer alteração anatómica do VIII par craniano.

Amostra

A amostra utilizada no trabalho foi constituída pelos doentes acompanhados nos últimos três anos (2011 a 2014), no serviço de saúde auditiva de hospital universitário em questão, com o diagnóstico clínico de NA.

Foram incluídos na amostra apenas os doentes que realizaram testes audiológicos e eletrofisiológicos com a nossa equipe de fonoaudiologia (audiologistas e terapeutas de fala), tendo sido utilizados para todos eles os mesmos equipamentos.

Critérios de inclusão

Os critérios de inclusão foram: perda auditiva neurossensorial; otoscopia normal; ausência de doenças do ouvido médio; exames de imagem (ressonância magnética

ou tomografia computadorizada) evidenciando a presença do nervo vestibulococlear e excluindo alterações retrococleares; ausência de reflexo acústico; exames audiológicos compatíveis com o espectro clínico da neuropatia auditiva:

a) OEA presentes e PEATE ausente, ou

b) OEA ausentes, PEATE ausente e microfonismo coclear presente, ou

c) OEA ausentes e PEATE ausente com limiares tonais presentes e suspeita clínica de neuropatia auditiva.

Avaliação Audiológica

Foram realizados os testes audiológicos, incluindo a impedanciometria, audiometria tonal e vocal. Os testes foram realizados com um audiómetro AC30-SD25, calibrado de acordo com a ISO 389/64.

Para as OEA e os PEATE, estes últimos repetidos pelo menos duas vezes, utilizou-se o dispositivo AT-235 (*Interacoustics*).

As OEA por produtos de distorção foram realizadas nas frequências de 700 a 8.000 Hz, com estímulos de 65 a 55 dBNPS, com razão de frequência de 1,22. As OEA foram consideradas presentes nas situações em que a relação sinal/ruído foi maior do que 6 dB, com reprodutibilidade maior ou igual a 70%.[19]

O PEATE foi estudado quanto à morfologia, latência (absoluta e interpicos), replicabilidade, amplitude e intervalo entre ondas das ondas I, III e V, sendo o intervalo interpicos I-V considerado normal até ou igual a 4,5 ms. As latências consideradas, das onda I a V respectivamente, normais foram, aproximadamente, 1.5, 2.5, 3.6, 4.9 e 5.6, estimulada a 100 dB. Para a amplitude foi considerado que quanto maior a intensidade do estímulo maio a amplitude, considerando que habitualmente a amplitude da onda V ser maior que a onda I. A diferença interaural de latências entre os intervalos intepicos foi menor que 0.3 ms. Limiar auditivo foi considerado quando há presença da onda V na menor intensidade. A avaliação eletrofisiológica foi feita nos 12 ms após os estímulos sonoros.[19]

Os testes dos PEATE e do MC foram realizados com fone de inserção. Para os PEATE, o estímulo utilizado foi de 100 dBNA, com frequências abrangidas entre 250 e 8.000 Hz, com duração de 100 microssegundos, e polaridades condensadas e rarefeitas, sendo realizados 2000 cliques para cada série, e tendo a pesquisa sido repetida duas vezes em cada intensidade. O teste foi considerado anormal nos casos em que houve ausência de formação de ondas ou se verificou alteração grave da morfologia das mesmas com até 100 dBNA de estímulo.[19]

As principais alterações de morfologia de ondas no PEATE foram relacionadas com deformidade ou mesmo com não formação da onda no período esperado, assim como duração menor ou maior, prolongamentos das ondas, exist6encias de mais de um pico ou a não existência de picos, a não reprodutibilidade das ondas. Isso foi confirmado com estímulos até 100 dB. Os valores normais de latência e amplitude foram adequados em acordo com a idade para a interpretação do PEATE.[19]

O MC foi avaliado nos exames dos PEATE, com o recurso à inversão da polaridade (condensada e rarefeita). Nos casos em que se detetou MC positivo com estímulos de 100 dbNA, foi pesquisado o seu limiar eletrofisiológico, de forma decrescente.[19]

A classificação da disfunção auditiva através da audiometria foi feita através da estratificação em disacusia leve, moderada, grave/severa ou profunda.[20]

De acordo com a interpretação profissional dos terapeutas da fala, o desenvolvimento de fala dos doentes avaliados foi classificado, de forma subjetiva, em três categorias (má, razoável ou boa).

Avaliação Genética

O DNA genómico foi extraído a partir de sangue venoso periférico, de acordo com os protocolos padrão. As mutações foram pesquisadas por sequenciamento direto dos genes avaliados (GJB2).[4,6,9,10]

Análise Estatística

Os dados foram analisados através de uma análise estatística simples, com cálculo das médias, medianas e desvios-padrão.

Para comparar os grupos da amostra, foi utilizado o teste do Qui-quadrado. Devido à reduzida dimensão da amostra em algumas das variáveis analisadas, o teste Exato de Fisher foi também utilizado para verificar a correlação entre os grupos.

O intervalo de confiança foi de 95%, e valor de $p < 0,05$ foi considerado significativo.

Aspetos Éticos

Este estudo foi aprovado pelo Comité de Ética em Pesquisa da instituição (parecer número 396/2006).

Tabela 1 - Distribuição dos doentes segundo o início dos sintomas auditivos e género

		n
Gênero	Masculino	23 (67%)
	Feminino	11 (33%)
Idade de aparecimento dos sintomas	Congênita	27 (80%)
	Infância	3 (9%)
	Adolescência	4 (11%)
	Adulta	0 (0%)
Total		34

Tabela 2 - Achados da avaliação eletrofisiológica

Avaliação Eletrofisiológica	Presente	Ausente
OEA Transientes	9 (26,5%)	25 (73,5%)
OEA Produtos de Distorção	11 (33%)	23 (67%)
Microfonismo Coclear	27 (79%)	7 (21%)
PEATE	0	34 (100%)

Tabela 3 - Grau de desenvolvimento da fala

Qualidade da Fala	n
Má	21 (61,5%)
Razoável	7 (21%)
Boa	6 (17,5%)

RESULTADOS

Após a criteriosa revisão dos processos clínicos, foram selecionados 49 doentes, porém foram incluídos no estudo apenas 34 doentes. Apesar de termos restringido o tamanho da amostra, a qualidade dos dados desses doentes tornam os resultados mais fidedignos.

A Tabela 1 mostra os principais dados clínicos dos doentes da amostra: 67% do sexo masculino e 80% com doença congénita.

Relativamente aos achados da pesquisa bilateral das otoemissões acústicas (Tabela 2), os PEATE foram considerados anormais em todos os doentes que apresentaram ausência de PEATE ou alterações significativas na morfologia das ondas. Em todos os casos com ausência de OEA (transientes ou por produtos de distorção) foi identificado microfonismo coclear, fundamental para a suspeita diagnóstica.

Através da análise da equipe de fonoaudiologia / terapia da fala, a qualidade da fala dos doentes foi classificada em má, razoável ou boa (Tabela 3). Cerca de 62% dos doentes foram classificados como tendo má qualidade da fala.

Em 12 doentes (35,3%) foram descritos os antecedentes gestacionais, perinatais e ambientais que estavam presentes, conforme demostrado na Tabela 4.

Os achados da avaliação genética dos doentes são apresentados na Tabela 5. Nenhum dos achados tem relação descrita com a NA. O único caso de homozigotia encontrado foi a mutação 35delG no gene GJB2, cuja relação com a NA não está ainda estabelecida na literatura. Nenhum dos doentes com alterações genéticas detetadas tinha antecedentes familiares de surdez relatados.

A avaliação audiométrica dos doentes, bem como a relação entre os achados audiométricos e a situação eletrofisiológica em que os doentes foram diagnosticados com NA, está descrita na Tabela 6.

A maioria dos doentes, 53% dos casos, apresentou disacusia severa/profunda (11 casos severa; sete casos profunda). Em 38% (n = 13) dos casos objetivou-se disacusia moderada e em 9% (n = 3) disacusia leve.

Não foi estabelecida uma correlação entre o tipo de disacusia e os achados eletrofisiológicos ($p > 0,05$).

Tabela 4 - Antecedentes gestacionais, perinatais ou ambientais

Antecedentes	n
UTI neonatal	10 (29,4%)
Prematuridade	6 (17,5%)
Antecedentes Familiar de Surdez	4 (11%)
Icterícia	2 (6%)
Kernicterus	1 (3%)
Meningite Neonatal	1 (3%)
Toxoplasmose gestacional	1 (3%)
Pneumonia neonatal	1 (3%)
Síndrome Genética (CHARGE)	1 (3%)
Atrofia Muscular Axial Ascendente	1 (3%)
Comorbilidades Sistémicas	0 (0%)
Ausentes	22 (64,7%)

DISCUSSÃO

A neuropatia auditiva é uma condição que tem vindo a ser estudada na última década, por diversos prismas, sendo que atualmente os exames eletrofisiológicos são ferramentas essenciais na avaliação diagnóstica desta condição.

Na nossa casuística, 35,3% dos doentes apresentaram antecedentes gestacionais, perinatais ou ambientais. Muitos estudos recentes descrevem os possíveis mecanismos causadores da fisiopatologia da NA, principalmente os fatores genéticos. Em cinco casos (14%) foram detetadas alterações genéticas em genes potencialmente envolvidos na génese da NA, porém, em nenhum dos casos do nosso estudo houve evidência clínica ou laboratorial de uma relação de causa e efeito.[19,21]

Estudos tanto clássicos como recentes defendem que a etiologia da NA está relacionada, em 42% das vezes com patologias neurológicas hereditárias, em 10% dos casos com situações diversas (tóxicos, metabólicas, infeciosas,

Tabela 5 - Achados genéticos dos doentes estudados

Local	Alteração	Frequência (n)	Genótipo	Significado Clínico
Exão 2	p.D43D (c.129C > T)	1	Heterozigose	Provavelmente não patogénica
Intrão 2	IVS2 + 28T > G	1	Heterozigose	Desconhecido
Intrão 4	IVS5 - 59T > C	1	Heterozigose	Desconhecido
Intrão 5	IVS5 + 10A > G	1	Heterozigose	Provavelmente não patogénica
GJB2	35delG / 35delG	1	Homogigose	Patogénico (relação com NA?)
GJB2	35delG / N	1	Heterozigose	Provavelmente não patogénica

Tabela 6 - Distribuição dos doentes de acordo com os achados audiométricos[20]

Disacusia	OEA + BERA -	OEA - BERA - MC +	OEA + BERA - MC+	MC +	Total (n)
Leve	3	0	2	2	3 (8,8%)
Moderada	6	7	0	7	13 (38,2%)
Severa	1	10	1	11	11 (32,4%)
Profunda	1	6	1	7	7 (20,6%)
Total (n)	11 (32,4%)	23 (67,6%)	4 (11,8%)	27 (79,4%)	34 (100%)

…) e que, nos restantes 48% dos casos, a NA é idiopática.[19,21-23]

Na nossa amostra, quase 40% dos doentes tinha história gestacional/perinatal ou antecedentes ambientais relevantes, tendo sido os mais prevalentes a prematuridade e a história de internamento UCI neonatal.

De entre os casos de NA Idiopática, é consensual na literatura que a maioria possa estar relacionada com distúrbios genéticos. Alguns autores consideram inclusivamente que alterações genéticas – sejam elas de origem sindrómica, não-sindrómica ou relacionadas com a herança mitocondrial – serão o principal fator causador da globalidade de patologias do espectro da NA.[22]

Os achados audiométricos de doentes com NA podem variar largamente, havendo desde doentes com limiares considerados normais até situações de disacusia auditiva severa. Assim, não está descrito na literatura um padrão de audiometria para estes doentes, mas antes uma grande variabilidade inter-sujeito.[19,21,22]

O presente estudo demostrou que a maioria dos doentes (53% dos casos) apresentava disacusia severa ou profunda. Verificou-se a presença de disacusia moderada em 38% dos casos, e de disacusia leve em 9%. Tal como está descrito na literatura, a amostra estudada apresentou uma grande variabilidade nesses achados, tendo na vasta maioria dos doentes sido objetivado uma perda auditiva significativa.

O diagnóstico de doença congénita foi muito frequente neste estudo, tendo ocorrido em 80% dos casos. Desses doentes, a maioria foi avaliada no nosso serviço após uma triagem auditiva neonatal com resultados irregulares. Embora a descrição de NA seja feita maioritariamente na população pediátrica, alguns autores defendem que a maioria dos doentes com NA seja diagnosticado em idade adulta.[21]

As OEAs são usadas para avaliar a função das células ciliadas externas e representam fenômenos préneurais relacionados com processos mecânicos da cóclea. A presença de OEAs depende de um sistema auditivo intacto e verificam o funcionamento de parte do Órgão de Corti, representado nessa situação específica pelas células ciliadas internas, e também do sistema eferente auditivo (células ciliadas externas).[23]

Com a progressão da condição patológica, a NA, a totalidade ou quase totalidade das células ciliadas externas são afetadas levando ao desaparecimento do MC e das OEAs. Tal situação pode ocorrer em até 30% dos doentes com NA, pelo que a ausência de OEA não é um fator de exclusão para o diagnóstico de NA.[8,24]

O MC é outra forma de verificar o funcionamento e integridade cóclea. Trata-se de um potencial de corrente alternada que aparece durante a estimulação sonora e reflete o movimento da membrana basilar. Esta é uma atividade pré-neural, que acontece antes das sinapses das células ciliadas com o nervo auditivo, ou seja, antes da onda I do PEATE.[28]

De certa forma, o MC avalia também a função das células ciliadas externas e da membrana basilar, o que torna essa avaliação fundamental no diagnóstico da NA e dos seus diagnósticos diferenciais. Como já foi referido, a pesquisa do MC é fundamental na avaliação desses doentes, principalmente na ausência de OEA.[8,24]

Em alguns programas de triagem auditiva neonatal, como o do *Newborn Hearing Screening Programme*, a presença do MC num exame de PEATE incita a investigação ativa de patologias cocleares, como a NA.[24]

Sabe-se que as células ciliadas externas são amplificadoras primárias da cóclea, e que podem auxiliar na modulação da sensibilidade das células ciliadas internas. A disfunção das CCE resulta numa disacusia moderada (~50 dB). Nesses casos, os doentes necessitam de ambientes de escuta favoráveis (silêncio, sem ruídos competidores) para terem bom desempenho auditivo, além de algum grau de amplificação sonora.[25]

Nas patologias das CCI, geralmente a audiometria cursa com disacusias severas a profundas. A inteligibilidade da fala (discriminação vocal) assim como o desenvolvimento da fala do indivíduo dependerão do grau de perda auditiva, do tempo de aparecimento da patologia (pré- ou pós-lingual) e do tempo de surdez/privação auditiva. A capacidade de discriminar sons e fala no ruído estão frequentemente afetadas, bem como o processamento auditivo.[25]

O MC estava presente em 27 (79%) dos casos da amostra, sendo que em 23 casos (67%) a situação encontrada foi OEA-PEATE-MC+, o que demonstra algum grau de integridade coclear, bem como CCEs funcionantes. Importa ressaltar que, também em 23 casos (67%), as OEA estavam ausentes, o que pode dificultar o diagnóstico de NA para uma equipe médica não especializada. Assim, e indo de encontro ao que está descrito na literatura, nem sempre a situação clássica de OEA+PEATE- é aquela que o doente com suspeita de NA apresenta.

A neuropatia auditiva classicamente cursa com prejuízo

na discriminação vocal.[21-23] Em indivíduos com défice auditivo congénito, há um evidente comprometimento do desenvolvimento da fala. Na amostra em estudo, 61,6% dos doentes com NA apresentava má qualidade da fala e apenas 17,5% dos doentes apresentavam um desenvolvimento satisfatório desta função, o que poderá estar relacionado com o facto de muitos desses doentes terem sua disfunção auditiva com origem congénita.

Acreditamos no amplo e continuo estudos relacionados com a NA para um melhor entendimento dessa situação clínica. Este manuscrito é parte de um projeto que envolve a NA em sua vertente clínica, genética e relacionada com o implante coclear e outras formas de reabilitação e os estudos relacionados serão estendidos.

Todos os 49 doentes selecionadas estão a ser mapeados geneticamente à procura de novas mutações, bem como de mutações já descritas como relacionadas com a surdez e com a neuropatia auditiva (conexina 26 e 30, mutações no gene OTOF e PJVAC e também um mapeamento completo do DNA desses doentes). Esperamos produzir mais resultados e informações científicas futuras com esses dados. Como a avaliação genética é mais demorada e tem custos mais elevados, principalmente quando orientados na nossa realidade, essa informação ainda não se encontra integralmente disponível para publicação.

A neuropatia auditiva apresenta um amplo espectro de alterações que podem ocasionar desde alterações leves até severas da funcionalidade da via auditiva afectando significativamente o desenvolvimento neurológico do indivíduo, e comprometendo a comunicação oral e o estabelecimento da linguagem.

CONCLUSÃO

Trata-se de um estudo de casos, logo os resultados apresentados nesse artigo são relacionados a amostra estudada e não devem ser extrapolados à toda população de doentes com neuropatia auditiva. Além do citado, a neuropatia auditiva apresenta uma grande heterogeneidade clínica.

Na amostra estudada nesse artigo, a vasta maioria dos doentes (80%) com NA terá desenvolvido doença de origem congénita e/ou apresenta MC. Pudemos também observar, na nossa amostra, que a grande maioria dos doentes, (91% dos casos) apresenta défice auditivo significativo, sendo que cerca de metade dos casos (53%) se enquadra na classificação de surdez severa ou profunda

A neuropatia auditiva é ainda um desafio, tanto no diagnóstico como na sua abordagem, tratamento e apresenta um amplo espectro de alterações que podem resultar em disfunções leves a severas no funcionamento da via auditiva.

PROTECÇÃO DE PESSOAS E ANIMAIS

Os autores declaram que os procedimentos seguidos estavam de acordo com os regulamentos estabelecidos pelos responsáveis da Comissão de Investigação Clínica e Ética e de acordo com a Declaração de Helsínquia da Associação Médica Mundial.

CONFIDENCIALIDADE DOS DADOS

Os autores declaram ter seguido os protocolos do seu centro de trabalho acerca da publicação de dados.

CONFLITOS DE INTERESSE

Os autores declaram não terem qualquer conflito de interesse relativamente ao presente artigo.

FONTES DE FINANCIAMENTO

Os autores declaram não ter recebido subsídios ou bolsas para a elaboração do artigo.

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6. CAPÍTULO 4

PERFORMANCE OF COCHLEAR IMPLANTS IN PEDIATRIC PATIENTS WITH AUDITORY NEUROPATHY SPECTRUM DISORDER

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Original Article

Performance of Cochlear Implants in Pediatric Patients with Auditory Neuropathy Spectrum Disorder

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OBJECTIVE: To describe the performance and results of CIs (cochlear implant) in patients with AN (auditory neuropathy) and to present a medical literature review.

MATERIALS and METHODS: Retrospective chart review of patients with AN who were treated with CI. The mesh terms used for the review in the Pubmed and Scopus databases were as follows: "hearing loss, cochlear implants, rehabilitation of persons with hearing impairment, auditory neuropathy". Statistical Analyses: The Mann-Whitney test was performed.

RESULTS: The sample consisted of 10 patients. The mean age at surgery was 4.3 years, range 2-16 years. The average length of CI use was 5.2 years. The comparison of hearing levels before and after CI use showed a significant improvement in all patients, with $p<0.05$. All of them also reported an increase in overall satisfaction 1 year after the procedure. A CI is the standard treatment for the hearing rehabilitation of patients with severe profound hearing loss who do not benefit from conventional hearing aids. There are diseases such as AN that also invoke a discussion in the literature regarding CI benefits.

CONCLUSION: Individuals with an demonstrated a significant gain in hearing levels and language use with CI.

KEYWORDS: Auditory neuropathy spectrum disorder, evoked auditory brainstem response, otoacoustic emissions, GJB2, hearing loss, cochlear implants

INTRODUCTION

Auditory neuropathy (AN) is a condition caused by a deficiency of synchronous neural activity of the cochlear nerve and is related to injuries that can affect the inner hair cell synapse, spiral ganglion, axon, the myelin sheath, and nerve dendrite [1-3].

The term has been used to describe a singular situation that affects both adults and children with diagnostic criteria that characterizes the normal functioning of outer hair cells, by examination of these otoacoustic emissions and the abnormal or absent operation of the cochlear nerve, through the presence of cochlear microphonic (CM) or by the absence or dyssynchrony of the waves generated in the auditory-evoked potential/brain response (ABR). Generally, patients still have difficulty in speech discrimination, inconsistent with the pure tone audiogram findings [1, 2].

The term AN was first used in 1996 and later changed to the AN spectrum disorder during the Conference of Consensus on Auditory Neuropathy/dyssynchrony in Como, Italy, in 2008 because of the different clinical forms of this peculiar disease [2, 4-6].

The pathophysiology of this condition remains unclear, although in recent years, the identification of genes involved in the pathogenesis of AN, both presynaptic and post-synaptic, has significantly contributed to the diagnosis and better understanding of the mechanisms involved in this clinical disorder [1, 6, 7].

In the past, AN patients were treated in different ways, depending on their degree of hearing loss. Thus, treatment ranged from a simple observance to the use of hearing aids and frequency modulation (FM) system with predominantly unsatisfying results, such as poor speech or hearing rehabilitation [8, 9].

As the conventional treatment of AN has proved, in most cases, refractory to conventional amplification, the cochlear implant (CI) approach is an alternative therapy for this condition [1, 3, 5].

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Cochlear implants (CI) currently has ample evidence for its efficacy in the treatment of various forms of deafness, from unilateral to bilateral cases with severe to profound hearing loss. It also has been shown as an alternative for hearing rehabilitation in many conditions, such as in deafness with residual hearing at low frequencies and AN. In AN cases, CI is an option when the patient has not had a good response to conventional hearing aids and an adequate development of speech [1, 3, 5, 10, 11].

Although it has been demonstrated that the earlier use of CI in children with AN with severe or profound hearing loss leads to an important benefit in most cases, the situation is still challenging in cases when the hearing loss is moderate [1, 3]. Thus, CI has shown conflicting results worldwide, probably because AN must be heterogeneous and have different etiologies [5].

A recent systematic review evaluated the improvement of speech development in children with AN treated with CIs and showed favorable results after CI regarding hearing and speech parameters but concluded that the evidence for CI is still weak and further studies are needed to clarify where and when CI could be applied as a good option for pediatric patients with AN [12].

Regarding the etiologic heterogeneity of AN, it is not surprising that the auditory performance of CI treatment in AN patients is also variable. Therefore, the use of CIs for individuals with AN has been relatively controversial in the literature [13, 14].

The identification of specific mutations involved with AN has implications not only for diagnosis but also for rehabilitation because a good outcome is expected with CI use when the disturbance is due to presynaptic mutations (OTOF gene) or postsynaptic mutations in the distal portions of the auditory fibers (DIAPH3 gene). However, it is expected that CIs will have limited benefit for all forms of AN involving the auditory nerve [14, 15]. Therefore, the genetic and molecular diagnosis of AN is also important in the choice of an appropriate treatment and follow-up of those subjects.

Cochlear implants (CI) is becoming a choice of treatment for patients with AN. This kink of the hearing disorder with mild-to-moderate hearing loss remains a challenge in terms of the treatment options, even to experts in this area.

Thus, this paper aims to study and demonstrate the performance and developing speech skills of pediatric patients with AN spectrum disorder treated with CI and to evaluate this device as a treatment option.

MATERIAL and METHODS

A cross-sectional and case series study was conducted, through a retrospective analysis of the records of patients diagnosed with bilateral hearing loss, suspected of AN spectrum disorder (NA), followed in the hearing center at a tertiary university hospital and undergoing CI surgery.

The parameters studied were age, sex, onset of hearing loss (congenital, childhood, adolescence, or adult), gestational/perinatal/genetic background, and the results of electrophysiological hearing tests, namely ABR, otoacoustic emissions by distortion product (OAE), and a search for CM.

Perinatal and gestational histories were any situations described in the past of the patients, such as prematurity, jaundice, kernicterus, neonatal intensive unit hospitalization, meningitis, severe neonatal infections (such as sepsis and pneumonia), genetic syndromes, concomitant neurological diseases, infectious diseases (such as rubella and toxoplasmosis), clinical systemic comorbidities (such as hypertension and diabetes mellitus), and a family history of deafness.

The age group was defined as the beginning of the onset of symptoms, as follows: congenital (up to 1 year old), childhood (between 1 and 10 years old), adolescents (between 11 and 18 years of age), or adult (over 18 years life). Adults were excluded from this paper.

The clinical diagnosis of AN was established as follows: ABR with missing or abnormal responses and the presence of OAE and/or of CM with no anatomical alteration of the VIII cranial nerve. All patients had no acoustic reflex, and the presence of the cochlear nerve was assessed by nuclear magnetic resonance (MRI) and computed tomography (CT).

Molecular Study

Genomic DNA was extracted from patients' peripheral venous blood according to standard protocols. GJB2 mutations were screened by a direct sequencing of the gene coding region [16, 17] and the exon 1 and flanking splice donor site [18].

Genomic DNA was extracted from patients' peripheral blood, according to standard protocols. All samples were tested for mutations in the GJB2 gene as well as for the deletions del(GJB6-D13S1830) and del(GJB6-D13S1854) in the GJB6 gene, the mitochondrial mutation m.A1555A>G in the MTRNR1 gene, and the p.Q829X mutation in the OTOF gene. Mutations in the GJB2 gene were screened by direct sequencing of the coding region of the gene [19, 20].

Multiplex-PCR methodology was used to detect the del (GJB6-D13S1830) and del(GJB6-D13S1854) mutations in the GJB6 gene [21, 22]. The investigation of the mutations m.1555A>G and p.Q829X was performed using PCR, followed by digestion with BsmAI (New England BioLabs, Inc.; Ipswich, MA, USA) and BfaI (New England BioLabs, Inc.) restriction endonucleases, respectively [23, 24].

Inclusion and Exclusion Criteria

Inclusion criteria were patients with AN submitted to CIs with bilateral sensorineural hearing loss, normal otoscopy, an absence of middle ear disease, and no acoustic reflex.

Clinical spectrum of AN was considered when audiological tests were compatible with the following:

A) OAE present and ABR absent, or
B) OAE absent and ABR absent and CMs present, or
C) imaging (MRI/CT) showing the presence of VIII cranial nerve and excluding retrocochlear alterations.

All patients who did not fulfill these criteria were excluded from the study.

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Sample: Patients with a clinical diagnosis of AN accompanied in the auditory hearing health care service of a tertiary care university hospital in the last 3 years submitted to CIs.

Only patients who had undergone audiological and electrophysiological testing with our team of speech experts with the same equipment were included in the sample.

Adults were excluded from this study and only pediatric patients were studied.

Audiological Evaluation

Audiological tests were performed including impedanciometry, speech, and pure tone audiometry. The tests were performed using an audiometer AC30-SD25 (Interacoustics; Copenhagen, Denmark), calibrated according to ISO 389 standards/64.

The Otoacustic Emissions (OAE) distortion products were performed at frequencies of 700-8,000 Hz with a stimulus at 65-55 dB SPL, with a frequency ratio of 1.22. OEA was considered present when the signal/noise ratio was greater than 6 dB, and with a reproducibility ≥70%.

The tests from ABR and CM were performed with insert earphones. A stimulus of 100 dB HL was used for ABR covered with frequencies between 250 and 8.000 Hz, with a duration of 100 microseconds, and condensed and rarefied polarities. The abnormality of ABR was defined as the absence of wave formation or severe changes in morphology of the same with up to a 100 dB HL stimulus.

Cochlear Microphonism (CM) was evaluated in tests from ABR, with the feature of inverting the polarity (condensed and rarefied). When CM was positive with a stimulus of 100 dB, the HL electrophysiological threshold, in decreasing order, was researched.

For ABR, which was repeated at least twice, the AT-235 device (Interacoustics; Copenhagen, Denmark), was used.

Hearing loss impairment was classified through audiometry stratification into mild, moderate, severe/severe, or profound hearing loss [25].

Speech perception tests: During preoperative evaluation, all subjects underwent a speech perception test on the same day of their surgery. The speech perception test was based on several studies in the English language, adapted and developed for Portuguese language by Bevilacqua et al. [26]. Patients performed the tests with hearing aids, in a quiet and peaceful place (best aided condition).

Postoperatively, all subjects repeated the speech perception test with at least 1 year experience with the CI. The tests were performed using CI. The same audiologist performed all the tests (pre- and postoperative).

Three protocols to evaluate the patients' performance language and hearing were used because most of them were children and pre-lingual developed. The scales used were as follows: IT MAIS (Meaningful Auditory Integration Scale for Young Children), MUSS (questionnaire for assessing oral language), and GASP (The Glendonald Auditory Screening Procedure, review of speech perception in profound deaf children from five years old) [27-29].

Such scales are widely used in this age group of patients and were adapted to the Portuguese language. IT MAIS and MUSS are protocols that are answered by the parents, but these scores are determined by the inquiring professional based on the examples that parents give for each question. It is a way of assessing patients in the first year of implant because they are still in the receptive language stage and have no spoken language, highlighting that IT MAIS has a greater focus on hearing, whereas the MUSS score has a greater focus on language acquisition [27-29]. The GASP has been used in patients aged more than 5 years.

Subjective evaluations: When patients did their postoperative speech tests, they were asked to rate the quality of their experience with CI compared to last year on a Likert scale ranging from 0 to 10, similar to the visual analog scale. A score of 0 indicates that the user regretted the intervention and would not recommend it to others and felt that they were better off before with just their hearing aids. A score of 10 indicates that the user was completely satisfied with the work and highly recommended it.

Statistical Analysis

The data were analyzed using descriptive analysis, with the mean, median, and standard deviation tabs. The software SIGMA XL was used to perform all statistical analysis (SigmaXL Inc.; Kitchener, Ontario, Canada).

The Chi-square and the Mann-Whitney test were used to compare the groups of samples as they are nonparametric tests applied for two independent samples.

The confidence interval was of 95%, and a p value of <0.05 was considered statistically significant.

Ethical Considerations

This study was previously approved by the Research Ethics Committee of the Faculty of Medical Sciences of the University of Campinas (Report number 396/2006).

RESULTS

A total of 19 patients were selected, but only 10 patients completed the full inclusion criteria presented above. Table 1 summarizes the main clinical demographics findings of these patients.

This table (Table 1) highlights that all the selected cases were pediatric, pre-lingual, and not oralized. In 50% of the cases, it was found that there were a presence of otoacoustic emissions with absence/abnormalities at ABR, and in the rest of the patients, AN was suspected and supported by the presence of CM.

Figure 1 shows the performance of patients according to the language, speech, and hearing evaluation through the application of the scales IT MAIS, MUSS, and GASP. In patients over the age of five (5) years, the GASP was used to assess the language, and in the younger ones, the MUSS was selected to evaluate this topic. IT MAIS had a focus on the largest review of the hearing improvement.

In The Glendonald Auditory Screening Procedure (GASP) analysis ($p=0.002$), there was a statistically significant improvement with the

Table 1. Demographic clinical data of the patients

Age at surgery time	4 years and 4 months variation: 2 years and 6 months to 6 years and 1 month
	Average: 4.31 years
	Median: 4.15 years
	Standard deviation: 1.4726 years
CI - duration of use	5 years and 3 months variation: 1 year and 1 month to 13 years and 6 months
	Average: 5.24 years
	Median: 2.92 years
	Standard deviation: 4.5319 years
Gender	7 M: 3 F
OAE	50% presents
CM	50% presents
ABR	100% absent or abnormal
Genetics findings	30% - mutations - homozygous at GJB2 gene (c.35delG/c.35delG) 70% - no mutations (normal)
Onset of symptoms	100% at congenital age
Anatomical changes (CT and MRI)	Normal - No abnormalities at 100% of the cases
Etiologic factors	Prematurity - 60% (mean 33 weeks)
	NICU - 60% (average 25 days)
	Family background - 10% brother with HNS
	Neonatal jaundice - 10%
	Cardiopulmonary stop- 10% (neonatal)
	Meningitis - 0 cases
Postoperative satisfaction (VAS - visual analog scale)	Average: 7.7 points
	Median: 8 points
	Standard deviation: 1.1595 points
Total	10 patients

CI: cochlear implant; OEA: otoacoustic emission (distortion product); ABR: auditory brain stem response; CT: computed tomography; RNM: nuclear magnetic resonance

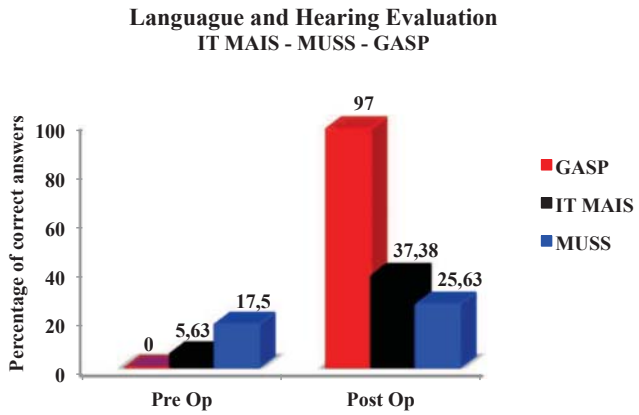

Figure 1. Performance of patients on the scales of IT MAIS, MUSS, and GASP

use of CI. There was better performance trend with the CI on the assessment by IT MAIS (p=0.054). Regarding the MUSS, no relevant statistical significance (p=0.35) was obtained in this topic evaluation.

Table 2 demonstrates the audiometric data of patients preoperatively, with the use of hearing aids (individual sound amplification devices), and after surgery with the use of CIs. Statistical evaluation compared the preoperative time (with the use of hearing aids) with the postoperative time (with the use of CI). There was a statistically significance at all frequencies analyzed, with $p<0.05$.

The patients were divided into two groups, regarding the age when they were submitted to CI surgery. Group 1 was implanted before 4-years old and Group 2 patients were implanted at more than 4-years old. Each group comprised five patients and we did not find any difference between the groups ($p>0.05$) in all the analyses, except that the Group 1 patients had more time using the CIs, which is normal because they were submitted to the surgery earlier than the other patients.

We also tried to perform two groups regarding the residual hearing. One group involved patients that had residual hearing (low/moderate hearing loss), while the other group had no residual hearing (severe/profound hearing loss), and we did not find any difference between the groups ($p>0.05$) in all the analyses.

DISCUSSION

The Auditory Neuropathy (AN) is a form of sensorineural hearing loss recently described and it is estimated that its prevalence is about 10-15% of newborns with sensorineural hearing loss [30].

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Table 2. Audiometric findings of the patients

Subject	Time	250	500	1	2	3	4	6	8	SRT	SRI
1	Pre-op	70	90	120	120	120	120	120	120	100 dB	68%
	Pre-op with HA	50	70	90	120	120	120	120	120	90 dB	NA
	Post-op with CI	20	30	25	25	25	25	20	NA	65 dB	92%
2	Pre-op	120	50	65	65	120	70	120	120	NA	NA
	Pre-op with HA	120	40	45	50	120	55	120	120	80 dB	92%
	Post-op with CI	25	25	25	20	25	25	20	25	55 dB	94%
3	Pre-op	45	50	85	100	120	120	120	120	NA	NA
	Pre-op with HA	30	35	45	45	70	90	120	120	NA	NA
	Post-op with CI	25	25	20	20	30	30	25	60	70 dB	68%
4	Pre-op	120	80	85	90	75	75	120	120	NA	NA
	Pre-op with HA	120	55	55	60	65	65	120	120	NA	NA
	Post-op with CI	25	30	35	35	30	35	45	75	70 dB	74%
5	Pre-op	120	70	70	80	70	60	120	120	NA	NA
	Pre-op with HA	120	60	50	55	55	55	120	120	90 dB	NA
	Post-op with CI	20	25	20	20	20	25	15	35	60 dB	80%
6	Pre-op	120	110	110	120	120	120	120	120	NA	NA
	Pre-op with HA	120	60	55	80	120	85	120	120	NA	NA
	Post-op with CI	45	35	35	30	30	30	25	35	85 dB	68%
7	Pre-op	120	120	120	120	120	120	120	120	NA	NA
	Pre-op with HA	120	120	120	120	120	120	120	120	NA	NA
	Post-op with CI	20	25	40	30	35	40	60	65	NA	42%
8	Pre-op	75	95	120	120	120	120	120	120	NA	NA
	Pre-op with HA	120	60	55	85	95	120	120	120	80 dB	NA
	Post-op with CI	35	35	30	40	35	50	55	NA	60 dB	84%
9	Pre-op	55	100	120	120	120	120	NA	NA	NA	NA
	Pre-op with HA	20	40	50	60	70	75	NA	NA	NA	NA
	Post-op with CI	25	30	30	30	30	35	40	45	NA	NA
10	Pre-op	75	95	115	110	120	120	120	120	95 dB	70%
	Pre-op with HA	75	95	115	110	120	120	120	120	85 dB	70%
	Post-op with CI	55	50	45	50	50	40	50	85	70 dB	82%

HZ: frequencies in Hertz; SRT: speech recognition threshold; SRI: speech recognition index; NA: not applicable/unrealized; HA: hearing aids; CI: cochlear implant

The degree of hearing loss found in patients with AN varies from moderate to severe and the treatment of these patients is a special challenge for physicians and speech therapists, since the audiometric thresholds tend to fluctuate, as well as the measurements of speech performance. Those described situations and parameters above in many times do not correspond to the degree of hearing loss [30].

Classic and accepted CI criteria do not include AN as one of their treatment options, especially in cases where there is AN with pure tone thresholds consistent with mild to moderate hearing loss, which could be considered as a nonsense indication.

Recently, several groups of specialists in hearing surgical rehabilitation began to indicate CI to their patients with NA. Those patients were submitted to CI when they did not show improvements with the standard "medical treatment" (speech therapy and/or hearing aids). This resulted in a change of concepts and paradigms and generated further discussion among professionals involved and about the interfaces with the AN patients.

The discussions and arguments are very complex, since most patients are pediatric and naturally not oralized. This is a great difficulty because it does not allow simple and objective assessments of the indication of CIs used in oralized deaf patients (post-lingual), such as the speech perception test.

This test (speech perception) has a fundamental importance for indicating and monitoring patients that are to be submitted to CIs, but it has extremely limited use in not oralized patients.

So, for not oralized patients, subjective rating scales are used that focus on language and hearing gains. Such scales are for the most part subjective and often the application depends on the support and active participation of parents/carriers, since these patients are children and are not oralized.

Several reports indicate that child users of CI show great variability in relation to its results. A possible cause for this variation is the impact of cognitive impairment or the present development within one-third of the patients with AN and this can affect the performance results with CI [31].

In Budenz et al. study [31], children with the diagnosis of AN without these confounding factors and who had received CI presented language development comparable to that of other children with sensorineural hearing loss also treated with CI.

Another recent paper highlighted 17 children who had just AN, while another nine had some cognitive or associated development deficits. The two groups were compared with patients treated with CI due to sensorineural hearing loss of cochlear origin, and it was shown that children with AN without other factors and the cochlear loss group showed similar results regarding hearing with CI surgery, while nine patients with associated disease showed some benefit with CI, but this was dependent on non-aural ways of communication (signal/gesture language) [31, 32].

In an Asian analysis, two patients with AN, with post-lingual/oralized hearing loss (one moderate and one severe), underwent unilateral CIs and were followed for 6 months. After 6 months of CI use, the patients showed an improvement in auditory thresholds with the use of the CI, with an average of 35 and 44 dB, respectively, and also had improved speech recognition [33].

The Glendonald Auditory Screening Procedure (GASP) rating scale shows that if the treatment is beneficial, the patient approaches an ideal result (close to 100%) in the first year of CI use. A delay of around 24 months was necessary to achieve this goal (hit rate close to 100% in GASP) in the patients studied in this study, and showed a not so great improvement in the first year of CI use.

The performance in IT MAIS was better in patients compared to the performance by MUSS; however, it needs to be pointed out that IT MAIS has a greater focus on evaluation of the hearing, while the MUSS has a greater focus on oral language.

We believe that, despite these results in these rating scales, this should not be viewed with pessimism. There are publications of patients with NA achieving good results but more slowly with good recognition and categorization of words with increased CI usage time [8, 31, 32].

An important longitudinal prospective study that followed up to 140 children with AN, in which 52 (37%) of them received CIs, highlighted that AN is a very heterogeneous clinical situation with an ample diversification of impairments. However, cochlear implantation could be a very good option for helping those children to achieve great hearing rehabilitation and speech development. Poor prognosis is related to central nervous diseases and multiple deficiencies. Here electrically-evoked intracochlear compound action potential testing may be helpful to select patients with AN as potential good candidates to receive a CI [34].

It should be noted that the measure of pure tone thresholds, the recognition rate of speech, and the speech perception test are objective measures of great importance in indicating and monitoring patients with CI, particularly with oralized patients (post-lingual). But these tests have limitations, especially in not oralized patients.

This study showed improvement in all parameters of pure tone audiometry and the speech preoperative recognition index (with the use of hearing aids) and postoperative (with CIs), with a statistical significance ($p<0.05$).

Such data in pre-lingual and not oralized patients, usually found in children and also in cases of AN, as well as all other cases in this article, are secondary and only corroborate and support the benefits of language development of this therapeutic modality. Such benefits are valued mostly for subjective evaluations, often with the support and use of information from parents and/or caregivers and through scales that measure hearing and language improvements as used (GASP, IT MAIS, and MUSS).

Speech therapy before and after CI surgery is very important for the acquisition and development of speech. Our patients underwent this treatment in their hometown cities and there was no standardization of how this therapy occurred. With a standardized and regular speech therapy, it is possible that our results would be even more satisfying.

Cochlear Implantation is becoming a choice of treatment for patients with AN. This kind of hearing disorder with mild to moderate hearing loss is still a challenge regarding the treatments options, even to experts in this area.

This paper adds data to support CI as a treatment for AN patients and is the first Brazilian-Latin American review on this topic, and also has a greater importance for a better reflection and emplacement of clinical and management decisions. It is still necessary to study more patients and a larger study group to gain more significance data to support the conclusions of this study.

Auditory Neuropathy (AN) is a heterogeneous situation and is very difficult to study and analyze patients and outcomes. Besides, this is also a very rare condition. We did not find any difference between the groups ($p>0.05$) in all the analyses related to the age of implantation, except that the Group 1 patients (CI before 4-years old) had more time of use with their CIs, which is normal because they were submitted to the surgery earlier than the others patients. Regarding residual hearing (low/moderate hearing loss vs. severe/profound hearing loss), we also did not find any difference between the groups ($p>0.05$) in all the analyses.

The statistical significance demonstrated through the GASP analysis and audiometric findings, and the correlation trend with the IT MAIS, are the strong indicators of the benefits of using a CI in patients with AN.

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The conclusion of this paper is that CI is an effective treatment for auditory rehabilitation and language development in children with AN.

Ethics Committee Approval: Ethics committee approval was received for this study from the ethics committee of School of Medical Sciences of Campinas University (UNICAMP, São Paulo, Brazil) /Report number 396/2006.

Informed Consent: Written informed consent was obtained from patients who participated in this study.

Peer-review: Externally peer-reviewed.

Author Contributions: Concept - G.M.D.C.; Design - G.M.D.C.; Supervision - A.M.C., E.L.S.; Resources - G.M.D.C., P.Z.R.; Materials - G.M.D.C.; Data Collection and/or Processing - G.M.D.C., P.Z.R.; Analysis and/or Interpretation - G.M.D.C., A.C.G.; Literature Search - G.M.D.C., P.R., A.C.G.; Writing Manuscript - G.M.D.C., P.R., A.C.G.; Critical Review - G.M.D.C., A.C.G., E.L.S.; Other - G.M.D.C.

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7. CAPÍTULO 5

SATISFACTION OF CHILDREN WITH AUDITORY NEUROPATHY AND COCHLEAR IMPLANT

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Original Article

Satisfaction of Children with Auditory Neuropathy and Cochlear Implant

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OBJECTIVE: In auditory neuropathy (AN) a dyssynchrony in the nerve conduction of the auditory nerve fibers is observed. Typically, patients with AN exhibit moderate to profound sensorineural hearing loss, and treatment using cochlear implants (CIs) or hearing aids should be performed as early as possible for a better hearing rehabilitation. The aim of this study is to evaluate the satisfaction level of patients with AN spectrum disorder treated using CIs. The Satisfaction with Amplification in Daily Life questionnaire was selected to evaluate 10 patients with AN treated using CIs.

MATERIAL and METHODS: Clinical study of patients with AN spectrum disorder submitted to CI. A retrospective data analysis, genetic and clinical evaluation in a tertiary referral center was done.

RESULTS: The means of the subscales for positive effects, services and costs, negative factors, and personal image were 6.15, 4.6, 3.26, and 3.33, respectively.

CONCLUSIONS: Patients with AN treated using CIs consider themselves satisfied.

KEYWORDS: Auditory neuropathy spectrum disorder, evoked auditory brainstem response, otoacoustic emissions, GJB2, hearing loss

INTRODUCTION

Hearing Loss (HL) is one of the most common disorders that affect newborns in developed societies, with an incidence of 1:1000 births [1]. Normal speech development is a desirable goal for the family of these patients [2].

Currently, cochlear implants (CIs) are widely accepted for the treatment of severe to profound sensorineural hearing loss (SNHL), and since its approval in 1990 by the Food and Drug Administration for the treatment of deafness in children above 2 years of age, the number of implants in children has been growing tremendously [3]. The indications of cochlear implantation in patients are divided into pre- and postlingual deafness.

For children under 6 years, the indications are as follows: bilateral severe to profound SNHL, no benefits from conventional hearing aids (HA), and adequate family motivation [4, 5].

For children between 7–12 years of age, the following indications in addition to the above criteria are considered: sentence recognition result ≤50% in the open format using HA, with worse hearing and sentence recognition result <60% with better hearing and the presence of linguistic code set [4, 5].

Auditory neuropathy (AN) is one of the many causes of deafness. It is a hearing disorder caused by a change in the function of the inner hair cells and/or the involvement of the auditory nerve fibers with dyssynchrony in this nerve conduction [4, 5].

Auditory neuropathy is considered as a spectrum of disorders affecting the auditory pathway. The knowledge about their actual pathophysiological origins is limited [6, 7].

A study by Manchaiah et al. [8], which looked into the causes of auditory neuropathy spectrum disorders (ANSD), showed that in 42% of the patients the disorder was associated with hereditary neurological disorders, in 10% of the patients it was associated with toxic, metabolic, immunological, and infectious causes, and in 48% of the patients the cause was unknown [8].

Presented in: This study was presented at the 13th International Conference on Cochlear Implants and Other Implantable Auditory Technologies, 18-21 June 2014, Munich, Germany.

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Patients with AN may present several degrees of HL and changes in speech discrimination. Otoacoustic emissions (OAEs) are usually normal with the absence of Brainstem Evoked Response Auditory/Auditory BrainStem Response (BERA/ABR), which is the most frequent clinical findings/phenotypes. The cochlear microphonic may be present and acoustic reflexes absent [9]. Typically, patients with AN exhibit moderate to profound SNHL, present worse speech discrimination, and have severe difficulties in speech perception in a noisy environment.

The treatment of patients with AN using CIs or HAs should be performed as early as possible to achieve better hearing rehabilitation [10].

Speech perception testing has become the gold standard for objectively measuring CI outcomes in adults. There are two basic ways to measure speech perception in individuals who have already learned to speak: (A) word recognition tests and (B) sentence recognition tests [11].

For children who are too young to speak, observations of the child's hearing-related behavior are made and quantified. For example, a commonly used test, the Infant-Toddler Meaningful Auditory Integration Scale (IT-MAIS), uses a structured parent interview to assess auditory behaviors, such as the child's behavior while using an HA or CI, the child's response to his or her name, and the child's awareness of environmental sounds [12, 13].

The hearing rehabilitation of patients with AN should be held as earlier as possible so that they can achieve the best possible outcomes that can be achieved using speech therapy, CIs, or HAs [6].

In most cases of AN with severe to profound HL, benefits has been demonstrated when treated with early cochlear implantation. However, in AN with mild to moderate HL, these benefits are uncertain, and the management of these cases remains a challenge [7, 14]. The outcomes of CI are conflicting, and the most plausible explanation for this is the heterogeneity of the AN etiology [15].

The improvement of speech development in children with AN using CIs was evaluated by a recent systematic review that showed favorable results after the cochlear implantation; however, it concluded that the evidence in the favor of CIs is weak and more studies are needed to clarify the best situations to perform cochlear implantation in patients with AN [16].

It is not surprising that the auditory performance of cochlear implantation in patients with AN is variable, considering the AN etiological heterogeneity. Therefore, the use of CIs for patients with AN has been reported to be relatively controversial [17].

The aim of this retrospective study was to evaluate the satisfaction levels of patients with AN treated using CIs.

We believe that these findings are of great importance to all professionals who work with patients with AN, such as pediatricians, otolaryngologists, audiologists, geneticists, and researchers.

The underlying causes and treatment options of AN are very discussed in the literature and remain uncertain; therefore, the study of satisfaction levels of patients with AN with CI can help to guide new studies, new discoveries and the interface of these disease with new discoveries.

MATERIAL and METHODS

We conducted a cross, retrospective, observational study through the analysis of medical records of patients with bilateral HL and clinical diagnosis of AN, accompanied in the auditory health care service of a tertiary care university hospital which undertook a CI surgery.

The analyzed variables were age, gender, HL onset (congenital, childhood, adolescent, or adult), previous pregnancies, perinatal and genetic data, and electrophysiological test results [ABR, transient and distortion product OAEs, and the search of cochlear microphonism (CMs)].

Perinatal and pregnancy history or any other situation such as prematurity, jaundice, kernicterus, admission to neonatal intensive care unit, meningitis, severe neonatal infections (such as pneumonia and sepsis), genetic syndromes, concomitant neurological diseases, infectious diseases (such as measles and toxoplasmosis), clinical systemic comorbidities (such as hypertension and diabetes), and a family history of deafness were described in the background of these patients.

Age groups, based on the age reported at the early onset of symptoms, were defined as follows: congenital (up to 1 year old), childhood (between 1 and 10 years old), adolescent (between 11 and 18 years of life), and adult (over 18 years of life).

The clinical diagnosis of AN was established as follows: absent or abnormal ABR with the preservation of OAEs and/or CMs. All patients had no acoustic reflex, and the presence of the cochlear nerve was assessed using nuclear magnetic resonance (MRI) and computed tomography (CT).

Inclusion and Exclusion Criteria

Inclusion criteria were as follows: patients with AN who underwent to CI, with bilateral SNHL, normal otoscopy, absence of middle ear disease, and no acoustic reflex.

Clinical spectrum of AN was considered when audiological tests were compatible with either of the following:

A) OAE present and ABR absent
B) OAE absent, ABR absent, and CMs present
C) OAE absent, ABR absent, hearing thresholds present, and a clinical suspicion of AN.

Imaging (MRI/CT) showing the presence of cranial nerve VIII and excluding retrocochlear alterations.

Patients who did not fulfill these criteria were excluded from the study.

Sample: Patients with a clinical diagnosis of AN who presented to the auditory health care service of a tertiary care university hospital in the last 3 years submitted to CIs.

Only patients who underwent audiological and electrophysiological testing with with the same professionals, with the same equipment were included in the sample.

Audiological Evaluation
Audiological tests including impedanciometry, speech and pure tone audiometry were performed. The tests were performed using an audiometer AC30-SD25 (Interacoustics; Copenhagen, Denmark), calibrated according to ISO 389 standards/64.

The OAEs distortion products were performed at frequencies of 700–8000 Hz, a stimulus of 65–55 dB SPL, and a frequency ratio of 1.22. OEA was considered to be present when the signal/noise ratio was >6 dB and with a reproducibility ≥70%.

The tests from the ABR and CM were performed with insert earphones. A stimulus of 100 dB HL was used for the ABR covered with frequencies between 250 and 8,000 Hz, with a duration of 100 microseconds, and condensed and rarefied polarities. The abnormality of ABR was defined as the absence of wave formation or severe changes in morphology of the same with up to 100 dB HL stimulus.

Cochlear microphonism was evaluated in ABR tests, with the feature of inverting the polarity (condensed and rarefied). When CM was positive with stimuli of 100 dB HL electrophysiological thresholds, in decreasing order was researched.

For ABR, which was repeated at least twice, the device AT-235 (Interacoustics) was used.

Hearing loss impairment was classified through audiometry stratification in mild, moderate, severe/severe, or profound HL [18].

Speech perception tests: During preoperative evaluation, all patients underwent a speech perception test on the day of their surgery. The speech perception test is based on several studies in the English language adapted and developed for the Portuguese language by Bevilacqua et al. [19]. Patients performed the tests using HAs, in a quiet place (best aided condition).

Postoperatively, all subjects repeated the speech perception test at least one year experience with CI. The tests were performed using CI. The same audiologist performed all tests (pre-and postoperative).

Three protocols were used to evaluate the patients' speaking and hearing performance because most of them were children and pre-lingual developed. The scales used were as follows: IT-MAIS, a questionnaire assessing auditory outcomes, the Meaningful Use of Speech Scale (MUSS), a questionnaire for assessing oral language, and Glendonald Auditory Screening Procedure (GASP), which reviews speech perception in profound deaf children ≥5 years old [20-23].

Such scales are widely used in this age group of patients and are adapted to the Portuguese language. IT-MAIS and MUSS are questionnaires that are answered by the parents, but the evaluator determines the score based on the examples that the parents give for each question. It is a way of assessing patients in the first year of implant because they are still in receptive language and has no spoken language, pointing out that the IT-MAIS has a greater focus on hearing and the MUSS score on language acquisition [19-22]. The GASP has been used in patients with more than five years.

Subjective evaluations: After the postoperative speech tests, patients were asked to rate the quality of their experience with CI compared with the experience prior to the implantation on a Likert scale ranging from 0 to 10, similar to the visual analog scale. A score of 0 indicates that the user regretted the implantation and does not recommend it to others and that he/she had been better before the implantation, with their HAs. A score of 10 indicates that the user was completely satisfied with the work and highly recommends it to others.

Satisfaction with Amplification in Daily Life (SADL)
The SADL questionnaire was selected to evaluate the study sample. It was translated into Portuguese and adapted to our cultural aspects by Mondelli et al. [23]; it was validated by Danieli et al. [24]. The questionnaire results in an overall satisfaction score and a profile of subscales that address positive effects, service and value, negative characteristics, and personal image.

Satisfaction with Amplification in Daily Life questionnaire was prepared to assess overall patient satisfaction with the use of HA. By identifying the factors that contribute to satisfaction and to try to confirm these attributes to the processes involved, this test has the potential to qualify and analyze the quality of health services [13, 24, 25].

The SADL questionnaire has 15 questions, divided into four subscales, reflecting overall satisfaction. 1) Positive effects: six items related to acoustic and psychological benefit. 2) Service and value: three items related to professional competence, product price, and number of repairs. 3) Negative factors: three items related to environmental noise amplification and phone use. 4) Personal image: four items related to esthetics and the stigma of HA use [25].

Items are rated such that satisfaction is reflected by the high score. A score is generated for each of the four subscales and each subscale score is computed from the average of the answers to the questions.

To answer the 15 questions, a scale of 7 points from the same period that corresponded to a categorical scale from "not at all" to "very much" satisfied was used. For 11 questions, "very much" indicated total satisfaction and was scored 7, whereas "not at all" indicated complete dissatisfaction and was scored 1. The other four questions were inverted, where "very much" indicated complete dissatisfaction and scored 1, whereas "not at all" indicated overall satisfaction and scored 7 [13, 25, 26].

Molecular Study
Genomic DNA was extracted from patients' peripheral venous blood according to standard protocols. GJB2 gene mutations were screened by direct sequencing of the gene coding region [13, 14, 27, 28] and the exon 1 and flanking splice donor site [29, 30].

Genomic DNA was extracted from the peripheral blood of patients, according to standard protocols. All samples were tested for mutations in the GJB2 gene, including deletions del(GJB6-D13S1830) and del(GJB6-D13S1854), mitochondrial mutation m.A1555A>G in the MTRNR1 gene, and p.Q829X mutation in the OTOF gene.

Mutations in the GJB2 gene were screened by direct sequencing of the coding region of the gene [13, 14, 29, 31]. Multiplex-PCR method-

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ology was used to detect the del (GJB6-D13S1830) and del (GJB6-D13S1854) in the GJB6 gene [29, 30]. The investigation of mutations m.1555A>G and p.Q829X was performed using PCR amplification followed by digestion with BsmAI and BfaI restriction endonucleases, respectively [13, 14, 29, 31].

Statistical Analysis

The data were analyzed using descriptive analysis, with the production of means, medians, standard deviation tabs. The software SIGMA XL was used to perform all statistical analysis (SigmaXL Inc.; Kitchener, Ontario, Canada)

The Chi-square text was used to compare the groups of our sample. Because of the small size of some of the variables analyzed, Fisher's Exact test was also used to check the correlation between the groups.

The confidence Interval was of 95%, and a p value of <0.05 was considered statistically significant.

Ethical Considerations

This study was approved by the Ethics Research Committee of the Campinas University (FCM–UNICAMP, Report number 396/2006).

RESULTS

Nineteen subjects were initially selected for the study, but only 10 (seven males and 3 females) passed all the inclusion and exclusion criteria.

The average age of the patients at the CI surgery was 4 years and 4 months, ranging from 2 years and 6 months to 6 years and 1 month. The average CI use was for 5 years and 3 months. AN was identified in 50% of the cases because patients had OAE present with ABR being abnormal or absent. The others 50% of patients have CM present with ABR being abnormal or absent.

Pathogenic variants that accounted for HL were detected in three patients (30%), all of them homozygous for the c.35delG mutation, at conexin 26 (GJB2) gene.

In all cases, the onset of symptoms appeared at birth. The past history of those subjects was as follows: 60% were premature (average, 33 weeks of pregnancy), 60% ate Neonatal Intensive Care Unity (average, 25 days), 10% had neonatal jaundice, 10% had cardio respiratory failure, 10% had family history of SNHL (brother with SNHL). No cases had meningitis.

The overall satisfaction score (Likert scale, ranging 0–10) was 8 (average). The median and mode were both 8, and the standard deviation was ±1. The minimum score was 7 and the maximum score was 9, one patient at each score (Tables 1, 2).

The SADL results are shown in Figure 1.

DISCUSSION

This is an important topic given the ambiguity of the current outcome data and clinical guidelines, the invasiveness of the therapy combined with significant potential, and the fact that it is generally the proxy decision-makers who decide whether to perform a cochlear implantation on children.

Table 1. Distribution of the mean results to the SADL questionnaire subscales

Subscale	Mean	Median	Standard deviation	Percentile 20	Percentile 80
Positive effects	6.15	7	1.09	5	7
Services and costs	4.6	5	2.16	2.8	7
Negative factors	3.26	3	1.50	2	5
Personal Image	3.33	2.5	2.41	1	6

SADL: satisfaction with amplification in daily life

Table 2. Distribution of the mean value results between the first and the second application of each question in the SADL questionnaire as to the mean/average, media, minimum value, maximum value, and standard deviation

SADL question	n (s)	Mean	Median	Min	Max	SD
1	10	6.4	7	4	7	1.26
2	10	3.8	2	2	6	1.68
3	10	6.6	7	5	7	0.84
4	10	3	2.5	1	7	2.26
5	10	6	6	5	7	0.94
6	10	6.3	7	4	7	1.05
7	10	2.9	3	2	3	0.31
8	10	4.9	6	1	6	2.07
9	10	6.3	7	4	7	1.25
10	10	5.3	6	4	6	0.94
11	10	3.1	2	1	7	2.02
12	10	6.7	7	6	7	0.48
13	10	2.1	1	1	7	2.33
14	10	2.3	1.5	1	4	1.49
15	10	4.8	5	3	7	1.54

s: subjects; min: minimum; max: maximum; SD: standard deviation; SADL: satisfaction with amplification in daily life

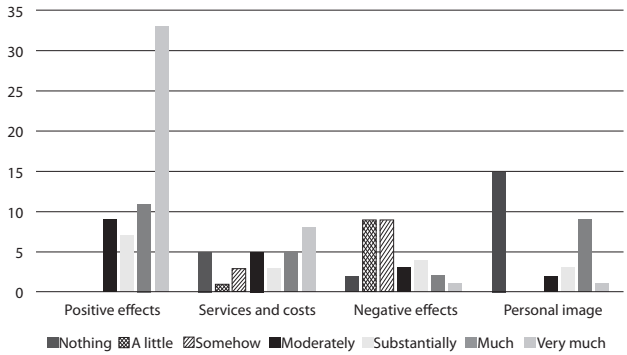

Figure 1. Illustration of the mean results to the SADL Questionnaire subscales
SADL: Satisfaction with Amplification in Daily Life

Satisfaction with Amplification in Daily Life is a questionnaire that was developed to evaluate the degree of satisfaction attributed to a hearing device for people with hearing disorders. This questionnaire evaluates several factors and is very useful to define how satisfied the patient is in daily life situations after the use of a hearing device.

In this study the SADL questionnaire was used to evaluate the satisfaction of children with AN after at least one year of cochlear implantation, the results showed that the positive effects were very high, with very low negative factors, costs or negative effects on personal image of the patients, indicating that cochlear implantation proved to be good for these patients.

Modern CI systems have become highly effective in helping patients to perceive and understand speech. Compared with other disabled people, those with severe to profound deafness have historically the lowest median education achievement level, the lowest median annual family income, the lowest rate of participation in the labor force, the lowest rate of persons in professional and technical jobs, and the poorest self-rated general health [30].

There is clear evidence that children who undergo cochlear implantation at a younger age have significantly better hearing outcomes than those undergoing at an older age. It is thought there is a window of time in the first few years of life during which implantation is critical to achieve maximum benefit. Beyond which the child's brain becomes less plastic or adaptable, and it begins to lose its ability to develop new neural pathways in response to the new auditory input that a CI provides [32].

Cochlear implantation has dramatically changed the outcomes for these patients because it provides auditory information that was available through conventional HA technology. In a study conducted by Geers et al. [33] comparing both technologies, a large percentage of young patients who have underwent cochlear implantation had auditory characteristics within the normal range [34].

Treatment CI not only improves the ability to hear but also improves the ability to acquire speech and language skills; it brings about greater success within the education system, better employment status, and improves the patient's quality of life. In addition, CIs provide benefits to the society in terms of decreased educational costs and restoration of work productivity potential [35].

A positive impact on a deaf child's quality of life is profoundly related to CI, with benefits seen more clearly in the preteen and teenage years. Quality of life (QOL) is defined as an individual's contentment or satisfaction with life [34].

Despite the first disappointing results of CI in children with AN, lately there has been an increasing number of studies showing a promising outcome of CI in such patients. Children with AN without any benefits from HAs seem to achieve significant benefits from CI. A reasonable explanation would be that CI provides adequate stimulation of the auditory pathway to overcome the existing dyssynchrony [36, 37].

It seems that in many cases acoustic stimulation, even when amplified by a conventional HA, will not be sufficient to overcome the auditory dyssynchrony. Thus, some of these children's speech perception will be very poor, resulting in disappointing language development skills. On the other hand, most of these children will achieve satisfactory speech perception and development following CI probably because the electric stimulation through the CI overcomes the existing auditory dyssynchrony [38].

Altogether, hearing rehabilitation with electronic devices such as HA, CI is beyond the HA performance, showing benefits to the patient's perceptions in everyday communication, self-esteem improvement, social life activities, and overall health [39-42].

Mutations in the connexin 26 (GJB2) gene are a common cause of genetic SNHL in many populations [29, 31]. However, there is no scientific evidence to support the real relationship between AN and mutations in the connexin 26 gene. Two studies detected mutations in this gene among patients with AN [43-45].

The auditory performance of cochlear implanted patients with mutations in the GJB2 gene is controversial in literature. Some studies report better results for GJB2-related HL, whereas others find no difference when compared to other causes [46, 47].

Genetic testing combined with clinical and audiological exams allows an accurate diagnosis, as well as the development of specific treatments and genetic counseling for patients and/or families.

Many subjective and psychosocial factors are related to the adaptation of HAs and CIs. The professionals involved need to know how to evaluate and validate them to make the adaptation easier and faster for the patients. Patients and their families must be informed that the device will not restore normal hearing, but it will support the acquisition of more acoustic information. Furthermore, hearing rehabilitation activities should be introduced when the patient starts using the device.

Although the benefits of CIs are well stablished and demonstrated, its cost-effectiveness must be considered. This device is more expensive than other forms of hearing rehabilitation devices, which can limit its use at the public health system. In some cases, patients who could benefit from this technology have to wait for a considerable time to undergo surgery if they cannot afford the procedure in a non-public hospital. We hope that in the near future, with the spread of this technology, CIs become less expensive and more accessible [48, 49].

Cochlear implants can positively impact the life of hearing impaired subjects, which has been demonstrated in multiple reports. Satisfaction surveys through questionnaires have been performed for evaluating these positive effects [50-53].

It can be considered a challenge to evaluated this topic using developing methodologies to systematically assess outcomes, particularly for moderate AN in young children. The small sample size of this study does not render the results insignificant, the bias of this study is the limited number of patients (n=10) which needs to be pointed out when analizing the results and conclusions.

Another difficult of these results is that the measurements used, particularly on the young children, it is a combination of objective measures and parental observation reports. This may a cause a bias because it is difficult to make a safe way of how integrated these information, and particularly about the parental observation can be very subjective.

This study concludes that the assessed patients with AN were satisfied with the CI intervention as analyzed using the SADL questionnaire .

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8. CAPÍTULO 6

AUDITORY NEUROPATHY SPECTRUM DISORDER: CLINICAL AND THERAPEUTIC CHALLENGES

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Editorial

Auditory Neuropathy Spectrum Disorder: Clinical and Therapeutic Challenges

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Editorial

The auditory neuropathy (AN) or the auditory neuropathy spectrum disorder is a disease that is not yet well defined and do not have an accurate diagnosis [1,2].

It is believed to be a sensory disorder of the inner ear on its interface with the brain stem and/or in the auditory cortex. Several groups studying this issue still disagree with the diagnoses parameters as well as with the treatment and it is still a challenge for physicians to diagnosis AN [1,3,4].

The diagnosis accepted by the majority, is based in the analysis of complementary tests, auditory evoked potentials that connote an activity cochlear present and absence or severe abnormalities of neural function. Classically it is observed the presence of otoacoustic emissions and the lack of response in auditory brainstem response ABR, which would be a "paradox" [1,4,5].

However there are many situations and circumstances in which the diagnosis can be very difficult, such as in cases of patients that have a deafness and AN, in which the presence of emission otoacoustic would not be identified. In these cases we have to look for the research of cochlear microphonic [1,5,6].

It is also known that the above diagnostic tools are often insufficient to diagnose and a large investigation may be needed to diagnose, such as the genetic evaluation. The genetic evaluation still is a very difficult to access, because it has shown great variability and no consensus on what mutations are related to this disorder, beyond the classical mutations already described [1].

The common diagnostic parameters are cases in which the otoacoustic emissions are present with absent or abnormal ABR which is the typical case and unquestionable AN. When the otoacoustic emissions are absent and there is an suspicion of AN the cochlear microphonic is used to support the diagnosis [3,6].

Some more atypical and challenging cases would be those where the subjects have any clinical suspicious of AN and audiological evaluation finds pure tone threshold present (sometimes close to the normal references) with the absence of ABR, the otoacoustic emissions and cochlear microphonic which is classically not considered as AN. The clinical suspicious in these cases would be through to phenotype speech, behavior and development, personal, pregnancy and perinatal antecedents [2,3,6,7].

An immense diagnostic difficulty can be noted, which turns very hard the indication of a treatment, because the uncertainty of the diagnosis, and that is one reason why the treatment becomes even more complex [3,6,7].

Basically, the treatment consists in speech and language therapy and auditory training, developing the speech and understanding skills, and it is supported by conventional hearing aids and even by the cochlear implant when necessary [6,7,8].

The classical criteria to support cochlear implant as a treatment normally does not include AN as an indication, even in cases where there is AN with tone thresholds compatible with mild to moderate hearing loss, which is somewhat questionable [2,6,8].

After many uncertainties, many groups, mostly from US and Europe, were cutting edge and started submitting their patients with AN, that did not improve with "medical therapy" (therapy and/or appliances hearing), to cochlear implants [2,6,8].

This resulted in a change of concepts and paradigms and brought further discussions between the professionals involved. The discussions are still more complex, since most patients are the pediatric age group, and of course pre-lingual (without spoken language) [1,2].

The indication of cochlear implants for AN patients is discussed because it does not allow simple and objective assessments of the indication of cochlear implant as used in deaf patients with previous knowledge of spoken language (post- lingual) as is the case of speech perception test. This test is of fundamental importance indication and follow-up of patients with cochlear implant use is very limited pre-lingual patients [1,2,6,8].

Then used subjective rating scales that focus on earnings speech and hearing impairments. Most of these scales are subjective and its application often depends on the support of parents and involvement of the family, since most of the patients with AN are children and pre-lingual [6,8].

In conclusion, the medical literature needs more studies from all over the world to help professional understand better this situation and be able to get more support for these patients.

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9. DISCUSSÃO GERAL

A neuropatia auditiva (NA) é uma afecção que ainda não está bem definida e não tem um diagnóstico preciso estabelecido (Hayes *et al.*, 2008; Roush *et al.*, 2011). Acredita-se que seja decorrente de algum distúrbio sensorial da orelha interna na sua interface com o tronco cerebral e / ou no córtex auditivo. Ainda não há consenso entre os especialistas do tema relacionado aos parâmetros diagnósticos, bem como com o tratamento, o que torna a NA uma situação desafiadora. (Rance *et al.*,1999; Shallop *et al.*, 2004; Hayes *et al.*, 2008).

O padrão diagnóstico mais consensual entre os profissionais que lidam com NA baseia-se na análise de dados clínicos, dos exames complementares, potenciais evocados auditivos. Nessa avaliação observa-se uma atividade coclear presente e uma ausência ou anomalias da função neural. Classicamente, o descrito acima, é observado quando há a presença de emissões otoacústicas e a ausência/anomalias na resposta auditiva de tronco cerebral (BERA/PEATE), o que seria um "paradoxo" auditivo (Rance *et al.*,1999; Hayes *et al.*, 2008; Sanyelbhaa *et al.*, 2009).

No entanto, existem muitas situações e circunstâncias em que o diagnóstico pode ser muito difícil e complexo, tais como em casos de pacientes que têm uma surdez concomitante com a NA, pois assim, tais pacientes, pela surdez em si, já não apresentam a presença das otoemissões acústicas. Nestes casos, é necessário atentar para a pesquisa de microfonismo coclear, para tentar identificar a presença de atividade coclear da orelha interna (Hayes *et al.*, 2008; Sanyelbhaa *et al.*, 2009; Jeon *et al.*, 2013).

Já é muito descrito e de conhecimento geral que as ferramentas de diagnóstico acima mencionadas são muitas vezes insuficientes, fazendo-se necessários outros recursos, tais como a avaliação genética. A avaliação genética ainda é de muito difícil acesso, pois ela tem mostrado grande variabilidade, custo econômico elevado, e não há consenso sobre quais mutações estão relacionadas com esta desordem, para além das mutações clássicas já descritas (Yasunaga *et al.*, 1999; Hayes *et al.*, 2008, Santarelli *et al.*, 2008; Manchaiah *et al.*, 2011).

Alguns casos mais atípicos e difíceis de NA seriam aqueles em que os pacientes têm qualquer suspeita clínica de NA e na avaliação audiológica encontra-se limiar tonal puro audiométrico presente, por vezes perto das referências normais, associado com a ausência/anormalidade do BERA/PEATE, das emissões otoacústicas e do microfonismo coclear, que classicamente não é considerada como um caso de NA, onde também acontece esse “paradoxo auditivo”.

A suspeição clínica nesses casos seria por meio de discurso/padrão vocal estereotipado (um “fenótipo/esteriótipo” vocal de NA), comportamento e desenvolvimento de fala, antecedentes pessoais, antecedentes gestacionais e perinatais (Shallop *et al.*, 2004; Manchaiah *et al.*, 2011; Roush *et al.*, 2011; Jeon *et al.*, 2013).

Devido a existência de incertezas no diagnóstico da NA, a seleção de um tratamento adequado é por vezes desafiante, demonstrando a complexidade dessa condição clínica (Shallop *et al.*, 2004; Manchaiah *et al.*, 2011; Jeon *et al.*, 2013).

Conceitualmente, o tratamento consiste em terapia de fala e de linguagem, além de treinamento auditivo. O foco desses tratamentos é o desenvolvimento e compreensão da fala, desenvolvimento de habilidades comunicativas e interpretativas. Para tal, pode ainda ser necessário o suporte com tecnologias auditivas para estimulação sonora, seja por aparelhos auditivos convencionais e até mesmo pelo implante coclear (Walton *et al.*, 2008; Manchaiah *et al.*, 2011; Jeon *et al.*, 2013).

Os critérios clássicos, que sustentam cientificamente e clinicamente o implante coclear como tratamento de distúrbios auditivos, normalmente não englobam pacientes com NA. O uso do implante coclear na NA, ainda mais em casos de pacientes que apresentam limiares tonais compatíveis com perda de audição leve e moderada, são bastante polêmicos e questionáveis (Walton *et al.*, 2008; Manchaiah *et al.*, 2011; Roush *et al.*, 2011; Jeon *et al.*, 2013).

Apesar dessas variáveis e incertezas, alguns grupos de especialistas, principalmente dos EUA (Estados Unidos da América) e da Europa, apresentaram dados clínicos e científicos positivos de pacientes com NA tratados com o implante coclear. Esses pacientes, com NA e submetidos a implante coclear, eram aqueles que falhavam com o “tratamento clínico clássico” (terapia da fala/treinamento auditivo e/ou aparelhos de audição convencionais), (Walton *et al.*, 2008; Manchaiah *et al.*, 2011; Roush *et al.*, 2011; Jeon *et al.*, 2013).

As evidências citadas acima resultaram em mudanças de conceitos e paradigmas, trazendo novas discussões e possibilidades a serem avaliadas entre os grupos profissionais que lidam com a NA. Para tornar ainda mais complexo, a maioria desses pacientes encontram-se na faixa etária pediátrica

e, são, pré-linguais e ainda não oralizados, o que torna esses dados e avaliação menos quantitativas e, portanto, mais subjetivas (sem linguagem falada desenvolvida) (Hayes *et al.*, 2008; Roush *et al.*, 2011).

A indicação de implante coclear para pacientes com NA ainda é tema de controvérsias, principalmente por não permitir avaliações simples, quantitativas e objetivas dos ganhos relacionados com o implante coclear, como é normalmente utilizado em pacientes surdos que já são oralizados (pós-lingual/ com conhecimento prévio da linguagem falada), como os testes de percepção de fala padronizados. Esses testes têm importância fundamental na indicação e no acompanhamento de pacientes com uso do implante coclear em pacientes já oralizados, o que não acontece com os pacientes pré-linguais. (Hayes *et al.*, 2008; Walton, 2008 *et al.*; Manchaiah *et al.*, 2011; Roush *et al.*, 2011; Jeon *et al.*, 2013).

Para os pacientes pré-linguais, não oralizados, que em são em sua maioria pediátricos, são aplicados testes que avaliam ganhos na aquisição de linguagem e no desenvolvimento de fala. A maioria desses testes são subjetivos e de resposta parental, e sua aplicação depende muitas vezes do apoio dos pais e envolvimento da família e desenvolvimento cognitivo do paciente (Walton *et al.*, 2008; Jeon *et al.*, 2013).

Os resultados que apresento nos diversos artigos também sofreram vieses de difícil transposição. A metodologia do estudo retrospectiva, por si só, já limita as análises e as conclusões que podem ser realizadas.

Ressalta-se também que os testes diagnósticos realizados para a identificação da neuropatia auditiva são dependentes da interpretação e

treinamento dos profissionais que os executam, tornando-os subjetivos. Portanto, podemos ter uma situação na qual não se sabe a quantidade de casos identificados corretamente.

Uma porcentagem considerável dos pacientes desse estudo eram pacientes pediátricos e não oralizados, e muitas das avaliações de ganhos e benefícios foram aplicados sobre essa população. Isso é um fator limitante, pois as avaliações nessas situações são muito mais objetivas e não há testes quantitativos que nos permitam dizer quais as evoluções e ganhos desses pacientes. A maior parte dos testes aplicados tem a necessidade do apoio das respostas dos pais (parenteral), sendo então também de avaliação indireta e um tanto quanto subjetiva.

O resultado e os ganhos de tratamentos como o implante coclear, dependem em muito da motivação do paciente e de sua família. Uma criança com neuropatia auditiva pode ter treinamentos mais intensos e receber mais atenção e também por isso que os resultados são favoráveis. O mesmo também pode ser dito por questões sócio econômicas de cada família/paciente.

A avaliação de tratamentos que envolvem procedimentos cirúrgicos, como o caso do implante coclear, também é uma limitação metodológica por si só. O tempo de seguimento e a avaliação tardia de pacientes submetidos a tal tratamento são necessários para demostrar evidências científicas relevantes. Além disso, em algumas situações, nos deparamos com dilemas éticos na elaboração metodológica de estudos científicos, o que também limita a evidência científica desses estudos.

Os treinamentos auditivos e terapias de fala tem protocolos muito variados e personalizados, portanto sendo adaptados caso-a-caso. Isso dificulta ainda mais a comparação entre os resultados desses pacientes. Entretanto, trata-se de um viés de difícil superação e não impede a análise e investigação, mas certamente deve ser ponderado.

Acreditamos também que seria importante ter um grupo controle, para que pudéssemos ter dados comparativos mais evidentes. Porém acreditamos também ser muito difícil ter um grupo controle padronizado, principalmente pois os pacientes de implante coclear são muito heterogêneos, o que poderia ser um outro viés para nossos estudos.

Outro grande viés, que também limita estudos de neuropatia auditiva, é o próprio espectro da neuropatia auditiva. Fica evidente que a NA é heterogênea, não há consenso diagnóstico, nem da fisiopatologia e nem dos tratamentos, e os diversos estudos apresentarem resultados e achados discrepantes entre si.

Os aspectos econômicos de estudos laboratoriais, genéticos e moleculares, também devem ser considerados, principalmente em países em desenvolvimento como o Brasil. Atualmente, é muito caro e dispendioso a investigação genética, ainda mais em doenças raras, como o caso da neuropatia auditiva. A avaliação genética com a realização de mais testes é de extrema importância, mas sempre há a interposição de limitações econômicas, dificultando assim a eficiência das investigações.

O diagnóstico genético na neuropatia auditiva, assim como em outras situações relacionadas com a investigação etiológica da surdez, ainda é muito

incipiente, não há padronização e existem poucos consensos estabelecidos. Há a descrição de mutações que não se repetem em populações distintas, há falta de correlação entre alguns achados e a significância clínica, o que demostra haver ainda um longo caminho científico a ser percorrido.

Fica nítido a complexidade desse tema e a necessidade de uma maior investigação científica a cerca de todos esses parâmetros relacionados com a NA, assim haverá mais hipóteses e ferramentas para a compreensão e avaliação dos pacientes com NA, proporcionando melhores soluções e terapias para os pacientes.

10. CONCLUSÃO GERAL

A mutação do gene *GJB2* está presente em 7,5% dos pacientes estudados . Nenhuma mutação do gene *OTOF* foi identificada, porém é necessário maior aprofundamento no estudo desse gene, uma vez que muitas mutações descritas ainda não tem correlação clínica estabelecida.

A maioria dos pacientes têm sintomas no primeiro ano de vida, apresentam-se com grande transtorno auditivo, sendo que em 53% tinham sido classificados como portadores de surdez severa/profunda.

O tratamento deve focar no desenvolvimento de meios para que o paciente possa conseguir interpretar os sinais auditivos e se comunicar de forma adequada. O treinamento auditivo e a estimulação sonora são fundamentais nesse processo.

O implante coclear é um tratamento que deve ser considerado quando o ganho de linguagem e o desenvolvimento da fala forem insatisfatórios, certificando-se que a terapia fonoaudiológica foi realizada de forma adequada. Além disso, as evidências científicas que suportam o tratamento com implante coclear em casos de NA tem aumentado sistematicamente.

A neuropatia auditiva ainda é um grande desafio diagnóstico e terapêutico, tendo sua etiologia genética e ambiental ainda incertas. Acreditamos tratar-se de um conjunto de afecções onde a avaliação eletrofisiológica auditiva e genética podem ser grandes ferramentas de auxílio da identificação e seleção dos pacientes com esse transtorno. A neuropatia auditiva é uma situação complexa e deve ser estudada e seguida de forma multidisciplinar.

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ANEXOS

1. Parecer do Comitê de Ética em Pesquisa (CEP) da Faculdade de Ciências Médicas (FCM) da Universidade Estadual de Campinas (UNICAMP) – Parecer CEP n° 396/2006.
2. Cartas de Permissão/autorização das editoras dos artigos/capítulos de livro para a devida inclusão desse material científico na presente tese de dissertação de doutorado, em atendimento à legislação que rege o direito autoral.

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PRP
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CEP, 01/09/17.
(PARECER CEP: Nº 396/2006)

PARECER

I – IDENTIFICAÇÃO:

PROJETO: **"ESTUDO MOLECULAR DA PERDA AUDITIVA".**

ADENDO: **"ESTUDO GENÉTICO-CLÍNICO DE PACIENTES COM O ESPECTRO DA NEUROPATIA AUDITIVA".**

PESQUISADOR RESPONSÁVEL: Prof.ª Dra. Edi Lúcia Sartorato

II – PARECER DO CEP.

O Comitê de Ética em Pesquisa da Universidade Estadual de Campinas (Unicamp) aprovou a inclusão do pesquisador Guilherme Machado de Carvalho junto ao projeto principal supracitado, e também a inclusão do subprojeto intitulado "ESTUDO GENÉTICO-CLÍNICO DE PACIENTES COM O ESPECTRO DA NEUROPATIA AUDITIVA", com fins de doutorado do aluno Guilherme Machado de Carvalho.

III – DATA DA REUNIÃO.

Aprovado "ad referendum" em 01 de setembro de 2017.
A ser homologado na VIII Reunião Ordinária do CEP/UNICAMP, em 26 de setembro de 2017.

Dra. Renata Maria dos Santos Celeghini
COORDENADORA DO COMITÊ DE ÉTICA EM PESQUISA
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CEP, 19/10/06.
(Grupo II)

PARECER PROJETO: N° 396/2006 (Este n° deve ser citado nas correspondências referente a este projeto)
CAAE: 0.0.146.000-06

I-IDENTIFICAÇÃO:

PROJETO: **"ESTUDO MOLECULAR DA PERDA AUDITIVA"**
PESQUISADOR RESPONSÁVEL: Edi Lúcia Sartoro
INSTITUIÇÃO: CEPRE/UNICAMP
APRESENTAÇÃO AO CEP: 07/08/06

II - OBJETIVOS

Estudo da prevalência e da etiologia genética da deficiência auditiva.

III - SUMÁRIO

O estudo terá 50 indivíduos com surdez sensorioneural não sindrômica provenientes do Centro de Estudos e Pesquisas em Reabilitação "Professor Dr. Gabriel Porto" (CEPRE), selecionados pela geneticista Prof. Dra. Andréa Trevas Maciel Guerra. Será retirada amostra de sangue periférico dos indivíduos (10-15ml). Será feita a extração do DNA genômico a partir de leucócitos do sangue. Será feita a detecção da mutação 35deLG, das deleições, amplificações do gene GJB2 e rastreamento de mutações, rastreamento de mutações no gene SLC26A4 (PDS), detecção de mutações em mitocondriais, sequenciamentos dos genes e rastreamento de mutações pela técnica de SSCP (Single-Starnd Conformation Polymorfhisms).

IV - COMENTÁRIOS DOS RELATORES

O estudo está adequado. Não está descrito a idade dos sujeitos selecionados. Oferece risco ao sujeito quanto a retira de amostra sanguínea periférica. O risco está descrito no TCLE. O resultado do estudo trará informações importantes a respeito da genética das perdas auditivas, incluindo o estudo de genes de suscetibilidade, com enorme aplicação nas políticas públicas da surdez. Haverá facilidade n o diagnóstico, prognóstico e aconselhamento genético.

Recomendação: Incluir no TCLE assinatura do pesquisador e a informação que forneceu cópia do TCLE.

V - PARECER DO CEP

O Comitê de Ética em Pesquisa da Faculdade de Ciências Médicas da UNICAMP, após acatar os pareceres dos membros-relatores previamente designados para o presente caso e

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atendendo todos os dispositivos das Resoluções 196/96 e complementares, resolve aprovar **com recomendação** o Protocolo de Pesquisa, bem como ter aprovado o Termo do Consentimento Livre e Esclarecido, assim como todos os anexos incluídos na Pesquisa supracitada.

O conteúdo e as conclusões aqui apresentados são de responsabilidade exclusiva do CEP/FCM/UNICAMP e não representam a opinião da Universidade Estadual de Campinas nem a comprometem.

VI - INFORMAÇÕES COMPLEMENTARES

O sujeito da pesquisa tem a liberdade de recusar-se a participar ou de retirar seu consentimento em qualquer fase da pesquisa, sem penalização alguma e sem prejuízo ao seu cuidado (Res. CNS 196/96 – Item IV.1.f) e deve receber uma cópia do Termo de Consentimento Livre e Esclarecido, na íntegra, por ele assinado (Item IV.2.d).

Pesquisador deve desenvolver a pesquisa conforme delineada no protocolo aprovado e descontinuar o estudo somente após análise das razões da descontinuidade pelo CEP que o aprovou (Res. CNS Item III.1.z), exceto quando perceber risco ou dano não previsto ao sujeito participante ou quando constatar a superioridade do regime oferecido a um dos grupos de pesquisa (Item V.3.).

O CEP deve ser informado de todos os efeitos adversos ou fatos relevantes que alterem o curso normal do estudo (Res. CNS Item V.4.). É papel do pesquisador assegurar medidas imediatas adequadas frente a evento adverso grave ocorrido (mesmo que tenha sido em outro centro) e enviar notificação ao CEP e à Agência Nacional de Vigilância Sanitária – ANVISA – junto com seu posicionamento.

Eventuais modificações ou emendas ao protocolo devem ser apresentadas ao CEP de forma clara e sucinta, identificando a parte do protocolo a ser modificada e suas justificativas. Em caso de projeto do Grupo I ou II apresentados anteriormente à ANVISA, o pesquisador ou patrocinador deve enviá-las também à mesma junto com o parecer aprovatório do CEP, para serem juntadas ao protocolo inicial (Res. 251/97, Item III.2.e)

Relatórios parciais e final devem ser apresentados ao CEP, de acordo com os prazos estabelecidos na Resolução CNS-MS 196/96.

VII - DATA DA REUNIÃO

Homologado na VIII Reunião Ordinária do CEP/FCM, em 22 de agosto de 2006.

Profª. Drª. Carmen Silvia Bertuzzo
PRESIDENTE DO COMITÊ DE ÉTICA EM PESQUISA
FCM / UNICAMP

UNIVERSIDADE ESTADUAL DE CAMPINAS
FACULDADE DE CIÊNCIAS MÉDICAS
COMISSÃO DE PÓS-GRADUAÇÃO

Campinas, 24 de março de 2017.

Spandidos Publications
Journal Title: Molecular Medicine Reports

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Carvalho GM, Ramos PZ, Castilho AM, Guimarães AC, Sartorato EL. Molecular study of patients with auditory neuropathy. Mol Med Rep. 2016 Jul;14(1):481-90. doi: 10.3892/mmr.2016.5226.

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Best regards

Guilherme Machado de Carvalho
guimachadocarvalho@gmail.com

Guilherme Machado de Carvalho
Aluno Doutorado

Profa. Dra. Edi Lúcia Sartorato
Orientadora

Gmail Guilherme Carvalho <guimachadocarvalho@gmail.com>

Spandidos Publications Esub no. : 152330

mmr@spandidos-publications.com <mmr@spandidos-publications.com> 27 de março de 2017 11:38
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Dear Professor Guilherme Carvalho,

Our reference: MMR-7479-152330-01

Title: MOLECULAR STUDY OF PATIENTS WITH AUDITORY NEUROPATHY

By: Guilherme Machado de Carvalho et al

Thank you for the information. Permission is granted.

Yours sincerely,

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Campinas, 24 de março de 2017.

Bentham Science
Journal Title: The Open Neurology Journal

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de Carvalho GM, Z Ramos P, M Castilho A, C Guimarães A, L Sartorato E. Relationship Between Patients with Clinical Auditory Neuropathy Spectrum Disorder and Mutations in Gjb2 Gene. Open Neurol J. 2016 Sep 30;10:127-135.

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PhD Thesis Request - The Open Neurol Journal

Bentham Open- Ambreen Irshad <ambreenirshad@benthamopen.com> 7 de abril de 2017 03:03
Para: guimachadocarvalho@gmail.com

Dear de Carvalho

This is fine.

With kind regards,

Sincerely,

AMBREEN IRSHAD

Senior Editor - Bentham Open
Email: ambreenirshad@benthamopen.com

URL: http://benthamopen.com/

From: Guilherme Carvalho [mailto:guimachadocarvalho@gmail.com]
Sent: Saturday, March 25, 2017 4:08 AM
To: sadaf@benthamscience.org; oacomposing@benthamscience.org; The Open Neurology Journal; Gul Ahmed
Subject: PhD Thesis Request - The Open Neurol Journal

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COMISSÃO DE PÓS-GRADUAÇÃO

Campinas, 24 de março de 2017.

Revista Oficial da Ordem dos Médicos de Portugal
Journal Title: The Open Neurology Journal

Dear Editorial Office of **Acta Medica Portuguesa**,

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Carvalho GM, Leão BP, Ramos PZ, Guimarães AC, Castilho AM, Sartorato EL. Auditory Neuropathy: Clinical Evaluation and Diagnostic Approach. Acta Med Port. 2016 Jun;29(6):353-359. doi: 10.20344/amp.6942.

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Orientadora

ACTA MÉDICA PORTUGUESA
Revista Científica da Ordem dos Médicos

DECLARAÇÃO

A Acta Médica Portuguesa cede ao Senhor Dr. Guilherme Machado de Carvalho os direitos de utilização do artigo "Neuropatia Auditiva: Avaliação Clínica e Abordagem Diagnóstica", em anexo, na sua tese de Doutoramento junto da Universidade Estadual de Campinas – UNICAMP, Campinas, SP, Brazil - http://www.unicamp.br.

A utilização do artigo em questão, da autoria de Guilherme Machado de Carvalho, Beatriz Prista Leão, Priscila Zonzini Ramos, Alexandre Caixeta Guimarães, Arthur Menino Castilho, Edi Lúcia Sartorato, é gratuita mas condicionada à referenciação clara e inequívoca de que o mesmo artigo, foi originalmente publicado na Acta Médica Portuguesa [Acta Med Port 2016 Jun;29(6):353-359 - http://dx.doi.org/10.20344/amp.6942, e destina-se apenas a utilização académica conforme referido no parágrafo anterior. .

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Lisboa, 29 de Março de 2017

Carla de Sousa

Coordenação Editorial

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The Mediterranean Society of Otology and Audiology
Journal Title: The Journal of International Advanced Otology.

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de Carvalho GM, Ramos P, Arthur C, Guimarães A, Sartorato E. Performance of Cochlear Implants in Pediatric Patients with Auditory Neuropathy Spectrum Disorder. J Int Adv Otol. 2016 Apr;12(1):8-15. doi: 10.5152/iao.2016.2232.

de Carvalho GM, Zago TM, Ramos PZ, Castilho AM, Guimarães AC, Sartorato EL. Satisfaction of Children with Auditory Neuropathy and Cochlear Implant. J Int Adv Otol. 2015 Dec;11(3):229-35. doi: 10.5152/iao.2015.1695.

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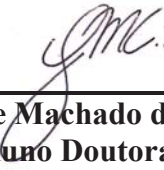

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PhD Thesis Request - / The Journal of International Advanced Otology / About your manuscript

Ali Şahin - AVES <ali.sahin@avesyayincilik.com> 22 de maio de 2017 05:54
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Cc: İbrahim Kara - AVES <ibrahim.kara@avesyayincilik.com>, Betül Çimen - AVES <betul.cimen@avesyayincilik.com>, "Dr. O. Nuri Özgirgin" <ozgirgin@politzersociety.org>, Buse Şenay - AVES <buse.senay@avesyayincilik.com>

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Tarih: 14 Mayıs 2017 Pazar 22:57
Kime: Ali Şahin - AVES <ali.sahin@avesyayincilik.com>
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