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Effect of GST gene polymorphism on cisplatin caused acute ototoxic side effects

PhD thesis

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List of Abbreviations

ABR	Auditory brainstem responses
AEP	Auditory Evoked Potential
AFP	alfa-feto-protein
ANOVA	Analysis of Variance
ASSR	Auditory Steady-State Response
BAEPs	Brainstem Auditory Evoked Potentials
BERA	Brainstem Evoked Response Audiometry
BM	Basilar Membrane
BSC	Best Supportive Care
CAEP	Cortical Auditory Evoked Potentials
CERA	Cortical Evoked Response Audiometry
CI	Confidence Interval
COMT	catechol-O-methyltransferase
CT	Computed Tomography
CYP	cytochrome P450 enzyme families
dB	decibel
DNA	deoxyribonucleic acid
DP	Distorsion Product
DPOAE	Distorsion Product Otoacoustic Emission
ECochG	Electrocochleography
EDTA	Ethylenediaminetetraacetic Acid
ERCC	excision repair cross-complementing group
GST	glutathione S transferase
hCG	human chorion gonadotropin
IL	interleukin
LDH	lactate dehydrogenase
OAE	otoacoustic emission
OR	Odds Ratio
PBMC	Peripheral Blood Mononuclear Cells
PCR	Polymerase Chain Reaction
PCR-RFLP	PCR-Restriction Fragment Length Polymorphism

PhD	philosophiæ doctor
PIC	pro-inflammatory cytokines
PTA	Pure Tone Audiometry
RNA	ribonucleic acid
ROC	Receiver Operating Curve
SFOAE	Stimulus-Frequency Otoacoustic Emission
SNP	Single-Nucleotide Polymorphisms
SOAE	Spontaneous Otoacoustic Emission
TEOAE	Transient-Evoked Otoacoustic Emission
TNF	Tumor Necrosis Factor
TPMT	thiopurine S-methyltransferase
WHO	World Health Organization
USA	United States of America
XME	xenobiotic-metabolizing enzymes

1. INTRODUCTION

The WHO constitution states: "Health is a state of complete physical, mental and social well-being and not merely the absence of disease or infirmity." (basic documents forty-ninth edition). This is our goal. When we treat a patient, especially a cancer patient, we have to consider the side effects. So, we always have to weigh the costs and benefits. We have four options for a cancer patient. Surgery, irradiation, systemic therapy or the combination of these. The fourth possibility is the BSC (best supportive care), when the patient is not fit enough to get any therapy. Every treatment has side effects.

Testicular germ cell tumors are one of the most common malignant diseases in men aged 15-44 in the USA (1). Even the advanced stages can also be successfully treated with combinations of chemotherapy regimens (2). These combinations are platinum based, and the major dose limiting side effect is the ototoxicity (3). However, platinum has other side effects, these can be much more preventable than ototoxicity. As an example, nephrotoxicity is solved by increasing saline hydration and mannitol diuresis, but there is no prevention against ototoxicity (3). The literature data place the probability of ototoxicity with cisplatin between 9-91%. These studies differed in many ways. The differences were in the chemotherapy protocol used, the characteristics of the patients, and the definition of ototoxicity. Successful researches are ongoing with otoprotective drugs to prevent ototoxicity, like amifostine N-acetylcysteine, multivitamins, dopamine receptor agonists (4-8) or Selegiline (9), but there is no proven medicine in clinical use to prevent ototoxic side effects. We can use a less ototoxic platinum derivative, such as carboplatin (3), we can reduce the dose of cisplatin (10), we can use otoprotective agents. In addition to all of this, it is critical to avoid other ototoxic drugs and hearing-damaging effects, such as excessive noise exposure.

Research into otoprotective agents is ongoing, and clinical implementation is expected in the near future. Determining which patients are more likely to experience ototoxic side effects is important to inform patients as accurately as possible about the benefits and drawbacks of the treatment they are receiving, and may also help in the judicious use of otoprotective agents.

However, if the patient experiences ototoxic side effects, steroid treatment is considered according to the Clinical Practice Guideline of Sudden Hearing Loss (11). This can be given intratympanically, orally, or intravenously. In our clinical practice, we prefer

intratympanic administration to avoid negatively influencing the effects and possible side effects of chemotherapy treatment.

Our working group is researching the ototoxic side effect caused by cisplatin. Individual differences in the appearance of the side effect were noticed. We aimed to explore one of the possible reasons for this. The main topic of the thesis is the investigation of the ototoxic side effect caused by cisplatin in patients with testicular tumors in the light of the GST gene polymorphism. Among other things, the GST enzyme is responsible for the degradation of cisplatin, so we can expect different ototoxic manifestations in the case of its variants with different efficacy.

1.1 PHYSIOLOGY AND ANATOMY OF HEARING

The sense organ of hearing is the ear, which converts sound vibrations into electrical signals and transmits them to the brain for processing through the auditory pathway. The ear can be divided into three parts: the outer, middle and inner ear. (figure 1.)

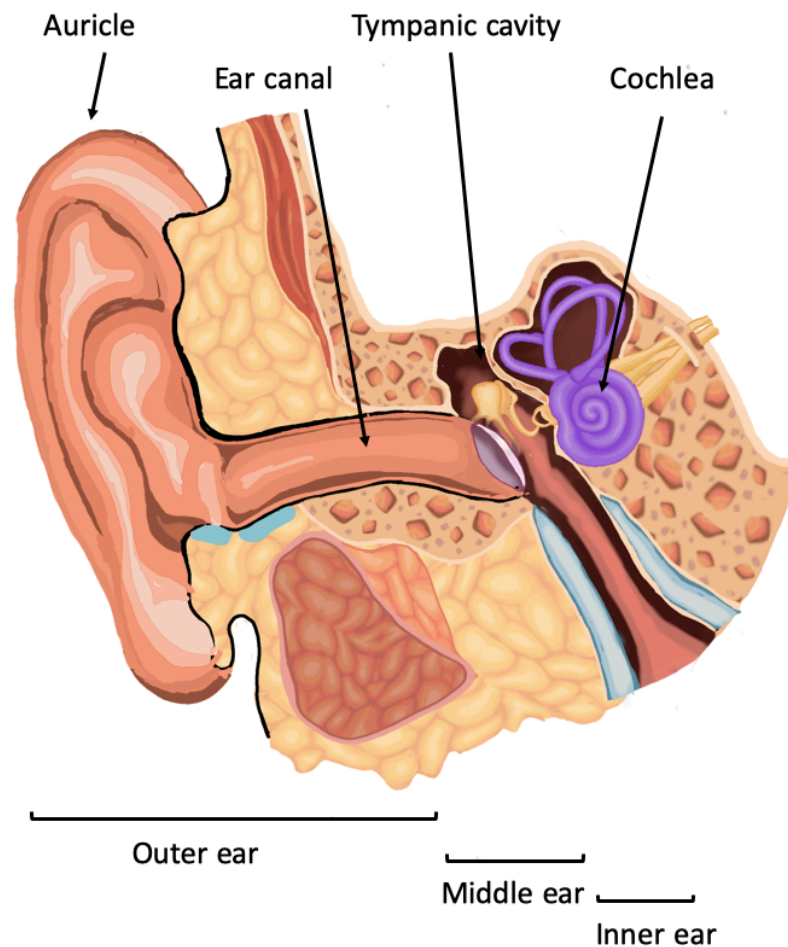


Figure 1.: parts of the ear

Sound travels through the external auditory canal and vibrates the eardrum. This vibration is transmitted to the auditory ossicles of the middle ear, which (as a two-armed lever) amplify the vibration just enough to compensate for the loss caused by the difference in surface area between the eardrum and the oval window, as well as reflection from the surface. The last auditory bone, the stapes, which is sitting in the oval window separates the middle and inner ear. Here, it transmits vibrations to the fluid space of the inner ear, eliminating most of the reflection that would otherwise (without auditory ossicles) occur at the air-fluid barrier. The auditory processing part of the inner ear is the cochlea. The labyrinth, the peripheral sensory system of the balance system, is located in the inner ear as well. (Figure 2.)

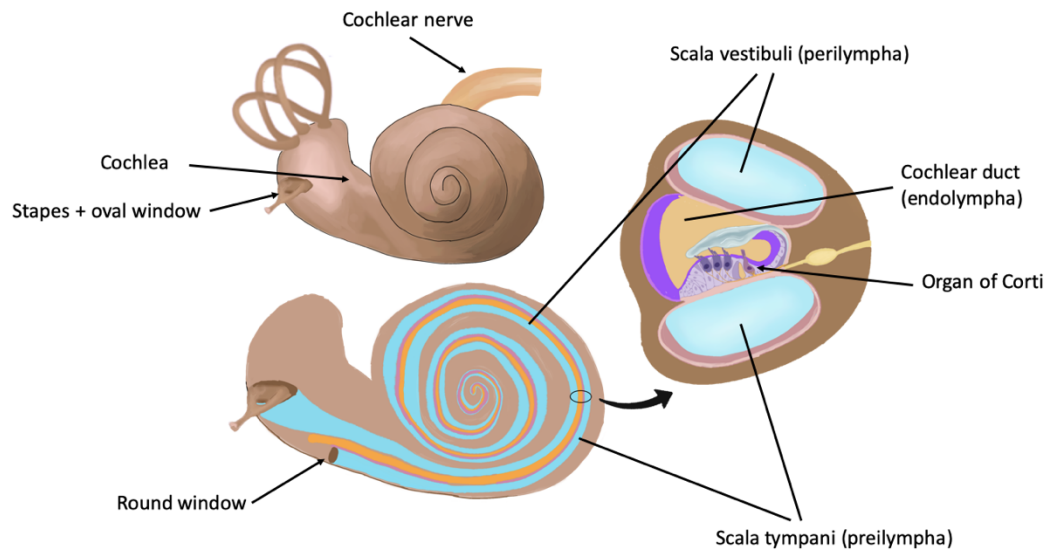
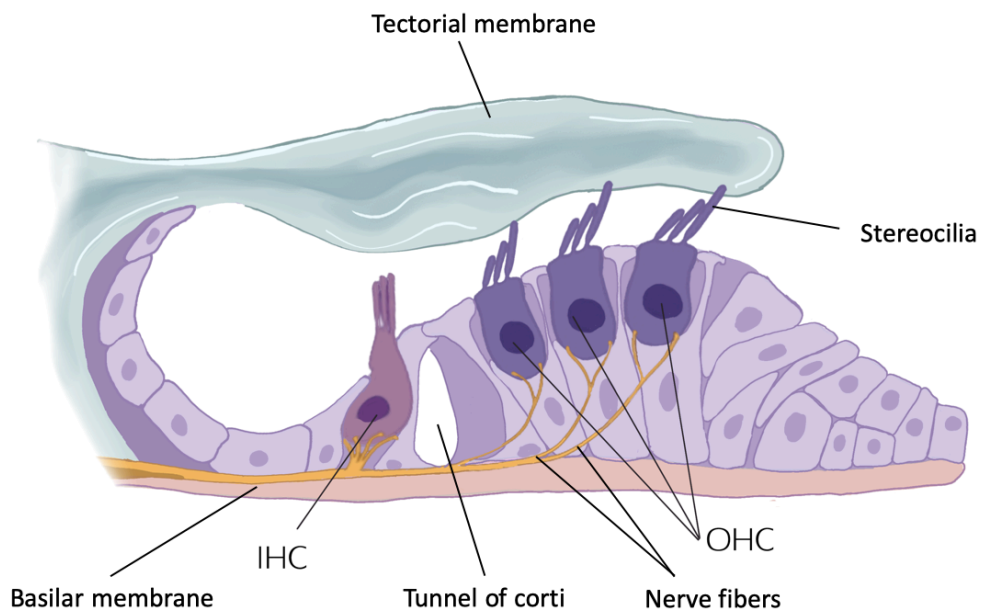


Figure 2.: the inner ear
 up-left: the cochlea and the labyrinth with their connections (stapes and nerve)
 down-left: longitudinal section of the cochlea
 right: cross section of the cochlea

The cochlea's job is to convert the vibrations of its fluid space into electrical signals that can be processed by the brain.

This happens in a fairly sophisticated way in a part of the cochlea called the organ of Corti. (Figure 3.)



3. ábra: organ of Corti

The vibration wave propagates from the footplate of the stapes through the fluid space of the scala vestibuli to the tip of the cochlea, where it turns into the scala tympani and travels in the other direction. To avoid continuous reflected waves at the end of the scala tympani, part of the energy returns back into the middle ear at the round window. But most of it is reflected. Pressure changes in the scala tympani cause deflections of the basilar membrane, which results in the displacement of the inner hair cells located on it. The stereocilia of the moving inner hair cells thus move relative to the tectorial lamina, which is closely connected to them. This movement is converted into an electrical signal through the opening of ion channels and is transmitted via the auditory nerve to the auditory cortex. The process is much more complex than this, and I still need to address one important element. Békésy György dealt with the physiology of hearing in the 1920s and established his model for this, the basilar membrane (BM) traveling wave theory. He published several works at that time under the title *Zur Theorie des Hörens*. For this, among other things, he later received the Nobel Prize in 1961. The theory went completely against the prevailing views on hearing, which Helmholtz published in 1863. He described the inner ear as a resonator, which cannot explain how the ear can distinguish approximately 1300 voice pitches, and all this while being able to distinguish between sounds arriving 0.1 second apart. Békésy builds a model, the physical characteristics of which he idealizes to the conditions of the cochlea. This work continued for years. Starting from the fact that the echidna's cochlea was approximately straight and its hearing is impeccable, for better observation, he designed his model, which was about 5 times larger than the cochlea, to be straight. He hoped that the changed geometry would not have affected the measurement results. The model represented glycerine-water spaces between copper frames and rubber membranes. The stapes was represented by a tuning fork operated by an electromagnet, the liquid vibrations were made detectable by carbon powder floating in it. The rubber membrane in the model represented the waves generated on the basilar membrane. The waves could be covered with an envelope curve. In the case of low sounds, the rubber membrane deflects most at the tip of the cochlea, the helicotrema. As the frequency increases and the sound becomes higher, the deflection of the membrane shifts towards the oval window. At the point of maximum deflection, a

vortex is created in both perilymph spaces. In order to make his observations more detailed, he drilled a hole in the axis of the straightened cochlea at the end of the helicotrema of the model and covered it with a glass plate. He did this in several places perpendicular to the axis and examined the movement of the carbon powder there with a microscope. The results of comparative checkups of the cochlea model and cochlear preparations were consistent, so the model worked well. However, despite its greatness and accuracy, Békésy perceived that the maximum of the traveling wave shifts with frequency change, but its deviation was low and flattened, so it could not model the frequency resolution ability of the ear.

It is obvious that the deflection of the basilar membrane stimulates the hair cells in a frequency-specific way, but in a too long distance according to the amplitude. Thus, in a longer section of the hair cells trigger electrical signals through the auditory nerve, not only the region, which belongs to the frequency of the voice. So, on the basis of this model, he couldn't illustrate, how the real signal becomes noise free.

One explanation was the vortex generation, which, if sufficiently intense, ruptured the basement membrane in the model. This rupture was relatively small compared to the amplitude width. The other theory is based on Mach's 1865 paper, in which he discusses the law of contrast named after him. In this experiment, he illuminated 3 rectangles made of the same material in such a way that the intensity of the middle one increases between its two edges. The two on right and on the left side, have the same intensity according to the central rectangle's edge value. As a result of this experiment, the brain visualizes a sharp line between the rectangles, one dark and one light, at the transitions. Thus, a subjective sensation is created that is different from the objective perception. On this basis, he justifies the physically unexplainable frequency discrimination.

Békésy did not find the theories completely credible, so he returned to the issue of frequency separation several times throughout his work.

Helmholtz's theory of hearing is replaced by Békésy's approach. At that time, frequency analysis was attributed to the activity of the auditory cortex.

Békésy received the Nobel Prize "for his discovery of the physical mechanism of the stimulus generated in the cochlea of the ear."

The issue of frequency separation could not be fully answered, based on evidence, because the experiments were performed on inanimate, cadaver cochleae, which could not detect and describe the now known active amplifier function.

In 1978, David T. Kemp described his complete explanation of the active amplification mechanism of the ear and presented the phenomenon of otoacoustic emission as measurable evidence (12). For this purpose, an earmold was created that can be placed in the ear and closes the external auditory canal, and which has an integrated speaker and microphone. For the registration a software was necessary, because he had to generate an input voice, and some millisecond later it had to be registered. It was the proof of otoacoustic emission, which is a measurable secondary product of the movement of the outer hair cells. The Corti-organ can tune the basilar membrane, in order to create the conditions of the known frequency separation with this outer hair cell movement. The traveling wave on the basilar membrane causes the hair cells to move. This movement is perpendicular to the basilar membrane and brings the hair cells closer to the tectorial membrane. When the stereocilium of the inner hair cells contact the tectorial membrane, an electrical signal is generated, which reaches the auditory cortex through the auditory pathway. This connection is modified by the movements of the stereocilia of the outer hair cells. As a result, the deviation of the wave amplitude generates an electrical signal in the inner hair cells over a much smaller area. Thus, the row of inner hair cells can become more sensitive to the little differences between frequencies. So, the organ of Corti becomes capable to detect the difference in the frequencies of the incoming sounds in a small area by the mechanical amplification of the fluid pressure wave transformed by the outer hair cells.

1.2 HEARING TESTS:

Depending on the method, a hearing test examines either the entire hearing (from the auditory canal to the auditory cortex) or a specific segment. If the entire system is examined and the result is good, then the patient's hearing is fine. If we get an impaired result, we do not know what part of hearing causes the patient's hearing loss. If we use a method that examines a certain section of the auditory system, then even if we get a good result, we don't know about the whole function, the patient may still have poor hearing

and speech understanding discrimination. Therefore, it is very important to know about each test, for what question the result may answer.

Hearing tests can be divided into two main groups. The first group is subjective hearing tests, which require the active participation of the patient, who must indicate what he or she can and cannot hear. Moreover, the result depends on the investigator too, for example, how quickly they change the volume and frequency.

Rarely, it also complicates the situation whether the person being tested is actually reporting their true hearing level. Some people want to get a bad result. This is a deliberate lie and it can be because of many reasons. For example, for tax return, to obtain some kind of certificate or it can be just a “crying for help”. It is called aggravation. Another group have good hearing, but their hearing problem is based on psychological reasons. They experience their hearing loss, but they don’t have any organic problems. This phenomenon is functional hearing loss. (13)

The second group is objective hearing tests. These are methods that do not require the active participation of the patient, the signal required for the measurement is based on unconscious reactions from the patient. The most important methods belonging to both groups are presented below to demonstrate why we chose the given method for our studies.

1.2.1 Subjective hearing tests

1.2.1.1 Speech audiometry

Whispered speech test: in the absence of these tools, we can draw conclusions about the patient's hearing from the understanding of whispered speech. It is expressed as how many meters away the patient can hear whispered speech.

1.2.1.2 Tuning fork tests

Quick, informative tests using a tuning fork.

1.2.1.2.1 Weber test

The tuning fork is placed on the midline of the skull and the patient is asked to indicate in which ear they hear the sound better, which reaches the inner ear through bone conduction. It is used to detect the difference in performance between the two ears. If the

hearing in both ears is equally good or bad, the patient will hear the sound equally strongly in both sides. If the hearing in one ear is worse than the other, then if the patient has conductive hearing loss, the sound will be heard better in the ear with poor hearing. If the patient has sensorineural hearing loss, the sound will be heard in the ear with good hearing.

1.2.1.2.2 Rinné test

In the given ear, air conduction is compared to bone conduction. The bent tuning fork is first held against the patient's ear, then the handle is pressed against the mastoid. In normal hearing, air conduction hearing - when held against the ear - is louder than bone conduction hearing - when touched against the mastoid. If conductive hearing loss is present, i.e. the hearing loss originates from the outer or middle ear, bone conduction is louder. Together with the Weber test, it can be determined with a fairly good degree of certainty whether the hearing loss is conductive or sensorineural.

1.2.1.3 Pure tone threshold audiometry

The classic hearing test, which is also featured in movies as a hearing test. The patient sits in a soundproof room and hears pure sounds through headphones, the frequency and volume of which are changed by the examiner. Thus, based on the patient's feedback, it can be determined at what frequencies and at what decibel level the patient is currently hearing, and this is plotted on an audiogram in comparison to normal hearing.

1.2.1.3.1 Pure tone air conduction test

If the earphone is a classic earphone, i.e. placed near the ear of the person being measured and the sound is introduced through the ear canal, then we can measure the patient's air conduction. We examine usually seven frequencies (125, 250, 500, 1000, 2000, 4000, 8000 Hz) so almost the entire hearing system.

1.2.1.3.2 Pure tone bone conduction test

If the earphone transmits the vibrations directly to the skull bone, bypassing the outer and middle ear, the vibrations reach the inner ear directly with the loudspeaker vibrating the mastoid. In this way, we can measure the patient's bone conduction, examining hearing

from the inner ear. The examined frequencies are: 250, 500, 1000, 2000, 4000 Hz. If the patient has conductive (ear canal, middle ear origin or third window syndrome) hearing loss, air conduction is abnormal, while bone conduction gives a healthy result. The difference between air and bone conduction is called the air-bone gap.

1.2.1.4 Suprathreshold auditory tests

In the presence of sensorineural hearing loss, suprathreshold subjective tests serve to differentiate between cochlear (sensory) and auditory pathway (neural) hearing loss. Thus, they answer the question of whether sensorineural hearing loss is of cochlear or auditory nerve origin.

Such hearing tests include speech audiometry, Békésy audiometry, short increment sensitivity index (SISI), tone decay test (TDT) or discomfort threshold measurement.

1.2.2 *Objective hearing tests*

1.2.2.1 Tympanometry

An earphone that hermetically seals the ear canal is placed in the ear, which also contains a microphone. The sound emitted by the earphone is reflected from the tympanic membrane, which is detected by the microphone. This earphone also contains a pump that can change the pressure in the closed ear canal. The sound, reflected from the eardrum, is of the lowest intensity when the pressure in the middle ear is equals atmospheric pressure, i.e. when the Eustachian tube is functioning properly (A-type diagram). In this case, the largest proportion of the sound energy reaches beyond the eardrum. If there is a malfunction of the eustachian tube and the pressure in the middle ear is lower than outside, then the lowest intensity reflection is shifted (C-type diagram). If there is fluid in the middle ear, most often due to inflammation, the reflection does not change when the pressure is changed, it is constant, so we get a flat curve (B-type diagram). In consequence, tympanometry provides information about the pressure conditions in the middle ear.

1.2.2.2 Evoked reflex test: stapedius reflex test

Strong sound stimuli can damage the inner ear. To protect against this, we have a protective reflex called the stapedius reflex. A muscle called the stapedius muscle runs to the stapes, which is innervated by the facial nerve, the nerve responsible for facial movement. If we hear a sound louder than 80 dB, the stapedius muscle tenses through reflex neural feedback, a signal arriving on the facial nerve, which causes the stapes plane to move, resulting in approximately 20 dB of sound attenuation. This is a complex reflex, requiring intact facial function, intact hearing, or a hearing loss not exceeding 40 dB, and a well-functioning auditory ossicle chain to be triggered. Pathological conditions that affect these functions result in changes in the stapedius reflex.

1.2.2.3 Evoked acoustic response: otoacoustic emission

David T. Kemp described stimulated otoacoustic emissions in 1978 in his paper: Stimulated acoustic emission from within the human auditory system (12): “Sound energy appears to be emitted by the auditory system, into the external ear canal, for some tens of milliseconds after impulsive acoustic excitation. The origin of this newly discovered phenomenon remains essentially unknown but a physiologically active mechanism in the cochlea is supported by the evidence that these acoustic emissions have nonlinear characteristics and are inhibited by damage to the sensory system such as occurs in cochlea deafness. Much more detailed study is both possible and desirable. If a cochlea origin is confirmed by experiments currently in progress, the technique developed in this study will provide a new avenue for investigation of the auditory system, with applications in both research and audiological medicine.”

Otoacoustic emissions are a byproduct of the active movement of the outer hair cells of the inner ear to amplify incoming sound. This is a special movement provided by a protein called prestin.

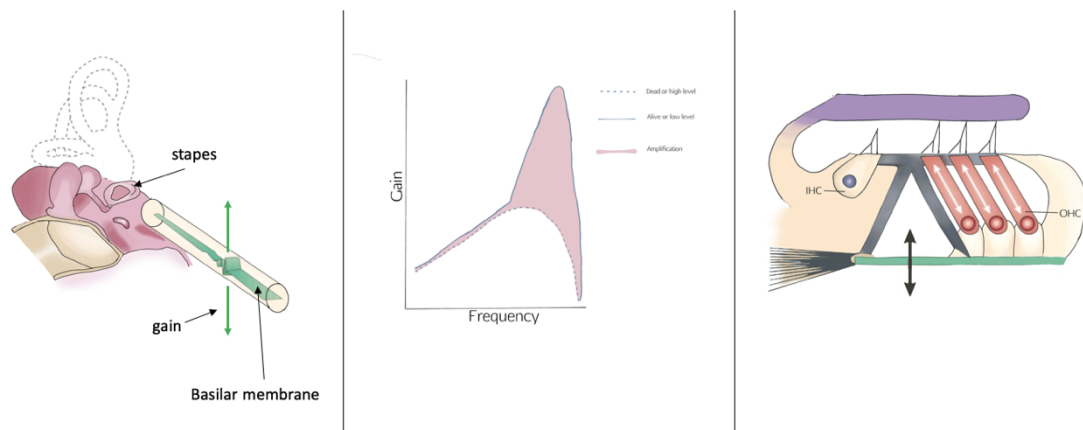


figure 4.:

left: the movement of the stapes causes the wave of the basilar membrane (green)

middle: frequency tuning curve–dotted line: without OHC amplification–continuous line: with OHC amplification

right: organ of Corti - black arrows: passive movements by pressure – white arrows: active movements by prestin

While skeletal muscles, which are faster than smooth and cardiac muscles, have a contraction time of 100-200 ms, prestin can contract at a frequency of up to 10,000/s. This makes the hair cell suitable for active movement that tunes the deflections of the basilar membrane to the frequency of sound. In this case, the tension of the basilar membrane changes, so that the frequency-specific stimulation of the inner hair cells is more focused. (figure 4.) This ensures frequency separation and frequency discrimination that could not be explained for a long time due to the size of the inner ear. Otoacoustic emission, as a secondary sound phenomenon, occurs when hair cells function properly. If the incoming sound reaches the inner ear with intact outer and middle ear function, then the otoacoustic emission also reaches the ear canal via this route and can be measured there. There are several types of otoacoustic emissions based on the triggering sound stimulus, or lack thereof. Of course, the detection also differs depending on the triggering sound stimulus.

1.2.2.3.1 Spontaneous otoacoustic emission (SPOAE)

Otoacoustic emission is measurable in the ear canal without an incoming sound stimulus. In the hearing-impaired population, it can be elicited in 70% of young adults (10) and 37% of young children (14). Their clinical utility has not yet been established.

1.2.2.3.2 Evoked otoacoustic emission

1.2.2.3.2.1 Stimulus-Frequency Otoacoustic Emission (SFOAE)

It is triggered by a continuous clear sound, but due to the complexity of the measurement and the lack of additional information, it has not become widespread in clinical practice.

1.2.2.3.2.2 Transient-Evoked Otoacoustic Emission (TEOAE)

We use click stimulation, i.e. short-term white noise, as the input signal. The emission is not as frequency-specific as in DPOAE. Nowadays it is used for newborn hearing screening.

1.2.2.3.2.3 Distorsion product otoacoustic emission (DPOAE)

As D. Konrad-Martin expresses (15): „Distortion product (DP) OAEs are generated when two BM traveling wave responses, elicited by two tones at closely-spaced frequencies, f_1 and f_2 , interact and undergo intermodulation distortion. This produces distortion products in the BM vibratory response at non- f_1 and f_2 frequencies that are arithmetically related to the primary frequencies. Energy at $2f_1-f_2$, the distortion product often measured clinically, is transmitted bi-directionally. It travels backward from the BM region that undergoes intermodulation distortion toward the tympanic membrane and forward toward its own relatively apical tonotopic place, where a reflection emission may be generated.” The most optimal is if the ratio of the two sounds f_1 and f_2 , f_2/f_1 , is 1,22 (16). In this case, the distortion sound is the most robust.

Distortion otoacoustic emission is a method suitable for examining the outer hair cells of the organ of Corti in the cochlea. Hair cells are located in the organ of Corti in the area corresponding to a given frequency and can be examined frequency-specifically with this method. We can use it successfully when we would like to assess the status of the inner ear, or we would like to detect its changes of function. Therefore, it is suitable for testing noise induced hearing loss (17) or ototoxicity, especially if hearing loss is due to damage to the hair cells of the organ of Corti.

Our research group therefore chose this hearing test method to investigate ototoxic side effects caused by cisplatin.

1.2.2.4 Auditory evoked potentials (AEP):

1.2.2.4.1 Electrocochleography (ECochG)

Electrocochleography (ECochG) is an electrophysiological technique used to measure electrical potentials generated in the cochlea and auditory nerve in response to acoustic stimulation. It provides valuable information about cochlear and auditory nerve function. There are two main types of ECochG based on the recording site: transtympanic ECochG, which provides higher amplitude responses due to closer proximity to the cochlea but requires local anaesthesia, and extratympanic ECochG, which is less invasive but yields smaller signals.

ECochG is clinically valuable in diagnosing conditions such as Ménière's disease, where an elevated SP/AP area ratio can indicate endolymphatic hydrops (18).

Nowadays, in most cases it has no clinical use, because its invasivity and products less information as BERA + OAE.

1.2.2.4.2 Auditory brainstem responses (ABR) / Brainstem Auditory Evoked Potentials (BAEP)

They are electrophysiological responses generated by the auditory nerve and brainstem nuclei following an auditory stimulus, typically a click or tone burst. These responses are recorded using surface electrodes placed on the scalp and mastoids and are commonly used in both clinical and research settings to assess the functional integrity of the auditory pathways up to the level of the midbrain.

The BAEP waveform consists of a series of waves, traditionally labelled I through VII, which occur within the first 10 milliseconds after the onset of the stimulus. Each wave is believed to reflect neural activity at specific anatomical sites.

1.2.2.4.2.1 ABR=BERA (brainstem evoked response audiometry) and ASSR (auditory steady-state response)

BERA and ASSR are objective electrophysiological techniques used to estimate auditory thresholds. As these are objective methods, we can use them in populations who cannot reliably respond behaviourally (e.g. infants, sedated patients). Each method has

advantages and limitations in terms of frequency specificity, threshold estimation, time-efficiency, and clinical utility.

Studies show strong correlations between ASSR thresholds and behavioural pure tone audiometry (PTA) in subjects with hearing loss. In adults with sensorineural hearing loss, multi-channel ASSR thresholds were within 15 dB of PTA in ~71-89% of cases at several frequencies.(19)

In newborn or infant screening, or cases where behavioural audiometry is not feasible, a combination of BERA for broad neural integrity and ASSR for threshold estimation may yield the best results.(20)

1.2.2.4.3 Cortical auditory evoked potentials (CAEP) / cortical evoked response audiometry (CERA)

Cortical Evoked Response Audiometry (CERA) is an objective electrophysiological technique used to assess auditory cortical function by recording cortical auditory evoked potentials (CAEP) in response to sound stimuli. It provides valuable information about the integrity of the central auditory pathways, particularly in individuals who are unable to provide reliable behavioural responses (21). CERA typically evaluates late-latency responses, such as the P1, N1, and P2 components, which reflect cortical processing of auditory information (22). While it is a complicated and time-consuming method it is used very rarely.

1.3 CISPATIN - OTOTOXICITY

Cisplatin is one of the oldest chemotherapy drugs. Platinum derivatives, such as cisplatin - cis-diamminedichloroplatinum, as the National Cancer Institute website states: "alkylating-like inorganic platinum agent (cis-diamminedichloroplatinum) with antineoplastic activity. Cisplatin forms highly reactive, charged, platinum complexes which bind to nucleophilic groups such as GC-rich sites in DNA inducing intrastrand and interstrand DNA cross-links, as well as DNA-protein cross-links. These cross-links result in apoptosis and cell growth inhibition." (<https://www.cancer.gov/publications/dictionaries/cancer-drug/def/cisplatin>).

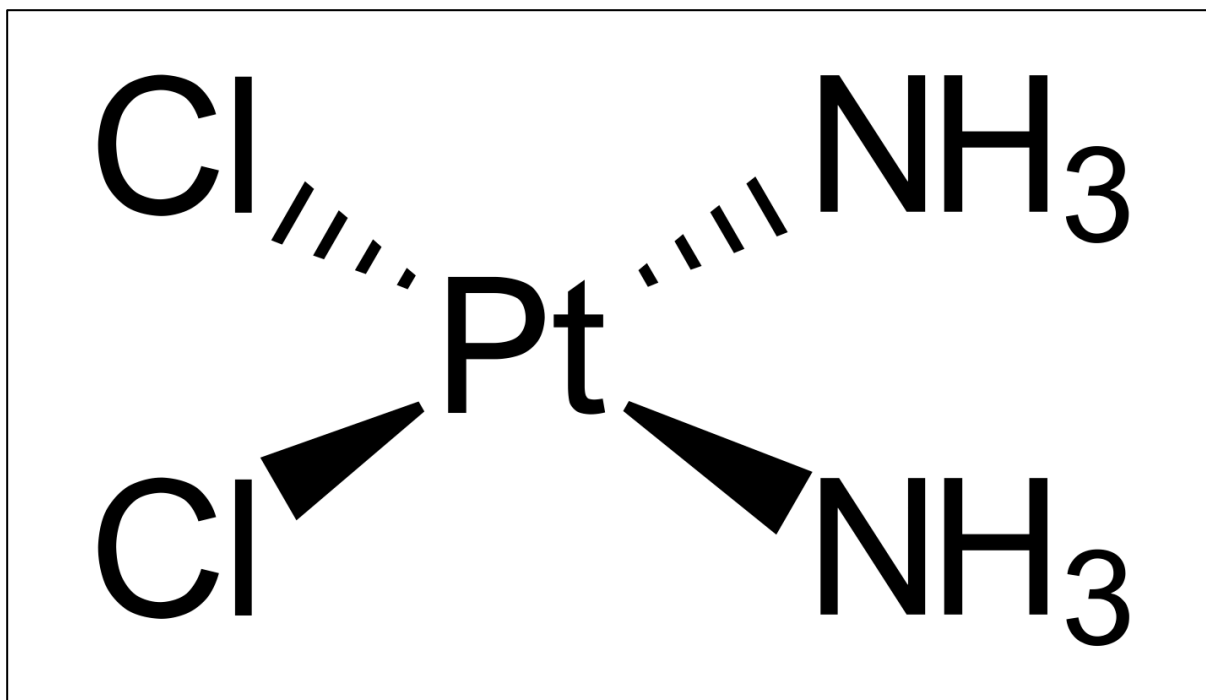


Figure 5.: the cisplatin molecule
(<https://en.wikipedia.org/wiki/Cisplatin>)

These antitumor effects also cause its side effects. It is a fundamental agent in the armament of anticancer therapy. Thus, it is important in the treatment of ovarian cancer, gastrointestinal cancer, testicular cancer, bladder cancer and head and neck tumors, among others. In connection with the increase in patient survival and the emphasis on quality of life, the reduction of side effects such as nausea, vomiting, kidney damage, neurotoxicity (neuropathy), hearing damage, and hematopoietic effects - myelosuppression - is becoming increasingly important. This is the aim of determining the dose to be administered, its method and circumstances.

Thus, we use antiemetics, selective inhibitors of type 3 [5-HT₃] serotonergic receptors, as an introduction to therapy. (23), (24). In addition, as an introduction to therapy, the patient is hydrated with 1-2 liters of fluid intravenously (25), which is continued during the treatment (4-6h: target diuresis 150-400ml/h) and afterwards (target diuresis: 18-24h 100-200ml/h) (26), (27), (28). In addition, sodium chloride should be added for supplementation. Cisplatin should be administered over a period of 6-8 hours, as stated in the drug's instructions for use, because rapid administration greatly increases the risk of kidney and hearing damage (29). The kidney damage was minimized by adequate hydration and increasing urine output 24 hours after administration.

Ototoxic side effects were also reduced by dose reduction and other preventive measures, but not to the desired level. Ototoxic side effects are dose-dependent and unilateral or bilateral. (30)

Cisplatin induces apoptosis of hair cells through activation of mitochondrial pathway is the major damage in the inner ear. Anyway, it can be targeted for otoprotection. (3, 31, 32) Subordinately, lesions of the type I neurons of the spiral ganglion and in stria vascularis have also been reported. (33, 34). Troung et al. published a review (35) published a review in which they suggested that cisplatin first causes damage to the marginal cells of the stria vascularis, which in turn affects the outer hair cells. They wrote that the damage to the stria vascularis is more transient than to the hair cells. If we accept this, DPOAE is still the most suitable for detecting and monitoring ototoxicity, since differences in endolymph composition due to damage to the stria vascularis also manifest themselves in changes in hair cell function.

Additionally, it activates the pro-inflammatory cytokines (PICs), namely IL-1 β , IL-6 and TNF- α in the cochlear spiral ganglion of rats after ototoxicity induced by cisplatin. (36)

Individual differences in the occurrence of cisplatin side effects are divided into genetic and non-genetic causes. One line of thought regarding the causal role of genetic differences is the single-nucleotide polymorphisms (SNPs) of genes that have been studied over time are especially those that encode enzymes or proteins (glutathione S-transferase = GTS; excision repair cross-complementing group = ERCC; thiopurine S-methyltransferase = TPMT; and catechol-O-methyltransferase = COMT). (37) These enzymes are responsible for the entry of cisplatin into the cell, its binding, degradation and elimination from the cell. Thus, differences in enzyme function cause variations in the risk of developing ototoxic side effects of cisplatin.

Since the ototoxic side effect of cisplatin primarily affects the inner ear, and within it, the hair cells in organ of Corti is most damaged, logically otoacoustic emission is the most useful method for detecting and monitoring this side effect.

The development of ototoxic side effect affects the patient's later quality of life, including their social relationships and work ability. In addition to correctly informing the patient and timely minimizing the side effect, research and studies are necessary to avoid ototoxic side effects caused by cisplatin.

Among other ototoxic drugs, the pathomechanism of aminoglycoside antibiotics has been extensively studied. In the fight against infections in newborns, we are often forced to use this known toxic agent. At the perinatal intensive care units, all newborn patients get aminoglycoside antibiotics. Aminoglycoside antibiotics are protein synthesis inhibitors, which work by binding to the 30S subunit of the 70S ribosome (specifically the S12 protein, thus preventing its normal function). The effect is concentration-dependent, as in small amounts, protein synthesis is impaired and slowed down; in larger doses, the ribosome cannot dissociate, so the finished protein chain does not detach from it; however, in significant amounts of antibiotic, chain initiation is also inhibited. Among the side effects, the nephrotoxicity, vestibulotoxicity and ototoxic side effects should be highlighted. These side effects are dose-dependent and generally occur with higher doses of treatment. McDermott et al (38) describe: „MT-RNR1 is a gene that encodes the 12S rRNA subunit and is the mitochondrial homologue of the prokaryotic 16S rRNA. Some MT-RNR1 variants (i.e., m.1095T>C; m.1494C>T; m.1555A>G) more closely resemble the bacterial 16S rRNA subunit and result in increased risk of aminoglycoside-induced hearing loss.” In this subgroup the ototoxic side effect occurs even at low dose of aminoglycoside administration. So, it's an example, when in case of a known genetic variation, the ototoxic side effect has a higher risk. It can be a basis of a different therapeutic decision, if it is possible.

1.4 TESTICULAR CANCER AND ITS THERAPY

Germ cell tumor of the testis accounts for approximately 1% of all male malignancies, but it is the most common malignancy in certain age and ethnic groups, such as young (15-40 years old) Caucasian men. Even at an advanced stage germ cell tumors can be successfully treated by the use of a combination of chemotherapeutic regimens. (2) In Hungary, the number of newly diagnosed patients fell between 547 and 637 per year between 2010 and 2017, according to the National Cancer Registry. The vast majority (about 95%) of malignant tumors of the testis are of germ cell origin. The majority of germ cell tumors occur in the testis, 35-45% are seminomas and 55-65% are non-seminomas.

The first step in treating testicular germ cell tumors is radical inguinal orchiectomy. This provides diagnosis, local control, and histopathological evaluation (39). Serum tumor

markers (AFP, β -hCG, LDH) should be measured before orchiectomy, and then post-orchiectomy to assess decline (40). Imaging (CT of chest, abdomen, pelvis) is used for staging. Brain imaging may be considered in selected high-risk cases (e.g. very high β -hCG) (39). Use of prognostic classification (e.g. IGCCCG: International Germ Cell Cancer Collaborative Group) for advanced disease is standard (41).

Post operative treatment of stage I seminoma may include observation, radiotherapy to paraaortic lymph nodes, or carboplatin treatment (39). In cases of stage II and III, the patients receive cisplatin-based chemotherapy with or without irradiation therapy.

In the case of non-seminoma, if patients receive treatment (surveillance may be recommended for the low-risk stage I patient group), they receive cisplatin-based chemotherapy with or without irradiation or lymph node dissection.

These chemotherapy combinations are: BEP (Bleomycin + Etoposid + Cisplatin), EP (Etoposid + Cisplatin), VIP (Vepesid + Ifosfamid + Cisplatin). These other drugs given in addition to cisplatin do not have ototoxic side effects. The protocol determines that the dose of one cycle of cisplatin treatment is 20mg/m²/day for five days. The cycles repeat 21 days.

The young age, exceptionally good survival, and treatment protocol of testicular germ cell cancer patients make them suitable for studies related to the ototoxic side effects of cisplatin.

1.5 GLUTATHIONE S TRANSFERASE - GST

Xenobiotic-metabolizing enzymes (XMEs) that degrade xenobiotics can be divided into two groups: phase I (functionalization) and phase II (conjugation) reactions. 75% of these belong to the Cytochrome P450 (CYP) enzyme families. 57 human genes are known in 18 families of CYP genes. Due to gene polymorphism, there are active and less active enzyme variations. When the cell has a less active phase I, metabolic activator enzyme, the metabolite provokes less damages in the cell. In case of a phase II protein, when it is less active, it provokes more damages, because the toxic metabolite stays in this form more time, then in case of a more active phenotype.(42)

Glutathione-S-Transferases are a family of phase II detoxification enzymes. They catalyse the conjugation of reduced glutathione to electrophilic compounds. So, they enhance the solubility of toxic agents, hereby the excretion and give protection against

cellular damage. Major GST cytosolic classes in mammals the GSTM, GSTT, GSTP families. The natural, most commonly occurring version of these enzymes is called wild type and is considered to have normal function. The different variants of enzymes are due to polymorphisms in the gene that encodes them. Enzymes encoded by less common gene polymorphisms have reduced function and are called null types.

Cisplatin is activated upon entering the cell (phase I). Due to the low chloride ion concentration inside the cell, water molecules bind to the platinum instead of chloride ions (hydrolysis). This is the activated form, which can now bind to DNA and drive the cell towards apoptosis. Conjugation with glutathione (GSH), which is carried out by the GST enzyme, is responsible for deactivation. The cisplatin-GSH conjugated form is released from the cell by active transport. (43)

In our present study, we examined the appearance of ototoxicity in the light of the different, individual-specific cisplatin degradation potential determined by these GST gene polymorphisms.

1.6 OUR PRECEDING STUDIES

I joined our working group in 2003, when we were curious to see, through a retrospective study, after how many cycles does the ototoxic side effect of cisplatin occur. Ototoxic side effects were detected using DPOAE and were determined by comparison with a healthy control group. (44, 45) 223 patients were included in the study. The results showed that ototoxic side effects could be detected after 4 cycles, i.e. a total dose of 400mg/m², years after treatment. (figure 6.)

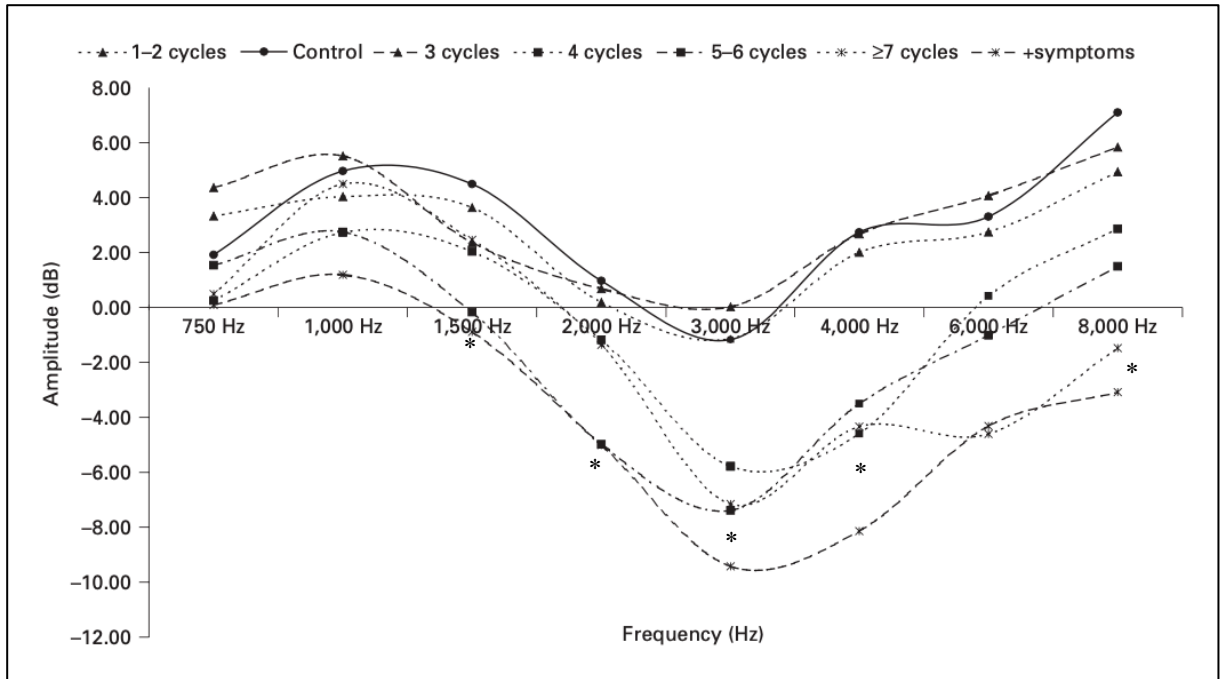


figure 6.: The 223 patients were divided into groups according to their cisplatin cycle and the mean DPOAE results are shown, compared to the control group. Significant difference can be measured after 4 cycles (marked with *) Biro et al. (45)

While working with patients, we noticed significant individual differences both among the patients in the study and in the patients, we treated with platinum derivatives acutely, directly around the time of treatment. Therefore, we designed prospective studies.

So, we started a prospective study involving 137 patients, during which we recorded the DPOAE results of the patients on the first day of their first cycle of treatment, before treatment, and then on the last day after treatment. (46) They got 100mg/m² cisplatin in combination with other, not ototoxic chemotherapy agents. Here, our aim was to investigate the acute ototoxic effect of cisplatin in case of 1 cycle. The results showed, that the subjective hearing complaints after the first cycle cisplatin increased significantly compared to the hearing complaints before treatment ($P = 0.003$). DPOAE measured before (B) and after (A) the first cycle of cisplatin at each frequency in right and left ear of patients resulted in significant differences (B-A) at low (750 Hz) and high frequencies (6,000 and 8,000 Hz). (Table 1.)

Table 1.: the values are expressed as DPOAE mean amplitude values (dB) at all measured frequencies, on both sides (right and left ears) A = after; B = before the chemotherapy. The next line the difference (B – A), then the CI = confidence interval and the significance values are listed. Noszek et al. (46)

Effect of First Cycle of Cisplatin on DPOAE of Patients With Germ Cell Tumor.								
	Frequency [kHz]							
	0.75	1	1.5	2	3	4	6	8
Right ear								
B (before cisplatin)	4.45	6.49	6.90	3.47	3.04	7.39	7.80	8.64
A (after cisplatin)	2.64	5.79	6.29	2.72	2.04	6.40	5.96	5.62
Change (B-A)	1.81	0.70	0.61	0.75	1.00	0.99	1.84	3.02
95% CI of B-A	0.66 to 2.96	-0.46 to 1.87	-0.44 to 1.65	-0.26 to 1.76	-0.34 to 2.34	-0.39 to 2.37	0.56 to 3.11	1.63 to 4.42
P (paired t test)	0.003	0.238	0.256	0.150	0.147	0.162	0.006	0.001
Left ear								
B (before cisplatin)	4.27	6.22	6.29	3.24	2.58	7.17	8.11	9.93
A (after cisplatin)	2.56	6.48	6.47	3.19	2.71	7.59	6.92	5.81
Change (B-A)	1.71	-0.26	-0.18	0.05	-0.13	-0.42	1.19	4.12
95% CI of B-A	0.38 to 3.04	-1.51 to 1.00	-1.17 to 0.81	-0.94 to 1.04	-1.26 to 1.01	-1.39 to 0.54	-0.15-2.52	2.61 to 5.63
P (paired t test)	0.013	0.687	0.499	0.915	0.828	0.387	0.085	0.001

The distribution of DPOAE amplitudes for B and A (all frequencies and both ears considered together) showed a shift toward lower values in case of A compared to B (Figure 7.)

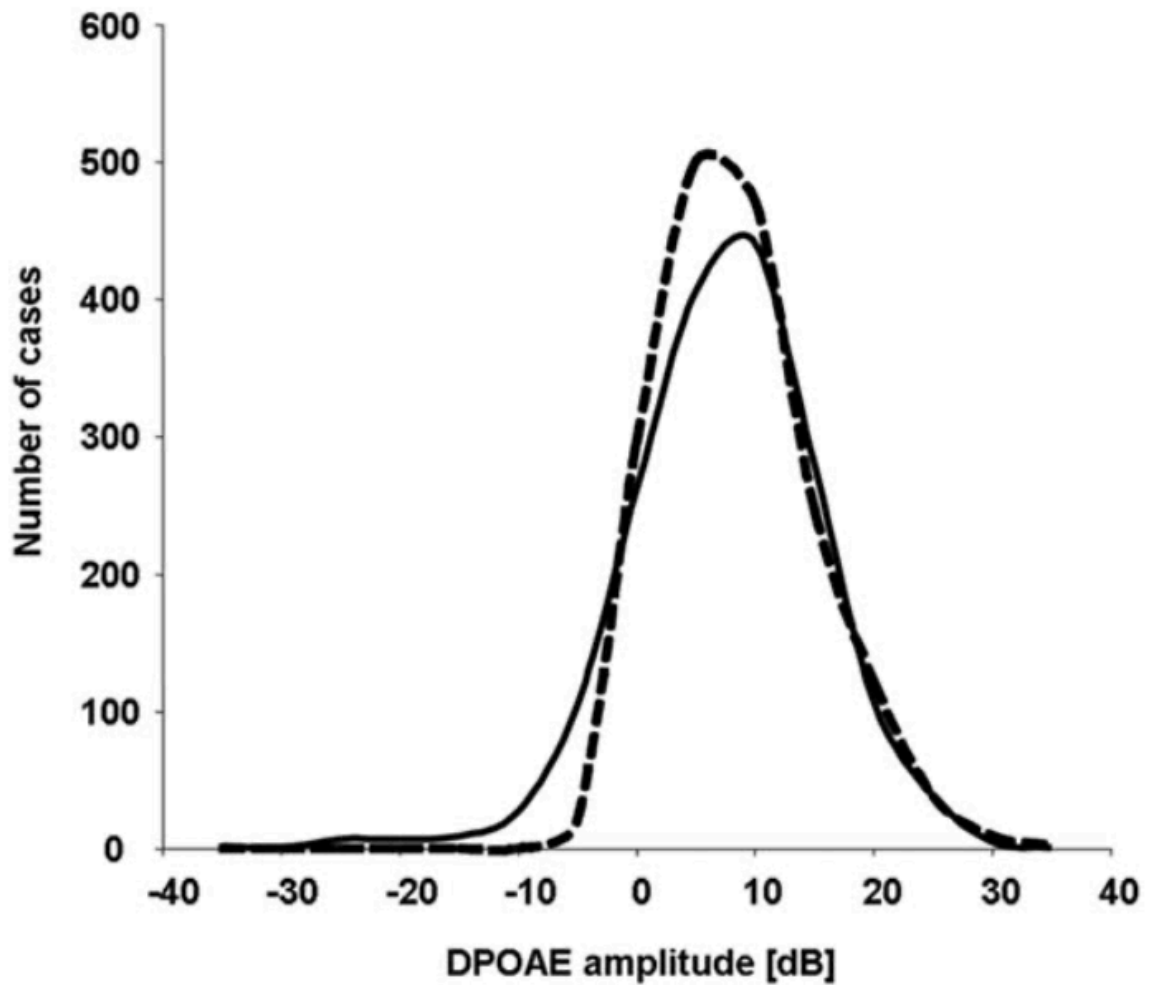


Figure 7.: distribution of DPOAE amplitude values of both ears of patients with testicular tumors before (--) and after (-) receiving the first cycle of platinum-based chemotherapy
Noszek et al. (46)

A similar shift was observed for each frequency in both ears. Thus, we were able to demonstrate the ototoxic effect of cisplatin already in the first cycle with distortion otoacoustic emission.

2 OBJECTIVES

The occurrence of cisplatin-induced ototoxic side effects is largely influenced by the combination of polymorphisms in different GST enzyme types, which are partly responsible for the degradation of cisplatin. (47), (48), (49). These studies were retrospective and used subjective hearing threshold tests.

Based on the information in the introduction, our working group considers distortional otoacoustic emission to be the most suitable hearing test for early detection of cisplatin ototoxic side effects, as it examines the element of hearing where cisplatin is most damaging. Early detection of outer hair cell dysfunction may result in the detection of changes in hearing before subjective hearing loss, which implies more accurate results.

If we are interested in the details of the ototoxic side effects of platinum derivatives, the testicular tumor population is an ideal patient material to study this. This is because they are young, so age-related hearing loss does not yet appear as a distorting factor in our measurements. The basic therapy is cisplatin-based chemotherapy. It means a combination therapy, cisplatin plus other chemotherapy materials, which are not ototoxic. Their survival is extremely high, they are a well-followed population, and side effects are also more prominent.

In order to explore the background of individual differences in the appearance of the ototoxic effect of cisplatin, we collected blood for genetic analysis from patients who were examined with DPOAE in the determination of the acute ototoxic effect detailed above, with the patients' information and consent.

The aim of this prospective study was to determine which GST gene polymorphisms are more likely to cause ototoxic side effects after 1 cycle of cisplatin, and what other predictive factors play a role in this. DPOAE studies were performed to detect ototoxic side effects. GSTT1, GSTM1, GSTP1 polymorphism studies, laboratory tests, and DPOAE studies were performed before and immediately after the first cycle of cisplatin treatment. Based on this patient selection, increased risk groups can be determined. The estimation of the risk of ototoxic side effects caused by therapy forms several groups from this perspective. If we get to know these risk groups, targeted, personalized dosing of otoprotective agents under development can be determined. In addition, the patient can be informed more precisely about the expected side effects.

3 METHODS

As a hearing test, we performed distortion otoacoustic emission testing immediately before and immediately after the patient's treatment. Thus, we examined the acute effects. Distortion was measured in the right and left ear of patients before (B) and after (A) the first cycle of chemotherapy (1–2 hours after the end of the 5th-day infusion).

The Institutional Ethical Committee and the Hungarian Medical Research Council approved the study (323-101/ 2005-1018EKU). All patients signed an informed consent. We reviewed the patient documentation focusing on the following: previous hearing complaints, present hearing complaints, noise injuries, exposure to noise pollution, risk factors for hearing loss, smoking habits. Before the DPOAE (Madsen Capella; GN Otometrics, Taastrup, Denmark) test (as in our previous studies), a physical examination of the ear by otoscopy and tympanometry, middle ear pressure testing was performed to exclude measurement errors of external and middle ear origin. Patients who's these tests were abnormal or whose DPOAE curve before treatment was in the abnormal range were excluded from the study.

3.1 HEARING EVALUATION AND IDENTIFICATION OF OTOTOXICITY

We examined the two ears of the patients as separate data and performed DPOAE tests immediately before treatment and after 100mg/m² cisplatin treatment, which was distributed over 5 days.

Since cisplatin exerts its ototoxic side effect primarily at high frequencies (50), DPOAE measurements were performed at 2, 3, 4, 6 and 8 kHz.

The intensities of f₁ and f₂ were 65 dB sound pressure level (SPL) and 55 dB SPL, respectively, and the f₂/f₁ ratio was 1.22. The criteria for the presence of DPOAE to each frequency were as follows: The signal-to-noise ratio was ≥ 6 dB SPL. Minimum amplitude with specific values for each frequency (considering the normal range of the DPOAE device) was 2,000 Hz: 25.3 dB; 3,000 Hz: 26.2 dB; 4,000 Hz: 22.1 dB; 6,000 Hz: 25.6 dB; and 8,000 Hz: 25.6 dB. Responses were classified as abnormal when these criteria were not met. In such cases, the test was repeated to confirm the result.

Dreisbach et al. (51) concluded that “overall, a 4-9 dB change in DPOAE level is considered statistically significant for short and long term monitoring of DPOAEs at frequencies lower than 8 kHz.” According to Reavis et al. (52), the standard error of

DPOAE measurement (SEM) was defined by test-retest changes, and the 90% DPOAE shift reference intervals were calculated for each frequency. The reference intervals were as follows: ± 5.47 dB at 2 kHz; ± 5.81 dB at 3 kHz; ± 6.14 dB at 4 kHz; ± 6.92 dB at 6 kHz; and ± 5.83 dB at 8 kHz. Based on the above statement and the calculated reference intervals, we defined acute ototoxicity at a ≥ 7 dB decrease in DPOAE amplitudes.

3.2 CLINICOPATHOLOGICAL PARAMETERS

In addition to patient's age, histology result (seminoma vs. non-seminoma), disease stage (1 vs. 2–3), and blood pressure (normotension vs. hypertension) at the beginning of treatment were used in the analysis. The following laboratory data were recorded before the treatment were used for the analysis: white blood cell and platelet count, serum level of haemoglobin and haematocrit, and levels of aspartate aminotransferase, alanine transaminase, gamma-glutamyl transferase, urea, creatinine, glucose, bilirubin, Na, K, Ca, and Mg. At the end of the first treatment cycle, the white blood cell and platelet count; serum level of haemoglobin, haematocrit, and urea; and creatinine levels were available for investigation.

3.3 GENOTYPING

Blood samples were taken from patients before chemotherapy. DNA prepared as Hitre et al. described earlier (53): „Peripheral blood mononuclear cells (PBMC) were extracted from anticoagulated (10 ml EDTA 15%) blood by Ficoll gradient. DNA from the PBMCs of the patients was isolated according to the manufacturer's instructions using the MasterPure DNA purification kit (Epicentre Technologies, Madison, Wisconsin, USA). The standard protocol and reaction conditions of the polymerase chain reaction (PCR) analyses was the same as Adleeff et al. reported previously (54) : „The buffy coat was washed and red blood cells freed by a lysis step. DNA was purified by the selective precipitation procedure according to the protocol provided. DNA from PMN was resuspended in TE buffer.

GSTT1 and GSTM1 genotypes and the GSTP1 Ile105Val (rs1695) polymorphism were evaluated by multiplex PCR and the PCR-RFLP method, respectively, as described in detail by Kiran et al (55): „Multiplex PCR method was used to determine GSTM1 and GSTT1 polymorphisms in the isolated DNAs. For the GSTT1 polymorphism, forward 5'-TTCCTTACTGGTCCTCACATCTC-3' and reverse 5'-

TCACCGGATCATGGCCAGCA-3' primers were used. For the GSTM1 polymorphism, forward 5'-GAACTCCCTGAAAAGCTAAAGC-3' and reverse 5'-GTTGGGCTCAAATAACGGTGG-3' primers were used. Albumin forward 5'-GCCCTCTGCTAACAAGTCCTAC-3' and reverse 5'-GCCCTAAAAAGAAAATCCCCAATC-3' primers were used as internal controls. Albumin 350 bp, GSTM1 219 bp and GSTT1459 bp PCR products were formed. PCR conditions required denaturation for 5 minutes at 94°C and then 35 cycles as follows: 1 minute at 94°C (denaturation), 1 minute at 58°C (annealing), one minute at 72°C (elongation) and finally 10 minutes at 72°C (final elongation). Genotypes were determined by migration of the products in agarose gel with added 2% ethidium bromide. GSTP1 (Ile105Val) gene polymorphism was determined using the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method. For the GSTP1 polymorphism, forward 5'-ACCCCAGGGCTCTATGGGAA-3' and reverse 5'-TGAGGGCACAAGAAGCCCCT -3' primers were used. To identify the GSTP1 (Ile105Val) gene polymorphism among the products, the Alw 26 I (Genemark, Russia) enzyme was used. In the analysis conducted in 4% agarose gel after cutting the enzyme, genotypes were determined as follows: if the 176 bp PCR product from the GSTP1 gene was cut into two distinct products of 85 bp and 91 bp, then the genotype was identified as Val/Val; if three distinct products formed as 176 bp, 91 bp and 85 bp, then the genotype was identified as Ile/Val; and if the product was 176 bp then the genotype was identified as Ile/Ile.

3.4 STATISTICAL ANALYSES

The statistical analysis was a major challenge because such a study had not been performed with otoacoustic emission before. As described in Section 3.1, acute ototoxic effects were defined as ≥ 7 dB. In addition, unilateral and bilateral involvement were also determined. Out of 118 patients, 23 bilateral and 45 unilateral acute ototoxic side effects were detected.

All continuous and categorical data were compared in the three subgroups with one-way ANOVA (or Kruskal-Wallis) and chi-squared (or exact) tests, respectively. Post hoc tests were applied to find all pairwise differences between the subgroups. In the case of

parameters available before and after treatment, the changes were also analysed. $P < 0.05$ value was considered statistically significant. A series of logistic regressions were applied to find independent markers of unilateral and bilateral, or any ototoxicity, and, moreover, to differentiate between unilateral and bilateral ototoxicity. All variables, with $P < 0.1$ of post hoc tests in univariate analysis, were introduced in the respective logistic regression analysis to determine the independent markers of acute ototoxicity. The Hardy-Weinberg equilibrium was tested for GSTP1 polymorphism on www.dr-petrek.eu/documents/HWE.xls. The NCSS program (NCSS 12 Statistical Software (2018). NCSS, LLC. Kaysville, Utah, USA, ncss.com/software/ncss.) was used for the statistical analyses.

4 RESULTS

We identified 118 testicular cancer patients who met the criteria and underwent chemotherapy with Cisplatin + Bleomycin + Etoposide (56).

The distribution of the clinicopathological parameters of the patients are presented in Table 2. In the case of the GSTP1, there was no deviation from the Hardy-Weinberg equilibrium ($P = 0.195$). The frequency of the null genotypes of GSTT1 and GSTM1 was 21.2% and 47.5%, respectively (Table 2).

Table 2.: Hearing test results and genomic and laboratory parameters of the patients according to ototoxicity (none, unilateral, or bilateral) measured by DPOAEs. (56)

CI confidence interval, hetero heterozygous, homo homozygous mutant, n no, NA not available, y yes

* $P = 0.008$ (0 vs. 2 $P = 0.037$; 1 vs. 2 $P = 0.002$)

** 0 vs. 1 $P = 0.032$

*** $P = 0.034$ (0 vs. 2 $P = 0.02$; 1 vs. 2 $P = 0.023$)

**** $P = 0.031$ (1 vs. 2 $P = 0.007$)

Parameters	Patients presenting ototoxicity		
	None (<i>N</i> = 50) Median (95% CI)	Unilateral (<i>N</i> = 45) Median (95% CI)	Bilateral (<i>N</i> = 23) Median (95% CI)
Age (range) [years]	31.5 (29 to 36)	34 (32 to 36)	33 (28 to 40)
Histology seminoma/non-seminoma	13/37	12/33	3/20
Stage 1/2-3	17/33	20/25	12/11
Former hearing complaints n/y/NA	36/2/12	27/5/13	11/2/10
Noise injury n/y/NA	24/14/12	19/13/13	6/7/10
Noise pollution n/y/NA	23/15/12	19/13/13	6/7/10
Hearing loss risk factors n/y/NA	30/9/11	20/14/11	7/7/9
Hearing complaints before the 1st cycle n/y/NA	36/0/14	30/1/14	12/0/11
Hearing complaints after the 1st cycle n/y/NA	31/6/13	26/5/14	11/2/10
Smoking ever n/y/NA	13/25/12	13/19/13	2/11/10
Hypertension before the 1st cycle n/y/NA	31/9/10	18/11/16	10/5/8
<i>GSTT1</i> wild type/null	40/10	40/5	13/10*
<i>GSTM1</i> wild type/null	31/19	18/27**	13/10
<i>GSTP1</i> wild type/hetero/homo	21/25/4	22/19/4	11/12/0
White blood cell count before the 1st cycle [G/L]	7.5 (6.5 to 8.4)	7.5 (6.8 to 8.5)	7.3 (6.5 to 9.1)
White blood cell count after the 1st cycle [G/L]	8.5 (7.7 to 9.5)	8.7 (8.1 to 9.6)	8.1 (7.1 to 8.7)
White blood cell count change [% of before]	6 (– 5 to 12.7)	13.3 (2.5 to 26.7)	8.1 (– 3.9 to 23.2)
Hemoglobin before the 1st cycle [mmol/L]	14.8 (14.4 to 15.1)	15.1 (14.6 to 15.4)	14.7 (13.9 to 15.5)
Hemoglobin after the 1st cycle [mmol/L]	13.7 (13.1 to 14)	13.9 (13.5 to 14.2)	13.6 (12.8 to 14.3)
Hemoglobin change [% of before]	– 6.7 (– 8.9 to – 4.9)	– 7.7 (– 9.4 to – 4.9)	– 6.5 (– 10.6 to – 3.5)
Hematocrit before the 1st cycle [L/L]	0.44 (0.44 to 0.46)	0.45 (0.44 to 0.47)	0.45 (0.43 to 0.47)
Hematocrit after the 1st cycle [L/L]	0.41 (0.4 to 0.42)	0.41 (0.4 to 0.42)	0.41 (0.38 to 0.42)
Hematocrit change [% of before]	– 7.3 (– 10.9 to – 5.4)	– 8.6 (– 10.6 to – 6.5)	– 8.8 (– 12.8 to – 4.9)
Platelet count before the 1st cycle [G/L]	254 (240 to 275)	249 (229 to 266)	247 (225 to 297)
Platelet count after the 1st cycle [G/L]	268 (234 to 296)	251 (219 to 270)	221 (208 to 282)
Platelet count change [% of before]	– 3.1 (– 38 to 4.9)	0 (– 2.6 to 4.7)	– 2.7 (– 9 to 2.5)
Creatinine before the 1st cycle [μmol/L]	89 (84 to 94)	89 (81 to 95)	89 (85 to 94)
Creatinine after the 1st cycle [μmol/L]	89 (78 to 92)	82 (77 to 89)	87 (79 to 98)
Creatinine change [% of before]	– 1.8 (– 10.1 to 2.5)	– 3.2 (– 12.9 to 0)	– 5.7 (– 11.7 to 1.4)

Urea before the 1st cycle [mmol/L]	4.2 (3.9 to 4.9)	4.6 (4.2 to 5)	5.2 (4.8 to 5.9)***
Urea after the 1st cycle [mmol/L]	5 (4.6 to 5.3)	5.6 (4.9 to 6.2)	4.9 (4.6 to 6.3)
Urea change [% of before]	12.2 (0 to 28.6)	15.5 (7.1 to 35.4)	- 1.7 (- 12.9 to 15)****
Aspartate aminotransferase [IU/L]	23 (21 to 27)	24 (23 to 26)	23 (21 to 26)
Alanine transaminase [IU/L]	26.5 (20 to 30)	28 (25 to 33)	25 (20 to 31)
Gamma-glutamyl transferase [IU/L]	33.5 (25 to 40)	41 (34 to 47)	33 (27 to 40)
Glucose [mmol/L]	5.2 (4.9 to 5.4)	5.1 (5 to 5.3)	5.2 (5.1 to 5.8)
Bilirubin [μmol/L]	11.2 (8.5 to 13.5)	11.3 (9.4 to 14)	10.6 (8 to 16.2)
Na [mmol/L]	138 (137 to 138)	138 (137 to 139)	139 (138 to 140)
K [mmol/L]	4.5 (4.3 to 4.6)	4.4 (4.3 to 4.6)	4.3 (4.2 to 4.5)
Ca [mmol/L]	2.5 (2.45 to 2.54)	2.5 (2.46 to 2.52)	2.5 (2.49 to 2.55)
Mg [mmol/L]	0.85 (0.83 to 0.89)	0.84 (0.82 to 0.87)	0.85 (0.8 to 0.87)

There was a statistically significant accumulation of patients (43%) with the GSTT1 null genotype presenting with bilateral ototoxicity, while the distribution of the null genotype was lower for unaffected or unilaterally affected patients (20% and 11%, respectively). The presence of the GSTM1 null genotype was significantly more frequent in patients with only one ear involvement (60%), while this value was 38–43% for the other subgroups.

In comparison with the pre-treatment measures, white blood cell count and urea level significantly increased at the end of first cycle, while the haemoglobin, haematocrit, and creatinine levels were decreased (Wilcoxon signed-rank test) in all subgroups, except for the white blood cell count and the urea level in patients with bilateral ototoxicity.

The initial level of urea was higher in patients with acute bilateral ototoxicity than in the other two subgroups ($P = 0.02$ and $P = 0.023$). Interestingly, this high initial level of serum urea remained unchanged even after the 1st cycle of chemotherapy, while in the other subgroups, a ~ 20% increase was observed.

The following variables were introduced in the logistic regression model of 0 vs. 1 involved ear: GSTM1, “urea after,” and aspartate aminotransferase. Only the presence of the GSTM1 null proved to be an independent marker of unilateral ototoxicity with OR = 3.62; (95% CI 1.3–10.5); $P = 0.018$. The area under the receiver operating curve (ROC) was 0.624.

The GSTT1, glucose, and urea levels were used as covariates in the logistic regression model for 0 vs. two affected ears. Only the GSTT1 null was an independent marker of bilateral ototoxicity: OR = 3.6; (1.1–11.4); P = 0.029. The area under the ROC curve was 0.617.

The model for unilateral vs. bilateral involvement included the following parameters: the GSTT1, urea, glucose, and “white blood cell counts after,” while “urea change” was excluded because of a multicollinearity. Only the GSTT1 null was found to be an independent predictor of acute bilateral ototoxicity compared with unilateral damage: OR = 4.31; (2.8–4.3); P = 0.033. The area under the ROC curve was 0.706.

If ototoxicity (either or both ears involved vs. non-affected patients) was considered and age and GSTM1 were used as covariates, logistic regression resulted in no significant variables.

Out of 118 patients, 23 presented with bilateral and 45 with unilateral ototoxicity (figure 8.). In both groups, 67% of patients showed significant changes in at least one of the two highest frequencies. In 46% of affected patients, a significant DPOAE shift was detected in two or more frequencies in at least one ear.

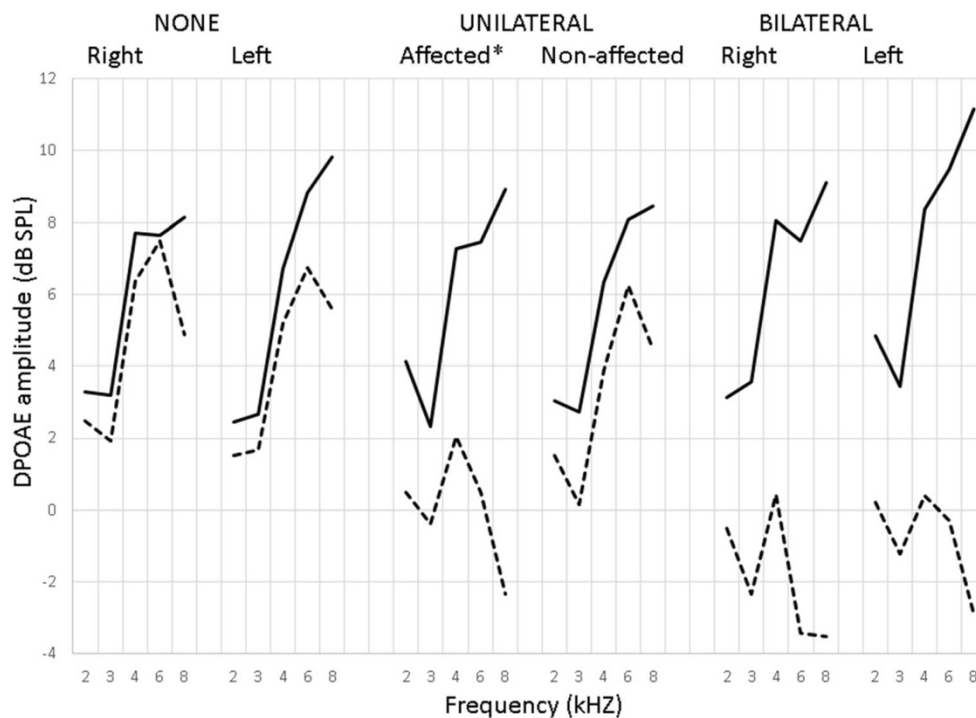


Figure 8.: results of DPOAE measurement according to the number of affected ears. The values are expressed as mean DPOAE before (solid line) and after (dotted line) the 1st cisplatin cycle. (56)

*In 23 patients the right ear and in 22 patients the left ear was affected.

5 DISCUSSION

In this study, we aimed to evaluate the potential predictive markers of cisplatin-caused acute ototoxicity as determined by DPOAEs to give further assistance to oncologists in designing individual therapies for patients at risk. DPOAE measures indicated earlier damage of outer hair cells compared with the outcomes of routine audiometric tests (57). In our previous study (46), acute ototoxicity was demonstrated by measuring DPOAEs before and after the 1st cycle of a cisplatin-based treatment in patients with testicular cancer. In the present report, we divided patients into three subgroups according to the nature of ototoxicity, i.e., no ototoxicity, unilateral toxicity, and bilateral ototoxicity. Data regarding hearing complaints, routine laboratory parameters, and three germline genetic polymorphisms (GSTM1, GSTT1, and GSTP1) were investigated. The frequencies of GSTM1 null and GSTT1 null genotypes were similar to those published for a Hungarian population (47.4% and 20.6%, respectively) (58). The distributions of GSTP1 genotypes were in Hardy - Weinberg equilibrium. We have observed that a GSTM1 null was associated with significant unilateral involvement, while the GSTT1 null was associated with a bilateral acute ototoxicity. It is likely that other non-investigated parameters can also contribute to the development of an ototoxicity, because the observed areas under the ROC curves for both markers were ~ 0.6 .

There are few reports, which have studied GST polymorphisms in cisplatin-induced acute toxicity (Table 3).

Table 3.: reviewed studies about GST polymorphisms in cisplatin-induced acute toxicity

ABR auditory brainstem response, *CHT* chemotherapy, *DPOAE* distortion product otoacoustic emission, *h* heterozygous genotype, *m* homozygous mutant genotype, *NS* non-significant, *PTA* pure-tone audiometry, *OR* odds ratio, *w*

homozygous wild genotype

*+/- vs others

**Multivariant analysis

^a Bilateral

^b Unilateral

Study	<i>n</i>	Ototoxicity	Cumulative dose m g / m ² cisplatin	Method	<i>GSTT1</i> null/null vs others	<i>GSTM1</i> null/null vs others	<i>GSTP1</i> w/h/m
Present	118	Early	100	DPOAE	OR = 4.1; <i>p</i> = 0.004 ^{a + b} (OR = 3.6; <i>p</i> = 0.029) ^{a**}	OR = 2.4; <i>p</i> = 0.032 ^b (OR = 3.6; <i>p</i> = 0.018) ^{b**}	NS
(51)	104	Early	200	PTA	NS	NS	NS
(37, 52)	54	Early	100	PTA	(OR = 6.4; <i>p</i> = 0.009)*	NS	NS
(37, 52)	38	Early	200	PTA	(OR = 6.3; <i>p</i> = 0.027)*	NS	NS
(53)	34	Any during CHT	75–600	PTA	NS	NS	–
(54)	72	Late	Median ~ 400	DPOAE+ PTA/ABR	–	–	(m vs others OR > 1; <i>p</i> = 0.03) (m vs others NS)**
(38)	173	Late	Median ~ 400	PTA	NS	NS (OR = 0.4; <i>p</i> = 0.022)**	<i>p</i> = 0.021 (m vs others OR = 0.2; <i>p</i> < 0.001)**
(56)	162	Late	Median 400	PTA	–	NS	NS
(64)	106	Late	Median 400	PTA	NS (OR = 3.5; <i>p</i> = 0.038)**	NS	<i>p</i> = 0.046 (w vs others OR = 3.8; <i>p</i> = 0.012)**
(51)	104	Late	600	PTA	NS	NS	NS
(67)	90	Late	Median 265	PTA	OR = 0.2; <i>p</i> = 0.01	NS	NS
(36)	68	Late	Median 526	PTA	OR = 0.2; <i>p</i> = 0.023 (OR = 0.1; <i>p</i> = 0.002)**	NS	–
(65)	42	Late	Mean 635	PTA	–	–	(w vs others NS, but at ≤ 4 kHz OR = 0.1; <i>p</i> = 0.02)
(55)	39	Late	Mean 236	PTA	NS	NS	NS
(37)	37	Late	600	PTA	NS (NS)*	–	–
(62)	238	Late	Median 397	Self-reported	NS	OR = 0.6; <i>p</i> = 0.025	(m vs others OR = 0.3; <i>p</i> = 0.008)**
(66)	69	Late	Median 300	Hearing aid	–	–	NS (w vs others OR = 0.3; <i>p</i> = 0.021)

To our knowledge, this is the first study investigating the role of *GSTM1*, *GSTT1*, and *GSTP1* polymorphisms in acute ototoxicity after the 1st cycle of cisplatin determined by DPOAEs in patients with testicular cancer. Khrunin et al. did not find any association between the *GSTM1*, *GSTT1*, or *GSTP1* gene variants, and ototoxicity measured audiometrically after the 2nd cycle of cisplatin (59). Talach et al. and Jurajda et al. measured ototoxicity by pure-tone audiometry and found significant associations with the

GSTT1 +/+ genotype both after the 1st and 2nd cisplatin cycles (48, 60). Barahmani et al. also investigated GST polymorphisms in patients presenting with a grade 3 audiologic toxicity during cisplatin treatment, but there was no correlation between development or time to the development of ototoxicity and the GST polymorphisms (61). After presenting these results, we can discuss those studies, which investigated the association between GST gene variants and late ototoxicity. Unfortunately, there are no investigations, which examined the association of these polymorphisms and the late-onset ototoxicity measured by DPOAEs. Olgun et al. (62) reported the use of DPOAEs; but in addition to pure-tone audiometry, unfortunately, the detailed results of DPOAEs were not reported and ototoxicity was considered based only on the audiogram. Peters et al. considered all cycles of cisplatin to define ototoxicity in 39 patients. However, even though the pure-tone audiometry was performed after each cycle, the rate of ototoxic cases was not reported separately after each treatment cycle (63). Aside from a consistently bilateral involvement, no association was reported for GSTM1, GSTT1, and GSTP1 polymorphisms. Similarly, Ross et al. (64) and Khrunin et al. (59) did not find any significant association between the above gene variants and late-onset ototoxicity. Among others, the significantly higher rate (69% vs 46% in non-affected patients) of concomitant treatments with ototoxic side effects (aminoglycosides and cranial irradiation) of patients with hearing loss (64), or patients' characteristics (women, median 52 years, ovarian cancer), and that cisplatin treatment was stopped if a grade > 1 of ototoxicity occurred (59) may mask the effect of GST polymorphisms. Talach et al. measured ototoxicity by pure-tone audiometry at the end of cisplatin treatment and found no association with any of the above polymorphisms (48). Interestingly, before and immediately after the 2nd cycle and before the 3rd cycle, they observed a significantly higher rate of ototoxic cases in patients with a wild type GSTT1 (+/+) genotype. If patients with at least one wild type allele were considered (as in our study), the association was not present. Unfortunately, unilateral and bilateral ototoxicity were not assessed. Based on our previous (65, 66) and present experiences and on reports from the literature (35, 67, 68), we presume that molecular processes that lead to acute or permanent (late) ototoxicity caused by cisplatin can differ. Therefore, comparison of results from studies on acute and chronic ototoxicity should be avoided as this may account for contradictory results in the literature. For example, Oldenburg et al. investigated hearing impairment

after > 4 years from the start of cisplatin treatment; thus, their findings (69) are not comparable with our results on acute ototoxicity. The contradiction between the results (GSTM1 or GSTT1 null genotype as a protector or facilitator for ototoxicity) relies on different physiological phenomena: early acute ototoxicity may be associated to damage of stria vascularis and supporting cells within the cochlea that have the potential to recover, whereas the damage to outer hair cells results in a permanent hearing loss (35). In some studies, ototoxicity was only assessed at the completion of chemotherapeutic treatment. Ototoxicity at that stage cannot be compared with acute ototoxicity, because the cumulated cisplatin doses ($4\text{--}6 \times$ higher than those for acute events) are similar to those presented for chronic events. The fact of incomparability was mirrored by the findings of Choeprasert et al. who measured ototoxicity by audiometry, just at the end of therapy, and the GSTT1 null proved to be protective for hearing impairment (47).

The difference between acute and late ototoxicity was also presented in studies by Talach et al. (48) and Jurajda et al. (60) where the early observed significant association of the GSTT1 genotype with ototoxicity disappeared at the end of treatment. According to the data of Table 3, the association between late ototoxicity and the GSTP1 polymorphism is very controversial. Both the homozygous mutant (49, 62, 70) and the wild genotype (71-73) were associated with ototoxicity (62, 71) or a protective effect (49, 69, 72, 73), or no effect was found (59, 63, 64, 74).

Interestingly, the GSTT1 null was found to be associated with hearing damage (presbycusis) at high frequencies, which was determined by audiometry in 50 adults (75), while the presence of the GSTM1 null genotype was associated with noise-induced hearing loss as determined by pure-tone audiometry in 889 Chinese workers (76). The latter result led us to hypothesize that our patients with the GSTM1 null genotype were also prone to noise-induced hearing loss, but a lower noise exposure threshold may be present, because of cisplatin treatment. This presumption should be investigated in further studies.

Regarding the high initial urea levels, acute dehydration can be excluded because the haemoglobin, haematocrit, serum sodium (Na^+), and potassium (K^+) levels of patients with further bilateral involvement were within the reference ranges (i.e., they were in the middle of the respective normal intervals). Impaired kidney function can also be excluded because the creatinine and the estimated glomerular filtration rate levels (calculated to

check the normal kidney function; data not shown) did not differ from those of other patients. Also, drugs (steroids, cytokines, tetracyclines, etc.), which may increase the urea levels of patients, were not initiated before chemotherapy.

Other factors, such as a high protein diet, presence of hidden infection, inflammation or stress, or other cause(s), may raise the urea levels. In order to verify if a systemic inflammation was present or not, we calculated the systemic immune-inflammation index (SII) (as the C-reactive protein was not available for our patients). There was no significant difference between patients with high vs. low urea level (data not shown), which suggested that high urea levels were not caused by systemic inflammation, or the SII was not a good marker for indicating cancer-caused inflammation in this patient group. Moreover, the yet unidentified urea increasing processes overcome the well-known urea increasing effect of cisplatin, which as a later process was observed in our patients. Further investigations should be conducted to reveal the nature of competing processes. On the other hand, a severe (bilateral) ototoxicity may be attributed to the additively enhanced reactive oxygen species formation due to existing high urea levels (70) and cisplatin treatment (77). None of the studied polymorphisms have been associated with urea level; however, our data shows that elevated level of urea at the initiation of therapy in patients, who later developed severe ototoxicity needs further prospective studies.

There are some limitations of this study. We have not investigated the combination of polymorphisms, as did other authors (49, 61), because the number of cases in the study groups would not allow enough statistical power for drawing such conclusions. There are several common laboratory parameters and markers related to ototoxicity (e.g., (64, 78)), which were not evaluated in our study, since these parameters were not available for all patients. On the other hand, the statistical power was strong in our analysis.

The role of the studied (and not evaluated in our current work) polymorphisms in predicting cisplatin-caused early ototoxicity is far from being clarified. In the future, the most important task would be to find a key to differentiate between those early ototoxic cases, which will recover, and those who are going to develop a permanent hearing impairment. It needs to be emphasized that early ototoxicity is prone to recovery in some patients (35). In contrast, other patients presenting with early ototoxicity will develop chronic hearing loss in spite of a dose reduction or changing to another platinum-

containing drug. Based on our present results, we may hypothesize that there may be several and different competing molecular mechanisms involving GST and supposedly other reactive oxygen species scavenger enzymes (77), for the development of unilateral and bilateral ototoxicity.

For the association of genotypes with unilateral and bilateral ototoxicity, we have only hypothetical explanations. The asymmetry and the genetic difference of the bilateral organs are also well-known; thus, an association of a genotype with unilateral ototoxicity is possible. Another option may be related to the unilateral noise-induced effect during treatment, which may result in hearing damage, because the ears during treatment are more sensitive to intense noise. However, this sensitivity may be related to some gene variants. Last, but not least, it is hypothesized that two molecular mechanisms with different speeds may cause ototoxicity. Due to the asymmetry of organs (which includes the asymmetry in the expression of enzymes), the slow toxicity becomes first unilateral and then bilateral. Our patients were also tested more than 1 year after treatment. According to our as yet unpublished preliminary results, in cases where the acute unilateral ototoxicity became bilateral, 50% of patients had GSTM1 null and 0% had GSTT1 null genotypes. So, this could be a GSTM1-dependent slow process. The rapid response is characteristic of GSTT1 null patients, who presented acute and also late (persistent) bilateral ototoxicity: with 67% of them being GSTT1 null. The situation is further complicated or explained by the fact that improvement is only seen in 12% of patients and only in unilateral cases, who exhibited 0% GSTT1 nulls and 67% GSTM1 nulls. Thus, the slow process involves also reversible ototoxicity, and perhaps this is why it seems slow in the development of bilateral damage. Further research is needed to clarify these phenomena.

6 CONCLUSIONS

In conclusion, GSTM1 null and GSTT1 null genotypes proved to be independent markers of unilateral and bilateral acute ototoxicity, respectively. The a priori identification of a high-risk group can serve as a basis for a better definition of individualized treatment and the targeted use of new otoprotective drugs.

In addition, we can say that distortion product otoacoustic emission is a suitable method for examining ototoxic side effects, if they occur in the inner ear, and monitoring these patients' hearing.

7 SUMMARY

I joined our working group in 2003, when we were curious to see, through a retrospective study, after how many cycles does the ototoxic side effect of cisplatin occur. We conducted our studies on testicular cancer patients treated with cisplatin because this is a young population treated with cisplatin-based chemotherapy, in whom age related hearing loss does not confound our results. We performed our hearing tests using distortion product otoacoustic emission. This is a quick test and does not require an anechoic room. But its greatest advantage is that it examines the function of the outer hair cells, which are the most critical target of the ototoxic side effects of cisplatin. 223 patients were included in the study. The results showed that ototoxic side effects can be detected after 4 cycles, i.e. a total dose of 400mg/m², years after treatment.

During our work, we noticed that, with significant individual differences, there are patients who experience hearing problems even after one cycle of cisplatin treatment. Therefore, we designed a prospective study in which we measured DPOAE in patients immediately before and immediately after 1 cycle of cisplatin treatment. 137 patients were involved. We investigated whether acute ototoxic side effects appear as a consequence of 1 cycle of cisplatin treatment. DPOAE measured before and after the first cycle of cisplatin at each frequency in right and left ear of patients resulted in significant differences 750, 6000 and 8000 Hz.

Subsequently, blood samples from 118 patients from this patient material were examined for GST gene polymorphisms. Glutathione-s-transferase is a phase II degradation enzyme, which is responsible for the degradation of cisplatin, among others. Due to the different degradation dynamics of its different efficacy phenotypes, this may be the reason for the individual differences in ototoxic side effects experienced by patients. Therefore, we examined the gene polymorphisms of the GSTP1, GSTM1 and GSTT1 enzymes in the 118 patients and based on this we created subgroups. The DPOAE results of the subgroups were compared. From our results we were able to conclude that, GSTM1 null and GSTT1 null genotypes proved to be statistically independent markers of unilateral and bilateral acute ototoxicity, respectively.

The a priori identification of a high-risk group can serve as a basis for a better definition of individualized treatment and the targeted use of new otoprotective drugs.

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9 BIBLIOGRAPHY OF THE CANDIDATE'S PUBLICATIONS

9.1 DISSERTATION-RELATED PUBLICATIONS

Budai B, Prekopp P, Noszek L, Kovacs ER, Szonyi M, Erdelyi DJ, et al. GSTM1 null and GSTT1 null: predictors of cisplatin-caused acute ototoxicity measured by DPOAEs. *J Mol Med (Berl)*. 2020;98(7):963-71.

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9.2 NOT DISSERTATION-RELATED PUBLICATIONS

Horvath A, Prekopp P, Polony G, Szekely E, Tamas L, Danos K. Accuracy of the preoperative diagnostic workup in patients with head and neck cancers undergoing neck dissection in terms of nodal metastases. *Eur Arch Otorhinolaryngol*. 2021;278(6):2041-6.

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