Tinnitus, hearing loss and inflammatory processes in an older Portuguese population

Abstract

Objective: Tinnitus is associated with various conditions such as presbycusis, infectious, autoimmune and many other diseases. Our study aims to identify an association between inflammatory markers and the presence of tinnitus or hearing loss (HL).

Design: Exploratory study including a structured interview, complete ENT observation, audiological and inflammatory markers evaluation.

Study Sample: 60 women and 54 men (55 to 75 years) from the Portuguese population, with or without sensory presbycusis and/or tinnitus.

Results: IL10 levels were significantly lower in participants with tinnitus than in those without tinnitus. Moreover, TGF-β was lower in older participants (p=.034), IL1α was higher in participants with tonal tinnitus (p=.033), and IL2 was lower in participants who reported partial or complete residual inhibition (p=.019). Additionally, we observed a negative correlation between tinnitus duration and IL10 levels (r=-.281), and between HSP70 levels and tinnitus loudness (r=-.377). TNF-α and HSP70 levels appears to be sensitive to the time when samples were collected (i.e. morning or afternoon).

Conclusions: The results of our study show fluctuations in inflammatory markers along the hearing loss process, reinforce the idea that inflammatory mechanisms are involved in hearing loss pathogenesis but also in tinnitus. IL10 levels appear significantly altered in tinnitus but not hearing loss.

Keywords: Tinnitus; Inflammatory biomarkers; ARHL (Age-Related hearing loss).

Introduction

Tinnitus is the perception of sound in the absence of acoustic stimulation and is frequently a consequence of hearing loss (HL) or activation of the somatosensory system (Mazurek et al. 2010; Shore 2011). It is frequently associated with conditions such as presbycusis, ototoxicity, infectious and autoimmune diseases, sleep disturbances, cognitive problems, psychological disorders, and many other problems and diseases (Heller 2003; Hoffman and Reed 2004; Seydel et al. 2013; Watts et al., 2018).

Tinnitus is most commonly associated with hearing loss. Many studies have linked chronic inflammation to age-related-hearing-loss (ARHL) and other age-related diseases also termed as inflammaging (Franceschi and Campisi 2014). Epidemiological studies in older adults have shown an association between long-term serum C-reactive protein (CRP) levels and hearing loss (Nash et al, 2013). Another study found a significant independent association between levels of circulating leucocytes and levels of hearing loss (Verschuur, Agyemang-Prempeh and Newman 2014). In brief, these studies show that this effect increases with age, with the strongest association among those over 75 years of age.

Inflammation occurs as the response of the organism to harmful stimuli. Inflammatory processes involve major cells of the immune system and are controlled by regulators such as cytokines and chemokines. Cytokines can be broadly classified according to their immune response as pro-inflammatory (interleukin 1 alfa and beta (IL1 α and IL1 β), IL 6, Tumour Necrosis Factor (TNF)-α and Interferon (INF)), and anti-inflammatory (IL-12, IL-10) (Turner et al. 2014).

Evidence suggests that frailty is due to a low-grade inflammatory response that persists for prolonged time, even in the absence of inflammatory stimuli, e. g. infection or injury (Hubbard et al. 2009; Leng et al. 2007; Qu, Walston et al. 2009; Qu, Yang et al.

2009). Thus, the mechanisms leading to frailty involve inflammation affecting the immune and neuroendocrine systems among others (Ferrari and Magri 2008; Poeggeler 2005; Walston et al. 2006) with inflammatory cytokines such as Interleukin (IL) 6, CRP and Tumor necrosis factor-α (TNF-α), playing an important role (Collerton et al. 2012; Leng et al. 2007; Qu, Walston et al. 2009; Qu, Yang et al. 2009).

The production of inflammatory cytokines is significantly influenced by oxidative processes (Bodamyali et al. 2000), wherein an increase in pro-inflammatory cytokines seems to be associated with a simultaneous increase in oxidative stress (Menardo et al. 2012). This influence of oxidative stress in the state of chronic inflammation can be associated with the development of premature hearing loss (Menardo et al., 2012).

Persistent tinnitus, as a consequence of hearing loss, can have a significant negative impact on quality of life originating major psychological distress (Bartels, Staal and Albers 2007). The relationship between tinnitus and distress is complex and manifests itself as the auditory attention focused on the tinnitus sound with consequent increased irritability, anxiety, depressive mood or somatic complains (Hiller and Goebel 1992; Tyler et al. 2014). Indeed, circulating levels of CRP, IL6 and TNF-α have been associated with psychological components of many disorders (Steptoe, Hamer and Chida 2007).

Tinnitus can also be regarded as a chronic stressor that affects cytokine production. Besides being associated with inflammatory or infectious diseases, changes in circulating levels of IL1 β, IL6 and TNF-α have been associated with aging, exposure to stress, and some neurological disorders (Zhang et al. 2013). In addition, serum concentrations of IL1 β (Szczepek et al. 2014) and TNF-α have been correlated to tinnitus-related distress (Szczepek et al. 2014; Weber et al. 2002). However, IL 6 does not seem to associate with tinnitus-induced distress (Szczepek et al. 2014).

The cochlear resident cells in the organ of Corti have immune competences and participate in the cochlear immune response to acoustic overstimulation (Cai et al. 2014). Disruption of gene expression related to pain and inflammation has been described as involved in noise-induced tinnitus and spontaneous hyperactivity in the cochlear nucleus (CN) (Manohar et al. 2016). Inputs from the CN leading to the disruption of the auditory-somatosensory pathway has also been suggested as a mechanism of tinnitus. This disruption results from maladaptive auditory-somatosensory plasticity, a form of axonal sprouting promoted by transforming growth factor (TGF-β) signaling, which can be inhibited by the anti-hypertensive drug losartan (Mun et al. 2018).

Cochlear and auditory nerve degeneration may elicit a chronic neuroimmune response (activation of microglia) and the up-regulation of proinflammatory cytokines such as IL1β (Fuentes-Santamaria et al. 2013) through the up-regulation of the glutamate transporter Slc17a6.

Animal studies have explored the association between Heat Shock Protein 70 (HSP-70) and the auditory system (Gong and Yan 2002; Trune et al. 1998), finding HSP-70 to be associated with an increase of autoimmune response in the inner ear (Gong and Yan 2002). Controversial results are published on the interaction between HSP-70 and HL, from no association (Trune et al. 1998), to its assumption as a prognostic marker of idiopathic sudden sensorineural hearing loss (ISSHL) (Düzer et al. 2014).

This study aims to identify associations between inflammatory markers and the characteristics, presence, or severity of tinnitus or HL, in an older Portuguese population, as potential diagnostic or prognostic markers.

Methods:

Participants

Our sample included 114 older individuals (n=60 women, n=54 men) consecutively recruited from ENT outpatient's consultation at Hospital Cuf Infante Santo, Lisbon, Portugal. Inclusion criteria were adults from the Portuguese population, of both genders, aged between 55 to 75 years, presenting with or without hearing loss and/or tinnitus. Presbycusis was defined as bilateral sensorineural hearing loss in downslope audiometric pattern, above 1000 Hz with poor speech discrimination (discrimination threshold > 40 dB SPL and 100% discrimination to 60 dB or worse).

Exclusion criteria comprised inability to understand and sign the informed consent due to a significant cognitive impairment, an uncompensated medical disorder that requires urgent evaluation, or the presence of a serious psychiatric disorder. Moreover, we excluded individuals with Ménière's disease, chronic otitis media, otosclerosis, tinnitus induced by occlusive exostosis, otitis externa, a history of immunologic, neurodegenerative or demyelinating diseases, ototoxic drugs use, massive noise exposure, or chemotherapy.

This study had the approval of the Ethical Committees from Hospital Cuf Infante Santo (26 the November, 2014), Nova Medical School (nº65/2014/CEFCM) and the National Department of Personal Data Protection (authorization number:1637/2016). The study was conducted in accordance with the Declaration of Helsinki.

Clinical Evaluation

Data collected from all participants comprised their personal clinical history (past and present), family history, and audiological assessment, including the rating of tinnitus intensity in a scale from 0 to 10 (being 10 the loudest possible). As part of the clinical evaluation, a complete ENT evaluation was performed. Epidemiologic data

(demographic, previous and present diseases, toxicological habits, and exposure to noise) were collected using a structured interview.

Audiological assessment

Pure Tone Audiometry:

Hearing thresholds were determined by pure tone audiometry (air and bone) according to ISO 8253 and 389. The exam was performed in a soundproof booth, (Model: IAC), using an Interacoustics® audiometer (Assens, Denmark; Model: AC40) and TDH39/HDA300 headphones fitted with noise-excluding headset ME70 and bone conductor B-71. Audiometry was performed at frequencies from 0.25 kHz to 16 kHz (standard tonal audiometry and extended high frequency). The category of hearing loss (HL) was defined according to the recommendations of the Bureau International d'Audiophonologie (BIAP): normal or subnormal hearing (below 20dB), mild hearing loss (21-40), moderate hearing loss (41-70), severe hearing loss (71-90), very severe hearing loss (91-119) or total hearing loss – cophosis (over 120). Pure tone average (PTA) was taken as the average threshold across 500 Hz, 1000 Hz, 2000 Hz and 4000 Hz. Frequencies not heard were recorded as 120 dB threshold. Retrieved May 15, 2018 from: http://www.biap.org/en/recommandations/recommendations/tc-02-classification/213-rec-02-1-en-audiometric-classification-of-hearing-impairments/file.

"High frequency" pure-tone average (HF_PTA) was calculated as the average thresholds across 2, 4, and 8 kHz (Newman et al. 2012).

All participants were submitted to immittance to rule out middle ear pathology (Model: Madsen Zodiac 901), so participants without type A tympanogram were excluded.

Tinnitus assessment

Psychoacoustic tinnitus evaluation:

This step was performed after audiometric testing in a soundproof booth using an Interacoustics® audiometer (Assens, Denmark; Model: AC40) and TDH39 headphones fitted with noise-excluding headset ME70. First, we checked whether the tinnitus percept was more similar to a tone or a noise, and the evaluation of tinnitus frequency was performed by offering frequencies from 125 to 16000Hz, two stimuli each time, asking the participants to choose which was more similar to their tinnitus sound. For the identification of tinnitus loudness (intensity), the elected frequency (from the previous step) was offered in an intensity similar to the hearing threshold, and loudness was gradually increased (5dB steps) until it reached the closest match to tinnitus percept.

Loudness discomfort levels (LDL):

This evaluation was performed for each ear individually, using pure tones (those from tonal audiometry) in an ascending method. The participant should state when the sound was uncomfortable (Goldstein and Shulman 2007).

Feldmann masking curves or Minimum Masking Levels (MML):

This test was performed at the frequencies where tonal audiometry was tested, using narrow band noises or pure tones (in case tinnitus was not masked by narrow band noises), using an ascending method, 5dB each step during 1-2 seconds, from hearing thresholds until the participant noticed he/she couldn't hear tinnitus. According to the spatial relation of the curves from hearing thresholds and tinnitus masking, this one was designated as: 1 - Convergent; 2 – Divergent; 3 – Congruent; 4 – Distant; 5 – Persistent (Lokenberg 2000).

Residual inhibition:

Procedure: at the identified tinnitus pitch (frequency), the participant was stimulated with a narrow band noise, 10dB above the tinnitus loudness for 1 minute. According to the responses of participants, 4 categories were possible: 1) complete (tinnitus is not audible); 2) partial (tinnitus became quieter); 3) negative (no change at tinnitus percept); and 4) "rebound" effect (tinnitus became louder). At categories 1 and 2 we measured the time that tinnitus was abolished or diminished (Coles and Hallam 1987; Goldstein and Shulman, 2007).

The severity of tinnitus was evaluated using the Tinnitus Handicap Inventory (THI) (Newman, Jacobson and Spitzer 1996). The THI is a self-administered questionnaire with good psychometrics properties (McCombe et al. 2001). It comprises 25 questions concerning tinnitus, with the response options "Yes", "Sometimes" and "No", respectively corresponding to scores of 4, 2 and 0, giving a total score that may vary from 0 and 100. The questionnaire has three sub-scales or dimensions: Functional (11 items - contributing 0-44 for the final score), Emotional (9 items - contributing 0-36 for the final score) and Catastrophic (5 items - contributing 0-20 for the final score). The total score of the responses allowed tinnitus classification according to its severity or impact in daily life: 0-16, Slight or no handicap (Grade 1); 18-36, Mild handicap (Grade 2); 38-56, Moderate handicap (Grade 3); 58-76, Severe handicap (Grade 4); 78-100, Catastrophic handicap (Grade 5).

Evaluation of inflammatory markers

Venous blood samples were collected into tubes without anticoagulant agents. Samples were allowed to coagulate for 30 minutes at room temperature and were centrifuged afterwards. After the separation from cells, sera were further divided in labeled aliquots of about 500 μL, which were frozen at -80º C until analysis. Each aliquot was used only once.

For the evaluation of IL1α, IL1β, IL2, IL6, IL10, IFN (Interferon)-γ and TNF-α, a BD CBA Flex Set (BD Biosciences, San Jose, CA, USA) bead based multiplex assay was used. The protocol was performed following strictly the instructions of the manufacturer. In brief, after the preparation of standards and other ancillary reagents, serum samples were incubated with specific capture beads for 1 hour at room temperature in flow cytometry tubes. The detection reagent was then added to the samples and incubated for 2 hours at room temperature in the dark. After a washing step, beads were resuspended and analyzed using BD FACS Canto II flow cytometer, previously set up according to the BD CBA Flex Set recommendations. A minimum of 300 beads were acquired for each cytokine in each sample. The FCAP Array Software (BD Biosciences) was used for data analysis. Standard curves covering a 0–2,500 pg/mL concentration range were generated after serially diluting reconstituted standards. To be accepted, all 10-point standard curves should present at least r^2 >99.90. Minimum detection levels were: 1.0 pg/mL for IL1α; 2.3 pg/mL for IL1β; 11.2 pg/mL for IL2; 1.6 pg/mL for IL6; 0.13 pg/mL for IL10; 1.8 pg/mL for IFN-γ; and 0.7 pg/mL for TNF-α.

A similar BD CBA Flex Set protocol was performed for TGF-β, using the Human TGF-β1 Single Plex Flex Set (BD Biosciences). The difference between this and the previous tests was that TGF-β requires activation of the latent TGF-β1 to its immunoreactive form. Therefore, the Sample Activation Kit 1 (R&D, Minneapolis, MN, USA) was used to acidify samples for 10 minutes with 1N HCL and then neutralize them using 1.2N NaOH/0.5M HEPES, according to the recommended procedure. After activation, samples were incubated with capture beads for 2 hours, washed and incubated with detection reagent. Acquisition and analysis were performed as described above. For TGF-β standard curves covered a 0–10,000 pg/mL concentration range, and minimum detection level was 14.9 pg/mL.

Finally, Heat Shock Protein 70 (HSP70/HSPA4) was assayed using the HSPA4 (HSP70) Human ELISA Kit (ThermoFisher, Frederick, MD, USA), a classical ELISA plate-based assay. Samples were assayed in duplicates, following the steps described in the manufacturer's instructions, including sample incubation with capture antibodies adsorbed in the plate, Biotinylated Antibody, Streptavidin-HRP Reagent, TMB Substrate and finally, Stop solution. After all washing and incubation steps, absorbances were assessed at 450nm in an ELISA plate reader (Stat Fax® 2100, Fisher Bioblock Scientific, France). Data were analyzed using Logit regression V21042005 free-software, available at www.xs4all.nl/~ednieuw. The Range for HSP70/HSPA4 was 2-600 ng/mL, and all mean values below the detection limit were evaluated as zero.

Statistical analysis

Descriptive analyses (absolute and relative frequencies, averages and respective standard deviations) were performed for the study sample and for the evaluated parameters. Subsequently, association analyses were performed between the presence of tinnitus and the evaluated inflammatory markers. The assumption of distribution normality was analysed with the Shapiro-Wilk test. Normal distribution was accepted for samples with a size greater than 30, and the Anova-One-Way test and the Pearson correlation coefficient were used as parametric tests. The assumption of homogeneity of variances was analysed with the Levene test. When the assumptions of the parametric tests were not satisfied, non-parametric tests were used as an alternative. Mann-Whitney (for two groups) or Kruskal-Wallis (for more than two groups) tests were employed to compare the presence and severity of tinnitus and the presence of HL. The level of significance considered was p=0.05. All the results were analysed through a logistic regression model, where age and gender where control variables. In the cases where we had missing data

we considered n=total of entries. Statistical analyses were performed in SPSS version 24.0.

Results

Sample Distribution

Our population included 114 adults with a median age of 63.0 (P_{25}=59.8, P_{75}=68.3) years old. Most of the individuals were female (n=60, 52.6%), presenting a median age of 63.5 (P_{25}=59.0, P_{75}=68.3) years old. For men (n=54, 47.4%), the median age was 63.0 (P_{25}=60.0, P_{75}=68.5) years old.

Participants were grouped primarily as 'tinnitus' versus 'no tinnitus', and secondarily, as 'with hearing loss' versus 'without hearing loss'. For some analyses, we further subdivided participants into subgroups (1) without hearing loss and without tinnitus (control group), (2) without hearing loss but with tinnitus, (3) with hearing loss but no tinnitus, and (4) with hearing loss and tinnitus (Table 1). As such we compared tinnitus (subgroup 2 + subgroup 4) with no tinnitus (subgroup 1 + subgroup 3).

[INSERT TABLE 1 HERE]

Audiological assessment

PTA and HF_PTA were higher in those with tinnitus than in those who did not have tinnitus

[INSERT FIGURE 1 HERE]

Figure 1. Pure Tone Audiometry (average curves) in the 4 subgroups.

Tinnitus characteristics

Table 2 shows clinical characterisation and the psychoacoustic characteristics of tinnitus. The mean tinnitus duration was 7.8 ± 8.6 years. Mean tinnitus intensity was 3.3 ± 1.6, on a visual analogue scale (VAS) of 1-10 (Table 2). For most participants tinnitus was central (i.e. perceived in the head) (47.8%) and tonal (53.2%). In most participants tinnitus was constant (87%). Tinnitus onset was gradual for 49% and abrupt for 19.5% of participants. Dizziness, often associated with tinnitus, was reported by 38% of participants with tinnitus, while 54.4% reported not having dizziness symptoms. In most participants, tinnitus worsened in situations where they were nervous (58.7%). Reduced sound tolerance was reported by 48.9% of participants, and 33.7% of participants with tinnitus had unprotected exposure to noise, while only four participants used protection when exposed to noise. Concerning psychoacoustic assessment, frequencies matched to tinnitus pitch ranged from 2000 Hz to 8000 Hz, with 4000 Hz being the most frequently matched. Loudness was matched to 0 dB (with a variation of + or – 5dB according to hearing threshold). Most participants reported central (52.4%) and pure tone (59.0%) tinnitus. Convergent (47.6%) and distant (29.8%) Feldmann's curve types were the most frequent. Residual inhibition was negative in 43.9% of participants and partial in 36.6%.

[INSERT TABLE 2 HERE]

Tinnitus severity was categorised by means of the THI scores. Mild handicap was the most prevalent (38 participants), followed by moderate handicap (22), slight or no

handicap (17), severe handicap (14), and only one participant had catastrophic handicap (Figure 2).

[INSERT FIGURE 2 HERE]

Figure 2. THI scores of tinnitus participants

Inflammatory characteristics

Table 3 demonstrates the mean values and standard deviation for each inflammatory parameter in the groups with and without tinnitus or hearing loss, and degree of hearing loss (n=112).

For analysis of HSP70 only 80 participants were included. The only significant difference between groups was for IL10 ($p = .025$). Between group differences in IL6 and TGF-β were not significant ($p = .052$ and $p = .064$, respectively).

[INSERT TABLE 3 HERE]

We analysed the inflammatory parameters in participants according to the presence or absence of deafness. The mean values and standard deviation of inflammatory parameters were presented according to the different degrees of deafness - normal, slight and moderate. Except for IL2 and IFN-γ, the values of the inflammatory parameters were lower in the moderately hearing-impaired group compared to the normal group and slight hearing impairment group. It is interesting to note that the mean value of several inflammatory parameters (IL1α, IL1β, IL10, IFN-γ, TNF-α and HSP70) decreased progressively as the degree of hearing loss increased. However, differences were not statistically significant.

Association tests concerning to inflammatory parameters:

Tinnitus and comorbidities

Concerning the comorbidities, only smoking was significantly associated with levels of IFN-γ (p=.041).

Clinical characterization and psychoacoustic assessment in tinnitus group:

In exploratory analyses we divided the participants according to those aged 55-64 and 65-75 years old. Between these groups there was a statistically significant difference in levels of TGF-β (U= 721.5, p= .034) (lower in the older group). There were also significant differences in IL1α (U= 577.000, p= .033) levels according to tinnitus type: IL1α values were statistically higher in patients with tonal tinnitus compared to those with narrow band tinnitus. Concerning residual inhibition, we found statistically significant differences inIL2 levels between those who did and did not experience it (H = 9,948, p = .019). Additionally, we observed a negative correlation between tinnitus duration and levels of IL10 (r = -.281, p = .007).

Correlations between matched tinnitus loudness and inflammation factors are shown in Table 4.

[INSERT TABLE 4 HERE]

There was a significant negative weak correlation between HSP70 and tinnitus loudness (r = -.397, p = .004). Because the coefficient is negative, this means that higher tinnitus loudness values were associated with lower levels of HSP70.

Presence of tinnitus and sample collection time

In a further exploration of the data, the study population was divided according to the time of collection (morning or afternoon), presence of tinnitus, and inflammatory

parameters. For 36 participants, blood samples were collected in the morning (before 11.30am) and for 78 participants blood samples were collected in the afternoon, (between 12 and 4.30pm (Table 5).

[INSERT TABLE 5 HERE]

Overall, only levels of TNF-α and HSP70 were significantly different (higher in the morning,) (Table 6).

[INSERT TABLE 6 HERE]

In the subgroup with tinnitus, IL10 and IFN-γ levels differed significantly between sample collection times (Table 5).

Modelling the data

Presence of tinnitus and inflammatory factors

Table 7 presents a logistic regression modelling inflammatory factors, age, gender, high frequency, IFN-γ and exposure to noise as confounding variables. This analysis was first performed for all participants, and then just for the 'afternoon' group. The dependent variable in the model was presence of tinnitus.

[INSERT TABLE 7 HERE]

High frequency hearing loss in both ears represented a significant risk of tinnitus in all participants and in the 'afternoon' group, 1.096 and 1.082 respectively.

Severity of tinnitus and inflammatory factors

In a logistic regression modelling inflammatory factors, age, gender, IL2 and residual inhibition were considered as confounding variables. The dependent variable in the model was severity of tinnitus, measured through THI (Table 8).

[INSERT TABLE 8 HERE]

The logistic regression revealed that residual inhibition ($p = .011$) had a significant effect on the probability of patients having severe or catastrophic tinnitus. Thus, the odds of a patient having severe or catastrophic tinnitus was higher in participants who had a negative or rebound residual inhibition, compared to those having partial or complete residual inhibition. The IL2 mean value was 0.62 pg/mL for participants with a negative or rebound effect of residual inhibition, and 0.36 pg/mL for those having a complete or partial effect of residual inhibition. Nevertheless, the difference was not significant ($p = .504$), which limits the use of this marker in the assessment of partial/complete residual inhibition.

Discussion

In this study, we have conducted an exhaustive audiological and inflammatory evaluation of older Portuguese aduls with or without hearing loss and/or tinnitus.

Studies have shown that inflammatory responses occur in the inner ear under various damaging conditions, including overstimulation with noise (Fujioka et al. 2006) and cisplatin-induced ototoxicity (Park et al. 2009). Several studies demonstrate possible relationships between inflammation and inflammatory mediators in the cochlea and the development of ear diseases such as deafness (Fujioka, Okano and Ogawa 2014).

Many inflammatory factors were measured in the current study but only IL10 emerged as significantly different, between those who do and do not have tinnitus. IL10 levels were

not significantly different between those who did and did not have hearing loss, or different levels of hearing loss, suggesting it may be a useful marker of tinnitus independent of hearing loss.

Analyses also identified some trends that warrant further investigation. Though statistical significance was not achieved, the mean value of several systemic inflammatory markers were lower (IL1α, IL10, TNF-α, and HSP70) or higher (IL2) with increasing hearing loss. Trends towards lower levels for most parameters was more pronounced in participants with more high-frequency hearing loss. Supporting this notion, in a study involving an older population, Doi and colleagues found an association between polymorphisms in the IL6 gene at region – 174G/C and susceptibility to tinnitus (Doi et al. 2015).In the current study IL6 levels were just short of significant, but there was a significant difference in IL10 levels. Epidemiologic prospective studies also confirm the association between inflammation and hearing loss (long-term serum C-reactive protein levels) in ARHL (Nash et al. 2013).

Our results have shown that tinnitus participants presented lower levels of IL10. The main source of IL10 are regulatory T cells and they target cells such as B cells and macrophages, promoting their anti-inflammatory functions by inhibiting cytokine production and the function of mononuclear cells. INF-γ is also mainly originated from T cells and influences various cells. This classical pro-inflammatory cytokine increases neutrophil and monocyte function, though according to the surrounding stimuli it may play both pro- or anti-inflammatory roles (Turner et al. 2014). Gilles et al (2017) in their genome-wide association study (GWAS) found through gene set enrichment analysis that several metabolic pathways, including those for oxidative stress, endoplasmic reticulum (ER) stress, and serotonin reception mediated signaling, may be implicated in tinnitus pathophysiology. The excessive production of ROS (Reactive Oxygen Species) and NO

can alter the ER and disrupt the electron-transport chain, causing ER stress and ROS production (Xu et al, 1999, 2004). This can activate calcium-dependent protein kinases, as well as JNK and NF-κB, leading to inflammatory responses and cell death (Malhotra and Kaufman 2007).

Finally, the statistical association between IL1α values and tonal type tinnitus may be related to specific pathophysiological mechanisms that warrant further confirmatory studies in larger study populations.

We have found more significant differences for the afternoon blood collection group, which may reflect different circadian paradigms depending on different inflammatory factors.

Several studies have shown heat shock transcription factor 1 (HSF-1) activation after injury, which in turn induces several HSP, these phenomena is diminished during ageing consequently reducing HSP cytoprotective action (Lobo et al. 2013; May et al. 2013). HSPs are present in different cell subsets. At the nervous and the immune systems, these proteins have intra- and extracellular functions with paracrine effects such as the activation of cytokines (Giffard, Macario, and de Macario 2013; Pujol and Puel 1999). On the other hand, conditions involving deficiency at the HSP system may lead to tinnitus in people with acute noise exposure (Dechesne et al. 1992). Our results open new therapeutic options regarding prevention or retardation of the mechanisms involved in ARHL and tinnitus that, although complex, are surely associated to inflammatory mechanisms. Nakamoto and colleagues suggested that the suppression of the proinflammatory cytokine HSF-1 in the cochlea by the administration of geranylgeranylacetone (GGA) may be an important way of protecting the inner ear (Nakamoto et al. 2012).

Study limitations

Blood samples in the current study were collected at different times during the day. , and for some variables this appeared to have an effect of the result. Hence, our participant samples may not be as 'homogenous' as first thought. Our results show that generally, levels of our tested inflammatory markers were higher in the morning than in the afternoon, and for TNF-α and HSP70 the differences were statistically significant. Petrovsky described a higher peak the cytokines IFN-gamma, TNF-alpha, IL-1 and IL-12 during the night and early morning (Petrovsky N., et al, 1998). Another study, regarding circadian rhythm included 30 different types of cytokines, has shown that plasma collected in the afternoon contains higher concentrations of cytokines and chemokines than serum and plasma collected in the morning (Altara et al., 2015). This apparently contradictory results reflects the need for further confirmatory studies regarding the more advisable time of the day for sample collection, which may vary according to the battery of cytokines to be studied.

Conclusions

Due to an increasing older population, it is estimated that in 2050 there will be two billion people older than 65 years of age. Results from the most recent World Health Organization (WHO) Global Burden of Diseases (2015) reports hearing loss as the fourth leading cause of years lived with disability. Given the strong links between hearing loss and tinnitus, tinnitus will surely follow this trend. In order to improve the quality of life in people with those disabilities it is imperative to invest in studies that aim to clarify the underlying causal mechanisms. Such studies will enable a more efficient prevention or treatment and avoid the progression to frailty and related mental health disabilities.

The results of our study clearly demonstrate that inflammatory mechanisms are involved not only in hearing loss pathogenesis but also in tinnitus. In addition, we have shown for the first time that the systemic concentration of IL10 is associated with the presence of tinnitus. Another interesting finding is that higher IL1α levels are associated with tonal type of tinnitus and HSP70 and IL10 are negatively correlated with tinnitus loudness and tinnitus duration respectively. Altogether our data reinforce the need for further research, not only to confirm our observations in larger samples, but also to address the pathophysiological mechanisms underlying this interplay, controlling possible confounding factors. Finally, a trend for negative correlations between many inflammatory markers and tinnitus characteristics makes it reasonable to hypothesise that inflammatory mechanisms are involved in the acute phase of tinnitus emergence.

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Tables

Table 1. Distribution of the individuals by subgroups.

Subgroup	*Audiological Characteristic*	*Gender*		*n (%)*	*Median Age, years (Median, 25th-75th percentiles)*
		Male	*Female*		
1	PTA≤20 without Tinnitus	5	12	17 (14.9%)	63.0 (59.8, 68.3)
2	PTA≤20 with Tinnitus	15	27	42 (36.8%)	
3	PTA >20 without Tinnitus	3	2	5 (4.4%)	
4	PTA >20 with Tinnitus	31	19	50 (43.9%)	
	Total	54	60	114	

PTA Pure Tone Average.

661 Table 2. Clinical characterization and psychoacoustic tinnitus assessment.

Clinical variables	*Participants with tinnitus (n=92)*
Tinnitus Duration (mean in years)	7.8 ± 8.6
Intensity of tinnitus (scale 1-10)	3.3 ± 1.6
Manifestation of tinnitus	
Constant	80 (87%)
Intermittent	7 (7.6%)
Pulsatile	4 (4.3%)
Omitted	1 (1.1%)
How did tinnitus begin?	
Gradual	45 (49%)
Abrupt	18 (19.5%)
Omitted	29 (31.5%)
Dizziness	
Yes	35 (38%)
No	50 (54.4%)
Omitted	7 (7.6%)
Does tinnitus gets worse when you're nervous?	
Yes	54 (58.7%)
No	37 (40.2%)
Omitted	1 (1.1.%)
Lower noise tolerance	
Yes	45 (48.9%)
No	47 (51.1%)
Noise exposure	
Yes, with protection	4 (4.3%)
Yes, without protection	31 (33.7%)
No	57 (62%)
Audiological measurements	*Participants with tinnitus (n=92)*
Pitch (n=83)	4000Hz (2000Hz; 8000Hz)
Loudness (n=83)	0 dB (0 dB; 5.0 dB)
Laterality	
Central	44 (47.8%)
Right	15 (16.3%)
Left	25 (27.2%)
Omitted	8 (8.7%)
Type	
Pure Tone	49 (53.2%)
Narrow Band Noise	34 (37%)
Omitted	9 (9.7%)
Feldmann's Curve	
Congruent	17 (18.4%)
Convergent	40 (43.4%)
Divergent	1 (1.1%)
Distant	25 (27.1%)
Persistent	1 (1.1%)
Omitted	8 (8.7%)
Residual inhibition	
Negative	36 (39.1%)
Partial	30 (32.6%)
Complete	13 (14.1%)
Rebound Effect	3 (3.3%)
Omitted	10 (10.9%)

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663 Table 3. Descriptive analyses of inflammatory parameters for tinnitus, hearing loss and deafness grade

Inflammatory parameters	*With Tinnitus*	*Without Tinnitus*	*p value (Mann-Whitney)*	*With hearing loss*	*Without hearing loss*	*p value (Mann-Whitney)*	*Hearing impairment grade*			*p-value (Kruskal-Wallis)*
							Normal <20dB	*Slight 21-40dB*	*Moderate 41-70dB*	
IL1α (pg/mL)	0.698±2.51	0.362±0.68	.300	0.736±2.64	2.741±17.22	.433	2.693±17.07	0.828±2.88	0.296±0.24	.768
IL1β (pg/mL)	1.424±5.40	0.810±1.85	1.000	1.535±5.68	4.637±28.02	.461	4.557±27.78	1.772±6.20	0.365±0.31	.539
IL2 (pg/mL)	0.464±1.62	0.227±0.70	.980	0.454±1.63	0.311±1.24	.171	0.306±1.23	0.428±1.74	0.657±1.01	.089
IL6 (pg/mL)	2.023±3.00	2.164±1.48	.052	5.339±19.45	1.937±3.49	.582	1.904±3.46	6.111±21.21	1.571±1.80	.647
IL10 (pg/mL)	1.175±1.30	1.843±2.51	**.025***	1.184±1.18	1.849±4.83	.470	1.827±4.79	1.273±1.24	0.747±0.66	.239
IFN-γ (pg/mL)	3.321±9.88	6.483±16.57	.116	3.985±12.29	7.461±17.60	.181	7.381±17.46	2.738±5.85	11.298±29.57	.302
TNF-α (pg/mL)	2.563±10.24	1.829±4.96	.841	2.573±10.46	5.424±26.93	.691	5.331±26.71	3.052±11.40	0.138±0.37	.391

HSP70 (ng/mL)	0.496±1.24	0.391±0.69	.827	0.396±0.96	0.531±1.24	.544	0.531±1.24	0.473±1.04	0.000	.333
TGF-β (pg/mL)	1450.609±775.71	1339.357±865.55	.064	1827.441±1254.80	1807.449±1102.73	.801	1819.252±1096.71	1861.179±1330.40	1550.385±780.86	.699

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Table 4. Correlations: inflammatory parameters and tinnitus loudness.

Inflammatory parameters	*r- value*
IL1α	-.018
IL1β	-.023
IL2	-.015
IL6	-.143
IL10	-.004
IFN-γ	.028
TNF-α	-.026
HSP70	-.397**
TGF-β	.115

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Table 5. Mean and standard deviation of the inflammatory markers in the morning and afternoon.

Inflammatory marker	*Morning period*		*Afternoon period*		
	Without tinnitus (n=2)	*With tinnitus (n=33)*	*Without tinnitus (N=20)*	*With tinnitus (n=57)*	*p- value (Mann-Whitney)*
IL1α (pg/mL)	0.745±0.96	5.131±22.73	0.307±0.65	0.346±0.54	
IL1β (pg/mL)	3.155±4.02	8.984±37.17	0.560±1.45	0.610±1.37	
IL2 (pg/mL)	0.000	0.556±2.03	0.239±0.72	0.343±1.24	
IL6 (pg/mL)	2.940±2.75	8.064±25.14	2.038±1.37	1.602±2.03	
IL10 (pg/mL)	6.300±8.72	2.186±6.11	1.347±0.60	1.032±0.87	**.032***
IFN-γ (pg/mL)	5.090±4.69	7.293±17.30	6.442±16.98	4.645±13.77	**.045***
TNF-α (pg/mL)	8.705±12.31	10.227±36.78	1.061±3.54	1.308±4.57	
HSP70 (ng/mL)	1.115±.95 (n=2)	0.682±1.12 (n=14)	0.315±0.65 (n=19)	0.438±1.28 (n=45)	
TGF-β (pg/mL)	694.370±315.22	2095.511±1402.92	1640.260±1349.39	1757.686±940.64	

667 Table 6. Inflammatory markers and collection time

	Morning		*Afternoon*		
	Mean	*SD*	*Mean*	*SD*	*Sig.*
IL1a	4.88	22.08	0.34	.57	.673
IL1b	8.65	36.09	0.60	1.39	.947
IL2	.52	1.98	0.32	1.13	.836
IL6	7.77	24.43	1.72	1.89	.845
IL10	2.42	6.20	1.11	.82	.463
IFNg	7.17	16.82	5.11	14.58	.056
TNFa	10.14	35.75	1.24	4.31	.038*
HSP70	0.74	1.09	0.40	1.13	.028*
TGF_BETA	2015.45	1401.51	1727.19	1053.51	.361

676 $^{*}p < .05$, $^{**}p < .01$, $^{***}p < .001$.

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Table 7. Logistic regression model applied to presence of tinnitus.

*Variable**	*B*	*Wald*	*OR*	*p- value*	*(95% IC)*
Sex	.015	.001	1.015	.978	(.345, 2.988)
Age	-.034	.481	.967	.488	(.878, 1.064)
High_frequency_PTA_OD_OE[a]	.092	6.502	1.096	.011*	(1.021, 1.176)
IFNg	.004	.051	1.004	.822	(.972, 1.036)
Exposure to noise	1.228	3.095	3.414	.079	(.869, 13.405)
Constant	1.416	.202	4.120	.653	
Sex	.109	.032	1.115	.858	(.337, 3.695)
Age	-.030	.310	.971	.577	(.875, 1.077)
High_frequency_PTA_OD_OE[b]	.079	4.099	1.082	.043*	(1.003, 1.168)
IFNg	.001	.002	1.001	.961	(.968, 1.035)
Exposure to noise	1.242	2.129	3.461	.144	(.653, 18.339)
Constant	1.080	.103	2.944	.749	

680 [a] whole group, [b] afternoon group * $p \leq 0,05$

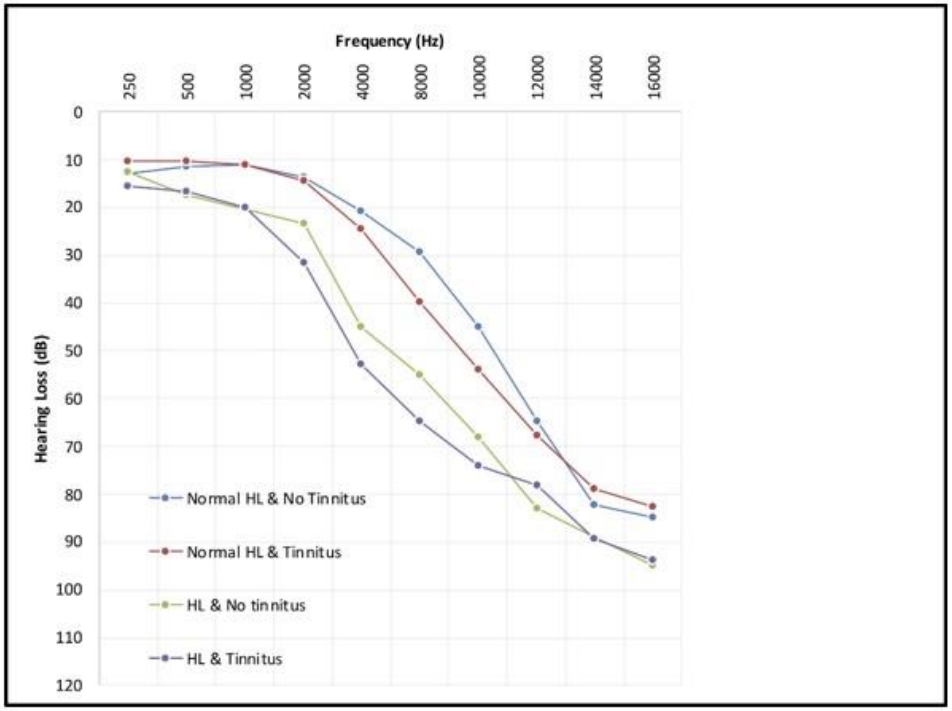
Table 8. Logistic regression model applied to severity of tinnitus and residual inhibition.

	B	Wald	OR	Sig.	*(95% IC)*
Sex (Female)	.813	.693	.535	0.367	(.138, 2.082)
Negative/rebound (1)	6.475	.728	6.381	0.011*	(1.531, 26.599)
Age	.176	.060	1.026	0.674	(.911, 1.154)
IL2	.110	.205	.934	0.740	(.625, 1.397)
Constant	1.084	3.889	.017	0.298	

681 ** $p \leq 0,05$*

Figures

Figure 1



693 Figure 2

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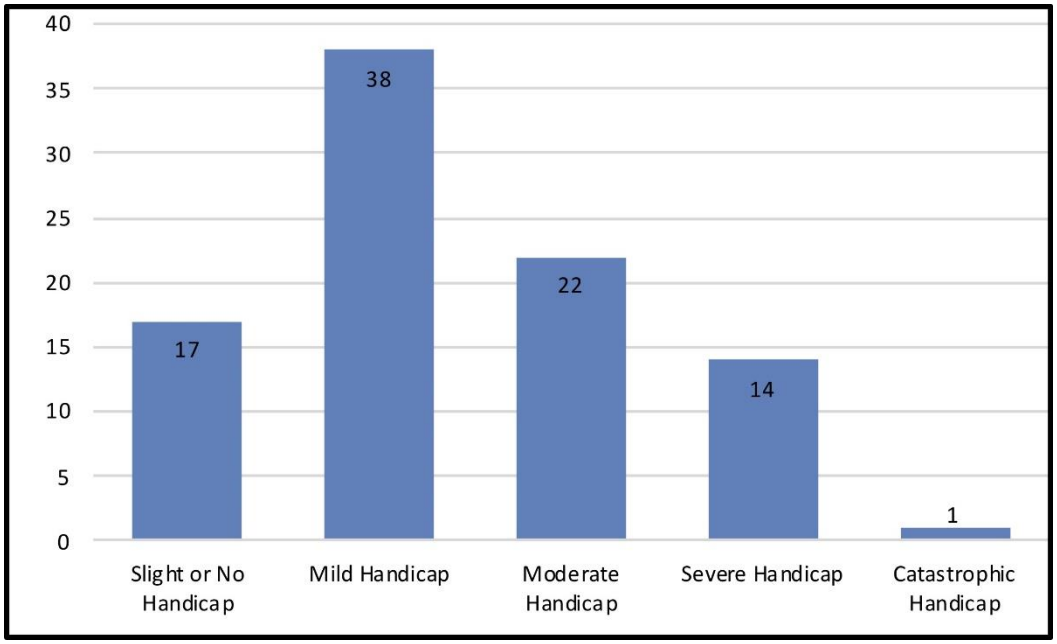

Figure Legends

Figure 1. Pure Tone Audiometry (average curves) in each of the 4 subgroups.

Figure 2. THI scores of tinnitus participants