UNIVERSITY OF SOUTHAMPTON

FACULTY OF ENGINEERING AND THE ENVIRONMENT

Hearing and Balance Centre (HABC)

The effect of inflammation in the progression of age-related hearing loss

by

Akosua Aboagyewaa Agyemang-Prempeh

Thesis for the degree of Doctor of Philosophy

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ABSTRACT

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THE EFFECT OF INFLAMMATION ON THE PROGRESSION OF AGE-RELATED HEARING LOSS

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Age-related hearing loss (ARHL) is the most common sensory deficit among older adults, with significant impact on communication, well-being and quality of life. The pathology is currently irreversible, with limited understanding of the biological mechanisms influencing it. There is evidence that a state of low-grade chronic inflammation associated with ageing is a key contributor to the progression of age-related diseases. The study investigates whether inflammation drives the ARHL pathology, as it may explain some of the variability observed in ARHL and may be the mechanism by which some chronic diseases and lifestyle factors contribute to ARHL.

The research consists of an exploration of the current literature on the mechanisms of ARHL, preliminary work with mouse model of ARHL, cross-sectional analysis of data and the first year of a three-year longitudinal study that characterizes inflammatory status among older adults to predict the progression of ARHL. The study also investigates the ability of extended high frequency audiometry and otoacoustic emission (OAE) to detect early decline in hearing function. Our findings demonstrate that high inflammatory status is associated with poor hearing in older adults and otoacoustic emissions can detect early decline in hearing function.

The findings from this research underscore the importance of monitoring and the control of inflammation in the elderly to limit the progression of ARHL and other age-related diseases. It also suggests a stratified medicine approach in the management of age-related hearing loss that takes into account individual aetiology and risk factors, and the use of OAE for screening and monitoring hearing loss in older adults. This body of work provides pilot data for a large-scale study that can further define the association between inflammation and age-related hearing loss.

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DECLARATION OF AUTHORSHIP

I, Akosua Aboagyewaa Agyemang-Prempeh declare that this thesis and the work presented in it are my own and has been generated by me as the result of my own original research.

Thesis title: The effect of inflammation in the progression of age-related hearing loss

I confirm that:

- This work was done wholly or mainly while in candidature for a research degree at this University;
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- Where I have consulted the published work of others, this is always clearly attributed;
- Where I have quoted from the work of others, the source is always given. With the exception of such quotations, this thesis is entirely my own work;
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- Where the thesis is based on work done by myself jointly with others, I have made clear exactly what was done by others and what I have contributed myself;
- Parts of this work have been published as: Verschuur C, Agyemang-Prempeh A, Newman TA. Inflammation is associated with worsening of presbycusis: Evidence from the MRC national study of hearing. *International Journal of Audiology* 2014; 53(7):469-75.

Signed: .....

Date: .....

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Above all, I thank God who has seen me through it all.

Abbreviations

ABR	Auditory Brainstem Response
ACTH	Adrenocorticotropic Hormone
AD	Alzheimer's Disease
ADL	Activities of Daily Living
ARHL	Age Related Hearing Loss
ASHA	American Speech Language Hearing Association
ATP	Adenosine Triphosphate
BBB	Blood Brain Barrier
CMV	Cytomegalovirus
CNS	Central Nervous System
CRH	Corticotropic Hormone
CRP	C-Reactive Protein
DAB	Diaminobenzidine
dB	Decibel
DP	Distortion Product
DPOAE	Distortion Product Otoacoustic Emission
EBV	Epstein Barr Virus
EDTA	Ethylenediaminetetraacetic Acid
EFH	Extended High Frequency
ELISA	Enzyme Linked Immunosorbent Assay
EP	Endocochlear Potential
GP	General Practioner

GTP	Guanosine Triphosphate
HAS	Hertfordshire Aging Study
HFA	High Frequency Average
HHIE-S	Hearing Handicap Inventory for the Elderly- Screening Version
HL	Hearing Level
HPA	Hypothalamic Pituitary Adrenal Axis
HPLC	High Pressure
ICAM	Intracellular Adhesion Molecule
IFN	Interferon
IHC	Inner Hair Cell
IL	Interleukin
iNOS	Indicible Nitrous Oxide
kHz	Kilo Hertz
LDL	Low Density Lipoprotein
LFA	Low Frequency Average
LPS	Lipopolysaccharide
MHC	Major Histocompatibility Complex
MRC	Medical Research Council
mRNA	Messenger Ribonucleic Acid
NSH	National Study of Hearing
OAE	Otoacoustic Emission
OHC	Outer Hair Cell
PET	Positron Emission Tomography
PKC	Protein kinase C
PTA	Pure-tone Audiometry

PTAv	Pure-tone Average Threshold
RIA	Radioimmunoassay
ROS	Reactive Oxygen Species
SGC	Spiral Ganglion Cell
SNR	Signal to Noise Ratio
TEOAE	Transient Evoked Otoacoustic emission
TNF	Tumour Necrosis Factor
U3A	University of Third Age
VEGF	Vascular Endothelial Growth Factor
WBC	White Blood Cell

To

My sons, Jayden and Zackry with love

Chapter 1: Ageing and hearing loss

This introductory chapter describes normal hearing and the pathology involved in age-related hearing loss. The ageing process together with certain risk factors, contribute to neurodegeneration in the auditory system, which is the hallmark of age-related hearing loss. The neurodegeneration is insidious and at a smaller scale than actual loss of nerve cells. This results in the priming of resident innate immune cells (microglia) in the auditory pathway. The presence of systemic low-grade chronic inflammation, which is associated with ageing (inflammaging) cause the primed microglia to respond in an exaggerated manner, resulting in further degeneration in the auditory pathway and the progression of age-related hearing loss.

1.1 Introduction

Age-related hearing loss (ARHL) is thought to be the combination of physiological degeneration, genetic factors and accumulated environmental insults on the auditory system (Huang & Tang 2010). It is the most common sensory deficit among older adults, characterised by reduced hearing sensitivity and speech understanding, diminished ability to localise sounds and reduced central processing of sound information (Gates & Mills, 2005). Hearing function, measured by pure-tone audiometry (PTA), is a measure of the lowest sound level a person is able to detect at octave frequencies ranging from 0.25 to 8 kHz (Stach 2010). Pure-tone average threshold, which is the average hearing threshold at frequencies 0.5, 1, 2 and 4 kHz, is commonly used to describe the degree of hearing loss, which ranges from range from normal hearing to profound hearing loss (figure 1.1). Age-related hearing loss typically presents as a progressive bilateral high frequency sloping sensorineural loss on the audiogram (figure 1.1), which over time extends to involve the lower frequency regions. The sensorineural nature means that the pathology affects both the sensory and neural elements of the auditory pathway and it presents on the audiogram as no air-gap difference between air conduction and bone conduction thresholds (figure 1.1). The loss of high frequency sounds affects the ability to hear speech in noisy environments, and when hearing loss progresses to affect mid frequency sounds, speech understanding in any listening condition becomes affected. People with ARHL will have difficulty hearing voiceless sounds such as ‘s’, ‘k’, ‘t’, ‘f’ and ‘th’ which are within the range of 2 to 8 kHz, (figure 1.1). For example they may have difficulty distinguishing between words like ‘bat’, ‘bath’ and ‘bus’, failure of which may cause miscommunications. Hearing loss has effects on an individual’s social interactions and mental health. Social isolation, loss of self-esteem and depression are some of the problems that

can result from ARHL (Gates & Mills, 2005), especially since people in such age brackets may be facing significant life changes including retirement or death of a partner.

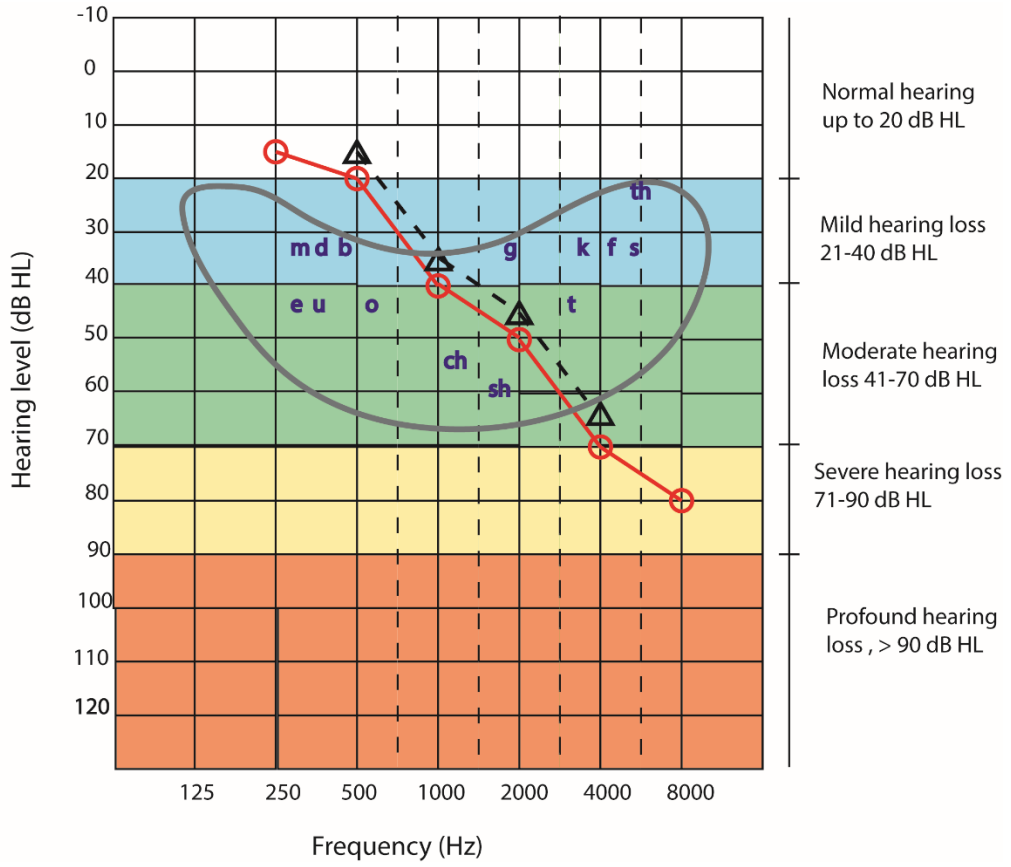

Figure 1.1: Audiogram showing high frequency sloping sensorineural hearing loss typical in ARHL. The y-axis indicates hearing level (dB HL) and x-axis frequency at octave intervals (Hz) Red circular plots represent air conduction thresholds and black triangular plots represent bone conduction thresholds. Speech banana within the audiogram shows the frequency range of common speech sounds (250 – 8000 Hz).

To date there are no ways to completely restore hearing or to slow down the progression of ARHL. Age-related hearing loss is commonly managed with hearing aids. Hearing aids are devices that amplify sounds based on a person's hearing loss configuration. The device has a microphone that picks up sound signals, which are then electronically processed and sent to a receiver or speaker to amplify the sound for the user. Although there is evidence that hearing aids improve audibility and improve the quality of life of people with hearing impairment (Kokx-Ryan et al. 2015), they do not reverse hearing loss. Moreover, there is evidence that only a third of elderly people who are eligible for hearing aids actually have them and 25% of elderly people who have hearing aids fail to use them (Mizutari et al. 2013). Some common reasons for non-usage of hearing aids include, users not deriving much benefit, finding hearing aids uncomfortable to wear, financial reasons and the perception that hearing aids make a person look old (McCormack &

Fortnum 2013). Understanding of the mechanisms involved in ARHL may lead to new forms of treatment that can potentially reduce the progression of the condition.

One of the reasons why there is no cure ARHL or modalities to reduce its progression is because the biological mechanisms involved in ARHL remains poorly understood. Although hearing loss is common during ageing, about a third of elderly individuals do not have hearing loss (Action on Hearing Loss 2015). This suggests mechanisms that drive ARHL are not inevitable. This suggests that the occurrence and progression of ARHL is variably expressed (Davis 1989). Age-related hearing loss is thought to be influenced by known factors including environmental noise, male gender, smoking and ototoxic medication (Huang & Tang 2010). People with chronic disease including diabetes, dementia and cardiovascular diseases are known to have a high incidence of ARHL (Friedland et al. 2009; Mitchell et al. 2009) and these chronic diseases are thought to be driven by the age-associated state of low-grade chronic inflammation, known as inflammaging (Franceschi et al., 2000). Inflammation may explain the link between ARHL and chronic diseases and give insights to the variability observed with ARHL. If inflammation is found to be a mechanism, through which ARHL is propagated, then controlling or preventing inflammaging may be a way of reducing the progression of ARHL. This suggests that in the management of ARHL a stratified approach that takes into consideration risk factors and biological factors should be considered.

1.2 Anatomy of the auditory system

The auditory system, which is responsible for the detection of sound, consists of the peripheral and the central auditory subsystems. The peripheral auditory system comprises the outer, middle and inner ear (figure 1.2). The outer and the middle ear has little contribution to age-related hearing loss therefore the focus of the peripheral system will be on the inner ear or cochlea (Chisolm et al. 2003).

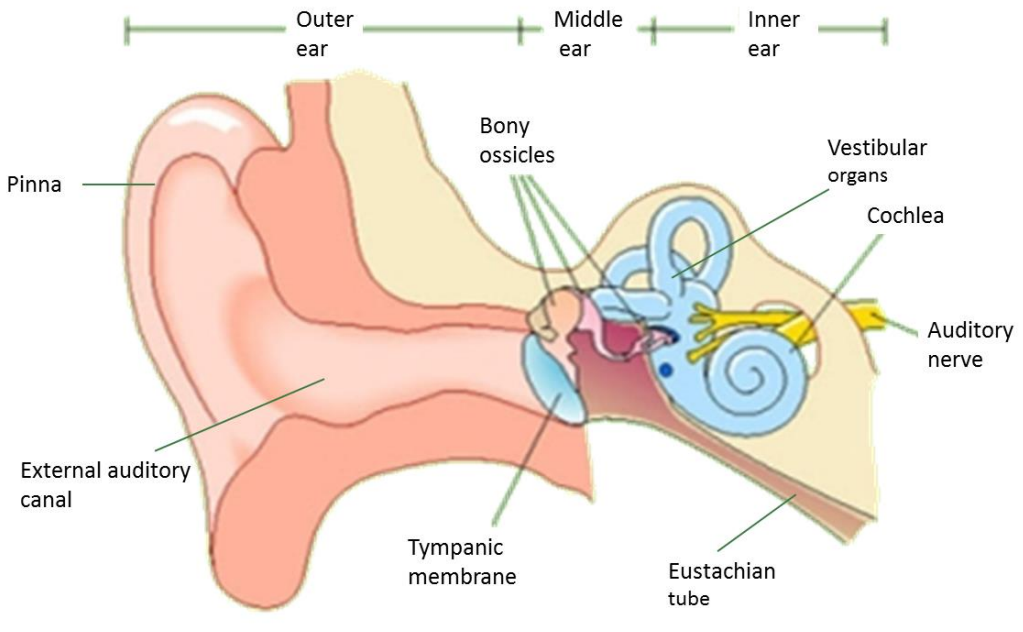

Figure 1.2: A diagram of the peripheral auditory system. The external ear consists of the external auditory canal and the pinna. The outer ear comprises the tympanic membrane and the bony ossicles. The inner ear comprises the cochlea, the auditory nerve and the vestibular (balance) system. Figure adapted from (Http://www.sltinfo.com/speech-perception/)

The cochlea is a fluid-filled coiled structure located within the temporal bone. In humans, the cochlea is about 2 3/4 turns, with a stretched out length of about 35 mm (Gelfand 2010). The cochlea is formed by a bony labyrinth, which encloses a membranous labyrinth. There are three fluid filled compartments: the scala tympani, the scala vestibuli and the scala media. The scala media contains endolymph, which is an intracellular-like fluid with high potassium concentration and low sodium concentration. The scala media is separated from the scala vestibuli by the Reissner's membrane, and the basilar membrane separates the scala media from the scala tympani. Both the scalae vestibuli and tympani contain perilymph, which is similar to extracellular fluid; it has a high sodium concentration and a lower potassium concentration. The scalae vestibuli and tympani, meet at the apex of the cochlea known as the helicotrema. A cross-section of the cochlea (figure 1.3) shows the scala media to be made of the organ of Corti, the spiroganglion cells making up the auditory nerve and the lateral wall.

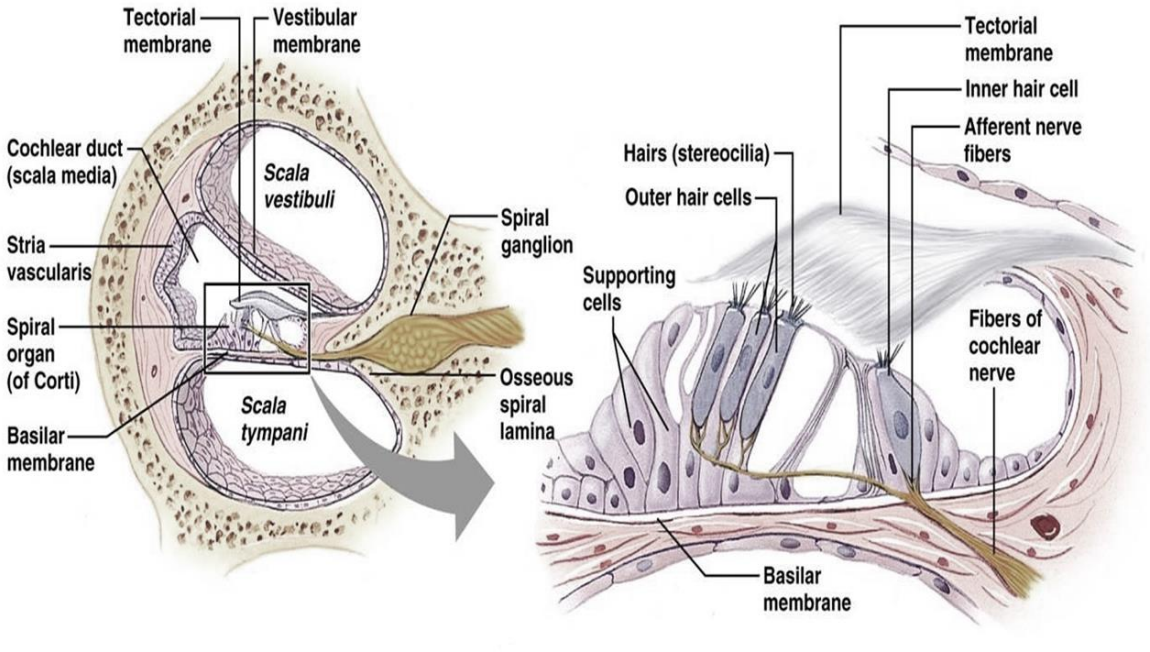

Figure 1.3 Coronal section of the cochlea. The cochlea has three main sections: the scala vestibuli, the scala tympani and the scala media. The main structures that undergo degeneration in ARHL are the hair cells, the stria vascularis and the spiral ganglion cells. The organ of Corti is zoomed out from the scala media to show its components, including hair cells and supporting cells. Figure adapted from (Cummings 2001) http://www.apsubiology.org/anatomy/2010/2010_Exam_

The lateral wall of the scala media comprises the stria vascularis medially and the spiral ligament laterally. The stria vascularis is made of three types of cells; marginal cells at the medial end, intermediate cells and basal cells at the lateral end. The marginal cells are epithelial cells derived from the membranous labyrinth. They are hexagonal shape and have their apical surface covered with microvilli. The microvilli are associated with the absorption and secretion of the endolymph to maintain the ionic composition of the endolymph (Raphael & Altschuler 2003). Their basal region is in close contact with blood vessels, which provide nutrients to drive the metabolic process. The intermediate cells are in between the marginal and the basal cells. They are thought to be important in the generation of endocochlear potential (EP). The basal cells, the most lateral cells, maintain a bridge between the stria vascularis and the spiral ligament (Raphael & Altschuler 2003).

The spiral ligament is a layer of connective tissue close to the bony labyrinth that contains vasculature that supplies the ear. It also provides mechanical support for the stria vascularis as well as holding the lateral aspect of the basilar membrane in place. The spiral ligament extends from the scala tympani to the scala vestibuli and pumps K^+ from the perilymph and transports it

to the endolymph to maintain the ionic concentration of the two fluids (Raphael & Altschuler 2003).

The organ of Corti is where the transduction of sound signals into electrochemical impulses takes place. The organ of Corti, which is made up of hair cells and supporting cells, lie on a bed of basilar membrane and connected to nerve fibres. There are two types of hair cells, outer hair cells (OHC) and the inner hair cells (IHC). The human cochlea has about 12000 OHC and 3500 IHC (Gelfand 2010). Each hair cell has projections at the tip known as stereocilia that moves in response to sound waves transduced along the basilar membrane. The main proteins that make up the stereocilia are actin, myosin VIIA and stereocilin. The stereocilia are linked to each other at their tips by tip-links and at their sides by side-links. The OHC and IHC are different both morphologically and functionally. The OHC are cylindrical in shape and more numerous while the less numerous IHC are flask-shaped. In addition, the tallest stereocilia of the OHC, the kinocilium, is embedded in the membrane that overlay the hair cells known as the tectorial membrane. The stereocilia of the IHC on the other hand are not embedded in the tectorial membrane. Functionally, the IHC is responsible for sending impulses to the auditory nerve fibres. The OHC enhance and modulate the sound signal that reaches the IHC. Hair cells are in contact with supporting cells such as Deiter's cells and Henson's cells, phalangeal cells and pillar cells. They provide mechanical support for the hair cells as well as participate in the regulation of ionic exchange within the cochlea (Raphael & Altschuler 2003). Hair cells synapse onto auditory nerve fibres to which they transmit neurotransmitters.

The organ of Corti sits on the basilar membrane. The basilar membrane is a unique connective tissue, it is less wide (150 μm) and stiffer at the base of the cochlea and gradually increases in width (450 μm) as well as reduces in stiffness as it reaches the apex. This characteristic of the basilar membrane allows it to vibrate maximally at the characteristic frequency of the travelling sound wave (Raphael & Altschuler 2003). The basilar membrane has a lateral portion known as the pectinate zone, which is the part that vibrates in response to sound. The medial portion, the arcuate zone, is partly enclosed in the spiral lamina, therefore restricted from vibrating. The arcuate zone has perforations known as habenula perforate, which houses the auditory nerve.

The auditory nerve is composed of cell bodies called spiral ganglion cells (SGCs), found in the centre of the cochlea, the Rosenthal's canal. There are two types of SGCs. Type I, which form 90-95% of the SGC population, are large, myelinated, bipolar, and are in contact the IHCs. The IHC and the SGC form a glutamatergic synapse to transmit sound information to the auditory nerve. The IHC receives 10-30 processes from type I SGCs. The much smaller, less numerous non-

myelinated type II, are in contact with the OHCs. They form part of the efferent feedback loop during sound transduction (Raphael & Altschuler 2003).

1.2.1 Normal hearing

The cochlea is tonotopically arranged, that is, each part of the cochlea detect sound of a specific frequency. High frequency sounds are detected at the base of the cochlea and low frequency sound at the apex. A sound wave arriving from the ear canal is transmitted through the air-filled middle cavity into the cochlea. The sound causes the basilar membrane to vibrate along its length, creating a travelling wave. The basilar membrane vibrates maximally at the point where the frequency of the incoming sound wave matches the characteristic frequency of the basilar membrane (Bekesy 1972). Displacement of the basilar membrane causes stereocilia on the hair cells to deflect and to allow ion channels to open for influx of K^+ and Ca^{2+} to generate a transduction current. The current generated opens voltage gated K^+ and Ca^{2+} channels in the IHC to cause depolarization of the cell with the release of the neurotransmitter glutamate into the synapse. Movement of the stereocilia in the opposite direction stops the release of neurotransmitter and repolarises the cell. The opening of voltage-gated channels elicits two types of response from the OHCs. First is the release of glutamate similar to the IHC, but to a lesser extent. The second response is contraction and elongation of the OHC which amplifies the movement of the basilar membrane, in effect enhancing the transduction of the IHC at that region (Kemp 2002). Glutamate receptors on the dendritic processes of the SGC receive the information and transmit it along the auditory nerve to the cochlear nucleus. At the level of the superior olivary complex, the sound information becomes binaural. As the sound travels through the central pathway, localization and temporal cues are added to the sound information until it reaches the auditory cortex where sound interpretation takes place.

For normal hearing to be maintained, all the components of the auditory system, must work efficiently. However, for many people, as they age, changes occur in structures of the auditory system that results in hearing loss. Due to the central role the cochlea plays by converting mechanical sound energy to nerve action potential, most of the studies that have investigated changes in ARHL have focused on the cochlea. However, there is evidence that the central auditory pathway also undergoes age-related changes that contributes to ARHL (Frisina & Walton, 2006).

1.3 Age-related changes in the auditory system

The outer and middle ear, which are responsible for mechanical conduction of sound, undergo minimal age-related changes. Notable changes include increased incidence of ear canal wax impaction, often contributed to by a reduction in the number of active cerumen glands resulting in the production of drier wax (Ruby 1986). In addition, thicker, longer hair follicles, seen especially in elderly males, may also obstruct epithelial migration and the process of wax elimination (Ruby 1986). Decrease in collagen and skin elasticity with ageing, may result in the cartilaginous portion of the external canal becoming more collapsible when earphones are placed on the pinna. Schow and Goldbaum have reported the incidence of ear-canal collapse in elderly subjects between 60-90 years as 41%, over estimating hearing thresholds by 2-8 dB (Schow & Goldbaum 1980). Notable changes in the middle ear include stiffening of the tympanic membrane and the ligaments of the ossicular chain (Rosenwasser 1964). Although age-related changes in the outer and middle ear are common, they appear to contribute very little to ARHL (Chisolm et al. 2003), which is essentially a sensorineural hearing loss .

Studies have shown changes in the cochlea that contribute to ARHL, many of the findings are based on the work of Schuknecht (1955, 1969, and 1993). Schuknecht described four structures they thought to be important for cochlea function; the organ of Corti, the stria vascularis, the auditory neuron and the vibrating portion of the organ of Corti, the basilar membrane (Gacek & Schuknecht 1969). Age-related degenerative changes to these structures led to the classification of three main pathological types of ARHL, sensory (degeneration of the organ of Corti), metabolic (degeneration of the stria vascularis) and neural (degeneration of spiral ganglion cells of the auditory nerve) (figure 1.4). The fourth pathological type, cochlear conductive, which was linked to stiffness of the basilar membrane was later found to have no scientific basis (Schuknecht & Gacek 1993). Schuknecht's work was based on the examination of ten post mortem human temporal bones using light microscopy, which he compared to their pre-mortem audiogram. The use of light microscopy for their study meant that lesions had to be substantially advanced to become detectable, therefore subtle changes in cells could have been missed. Despite this, their findings have provided the framework for other studies in this field to be built on.

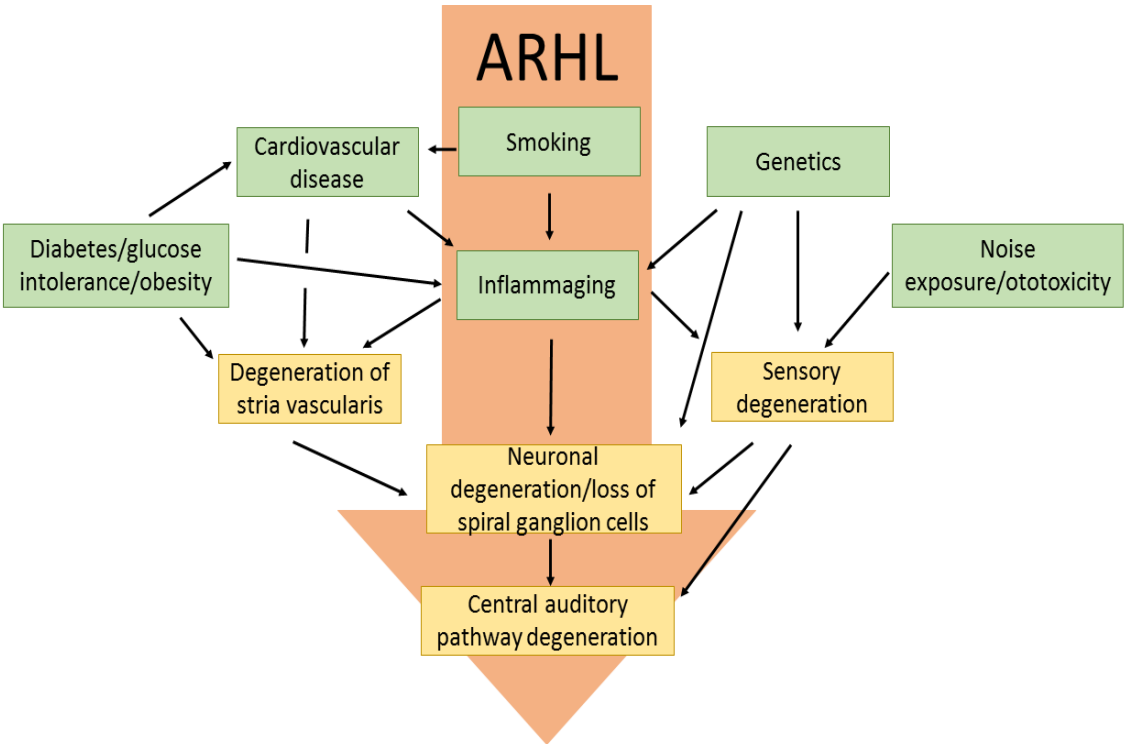

Figure 1.4: Schematic representation of how risk factors may drive the progression of the neurodegenerative process of ARHL. Different risk factors (green boxes) contribute to degeneration of the hair cells, stria vascularis, spiral ganglion cells and the central pathway, representing the pathological types (gold boxes) of ARHL to drive the progression of hearing loss.

1.3.1 Sensory ARHL

The sensory region of the cochlea is the organ of Corti, which comprises the inner hair cells (IHC) and the outer hair cells (OHC) together with their supporting cells. The OHC are responsible for the active non- linear amplification of sound while the IHC are the sensory receptors that release neurotransmitters to the auditory nerve (Kemp 2002). Degeneration of the sensory and supporting cells affect sound transduction and may also result in degenerative changes in the auditory nerve (Sugawara et al. 2005). Sensory ARHL is limited to the basal portion of the cochlea and presents on the audiogram as a steep sloping high frequency hearing loss (Schuknecht & Gacek 1993).

Sensory ARHL often starts at middle age and progress slowly through life and Schuknecht observed that subjects that fell into this category were men with histories of noise exposure (Gacek & Schuknecht 1969). Noxious agents including noise exposure and ototoxic medication, generate reactive oxygen species (ROS) in the organ of Corti, which contributes to degeneration of the hair cells (Sha et al., 2001). Reactive oxygen species (ROS) cause damage to proteins including prestin, an important constituent of OHC plasma membrane responsible for the contractility of the OHC (Chen & Zhao 2007). Since ageing is associated with increased generation

of free radicals and ROS, it is possible that ageing contribute to sensory ARHL through the generation of ROS. However, some researchers suggest sensory ARHL is the result of accumulation of lifetime environmental noise and does not truly reflect ageing (Gates & Mills, 2005; Schmiedt, 2010). The basis of this theory is that aged laboratory animals without genetic predisposition to hair cell loss that have been raised in quiet environment, have shown intact hair cells (Mills et al., 2006; Schmiedt, 2010). Suggesting a combination of genetic predisposition and noise are the cause of sensory ARHL. However, there is evidence from other studies that mice species without any genetic mutations for hair cell loss including the CBA mice show significant OHC loss as a result of ageing (Spongr et al., 1997). This relates to the wider debate in ageing research about whether ageing is as a result of pre-programmed mechanisms that are genetically determined versus whether ageing occurs as a result of damage to essential molecules needed for survival (Troen 2003). Since high frequency hearing loss, which is the main feature of sensory ARHL, is also common in ARHL, it is possible that for a group of individuals sensory loss will be the main feature of ARHL.

1.3.2 Neural ARHL

According to Schuknecht, neural ARHL was characterised by loss of spiral ganglion cells and auditory nerve fibres. Neural ARHL is believed to be influenced by genetic factors, which may also affect neurones in other CNS sites (Gacek & Schuknecht 1969). The hearing loss becomes apparent in later life when more than 50% of SGC have undergone degeneration. It is characterised by speech discrimination score that is worse compared to the audiogram.

Degeneration of SGC was previously believed to be as a consequence of retrograde hair cell loss (Glueckert et al. 2008). However, there is evidence that neurodegeneration of auditory nerve fibres can occur long before loss of hair cells or cell bodies of the SGC (Kujawa & Liberman 2009). Kujawa and Liberman (2009) have shown that CBA mice that have been exposed to noise have also shown a permanent neuronal degeneration at the level of IHC synapse even when hair cells recover. The degeneration showed as swelling of the cochlear nerve terminal 24 hours after noise exposure, loss of presynaptic ribbon and abnormal shape when ribbons were present, reduced number of postsynaptic fibres and reduced contact between presynaptic and postsynaptic terminals. In these specimens, hair cells were intact and functional measure of OHCs using otoacoustic emissions showed recovered thresholds. However, auditory brain response, which measures synchronized neuronal activity including the auditory nerve, showed reduced amplitudes (Kujawa & Liberman, 2009; Sergeyenko et al., 2013). It takes several months to years for SGCs to be completely lost, however progressive loss of neuronal structure and function (neurodegeneration) would have long begun. Similar observations have been made on old CBA

mice with ARHL (Sergeyenko et al., 2013). Therefore in the ageing ear, SGCs counts underestimate the degree of neurodegeneration (Kujawa & Liberman 2009).

1.3.3 Metabolic ARHL

Metabolic ARHL occurs as a result of degeneration of the stria vascularis. The stria vascularis being the part of the cochlea, which generates the endolymphatic potential (EP) means, its degeneration affects the cochlear amplifier (Schmiedt 2010). The resultant audiogram is a flat loss at the low frequencies and a gradually sloping high frequency loss (Schmiedt 2010). The high metabolic activities of the stria vascularis in maintaining the endocochlear potential renders it susceptible to age-related changes (Dubno et al., 2013). In addition, ageing itself is associated with chronic vascular diseases that potentially can affect vessels in the stria. Evidence from gerbils have shown the involvement of the vasculature in ARHL, stria atrophy in gerbils have been associated with areas of capillary loss (Gratton & Schulte, 1995). Studies have found a correlation between normal stria vasculature and endocochlear potential (Gratton, Schmied, & Schulte, 1996). This suggests that alterations in blood flow in the stria can affect the endocochlear potential. In a study, transient ischemia caused by 5 minutes occlusion of both vertebral arteries resulted in increased excitotoxicity from increased glutamate release, even after reperfusion (Hakuba et al., 1997). Such transient ischemic damage is known to also produce free radicals which also damage the sensory cells (Morizane et al. 2005). This could suggest that chronic ischemia associated with conditions such as atherosclerosis and type II diabetes, which are often associated with ageing, could potentially cause changes in endocochlear potential over time. This may be a mechanism by which vascular and inflammatory diseases are associated with ARHL.

Later work by Schuknecht (1993) showed that the subtypes of ARHL might co-exist in an individual; this led to another subgroup of ARHL known as the mixed ARHL. The features of some of the temporal bones Schuknecht studied did not conform to any of the subtypes were classified as indeterminate. The work of Schuknecht was limited to the cochlea however; recent evidence shows that central auditory pathway contributes significantly to ARHL.

1.3.4 Central ARHL

Central ARHL refers to age-related changes that occur in the central auditory pathway that results in decline of auditory perception or speech understanding (Humes et al. 2012). Neurodegeneration that occur in ARHL can be as a result of secondary degeneration from the peripheral pathway or can occur independently to peripheral changes (Humes et al. 2012; Mazelová et al. 2003). There is evidence of age-associated primary degeneration in structure and

function of the central auditory system (Gray et al. 2014). In CBA mice, the number of neurones responsible for minimal gap junctions in the inferior colliculus of older mice are less than for younger mice and this resulted in a slower response to stimulus in the old mice (Walton et al. 1998). This occurred without differences in peripheral auditory system between old and young mice (Walton et al. 1998). In addition, neurochemical changes have been shown in the cochlear nucleus, inferior colliculus and superior olivary complex of old macaque monkeys without peripheral changes in hearing function (Gray et al. 2014).

Some studies have suggested that neurodegeneration in the central auditory pathway is in response to loss of peripheral inputs (Frisina & Walton, 2006; Willott & Schacht, 2010). A method by which peripheral induced central degeneration may occur is through plasticity. Willot et al (2001) investigated the age-related changes in the inferior colliculus of the C57 mice. Their findings showed that in response to hair cell loss in the high frequency regions of the cochlea, neurons in the inferior colliculus of young mice that were sensitive to high frequency tones became less responsive with age. This resulted in the reorganisation of the tonotopic map of the inferior colliculus and the auditory cortex (Willot 2001). While plasticity here may be initially a beneficial compensatory action for the loss of high frequency sound input, it may become detrimental if it is unable to cope with further hair cell loss (Walmsley, 2006).

Assessing central auditory pathway deficits is difficult. Since this type of deficit does not show up on the audiogram, it is commonly assessed by speech test. However, poor speech understanding tests in the elderly could be attributed to either peripheral deficits, central auditory deficits or cognitive decline and the contribution between these three are not easily separable (Humes et al. 2012).

1.4 Age-related hearing loss in animal models

Animal models including mice, rats and gerbils have been studied extensively to give insights to the mechanisms involved in ARHL (Sergeyenko et al. 2013; Chen et al. 2009; Spongr et al. 1997). Unlike in human studies, animal models can be manipulated in a controlled way. Wide varieties of animal models enable investigations into several possible mechanisms underpinning ARHL. The characteristics of three commonly used ARHL animal models are discussed below.

1.4.1 Mouse model

Different inbred mouse strains have shown diverse ARHL pathologies, including degeneration of the organ of Corti with loss of hair cells, degeneration of the spiral ganglion cells, degeneration of the central auditory pathway and mixed pathologies (Kujawa & Liberman 2009; Spongr et al.

1997; Frisina & Walton 2006b). A common abnormality found in several inbred mice strains including the C57BL/6J is the *Ahl* (age-related hearing loss) allele on chromosome 10. Such mice are homozygous for the *Ahl* allele that codes for cadherin 23, a regulator of gated ion channels on the hair cells (Erway et al., 1993). The C57BL/6J mouse strain has been widely studied as a model of ARHL (Keithley et al. 2004; Someya et al. 2009; Stamataki et al. 2006)). The cochlea of the C57 mouse begins to undergo progressive degeneration very early in life. By age 6 months, the mouse shows significant high frequency hearing loss which progress to severe hearing loss involving all frequencies by 14 months (Kazee et al. 1995). The pathology seen is degeneration of the hair cells starting at the basal turn of the cochlea and progressing to involve the apical regions (Li & Borg 1991). Outer hair cells are more involved in the degenerative process compared to inner hair cells. In addition, loss of spiral ganglion cells are have been shown to occur throughout the life span (Li & Borg 1991). In the central auditory pathway, Kazee et al (1995) have shown progressive loss of principal neurones wit age and decrease in the number of synapses. Though these changes may result from loss in peripheral inputs, it is possible that some of the changes represent primary central auditory degeneration.

The CBA mouse on the other hand, do not show hearing loss until later in life, about 18 months (Spongr et al. 1997). They do not carry the *Ahl* gene. Loss of OHCs typically takes a U-shaped form, with greater losses at the apical and basal cochlea and minimal loss at the mid portions (Spongr et al. 1997). This is seen between months 18 to 26. The endocochlear potential remains stable till very late in life, 25 months old (Gratton et al. 1996). However, there is a steady decline of auditory brainstem response (ABR) threshold early in life without associated loss in hair cells (Fetoni et al. 2011). This is consistent with the findings that neural degeneration within the cochlea occur long before the onset of outer hair cell loss (Kujawa & Liberman 2009).

1.4.2 Rat model

The Fischer rat (F344) has been frequently used as a model of ARHL. Their relatively short life span of approximately 22 months and limited variability makes it a popular rat model for ARHL study (Chesky & Rockstein 1976; Bielefeld et al. 2010). Hearing loss begins at the high frequencies and progresses to involve the lower frequencies. Loss of OHCs is the main pathology seen in Fischer rats although degeneration of the stria occur in the *F344/DuCrl* sub strain. The loss of OHC has been shown to be as result of low prestin levels in the OHC of aged Fischer rats compared to younger rats (Chen et al. 2009). Prestin is a membrane protein present in the lateral membrane of OHC. It is responsible for the contraction and elongation that occurs during electromotility of the OHC to amplify sound. Low levels of prestin found in the OHC of aged rats is thought to be responsible for the reduced distortion product otoacoustic emission (DPOAE) response and

elevated hearing threshold (Bielefeld et al. 2010). The hearing loss pattern in the Fischer rat is consistent with sensory ARHL.

Other rat strains such as the Long Evans and the Sprague-Dawley, have been used in some studies to study the mechanisms underlying ARHL. Sprague-Evans strains exhibit loss of OHC that progresses with age and show significant spiral ganglion loss. Long Evans, which is a pigmented version of the Fischer rat, develop very little age-related hearing loss and have been used as controls in ARHL studies (Buckiova et al. 2006).

1.4.3 Mongolian gerbil

The gerbil undergoes ARHL that is consistent with strial/metabolic ARHL model (Schmiedt et al. 2002; Spicer & Schulte 2005). Gerbils develop hearing loss late in life, from 36 months; they show 15-35 dB reduction in threshold (Mills et al. 1990). The decline in hearing threshold is described as flat, with a slightly higher loss at the higher frequencies (Schmiedt 2010). The main pathology is the reduction in the endocochlear potential (EP) from 90 mV in the young gerbil to about 60 mV in the 36 month gerbil (Schmiedt et al. 2002). The generation of the EP is an active process that involves the active transport of K^+ into the endolymph using energy from Na K-ATPase pump (Schmiedt et al. 2002). The marginal cells of the stria consists of processes that produce large amounts of Na K-ATPase needed for active transport (Fetoni et al. 2011). In aged gerbils, the marginal processes become degenerative, limiting the surface area required for K^+ absorption and affecting ATP production (Spicer & Schulte 2005).

Although animal models provide insights into biological mechanisms, the results obtained are not always translatable in humans. Differences in genetic make-up may affect the ability to translate results of animal studies in human. Secondly, humans do not live in controlled environment therefore social and environmental factors may influence results. Genetic and environmental factors can combine in multiple and unpredictable ways to influence results. Therefore, in addition to animal studies, investigations involving human subjects are essential in the elucidation of human ARHL.

1.5 Evidence of Age-related hearing loss in humans

Many studies have investigated the prevalence and the progression of ARHL through both cross-sectional (Cruickshanks et al. 1998) and longitudinal studies (Brant & Fozard 1990; Davis 1989). Brant and Fozard investigated changes in hearing threshold in 813 men aged between 20 and 95 and followed up for a 15-year period. Subjects were distributed between seven age groups (20-35, 30-45, 50-65, 60-75, 70-85 and 80-95). Pure-tone thresholds of subjects were measured at

frequencies 0.125 to 8 kHz at two or more times within 15 years using Bekesy audiogram. The cross-sectional rate of hearing loss decline was estimated at 0.59 dB/year. The rate of hearing decline was highest in the 80-95 age group at 1.68 dB/year and lowest in the 50-65 age group at 0.69 dB/year (Brant & Fozard 1990). For elderly subjects, 70 years and above, the rate of decline was greatest at the speech frequencies (0.5- 2 kHz) rather than at high frequencies. A possible reason for this was that hearing was already poor at the highest frequencies at the start of the study such that further audiometric deterioration could not be measured over time. As this study was a longitudinal one, it had the advantage of overcoming possible cohort differences by measuring hearing change within individuals over time. However, no assessment of noise exposure was undertaken, as the investigators assumed that subjects were unlikely to have significant noise exposure since most of them had white-colour jobs. However, there is the possibility that occupations including army jobs and leisure activities including shooting could have impacted on hearing acuity making some of the subjects more vulnerable than others.

Research about the prevalence of ARHL has progressed from self-report studies which have reported the prevalence of hearing loss to be about 30% (Ries 1985) to epidemiological studies which have shown a much higher prevalence (Cruickshanks et al. 1998; Gates et al. 1990). One such epidemiological study is the Beaver Dam cohort comprising 3,753 participants aged 43 to 92 (Cruickshanks et al. 1998). The aim of the study was to determine the prevalence of hearing loss among adults. The average age of subjects was 65.8 years with 57.7% being female. Hearing examination included otoscopy, tympanometry, pure-tone audiometry for frequencies 0.25- 8 kHz and speech audiometry. Hearing loss was defined as average hearing threshold of greater than 25 dB at 0.5, 1, 2 and 4 kHz. The prevalence of hearing loss was estimated at 45.9% (Cruickshanks et al. 1998). Studies like Davis et al (1989) that included younger subjects (age range 17–80) had a lower prevalence rate of 25% (Davis 1989). The risk of hearing loss was found to increase with ageing and male gender. Hearing loss in males remained greater than for females after adjustments had been made for age, education, occupation and noise exposure. The audiogram configuration for the cohort was found to be sloping with increasing frequency above 1 kHz. The slope of the audiogram was found to be steeper in males compared to females. Interestingly, educational level was found to be inversely related to hearing loss; persons who did not go to college were 2.42 times likely to have hearing loss compared to people who had college education (Cruickshanks et al. 1998). This could be linked to the fact that the highly educated subjects would be likely to have white-collar jobs resulting in minimal chances of noise exposure. The study was able to highlight many important risk factors to age-related hearing loss such as age, gender, educational status, occupation, diet, smoking and chronic diseases

Another hearing epidemiological study involved 2293 subjects from the Framingham cohort (Mościcki et al. 1985). Subjects were aged 57 – 89 with females forming 59.2%. A conservative approach of hearing loss which was, hearing threshold greater than 20 dB for at least one frequency from 0.5, 1 2 and 4 kHz was used in this study. This contributed to the much higher prevalence of hearing loss of 83% that was obtained. Similar to the Beaver Dam study and other studies (Gates et al. 1990; Mościcki et al. 1985), females had better hearing compared to males. Hearing loss configuration was of a high frequency sloping nature starting above 1 kHz, which was more prominent for males than for females. Interestingly, as in the Beaver Dam study, the configuration and the differences between genders did not differ significantly after adjustments for noise exposure was performed (Mościcki et al. 1985).

1.5.1 Audiometric patterns in human ARHL

As earlier discussed, Schuknecht's work highlighted different pathologies that can exist in ARHL (Schuknecht 1955; Gacek & Schuknecht 1969; Schuknecht & Gacek 1993). Studies have found associations between cellular changes and environmental interactions that have reflected in the audiometric shape (Demeester et al. 2009; Eckert et al. 2013; Dubno et al. 2013). Auditory patterns differ among people with ARHL, and such differences potentially points to differences in pathophysiology and mechanisms of treatment. Classifying ARHL entirely on audiometric configuration is challenging as many other factors including gender, noise exposure, race and diet may affect hearing to different extents (Dubno et al. 2013).

In a study to find out the prevalence of the different audiometric shapes in ARHL, 1147 individuals age 55 - 65 comprising 52.1% females were recruited through population registry in Antwerp, Belgium (Demeester et al. 2009). Audiometric shapes were identified based on an audiometric configuration classification reported in Wuyts et al. 1998z. Flat audiogram was defined as audiogram with < 15 dB differences between mean thresholds at 0.25/0.5 kHz, 1/2 kHz and 4/8 kHz. High frequency gently sloping (HFGS) was defined as audiogram with mean threshold difference of 15-30 d B between 0.5/1 kHz and 4/8 kHz. High frequency steeply sloping (HFSS) was defined as > 30 dB difference between mean thresholds at 0.5/1 kHz and 4/8 kHz. The most commonly occurring audiogram pattern was the flat type, with 37% of subjects having this configuration, followed by HFGS pattern, found in 35% of subjects. The HFSS type occurred in 27% of subjects, 41% of which were males. The flat audiogram was commonly associated with female subjects (Demeester et al. 2009). Flat shaped audiogram has been linked to strial type of hearing loss and has been shown in studies to be common in females and have a high heritability (Gates et al. 1999). Similar to the findings in other studies (Brant & Fozard 1990; Cruickshanks et al.

1998), steep sloping audiogram pattern in males did not change after exclusion of subjects with history of noise and solvent exposure.

A second study by Dubno et al (2013) investigated the different audiometric shapes in humans based on the different audiometric configurations based on Schuknecht's work as well as configurations obtained from animal studies, and correlated the shapes obtained with subjects' demographic information. Analysis was performed on 1728 audiograms collected from subjects aged 50-97 years, of which 55.6% were females. Audiograms were classified as older-normal, pre-metabolic, metabolic, sensory and a mixed metabolic-sensory. The definitions are as follows:

- Older-normal: audiograms with thresholds ≤ 10 dB from 0.25 - 1 kHz and ≤ 20 dB at high frequencies.
- Pre-metabolic: thresholds ≤ 10 dB from 0.25 - 1 k Hz and 10-25 dB at high frequencies
- Metabolic: flat audiogram from 10 - 40 d B at lower frequencies and 30 - 60 dB at higher frequencies with slope 10-20 dB/octave.
- Sensory: thresholds at low frequencies ≤ 10 dB, thresholds at high frequencies 40 -70 dB with slopes >20 dB/octave.
- Metabolic-Sensory: thresholds at low frequencies 10 - 40 dB and a steep sloping high frequency loss with slopes >20 dB/octave.

Out of the 1728 audiograms, only 338 fit precisely into the four patterns (pre-metabolic pattern was dropped since only few subjects fitted into this category). Eleven percent of the audiograms were classified as older normal, 25% as metabolic, 23% as sensory and 41% as metabolic-sensory (Dubno et al. 2013). The following conclusions were drawn from their results: subjects in the older-normal and the sensory groups were younger than subjects in the metabolic and metabolic-sensory groups. Subjects in the sensory group were predominantly males and were more likely to have had a positive history of noise exposure. However contrary to the results of other studies (Gates et al. 1990; Cruickshanks et al. 1998), females did not have better hearing compared to men.

Some studies have shown a connection between flat audiometric shape and female gender (Eckert et al. 2013; Dubno et al. 2013; Gates et al. 1993). This has led to the suggestion that hormonal influences may play a part in strial type of ARHL. Konig et al (2008) have demonstrated in a study that megalin, an endocytic receptor for lipophilic metabolites including oestrogen, is strongly expressed within the marginal cells of the stria vascularis in the inner ear. In addition, homozygous megalin knock out mice showed severe ARHL (Konig et al. 2008). This suggests that oestrogen deficiency may have a significant role ARHL in post-menopausal women. This is further supported by the fact that post-menopausal women show greater hearing loss than

premenopausal women and women taking hormone replacement therapy (Hederstierna et al. 2007). There is evidence that cardiovascular disease is associated with hearing loss (Friedland et al. 2009). This is further supported by the fact that vascular and inflammatory health has an impact on ARHL (Eckert et al. 2013). Studies have shown that the association between vascular health and hearing loss is for low frequency hearing (Gates et al. 1993). This association appears to be present in females rather than males (Gates et al. 1993; Eckert et al. 2013).

1.5.2 Impact of age-related hearing loss

The high prevalence of ARHL, coupled with increasing ageing population has made ARHL a social and health problem. Presently, there are more than 11 million people living in the UK with hearing loss and more than two thirds are aged above 60 years (Action on Hearing Loss 2015). This number is expected to rise due to high life expectancies. Difficulty in communication associated with ARHL can lead to adverse effects including social withdrawal, anxiety, low self-esteem and depression (Gates & Mills 2005). The insidious nature of ARHL may allow people to adapt to the hearing loss by adding increments to volume without seeking professional help early (Carson 2005). There is also the perception that ARHL is a rite of passage for old age, which may prevent people from seeking help. Communication difficulties in ARHL are not restricted to the person with ARHL, partners, family members and friends are affected. This causes strain in relationships and may contribute to social isolation (Carson 2005).

Dalton et al. (2003) investigated the impact of ARHL on the quality of life using the Beaver Dam cohort. The prevalence of hearing loss among the 2,688 participants of average age 69 was 51%. Twenty-four percent had mild hearing loss and 23.8% had moderate to severe hearing loss based on average pure-tone thresholds at 0.5, 1, 2 and 4 kHz. Mild hearing loss was defined as average pure-tone thresholds between 25 and 40 dB, moderate hearing loss, 40 - 60 dB and severe hearing loss > 60 dB. The screening version of Hearing Handicap for the Elderly (HHIE-S) was used to assess the level of handicap caused by hearing loss and Activities of Daily Living (ADL) was used to assess quality of life of subjects. The study found that severity of hearing loss was significantly associated with a greater handicap in communication and a lower quality of life (Dalton et al. 2003).

There is evidence that hearing loss is associated with cognitive decline including Alzheimer's disease (AD) (Lin et al. 2011). In the Lin et al (2011) study, audiometric data were measured for 639 subjects between age 36 and 90 with no history of dementia. Follow up after 11 years showed that the risk of dementia, measured with a battery of mental capacity tests, was higher for subjects with greater baseline hearing loss compared with people with normal hearing (Lin et

al. 2011). This association was significant independent of other factors including age, gender, race, education, smoking and chronic diseases. The rate of cognitive decline in a separate study has been found to be 54% faster in people with hearing loss compared to normal hearing individuals (Gurgel et al. 2014). This has led to the suggestion that ARHL may be a risk factor to dementia. An explanation to these findings may be that ARHL and dementia may have common neuropathological pathways through which they occur. Others have suggested that depletion of cognitive reserve and social isolation may be another way in which ARHL and dementia are associated (Lin et al. 2011). Cognitive reserve is an individual's ability to cope with neuropathology (Stern 2002). It has been suggested that during hearing loss, a great portion of cognitive resources are allocated to auditory processing, resulting in loss of neural input into other cognitive processes such as memory. This results in the decline of cognitive reserve and possibly leads to early manifestation of dementia (Boyle et al. 2008). Social isolation associated with hearing loss may also be a contributor to the prevalence of dementia in people with ARHL, as there is evidence that perceived loneliness is associated with cognitive decline (Holwerda et al. 2014; Cacioppo & Hawkley 2009). Other factors including the possible over diagnosis of hearing loss in dementia patients and the presence of other risk factors such as vascular disease or trauma cannot be ruled out as the reason for the link between hearing loss and dementia.

1.6 Risk factors

Age-related hearing loss is viewed as the result of the combination of intrinsic ageing of the auditory system superimposed with genetics, gender and other environmental factors (Gates & Mills, 2005). The mechanisms by which these risk factors contribute to ARHL are intricately connected. Some of the environmental factors such as smoking and chronic diseases are believed to affect ARHL by contributing to the development of pro-inflammatory state. The interaction between genetics and environment can result in variable effects on the auditory system of the elderly (Schmiedt, 2010). An understanding of the contribution of the risk factors is essential for elucidating the mechanisms involved in ARHL. This section of the report explores some important predictive factors of ARHL.

1.6.1 Age

Age is the most important risk factor in ARHL and many studies have recorded progressive hearing loss with age (Gates, Couropmitree, & Myers, 1999; Helzner et al., 2005). Ageing results in changes at the cellular, tissue, systemic and organismic levels that differ from species to species (Troen 2003). Since ageing is associated with decreased physiological reserve and changes in

biochemical composition (Troen 2003), features of ARHL including loss of hair cells and loss of spiral ganglion cells and degeneration of sensory cells can be attributed to normal ageing.

The association of ageing with mitochondrial dysfunction and the formation of reactive oxygen species (ROS) may affect ARHL. Mitochondria are organelles found in the cells of eukaryotic organisms. They are the powerhouse of the cell since they supply the cell with energy required for amplification and transmission of sound. Therefore disruption in energy production, as seen in mitochondrial disorders have an adverse effects on the cochlea (Someya & Prolla 2010). This may be observed in the growing body of evidence that mitochondrial mutations is associated with ARHL (Someya & Prolla 2010; Crawley & Keithley 2011). In a study to investigate the effect of mitochondria mutations on cochlear pathology and hearing, transgenic D257A mice with polymerase deletion in their mitochondrial DNA were compared to wild type. Hearing and histological tests performed on groups of mice at monthly intervals from age 3 to 10 months revealed that by age 7 months, mice with mitochondrial mutations demonstrated a worse hearing threshold at all frequencies compared to the wild type. Also at age 10 months histological findings revealed a severe degeneration and clumping together of spiroganglia cells in the mice with mitochondrial mutations; none of the younger mice or wild type mice had showed such clumping (Crawley & Keithley 2011). The mechanisms through which mitochondrial mutations affect hearing remains to be elucidated. In addition, it was not clear from the study mitochondrial mutations had effects on hair cells or the stria.

Apart from mutations, there is evidence that ROS contribute to aging and ARHL. Reactive oxygen species are produced by both young and old cells and their effects are counteracted by antioxidants. However, in aging cells the production of ROS exceeds its elimination by antioxidants, leading to oxidative stress (Van Eyken et al., 2007). The mitochondrion is a site for ROS production. Reactive oxygen species cause damage to mitochondrial DNA and proteins required for respiratory chain reactions (Tanaka et al., 2012). A mitochondrial pro-apoptotic gene *Bak* has been found to be expressed in oxidative stress and deletion of this gene prevents apoptosis (Someya et al. 2009). A study by Someya et al (2009) found that in C56BL/6J mice with deletion of the *Bak* gene, their cochlear cells were resistant to cell death caused by increased production of superoxide ions compared to wild type (Someya et al. 2009). Tanaka et al., (2011) also showed that in Fisher rats, increased expression of genes that regulate oxidative stress in older rats, also correlated with hearing measurements (Tanaka et al, 2011). These studies suggest that ROS mediated damage may be associated with ARHL.

The fact that hearing loss becomes common with age means ARHL has been viewed as a ‘rite of passage’ of ageing. However, ARHL is not universal, as about a third of elderly individuals have

good hearing (Action on Hearing Loss 2015). Secondly, the rate of hearing loss is not linearly associated with age and can be highly variable. Age alone account for less than 10% of the variance (Gates et al., 1990). This suggests there are other factors apart from ageing that influence the progression of ARHL.

1.6.2 Genetics

Murine models have been used extensively in an attempt to unravel the genetic contribution of ARHL, this is mainly because of the similarity between the mouse auditory system and that of humans (Ohlemiller, 2006). In particular, the CBA and C57BL/6J mice strains have provided insights into genes involved in ARHL. Recessive alleles for three different loci which contribute to ARHL have been identified in the C57BL/6J mice, while CBA mice which have relatively good hearing until old age have none of these recessive alleles (Erway et al., 1996). Mutations of the *Ahl* allele on the Cadherin23 gene (Cdh23) occur in the C57BL/6J mice which makes it susceptible to ARHL (Erway et al., 1993). Cadherins are a family of intercellular adhesion proteins that provide adhesions via calcium dependent interactions (Bork et al. 2001). In the cochlea, cadherins hold the tip-links of the stereocilia together to allow for influx of ions during depolarisation of the hair cells. In humans, cadherin23 expression has been demonstrated in the retina and the cochlea, mutations of which causes non-syndromic deafness and Usher's syndrome (Bork et al. 2001). Presently, there is no evidence to show that mutations in cadherin cause ARHL in humans.

Many people who have ARHL often have a family member with similar condition. Gates et al (1999) investigated the heritability of ARHL by analysing hearing data, using PTA, for 1232 family members and 1079 unrelated subjects. Their findings revealed that hearing thresholds of genetically related people were more similar compared to unrelated people living within the same locality. Based on audiometric shape, they estimated 35-55% heritability for sensory ARHL and 25-42% for strial ARHL. Heritability was higher in female relations compared to male relations, especially low frequency or strial ARHL. The reduced inheritability of hearing loss in males was attributed to the fact that males were generally more likely to have exposure to occupational and recreational noise, which could have a greater impact on hearing (Gates, 1999). Although this study gives insights into genetic contribution to ARHL and uses a large sample size, it does not differentiate between purely genetic effect and interactions between genetics and environmental factors.

1.6.3 Gender

Evidence from literature shows gender differences in age-related hearing loss. Women have been found to have worse low-frequency hearing compared to men but better high frequency hearing (Gates et al., 1999; Helzner et al., 2005). Hearing loss is reported to be twice as fast in men as in women (Pearson & Morrell 1995). Decline in hearing has been shown to begin at age 30 at most frequencies in men and at age 40-50 in women (Pearson & Morrell 1995). Accelerated hearing loss in men has been attributed to the liklihood of increased noise exposure (Helzner et al. 2005) but even when subjects were screened for noise exposure and excluded from the study, men still had a steeper progression of hearing loss compared to women (Pearson & Morrell 1995).

There is some evidence that oestrogen may play a role in the gender disparities. Oestrogen receptors have been found in the inner hair cell, outer hair cell, stria vascularis and spiral ligament of rodents and humans which may have a protective effect in premenopausal women although the exact mechanism is not known (Stenberg et al. 2002). This is supported by the evidence of better auditory brainsten response (ABR) latencies in premenopausal women than in men but after menopause the female advantage is lost (Stenberg et al. 2002); a situation which cannot be explained by occupational noise in men Another reason for the suggested hormonal influence is found in Turner's syndrome, a chromosomal abberation resulting in dysfunctional ovaries and therefore no oestrogen production. In such individuals evidence of ARHL begins early in life (Wang et al, 2001). Similarly mice with a knockout of their estrogen receptor results in severe hearing loss (Wang et al. 2001).

1.6.4 Noise exposure

Accumulation of environmental noise exposure has been associated with ARHL (Gates et al., 2000; Kujawa & Liberman, 2009). Two factors, the characteristics of the noise and the sound transmission properties of the ear canal and middle ear determine the pattern of noise-induced hear hearing loss. Noise exposure typically appears on an audiogram as a notch in the 3-6 kHz region (Cooper & Owen 1976). A longitudinal study by Gates et al (2000) observed that people with hearing loss because of previous noise exposure had subsequent progression of hearing loss with age that greatly involved frequencies not affected by noise exposure. This pattern of ARHL was different from people with no previous noise induced hearing loss. The study compared audiometric findings of elderly subjects (mean age 64.1 years) at baseline to their hearing thresholds 15 years later (mean age 78.5 years). Notch depth at 3 – 6 kHz was used to classify subjects into three groups: no evidence of noise damage (notch depth < 15 dB), possible noise damage (notch depth between 15 and 35 dB) and noise damage (notch depth >35 dB). Their

results showed that subjects with greater noise depth showed very little change in frequencies 3-6 kHz; however, hearing loss progressed to involve lower frequencies (1-2 kHz) compared to subjects who had no noise exposure (Gates et al, 2000). There are two possible reasons for this, first, the high frequency damage is so great to notice any difference or secondly, once hearing loss pathology starts it sets the auditory pathway up for further damage. It is possible degeneration of OHC caused by noise exposure induces inflammatory changes in resident microglia in the cochlea (Hirose et al., 2005) to become primed for further damage . This suggests that damage from noise may persists even after the noise exposure has stopped. A limitation of this study was that it assumed that there was no noise exposure between the 15-year-period between tests. Secondly, the study assumed that noise notch was solely due to noise but there is evidence that notches on the audiogram can be observed in people without a history of noise exposure (Nondahl et al. 2009).

Physical effect of noise exposure are visible in the organ of Corti including breaking-off of hair cell stereocilia itself or their tip-links, fusion of the tip-links and damage to supporting cells including pillar cells and Henson's cells (Henderson et al., 2006). Damage to the OHCs result in altered function as mechanoelectrical transducers, initially, resulting in temporal threshold shift but may become permanent with persistent damage (Henderson et al. 2006). Although high noise intensity may rip-off stereocilia and hair cells, the damage is often more insidious. There is evidence that the mechanism of damage is through the generation of ROS (Ohlemiller & Dugan, 1999). Electromotility of OHC requires a lot of energy, during noise exposure, there is high demand from mitochondria to provide energy through aerobic respiration, which produces ROS as a by-product (Henderson et al. 2006). Reactive oxygen species build up over time and cause chain reactions of lipid peroxidation in the plasma membrane of OHC and other cells of the organ of Corti (Le Prell et al., 2007) resulting in degeneration of the organ of Corti. Evidence shows that apart from damage to the organ of Corti, noise exposure may also result in damage to neural tissues (Kujawa & Liberman 2009). Noise exposure causes production of high amount of glutamate release by IHC in response to multiple action potentials generated by excessive noise (Hakuba et al. 1997). This results in glutamate excitotoxicity, which may manifest as swelling, loss of presynaptic ribbons and rupturing of dendritic afferent nerve fibres (Kujawa & Liberman 2009; Henderson et al. 2006). Damage to neural fibres occur even when OHC seems intact and their effect is long lasting (Kujawa & Liberman 2009; Sergeyenko et al. 2013).

1.6.5 Chronic disease

A high incidence of ARHL has been associated with chronic diseases including dementia (Lin et al. 2011), type II diabetes (Mitchell et al. 2009) and cardiovascular diseases (Friedland et al. 2009).

Epidemiological studies have shown an association between hearing impairment and poor cognitive function (Lin et al, 2011; Lin 2013). In these studies, hearing impairment has been associated with increased risk of all-cause dementia. The associated risk is increased with severity of hearing loss (Lin et al., 2011). There is evidence of changes in auditory cortex volume in people with dementia. The mechanisms underlying the association have been speculative, they include a common neuropathological pathway between dementia and ARHL, hearing loss mediation through social isolation associated with dementia and exhaustion of cognitive reserve allocated for auditory processing (Lin et al. 2011). Interestingly, there is magnetic resonance imaging (MRI) evidence of auditory cortex atrophy in people with hearing loss (Lin et al., 2014).

Studies have found association between type II diabetes and hearing loss (Mitchell et al. 2009; Fukushima et al. 2006). Frisina et al (2006) observed that pure tone audiometry thresholds of diabetics were 7.9 to 12.3 dB higher (poorer) compared to age-matched non-diabetics. Interestingly, the difference was markedly higher at low frequencies. There are conflicting reports as to the site of auditory injury in diabetes. While some studies have shown loss of hair cells (Duck et al., 1997), others have observed loss of spiral ganglion cells (Lee et al., 2008) thickening of the basilar membrane and the vessels of the stria vascularis, and strial atrophy (Fukushima et al. 2006).

The effect of diabetes on the retina is well known and since both the retina and the cochlea receive end artery supply for oxygenated blood, in trying to understand the association between diabetes and ARHL, analogies may be drawn from the mechanisms involved in diabetic retinopathy. Diabetic retinopathy has inflammation as one of its mechanisms (Silva et al, 2007). In people with diabetic retinopathy, intracellular adhesion molecule (ICAM-1) is up-regulated in the retina and this promotes leukocyte infiltration into the retina (McLeod et al, 1995). In the presence of inflammation, it is known also that ICAM-1 is linked with microglia activation in the central nervous system (CNS) (Lee et al., 2008). Activated microglia may then produce cytotoxic inflammatory mediator such as ROS, nitric oxide (NO), tumour necrosis factor alpha (TNF-α) and interleukin-1(IL-1) which would promote more inflammation (Chao et al, 1995). Unpublished work within our laboratory group have also found microglia to be present in the cochlea, therefore it is possible the microglia modulate inflammatory changes in the cochlea of diabetics as occurs in the visual system.

Another mechanism by which diabetes affects ARHL are through the vasculature. Vascular complications of diabetes are macro and microvascular. Microvascular complications are seen in the kidneys, retina and vessels supplying neurons and it is likely that microvascular complications occur in the auditory system. Hyperglycaemia induces microvascular injury through at least four

mechanisms: increased glycation leading to increased formation of glycation end products (AGE), polyol mechanism, activation of protein kinase C (PKC) and increased activation of hexosamine pathway(Frisina et al., 2006). Hyperglycaemia results in elevated levels of by-products of metabolism including diacylglycerol that activates protein kinase C (Frisina et al., 2006). Protein kinase C upregulates production of vascular endothelial growth factor (VEGF) which results in thickening of basal membranes and a more permeable endothelium (McQueen et al. 1999). Increased porosity of the endothelium of the vessels in the stria could interfere with oxygen supply, nutrient uptake, electrolyte homeostasis and signal transduction in the cochlea (Frisina & Walton, 2006).

Studies have shown links between cardiovascular disease and hearing loss, and it has been suggested that hearing loss is a predictor of worsening cardiovascular disease (Friedland et al. 2009). Rosen et al (1970) made one of the earliest links between cardiovascular risk factors and hearing loss when they investigated the effect of saturated fatty diet on coronary heart disease and hearing loss in two sub-populations. One group was fed on food, which contained saturated fat, and in the second group with polyunsaturated fat. After a five-year period, the incidence of coronary heart disease was significantly lower in the group fed on polyunsaturated fat. Interestingly, the audiogram of the polyunsaturated group was better at all frequencies than the saturated group. When the group was swapped, the hearing of the group now being fed on polyunsaturated fat had better hearing threshold compared to the group on saturated fat diet (Rosen et al. 1970). Similarly associations between cardiovascular disease and hearing loss have been found in other studies (Friedland et al., 2009; Torre III et al., 2005).

Low frequency hearing loss is being regarded as a risk factor for cardiovascular events like myocardial infarction, transient ischemic attack and stroke (Friedland et al. 2009). A possible mechanism for the association between low frequency hearing loss and cardiovascular diseases is through ischemia of the cochlear vessels (Friedland et al. 2009). The stria vascularis houses the cochlear vessels. Arteries of the stria are terminal vessels, which makes it vulnerable during periods of spasms or occlusion. In addition, the sparse number of vessels at the apex, the region responsible for low frequency hearing, compared to the base makes it vulnerable to ischemia (Friedland et al. 2009). It is possible that ischemia from vascular spasm or atherosclerosis could be responsible for the low frequency hearing loss associated with cardiovascular diseases and type II diabetes.

1.6.6 Smoking

Smoking is known to be a risk factor in many diseases including stroke, myocardial infarction, atherosclerosis and lung cancer but its role in hearing loss is controversial. Cruickshank et al (1998) have found in a large population-based cross-sectional study of 3,753 subjects aged 48-92, that people who smoke were 1.69 times more likely to have a hearing loss compared to non-smokers. However, no association between smoking and hearing loss was found in another population-based study, the Framingham study (Gates et al., 1993). The lack of association between smoking and hearing loss could have stemmed from the fact that a higher cut-off of > 40 dB HL was used to classify hearing loss, which could have meant that people with mild hearing loss (21-40 dB HL) could have been classified as having normal hearing.

Inhalation and absorption of nicotine causes systemic release of adrenaline and noradrenaline which bind to α_1-adrenergic receptors on vascular smooth muscle to cause vasoconstriction (Nomura, Nakao, & Morimoto, 2005). In a healthy person, the vasoconstriction is counterbalanced by the release of the vasodilator nitric oxide to prevent platelet aggregation. Smoking impairs endothelium production of nitric oxide leading to damage to the vessels (Powell 1998). Vasoconstriction in the end arteries of the cochlea will limit supply of oxygen and nutrients for the cochlea and possibly contribute to atrial degeneration (Friedland et al. 2009). In addition, smoking promotes the formation of free radicals which catalyse oxidation of low density lipoprotein (LDL) (Bakhru & Erlinger 2005). The inflammatory process of atherosclerosis, which then ensues, involves the recruitment of leukocytes, macrophages and the release of cytokines (Bakhru & Erlinger 2005). Inability to resolve the inflammation causes increased adhesiveness of endothelium and migration of smooth muscle cell and the thickening of the arterial wall (Ross 1999) and possibly causing more damage to the cochlear vessels through chronic vasoconstriction.

1.7 Age-related inflammation (Inflammaging)

1.7.1 Ageing

All living organisms undergo ageing, which is defined as post-maturation progressive decrease in physiological capacity, and the reduced ability to respond to environmental stimuli that results in reduced homeostasis, increased susceptibility to disease and increased mortality (Troen, 2003). Ageing is a complex process characterised by changes in the composition of tissues, decline in physiological function, reduced ability to adapt to environmental stimuli, and increased susceptibility to disease and mortality (Troen, 2003). Healthy or normal ageing is physiological, however it can become associated with age-related diseases and become unhealthy ageing

(Troen, 2003). Due to the complexity of ageing, many different theories have proposed to explain the mechanism of ageing. Theories of ageing can be broadly categorised into stochastic theories and developmental-genetic theories (table 1.1).

Stochastic theories propose that ageing is as a result of accidental damage of essential molecules and accumulation of these damages causes decline in physiological capacity associated with ageing (Troen 2003). A number of sub theories make up stochastic theory, some of which are presented in table 1.1. For example somatic mutation proposes that exposure to radiations is associated with cell mutations which results in functional decline and death. Although exposure to radiation may cause cancers and result in shorter lifespan (Pierce & Preston 2000), there is not much evidence that it causes accelerated ageing. Another stochastic theory postulates that errors occur in the proteins that produce deoxyribonucleic acid (DNA) and end up with faulty DNA that is incompatible with life (Aubert & Lansdorp 2008). The production of free radicals have been associated with ageing, which is suggested to cause damage in DNA and proteins resulting in ageing and cell death (Weinert & Timiras 2003).

Developmental genetic theories propose that ageing is a genetically programmed and controlled spectrum which forms part of development and maturation (Troen 2003). A sub theory of this is the longevity theory which suggests the existence of longevity genes that promote healthy ageing and longevity, for example males of Sardinia, Italy are thought to express genes that promote longevity (Franceschi et al., 2007). This theory draws evidence from the existence of genetic diseases like Werner's syndrome and Down's syndrome in which genetic mutations cause accelerated atherosclerosis, metabolic diseases, ageing and reduced life span. As part of the developmental-genetic theory, cellular senescence postulates that intrinsic mechanisms in each cell allows cells to proliferate at their early stages and peak at a certain point. After a certain critical period or after a number of divisions have occurred cells will stop dividing. The number of divisions allowed per cell is unique to the type of cell and the organism, and after that, mutations occur in the cells that accelerate ageing. Neuroendocrine theory proposes that functional changes in neurones and hormones result in ageing. Central to the theory is the role of the hypothalamic-pituitary adrenal (HPA) axis, which is regulates development, reproduction, metabolism and many physiologic functions. Therefore, changes to the function of this axis promote to ageing. Immunosenescence and inflammaging form part of the developmental-genetic theory, and will be discussed in detail in this section. The mechanisms involved in ageing are so complex that it cannot be explained using a single theory. These theories are not mutually exclusive; therefore, a combination of theories may better explain the ageing concept.

Developmental-genetic theories	Stochastic theories
Longevity genes	Somatic mutation
Neuroendocrine	Error-catastrophe
Immunosenescence	Protein modification
Inflammaging	Free radical (oxidative stress)
Cellular senescence	Mitochondrial DNA

Table 1.1 Theories of ageing under two broad headings; developmental-genetic and stochastic

1.7.2 Inflammation

Inflammation is the body's response to harmful stimuli (Ferrero-Miliani et al., 2007). Inflammation is a protective measure for the body to eliminate harmful agents and initiate healing. An acute inflammatory response is triggered by infection or injury. Tissue macrophages, dendritic cells, and mast cells, all of the innate immune system, mediate the initial response. On these cells are pattern recognition receptors (PRR) which bind to pathogen-associated molecular patterns (PAMP) on the offending pathogen and damage-associated molecular patterns (DAMP) on injured host cells (Medzhitov 2008). Inflammatory mediators including chemokines and cytokines are secreted, and there is movement of plasma proteins and white blood cells, particularly neutrophils from circulation to the injury site. This results in the cardinal signs of inflammation; pain, redness, heat, swelling and loss of function. Inflammation is aimed at bringing the body back to homeostasis. However, the process may cause damage to surrounding host tissue. For example, neutrophils release toxic proteins including elastase proteinase 3 and ROS to kill offending pathogens but also results in host tissue damage (Medzhitov 2008). Both cells of the cellular and humoral immune system become activated in response to inflammation. Following elimination of the offending agent, repair of host tissue begin and this is mediated by macrophages. In the acute stages of inflammation, it takes the body a few days to restore homeostasis.

When the acute inflammation remains unresolved, chronic inflammation ensues. In chronic inflammation, there is continual destruction of tissues and attempt at healing occurring simultaneously. This process may continue for months or years. Chronic inflammation is characterised by the dominance of macrophages at the injured site. The toxins they release including reactive oxygen species cause by-stander damage to the body resulting in tissue destruction (Fujiwara & Kobayashi 2005).

1.7.3 Inflammaging

Evidence suggests that ageing is associated with a state of low-grade chronic inflammation referred to as inflammaging (Franceschi et al., 2000), characterised by upregulation of inflammatory mediators (Hunt et al., 2010a). It has been suggested that inflammaging plays a key role in the progression of age-related chronic diseases including type II diabetes, cardiovascular diseases and dementia (Giunta et al., 2008; Hunt et al., 2010b). This type of inflammation is not triggered by micro-organisms, it is thought to occur as a result of endogenous factors or environmental factors including hyperglycaemia or hypercholesterolemia that result in tissue injury (Medzhitov 2008). Cells of the immune system, particularly resident macrophages recognise injury to tissue and mount a response to restore tissue homeostasis (Fujiwara & Kobayashi 2005). The extent of tissue inflammation that occurs is dependent on the degree of injury.

Tissues are consist of many cells and are adaptable to changes or damage to some of the cells they contain. This suggests that tissues can malfunction to different degrees within the spectrum of normal state and gross malfunction based on the number of damaged or healthy cells. Normal tissue conditions are maintained by resident macrophages by sustaining neuronal integrity, clearing debris and dead cells (Xu et al. 2009). During stressful or harmful conditions, resident macrophages assume an adaptive state between normal and inflamed states, initially to restore the tissue back to homeostasis (Fujiwara & Kobayashi 2005). However, when the harmful condition persists, homeostatic set points begin to change, for instance blood pressure or blood sugar levels may become set at a higher than normal level and tissues are unable to adapt, resulting in the progression of chronic diseases (Medzhitov 2008). The resulting chronic diseases including type II diabetes and atherosclerosis are characterised by low-grade increase of inflammatory mediators (Giunta et al., 2008; Hunt et al., 2010b). According to the inflammaging concept, the extent of the inflammatory response varies for different individuals (Franceschi & Bonafè, 2003). In addition, different tissues have different microenvironments and varying abilities to cope with malfunction and therefore may malfunction at varying levels. This may explain the variability seen in the types and extent of age-related chronic diseases (Xu et al. 2009).

Although ageing is associated with inflammation, not all old people are ‘inflammaged’. Many studies have found elevated levels of inflammatory mediators in older populations compared to young people (Hearps et al., 2012; Shaw et al., 2013; Wenisch et al., 2000). However, in several of these studies, elderly subjects were analysed as a unit, therefore it is unclear whether there were subsets of elderly people who had normal inflammatory levels. The wide range of levels of inflammatory markers for the elderly (Krabbe et al. 2001; Fuchs, Werner, et al. 1992) suggests that there are some elderly individuals who have inflammatory markers comparable to younger

people. Many elderly people may have health related problems that could affect their inflammatory status, and therefore result in the variability in the levels of inflammatory markers. For instance, there is evidence of variability in the responses of elderly individuals to influenza and pneumococcal vaccination, while the vaccine is protective for many, there is a subset of people in whom the vaccine does not induce adequate levels of immunity (Goronzy & Weyand 2013). In studies that have investigated the effects of influenza vaccination in the elderly, there is evidence that elderly individuals with at least one chronic disease have increased risk of hospitalization and death from influenza (Nichol et al., 2007), highlighting the fact that chronic diseases and other lifestyle factors that promote inflammation, have deteriorating effect on the immune response. Therefore, inflammaging can be viewed as a spectrum that individuals will vary, based on a combination of some of the contributing factors including immune factors, hormonal, genetics, chronic diseases and infections as discussed in the following section (figure 1.5).

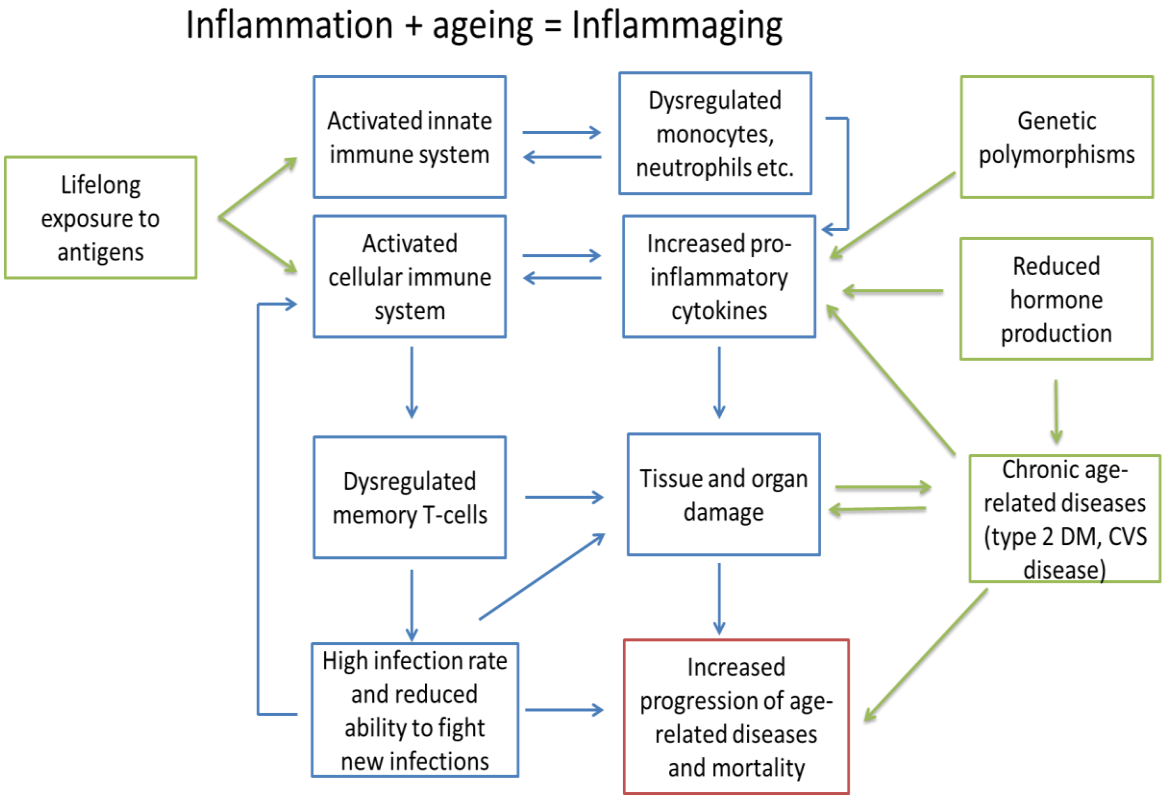

Figure 1.5 A schematic representation showing how age-associated inflammation (inflammaging) drives the progression of age-related diseases. Green boxes represent possible initiators of the inflammaging process, blue boxes represents resulting changes within the immune system and the red box represents the end-point of inflammaging.

1.7.3.1 Immunosenescence

Age-related changes in the immune system are primary contributors to inflammaging. Immunosenescence is a deterioration in the ability of the immune system to mount an

appropriate and effective response as a result of ageing (Targonski et al. 2007). Immunosenescence is characterised by the dominance of the innate immune system and altered adaptive immune system (Giunta et al., 2008; Hunt et al., 2010a). The altered adaptive immune system is largely due to exhaustion of T-cells (Giunta et al., 2008). Ageing results in reduced ability of the bone marrow to produce progenitor cells (Shaw, Goldstein, & Montgomery, 2013). Due to fact that the number of T-cells in healthy individuals remains stable over time, T-cells are turned over peripherally in the thymus for the replacement of T-cells. Involution of the thymus with ageing occurs, which is thought to contribute to the reduction of naïve T-cells and the contraction of T-cell repertoire (Hunt et al. 2010a). Although this assertion has been challenged by evidence that in humans, T-cell regeneration largely occurs peripherally rather than in the thymus (den Braber et al. 2012). In addition, as people become exposed to antigens repeatedly with age, there is increase in the number of antigen-experienced memory T-cells. With persistent antigenic load, these memory T-cells become less competent and dysregulated (Giunta et al., 2008; Goronzy & Weyand, 2013). The result in a subset of people is a less robust response to previously encountered pathogens, and a weakened response to new antigens.

Age associated B-cell changes also occur but on a smaller scale. The quality of humoral response observed is decreased with age due to low serum immunoglobulin concentrations, low antibody affinity and reduced B-cell repertoire (Desai et al. 2010). A low affinity of antibodies to their corresponding antigens results in an impaired inflammatory response, with the increased likelihood for autoimmunity and the development of B-cell tumours (Desai et al. 2010).

There is evidence shows that the innate system in most elderly people is in a pre-activated state, particularly cells of the mononuclear phagocytes (Giunta et al., 2008; Krabbe et al., 2001). Age-related changes have been observed in peripheral monocytes/macrophages (Krabbe et al. 2001) as well as CNS resident form, microglia (Norden & Godbout 2013). It is unclear if this is a compensatory response to the reduced effectiveness of the adaptive system. The monocytes/macrophages are the first to arrive at sites of injury. Their role includes initiation of the inflammatory response, phagocytosis of the offending pathogen and initiation of the adaptive response (Shaw et al., 2013). Studies from aged mice and humans have shown an increased pro-inflammatory response of mononuclear cells (Krabbe et al. 2004). For example, monocytes from older people have been shown to produce higher amounts of TNF-α upon stimulation with lipopolysaccharide (LPS) compared to young controls (Hearps et al. 2012). In a study, when monocyte concentration was measured after inoculation with *Escherichia coli*, the concentration of monocytes remained significantly elevated and for a longer duration than for younger subjects (Krabbe et al. 2001).

1.7.3.2 Hormonal influences

Reduced production of certain hormones with age affects the maintenance of homeostasis and as a result influences inflammaging (Hunt et al. 2010a). The gonadal hormones, oestrogen and testosterone have anti-inflammatory effects that depress inflammatory response. Elevated levels of serum cytokines including 1L-1β and 1L-8 have been observed with the decline of oestrogen after menopause (Malutan et al., 2014). Some suggested mechanisms by which oestrogen exert anti-inflammatory effects are through antioxidant effect on vasculature, generation of the vasodilator nitric oxide, and suppression of cytokines and the renin-angiotensin pathway (Chakrabarti et al. 2008).

The hypothalamic pituitary adrenal axis (HPA) is central in the maintenance of homeostasis in the body. Maintenance of the balance between corticotropic hormone (CRH), adrenocorticotropic hormone (ACTH) and cortisol, limits the inflammatory response to prevent potential damage that can be caused by unregulated inflammation (Hunt et al. 2010a). During a stress response, the hypothalamus produces CRH, which acts through ACTH to release cortisol, which has an anti-inflammatory effect. In elderly people with ongoing inflammaging, there is increase in the normal circadian levels of cortisol. The prolonged effect of free cortisol dulls its negative feedback on CRH and ACTH and results in cytokine dysregulation (Hunt et al. 2010a).

Reduction in the production of other hormones including growth hormone, parathyroid hormone and vitamin D also result in dysregulation of cytokines and an increase in inflammatory state.

1.7.3.3 Genetics

Genetics seem to play a role in the regulation of inflammatory state and this may affect longevity. Genes and different polymorphisms have been associated with the regulation of inflammation. The IL-10 polymorphic gene IL-10-1082GG, which has anti-inflammatory activity has been associated with longevity (Franceschi et al., 2007). Evidence also suggests that IL-6-174G polymorphism has reduced cardiovascular and mortality risk, on the other hand IL-6-174C polymorphism is associated with insulin resistance (Franceschi et al. 2007; Hunt et al. 2010a). It has been suggested that polymorphisms in toll-like receptor four (TLR4) which initiates release of cytokines and eicosanoids may cause some individuals to mount greater inflammatory responses, predisposing such people to chronic cardiac diseases (Hunt et al. 2010a). This suggests that a genetic propensity towards an anti-inflammatory state is protective against chronic diseases and promotes longevity.

1.7.3.4 Chronic diseases

The chronic systemic inflammation that accompanies ageing predisposes to the development and/or progression of chronic inflammatory diseases. Many of the chronic diseases including type II diabetes (Silva et al. 2007), cardiovascular disease (Ross 1999) and Alzheimer's disease (Perry et al. 2007) have similar inflammatory pathways. Increased levels of inflammatory markers have been found in these chronic diseases (Baylis et al., 2013; Giunta et al., 2008). It is difficult to ascertain whether the high inflammatory state results in chronic diseases or the presence of chronic diseases promotes high inflammatory state. The direction of causality is unknown; however, it is possible that both inflammaging and chronic diseases drive each other in both directions.

1.7.3.5 Infection

Infection releases a high level of inflammatory mediators including cytokines into circulation, therefore repeated and untreated infections contribute to the inflammatory state. This is especially relevant in subclinical infection, which may go untreated for a long time. Viruses including herpes virus and chlamydia pneumoniae and bacteria that cause periodontal disease have been found in atheromatous plaques, which stimulates the progression of atherosclerosis (Ross 1999). Although most of these infections are subclinical, they have been associated with coronary artery diseases and therefore contribute to the inflammatory state (Hunt et al. 2010a). Cytomegalovirus (CMV) and Epstein Barr virus (EBV) have been implicated in inflammaging (Franceschi et al., 2007; Giunta & Sergio, 2008). Cytomegalovirus is said to be present in 80-90% of people over age 65 (Pawelec et al. 2010). Older people with positive titres of CMV have been shown to have elevated c-reactive protein levels and high mortality as well as high levels of IL-6 and TNF-α (Bartlett et al. 2012). This has led to the view that CMV contributes to immune risk profile and may be a driver of inflammaging.

1.8 Inflammaging in age-related diseases

The presence of a chronic inflammatory state contributed by a dysregulated immune system, hormonal influences, genetics, chronic diseases and infections drives damage to tissues (Giunta et al., 2008). This can remain subclinical, but for a proportion of high responders or over time, this may develop to one or more age-related diseases including frailty, neurodegenerative diseases like Alzheimer's disease and possibly ARHL (Giunta et al., 2008; Leng et al., 2011).

1.8.1 Inflammaging in frailty

Frailty, a clinical syndrome in older adults is characterised by the presence of three or more of the following signs: slowed motor performance, poor endurance and energy, weakness, weight loss and low physical activity (Fried et al. 2001). Frailty is thought to stem from dysregulation in multiple physiological systems resulting in increased disability, hospitalisation and mortality (Leng et al. 2011). Studies which have investigated the role of inflammaging in frailty have found that people who were frail had high inflammatory mediators including IL-6, white blood cell count and monocyte count (Leng et al. 2009; Leng et al. 2011; Leng et al. 2005). Leng et al (2011) investigated the association between frailty and levels of cytokine IL-6 and neopterin, a marker of monocyte/macrophage activation, in 133 individuals above age 70. They used the frailty criteria to group subjects into frail (exhibiting 3 or more characteristics), pre-frail (one or two characteristics) and non-frail (having none of the frail characteristics). Their results showed that high neopterin level and IL-6 were independently associated with significantly increased odds of being frail, adjusted against age, gender and body mass index. Average neopterin levels were highest in frail individuals, followed by the pre-frail group. Subjects who were not frail had the lowest neopterin levels (Leng et al. 2011). These results are consistent with results from other studies (Leng, et al., 2007; Maggio et al., 2006). The mechanism by which inflammation contributes to frailty is not fully understood. It is thought to be as a result of an imbalance between production of catabolic cytokines due to chronic systemic inflammation, and the decreased production of anabolic hormones in old age, which results in decreased muscle mass and functional ability (Licastro et al. 2005). Evidence from a longitudinal study has shown that high baseline levels of inflammatory mediators including white blood cell, monocytes and neutrophils counts are associated with the 10 year likelihood of becoming frail (Baylis et al., 2013). This suggests that monitoring inflammation and keeping inflammation at lower levels can potentially reduce the risk of frailty.

1.8.2 Inflammaging in Alzheimer's disease

The dominance of the innate immune system, particularly cells of the mononuclear phagocytes manifests in Alzheimer's disease. Microglia, CNS resident macrophages play a key role in the progression of the disease. Microglia are macrophage-like cells which form part of the innate immune cells of the CNS. They originate from the yolk sac during embryogenesis and migrate to the brain, where they remain a relatively stable population throughout the individual's lifetime (Perry & Holmes 2014). They play a key role in development, homeostasis, plasticity and immune surveillance within the CNS (Norden & Godbout 2013). Microglia in the normal brain are described as being in a resting or quiescent state and they show a highly branched (ramified)

morphology, continually surveying their local microenvironment and quickly respond to any local disturbances (Perry et al. 2007). They continually monitor synapses by making contact with their processes, prune of synapses and phagocytose dead neurons and debris in the brain (Norden & Godbout 2013). In the absence of disease, microglia numbers and activity are tightly regulated. This may be because microglia can potentially damage brain tissue, which has limited regenerative ability (Perry & Holmes 2014). Ligands expressed on neurons, astrocytes and oligodendrocytes bind to receptors on microglia to inhibit and modulate the activity of microglia (Perry & Holmes 2014).

The effect of systemic infection on microglia has been studied in experiments in which mice with neurodegenerative disease were challenged with components of bacterial cell wall lipopolysaccharide (LPS). In a healthy CNS, when there is systemic inflammation, perivascular macrophages and microglia are signalled. This causes activation of the microglia to transiently produce cytokines and other inflammatory proteins to induce sickness behaviour (Perry et al. 2007). Sickness behaviour is a homeostatic mechanism that allows the body to adapt to fight infection, it is characterised by symptoms including malaise, apathy, somnolence, lethargy, decreased activity and decreased social interaction (Perry et al. 2007). An intact blood-brain-barrier (BBB) prevents transmission of microbial agents from the systemic circulation to the brain. During sickness behaviour, the BBB remains intact. Communication between systemic and central circulation occurs across an intact BBB. Proposed mechanisms by which systemic infection can reach CNS are first, inflammatory and cytokines interact with receptors at the cerebral endothelia, which signals to perivascular macrophages, which in turn signals to microglia to produce cytokines and sickness behaviour (Ek et al., 2001). A second mechanism is, cytokines and inflammatory mediators communicate directly with CNS macrophages at circumventricular organs, that is, organs that lack blood-brain barrier, which is then communicated to the microglia (Laflamme & Rivest 1999). In the third route inflammatory events are signalled from the thoracic abdominal cavity to the brain through vagal afferent nerves and information from the brain to the systemic circulation by the efferent nerves through secretion of acetylcholine (Tracey 2002).

Microglia play a central role in inflammation in chronic neurodegenerative diseases like Alzheimer's disease, Parkinson's disease and multiple sclerosis (Cunningham 2013). Neurodegenerative diseases are characterised by the presence of misfolded proteins and death of neurons. Loss of neurons means loss of the inhibition offered by the neurons resulting in priming of the microglia by altering their morphology when there is an insult to the brain such as injury or infection. Like other tissue macrophages, the phenotype they adopt depends on the kind of injury. Microglia then increase in number and upregulate their cell surface antigens including major histocompatibility complex (MHC) 1 and 2, complement receptor 3 (CR3) and CD68 (Perry &

Holmes 2014) (figure 1.6). In the setting of chronic neurodegeneration, when there is further systemic inflammation challenge, it results in an exaggerated response from the microglia (Perry et al. 2007). Microglia increase in number and release increased amount of cytokines including IL-1β, TNF-α, production of nitric oxide and increased levels of neurotoxicity (Perry & Holmes 2014) (figure 1.6). Therefore, microglial priming sets the stage for further degeneration by systemic inflammation. Evidence for microglia responses to neurodegeneration and inflammation have been mostly obtained from rodent studies in which LPS has been inoculated into rodent models of neurodegenerative diseases (Cunningham et al., 2005). The LPS doses that have been used in rodents in these studies are thousands of times higher than doses that can be tolerated by humans, and therefore may not translate in humans; however it shows that systemic inflammation can induce activation of microglia (Perry & Holmes 2014). In addition, evidence from positron emission tomography (PET) studies of humans with chronic systemic inflammatory diseases like diabetes and atherosclerosis who have mildly elevated levels of CRP and IL-6, show a corresponding increase in microglial activity (Drake et al. 2011). The evidence from these studies indicates that systemic inflammation, even low-grade inflammation can result in progression of neurodegeneration.

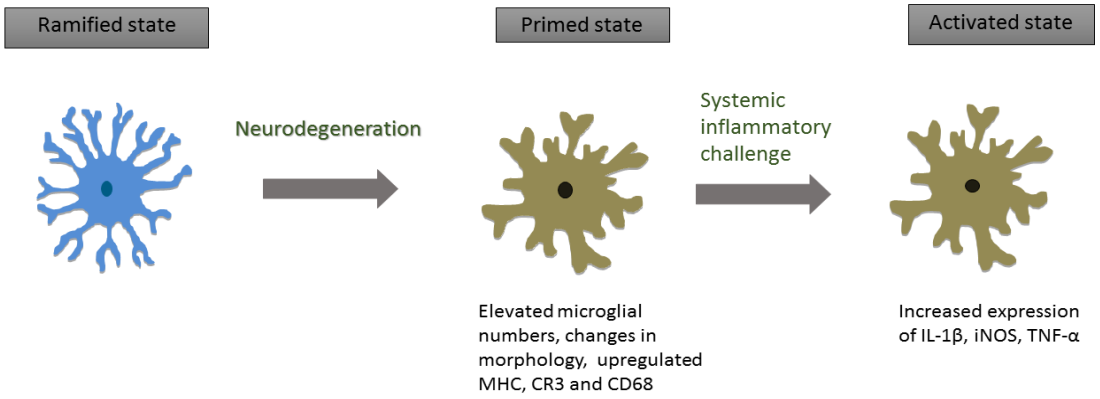

Figure 1.6 Microglia activity in neurodegenerative disease. In the resting stage, microglia assume a ramified morphology and surveying their microenvironment. During neurodegenerative pathology, the microglia become primed, resulting in increased microglial numbers, altered morphology and pro-inflammatory cell surface marker expression including MHC, CR3 and CD68. Primed microglia become activated following systemic inflammation resulting in an increased cytokine release including IL-1β, TNF-α, iNOS resulting in neuronal damage and death.

Although systemic inflammation contributes to neurodegenerative diseases, anti-inflammatory medication like non-steroidal anti-inflammatory drugs (NSAIDS) (de Jong et al. 2008) and statins (Sparks et al. 2005) have shown very little improvement of Alzheimer's disease. It is hypothesized that this may be due to decreased permeability of the blood-brain barrier to these drugs or its ability to supress the activity of cytokines (Perry & Holmes 2014). However, there is evidence that older people who undertake physical exercise, which is known to reduce pro-inflammatory state, have a reduced risk of Alzheimer's disease (Larson et al. 2006).

1.8.3 Inflammaging in ARHL

Inflammaging has been shown to be associated with ageing and the progression of age-related conditions including dementia (Giunta et al., 2008), cardiovascular disease (Lee et al., 2001) and diabetes (Savoia & Schiffrin 2007), which are themselves associated with ARHL (Hutchinson et al., 2010). Inflammation may explain the association seen between chronic diseases, lifestyle factors like smoking and ARHL. Inflammation may also be a contributing factor to the variability in occurrence and progression observed in ARHL.

Some studies have investigated the role of inflammation in ARHL have found elevated levels of inflammatory biomarkers to be associated with worse ARHL (Nash et al., 2014; Verschuur et al., 2012). The Hertfordshire Ageing Study (HAS) investigated the association between inflammatory markers and hearing threshold in 611 elderly (63-74 years) residents of Hertfordshire. Results showed an association between hearing threshold and inflammatory markers including WBC, neutrophil count, IL-6 and CRP. The found association was independent of age, positive history of noise exposure, smoking, and male gender (Verschuur et al., 2012). A second study, Nash et al. (2014) have also shown that consistently elevated CRP levels was associated with high risk of developing ARHL over the next ten years. These findings are consistent with the hypothesis that inflammatory status contributes to ARHL and that high inflammatory status, demonstrated by raised inflammatory markers is detrimental to hearing function.

Although the cochlea is not considered to be immune-privileged, it is still protected from gross infiltration by the blood labyrinth barrier (Harris & Ryan, 1995). Therefore, in a healthy cochlea, there will be few stimuli for microglia activation. Bone-marrow derived resident tissue macrophages have been identified in the spiral ligament and the spiroganglion of mice cochlea, the number of which were increased in response to surgical stress (Okano et al. 2008). Other studies, including unpublished work in our laboratory group have also described macrophage-like or microglia-like cells in the mammalian cochlea, which respond demonstrate inflammatory response to injury (Hirose et al., 2005). Furthermore, there is evidence that the release of

cytokines including IL-1β and IL-6 occurs in response to acoustic injury (Nakamoto et al. 2012), indicating the ability of the cochlea to mount an inflammatory response.

Loss of auditory input, whether due to hair cell loss or degeneration of the stria, results in subtle changes in neuronal elements (figure 1.4) (Kujawa & Liberman 2009). There is evidence that changes in neuronal elements of the auditory system starts at the synapses (Sergeyenko et al. 2013). Damage to synapses, shown in immunohistochemistry as loss of contact between presynaptic ribbons of the IHC and postsynaptic glutamate receptors of auditory fibres (Sergeyenko et al. 2013). This neurodegeneration occurs months to years prior to the death of hair cells or actual death of the SGC (Kujawa & Liberman, 2009; Sergeyenko et al., 2013). In addition, progressive damage to neuronal elements may result in disruption of the regulatory role neurones have on microglia. Glia may then become primed resulting in a shift from a supportive role to a more pro-inflammatory and destructive role. The idea of neurodegenerative damage to the cochlea is not a new one, there is evidence of loss in medial olivocochlear efferent innervation has been shown to occur following age-related loss of synapses (Fu et al. 2010). It is believed that such synaptic changes in the central nervous system precipitate symptoms such as tinnitus and hyperacusis, which frequently accompany hearing loss. Also, there is evidence of damage to topographically mapped areas in the cochlear nucleus following mechanical ablation of the organ of Corti (Morest et al. 1997). Although mechanical ablation is an extreme kind of injury to the cochlea and different from injury from ARHL, it can be inferred that injury in the auditory pathway can lead to progressive neurodegenerative changes. It is postulated that in ARHL, subtle and progressive neurodegeneration occur in the auditory system that may be on a smaller scale than neuronal death.

Microglia in the auditory pathway become primed as a result of neurodegeneration and any additional acute systemic acute or ongoing chronic inflammation will cause the microglia to be hyper-responsive and cause more neurodegenerative damage. Evidence has shown that systemic inflammation can induce an inflammatory reaction in the cochlea (Hirose et al., 2014). Hirose et al (2014) have also shown that systemic LPS inoculation is able to induce inflammatory reaction from macrophage-like cells within the cochlea. They went on to show that in mice that have been treated with LPS as well as ototoxic medication, there were a greater number of recruited macrophage-like cells in the cochlea. This also resulted in a greater hearing loss compared to mice that received only ototoxic medication (Hirose et al., 2014). This provides evidence that inflammatory state affects the extent of injury to the cochlea. This suggests in an individual with ongoing neurodegenerative damage in the auditory pathway, systemic inflammation is likely to result in increased damage.

The proposed mechanism of the progression of ARHL in this thesis therefore is, neurodegenerative changes occurring as a consequence of hearing loss or injury to cochlea, causes microglia in the auditory pathway to become primed. This results in the expression of intense response on further systemic inflammation challenge.

1.9 Hypotheses

1. Inflammation is associated with age-related hearing loss. People with high inflammatory load have poorer hearing.
2. People with persistently high inflammatory load will have a worse progression of hearing loss compared to people with low inflammatory load. This follows the theory that immune cells in the CNS respond to systemic inflammation by producing inflammatory mediators that contribute to the progression of neurodegeneration in the CNS.

Chapter 2: Preliminary work with mouse model of age-related hearing loss

2.1 Introduction

The work in this chapter was part of a larger study that investigated age-related hearing loss (ARHL) in the mouse model. The aim was to understand the mechanism of ARHL by investigating microglia response to hearing loss and inflammation within the cochlea using the C56BL/6J mice. The findings from this study were to provide basic biological background for our human study.

Age-related hearing loss affects over two-thirds of people over aged 70 years (Action on Hearing Loss 2015), making it the most common sensory deficit worldwide. Research into the pathology of ARHL has yielded evidence of different models including hair cell loss, degeneration of the spiral .ganglion cells and degeneration of the stria vascularis (Schuknecht & Gacek 1993). However, the underlying mechanisms in these models are yet to be fully understood.

Neurodegeneration is thought to play a key role in ARHL (Sergeyenko et al. 2013). Since micoglia being the immune cells in the CNS play a key role in neurodegeneration, it suggests that microglia are involved in ARHL. In the young, healthy CNS, microglia survey and respond to changes within the microenvironment. However, during neurodegeneration microglia can become primed. Primed microglia respond in a an exaggerated way in the presence of systemic inflammation by producing increased amounts of cytokines including IL-1β (Perry & Holmes 2014). The exaggerated response destroy inflammatory agent as well as CNS structures; contributing to further neurotoxicity. Since the auditory system forms part of the CNS, it is hypothesized that resident microglia in the cochlea and central auditory system, contribute to the progression of ARHL in this way.

C56BL/6J begin to develop high frequency hearing loss from 2 months and by 14 months, they are severely deaf (Kazee et al. 1995). Their hearing loss is predominantly peripheral, due to loss of hair cells, although there is evidence of loss of synapses in the inferior colliculus (Kazee et al. 1995). This study investigated microglia morphology in older versus younger C56BL/6J mice and their response to lipopolysaccharide (LPS) challenge.

2.1.1 Hypotheses

1. Age-related hearing loss pathology is associated with increased proliferation and changes in morphology (increase in size and branching) of microglia in the cochlea.

2. The hearing loss pathology primes microglia in the cochlea by increased responsiveness to inflammation by producing pro-inflammatory cytokine IL-1β and inducible nitrous oxide (iNOS).

2.2 Methods

2.2.1 Animal tissue

Female ex-breeder C57BL mice (Charles River, UK, bred in house) were used in this experiment. These mice are genetically predisposed to developing ARHL. Two age groups were used, 7 month old, which have minimal hearing loss and 14 month old mice which have severe hearing loss. Twenty animals for each age group were used. Animals were either inoculated intraperitoneally with 200 μg/kg of LPS in 0.9% saline or saline for control. The LPS dose was enough to cause a febrile illness without breech of the blood brain barrier (BBB). Animals were sacrificed 48 hours after the challenge.

2.2.2 Tissue preparation

The animals underwent terminal anaesthesia and transcardial perfusion with heparinised saline. This was followed by perfusion fixation with 10% buffered formalin. The animals were decapitated and the skulls were decalcified for 6 weeks in 10% EDTA solution. The brains and the cochlea were dissected and embedded in paraffin wax. With the use of microtome, the cochlea were cut into 10 μm sections. Sections of dentate gyrus, an area of the brain responsible for memory, were used as control slides. The Local Animal Welfare and Ethical Review Body approved the experimental procedure involving mice.

2.2.3 Immunohistochemistry

Sections were deparaffinised by incubating at 60 degrees and placing in xylene solution. Sections were rehydrated by placing them in decreasing concentrations of ethanol. Antigen retrieval process was completed by microwaving slides in citrate buffer. To prevent non-specific binding, a blocking agent was applied to the before incubation with the primary antigen. The primary antigens used were Iba-1 (Abcam, Cambridge, UK), IL-1β (PeproTech, London, UK), and iNOS (BD Transduction Lab, Oxford, UK). The sections were incubated with the appropriate secondary antibodies and visualised using ABC kit (Vector, Peterborough, UK) and diaminobenzidine (DAB) (Sigma Aldrich). The slides were then counterstained with haematoxylin.

2.2.4 Imaging and analysis

Images of the cochlea were taken using AxioVision 4.5 software system. We aimed to analyse microglia density by counting the number of Iba-1 positive cells and the expression of IL-1β and iNOS with the use of Image J and quantifying microglia morphology, measuring the soma area, cell diameter and number of branches. Statistical analysis using IBM SPSS version 20 was aimed to carry out independent t-test to analyse microglia differences due to age and LPS challenge. In addition, a binary group would have been created to access branching, with < 3 branches being minimally branched and ≥ 3 being highly branched, and use Pearson's chi square to compare groups.

2.3 Results

We were interested in analysing microglia morphology, including density, soma size and amount of branching in the cochlea of older mice (with greater hearing loss) compared to younger mice (with less hearing loss). In addition, we were interested in investigating whether challenge with LPS would prompt the microglia, particularly in the older mice to become more aggressive by producing pro-inflammatory cytokine IL1-β and iNOS.

However, on imaging, some of the cochlea samples had lost their integrity. This did not change when the protocol was altered to shorten the time for antigen retrieval. Upon examining the cochlea sections with intact spiroganglion cells and auditory nerve regions under the microscope, no Iba-1 positive staining for microglia was observed (figure 2.1).

However, Iba-1 staining worked on regions of the central auditory system. Other members of the laboratory group worked on the central auditory system. Their results showed that 14-month-old mice had significantly high microglia density in the cochlear nucleus compared to 7-month old mice, the size of change was greater than the change due to age alone. A significant increase in soma size was found in the auditory cortex that was greater than the effect due to age. In addition, significantly increased branching was found in the old mice compared to young mice. Increase in microglia density, soma size and branching are indications of a primed morphology. It was expected that following systemic LPS challenge, 7-month-old mice and to a greater extent 14-month mice will show a microglial response by producing cytokines and inducible nitric oxide. However, no measurable IL1-β or iNOS was expressed in any of the regions, including the cochlear nucleus, which had shown greater microglial density.

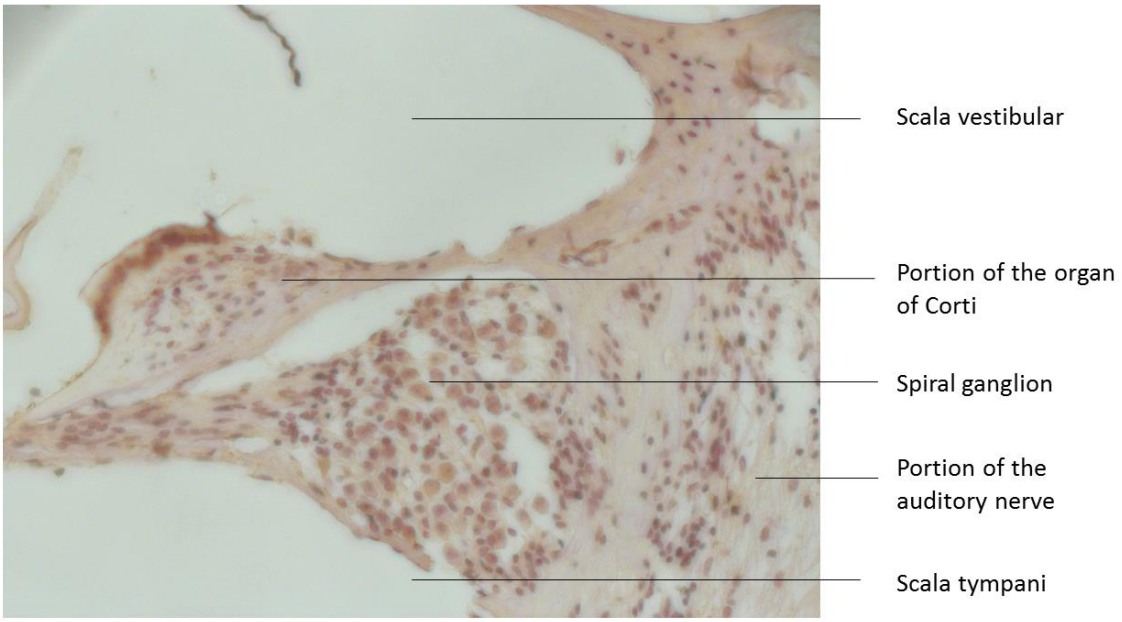

Figure 2.1: Cochlea section of C56BL/6J mouse. Immunohistochemistry with Iba-1 staining and counter stained with haematoxylin and eosin. Part of the cochlea section disintegrated following immunohistochemistry. No microglia observed. Scale 20 µm

2.4 Discussion

The aim of the study was to investigate the microglia characteristics in the cochlea of C56BL/6J mice at different stages of ARHL and to investigate microglia response to LPS challenge.

Two main problems were encountered; some of the cochlea tissues had disintegrated and it was impossible to locate the different regions of the cochlea. This was attributed to the antigen retrieval process, in which the sections were microwaved in citrate buffer for 5 minutes. Heating up tissues in microwave has the potential to evaporate the reagent and destroy the tissue (Tacha & Teixeira 2002). However, the situation was not rectified even when we modified the protocol by reducing the microwave time.

Secondly, we were unable to identify microglia staining in the cochlea with Iba-1 staining. Possible reasons are that formalin fixation of the tissues and the use of paraffin-embedded the tissues do not allow for optimum Iba-1 staining in order to visualise microglia in the cochlea sections. An alternative approach would be to use fresh frozen tissue (Lang et al. 2016). However, this could not have been performed at that time because the same animals were being used to investigate the central auditory system, which worked with paraffin-embedded tissue. It was also possible that the antigen retrieval process interfered with the antigen binding sites in the cochlea tissue and had prevented binding with the primary antigen. Following the results, the next stage to

progress the study was to obtain a fresh batch of mice species to process using frozen technique, however, a new batch of mice was not available at the time.

Although microglia could not be visualised in the cochlea sections at the time, it was unlikely that there was no microglia in the cochlea because other studies have shown evidence of resident microglia in avian and murine inner ear (Bhave et al. 1998; Hirose et al. 2005). More recently, microglia have been identified in cochlea sections of human temporal bone tissue of individuals with ARHL (Malley et al. 2015). Some members of our laboratory group have recently characterised microglia in the cochlea of C56BL/6J mice (18 months) and young normal hearing mice (3 months). This time, the tissues were processed differently; tissues were embedded with optimal cutting temperature compound, frozen and sectioned. The results showed a significant greater density in the auditory nerve and spiral ganglion of mice with ARHL compared to normal hearing mice. In addition, the basal part of the spiroganglion, which correspond to the region with greater hearing loss, showed a greater microglial density compared to the apical and medal spiral ganglion regions (Causon, PhD thesis 2016). This gives evidence that microglia in the inner ear responds to hearing pathology.

Within the central auditory pathway, the results of the current study showed a greater microglial density in the cochlear nucleus of older mice with greater ARHL compared to younger mice with lesser loss. Increase in microglia density is not associated with normal ageing (Tremblay et al., 2012), therefore it suggests that the microglia were responding to hearing loss pathology rather than ageing. In addition, the greater number of microglia observed in the cochlear nucleus of ARHL mice was greater than other non-auditory dentate gyrus in similar aged mice, suggesting that the microgliosis is not triggered by age. The inferior colliculus and the auditory cortex of the older mice did not show significant microglial density. A possible explanation could be that at 14 months, hearing loss had not progressed to involve higher regions of the central auditory system.

Per our hypothesis, it was anticipated that systemic LPS challenge would result in the expression of pro-inflammatory cytokines including IL-1β and the expression of inducible nitric oxide synthase (iNOS), however this was not observed in neither the cochlea samples nor the central auditory samples. Possible reasons for the lack of response following systemic challenge could be that the LPS concentration was not enough to elicit a response. Another reason could be that the response produced was at a sub-cellular level making it undetectable by the methods used.

The findings of increased microglia density, cell size and branching in the central auditory pathway support the hypothesis that ARHL pathology is associated with priming in the cochlea. Although LPS challenge did not result in cytokine production, the evidence of priming in central auditory pathway supported the hypothesis that inflammation play a role in ARHL.

2.5 Conclusion

Microglia priming is associated with greater hearing loss pathology and suggests the possibility of the involvement of microglia in the association between inflammation and ARHL.

Chapter 3: Inflammation is associated with age-related hearing loss: MRC hearing study

Chapter 2 reports on the analysis of data from the MRC national study of hearing. The chapter starts by reviewing current literature on the association between inflammatory markers and hearing threshold the elderly. In the study, 320 elderly adults age 60 to 89 with available audiometric data for frequencies 0.25 to 8 kHz and white blood cell (WBC) count were analysed. The hypothesis was elevated WBC count would be associated with worse hearing thresholds. Subjects were stratified into tertiles based on WBC count. The results provided corroborating evidence that inflammation was independently associated with hearing level in the elderly. White blood cell count was found to affect both pure-tone average threshold and low frequency average threshold but not high frequency average threshold. This suggests that inflammation acts on the stria vascularis, which evidence shows it effects are apparent on the lower hearing frequencies (Dubno et al. 2013). In addition, the study demonstrated that the association between WBC and hearing threshold was greater in the very old subgroup (age 75-89), which translated as 22 dB and 25.7 dB difference in pure-tone average and low frequency average thresholds respectively, between low and high WBC tertiles.

3.1 Introduction

Age is the primary risk factor in ARHL, however there are other influencing factors, suggesting why ARHL is not inevitable in every elderly person. Genetic and environmental factors including noise exposure, smoking and male gender have been implicated. Particularly interesting is the evidence that there is high prevalence of ARHL in people with chronic age-related diseases like type II diabetes (Frisina et al., 2006), cardiovascular diseases (Friedland et al., 2009; Gates et al., 1993) and dementia (Lin et al., 2011). At the same time ageing has been associated with a state of systemic low-grade chronic inflammation known as inflammaging (Franceschi et al., 2000). Inflammaging, often measured by the levels of inflammatory markers including CRP, IL-6 has also been found to be a key driver in the progression of many age-related diseases like dementia (Giunta, 2006), frailty (Baylis et al., 2013) and cardiovascular diseases (Hunt et al. 2010b). Therefore, it is possible that inflammaging is a link between ARHL and other age-related chronic diseases. If this is true, then inflammaging may drive the progression of ARHL.

Few studies have investigated the effect of systemic inflammatory markers on ARHL in elderly humans (Nash et al., 2014; Verschuur et al., 2012). The Verschuur et al. (2012) study, discussed in section 1.6.3, found cross-sectional association between hearing threshold and inflammatory

markers including WBC in the Hertfordshire Ageing Study (HAS) cohort. However, audiometric data measured in this study were between 0.5 and 4 kHz. The auditory information limited the investigation to the effect of inflammation on the mid-frequency range. The lack of information on low frequency and high frequency meant that the effect of inflammation the extreme speech frequencies could not be investigated. In addition, air conduction thresholds were measured, with no measurement of subjects' bone conduction thresholds. This suggested that subjects with conductive hearing loss, which is not a feature of ARHL, could have been included in the study. Thirdly, the age range of subjects was between 63 and 73, which did not allow investigation in older adults. Therefore the objective of our study was to investigate whether in another cross-sectional study, inflammatory status will be found to be associated with hearing status. Additionally, to investigate which hearing frequencies (low, mid and high frequency) will be closely associated with inflammatory status. There is some evidence that low frequency hearing loss is linked with metabolic ARHL (Eckert et al. 2013; Dubno et al. 2013). Metabolic or strial ARHL is thought to be as a result of degeneration of the stria vascularis resulting in reduction in endocochlear potential (EP) (Schmiedt, 2010). The stria contains many blood vessels to drive its high metabolic activities. Due to the high metabolic activities, the stria is susceptible to age-related changes resulting in a fall from the EP from its normal 90 mV (Schmiedt 2010). It has been suggested that chronic diseases and vascular diseases can affect the vessels at the stria to result in ARHL (Eckert et al. 2013). Since many of such chronic diseases like atherosclerosis are thought to be inflammatory, it presupposes that inflammation may have an effect on strial ARHL. The audiometric pattern here is a flat loss at the low frequencies (10-40 dB HL), which is often worse than other forms of ARHL, and a slightly sloping high frequency loss (Dubno et al. 2013). It has been suggested that strial ARHL is the true ARHL in people without history of noise exposure (Schmiedt 2010).

We were also interested in investigating the effect of inflammation on how hearing differs between different age groups: a relatively young subgroup (age 60-74) and a much older subgroup (age ≥75). It was envisaged that in the older group in which subjects were likely to have chronic diseases as comorbidities, inflammaging would play a greater role in their hearing loss compared to the younger subgroup.

To answer these questions, data from the Medical Research Council (MRC) National study of hearing (NSH) which took place 1980-1986 was examined. Data from the study had been collected at four different centres with the aim to assess the prevalence and distribution of hearing impairment in Great Britain (Davis 1989). Details of the study has been reported in Davis et al (1989). A section of the subjects had data on both inflammatory marker and hearing threshold. The inflammatory marker measured in the study was white blood cell (WBC) count.

White blood cells, also known as leukocytes are cells of the immune system that fight against infections and foreign bodies. They are produced by haematopoietic stem cells in the bone marrow and comprises five subtypes: neutrophils, lymphocytes, monocytes, eosinophils and basophils. White blood cells make up 1% of total blood volume, with normal count as 4-10 x 10^9/L and a life span of 10-15 days. White blood cell count is a marker of inflammation, elevated levels have been associated with increased cardiovascular disease risk (Horne et al. 2005), increased frailty (Leng et al. 2007) and mortality (Willems et al., 2010). Since elevated WBC count has been associated with age-related diseases, which are known to be driven by inflammaging, it can be inferred that WBC is a valid biomarker of inflammaging (Baylis et al., 2013). In the Verschuur et al (2012) study, WBC was found among other inflammatory markers including CRP and IL-6, to be strongly associated with hearing threshold among elderly subjects. Therefore, WBC was available for a subset of subjects, with their hearing data; we sought to determine if such association was present in a new cohort.

3.1.1 Hypotheses

1. There will be an association between WBC count and hearing status in a cohort of elderly subjects.
2. There will be increased association between WBC count and low frequency hearing compared to pure-tone average hearing and high frequency hearing.
3. The association between WBC and hearing threshold will be stronger for the much older subjects than for 'younger' subjects.

3.1.2 Aims

1. To analyse inflammation and hearing data in a cohort of older people to show any association between them.
2. To compare the findings of this study with previous findings in the HAS cohort

3.2 Methods

3.2.1 The MRC national study design

The MRC conducted a national hearing study from 1980 to 1986 to determine the prevalence and distribution of hearing impairment and disability in Great Britain. The study conducted at four centres: Cardiff, Glasgow, Nottingham and Southampton. The study comprised of two stages. At

the first stage, questionnaires were sent to 48 313 people. Questionnaire inquired about demography, hearing ability, tinnitus, hearing handicap and healthcare. Two thousand nine hundred and ten (2 910) people who responded to the questionnaire were entered in the second stage. The second stage included clinical interview, medical examination and audiological assessment. Pure-tone audiometry was performed in sound treated booths with two channel audiometer using supra aural earphones (TDH39 OR TDH49) to test for air conduction thresholds and bone vibrator (RadioEar B71) for bone conduction thresholds. Air conduction thresholds were measured at frequencies 0.25, 0.5, 1, 2, 3, 4, 6 and 8 kHz. Bone conduction thresholds were measured at 0.5, 1 and 2 kHz. All measurements were performed according to British Society of Audiology (BSA) protocols.

Blood measurements were obtained from 1692 subjects for WBC, haemoglobin, haematocrit, red cell count, mean corpuscular haemoglobin concentration, mean corpuscular volume, glucose, thyroxine, albumin, calcium, phosphate, sodium, potassium, chloride, bicarbonate, urea, bilirubin and alkaline phosphatase. The only inflammatory marker measured was WBC count. Subjects with both hearing and WBC data had only a one-time point.

3.2.2 Data refining

One thousand one hundred and thirty four (1134) subjects between ages 17 and 89 had both audiometric and WBC data. In order to analyse the link between ARHL and age-related inflammation (inflammaging), the analysis was limited to subjects aged 60 and over, which yielded 355 subjects. Subjects with asymmetric hearing were excluded subjects since ARHL is known to be symmetrical, and to exclude possible pathologies such as acoustic neuroma and Meniere's disease, which can be asymmetrical and different from ARHL. Asymmetry between ears was defined as more than 20dB between average air conduction hearing thresholds (0.25 to 8 kHz) of the left and right ears. The total number of subjects analysed was 320 (N=320).

The worse ear was defined as the ear with the highest pure-tone average threshold at 0.5, 1, 2 and 4 kHz. Based the worse hearing ear, three outcome measures used were pure-tone average threshold, low frequency average threshold (average air conduction thresholds at 0.25, 0.5 and 1 kHz) and high frequency average threshold (air conduction thresholds at 4, 6 and 8 kHz). The worse ear was analysed in order to create consistency with the Hertfordshire study since part of the aim of this study was to compare the two studies. Other studies that have investigated ARHL have used worse hearing ear for their analysis (Lin et al. 2012; Helzner et al. 2005). Secondly, analysis with the better ear did not give statistically different results from the worse ear.

Covariates of hearing loss used in the analysis were age and gender. Unfortunately, noise exposure history and smoking history were not available for the cohort.

3.2.3 Statistical analysis

Data was compiled using Excel 2010 and statistical analysis was performed using PASW SPSS version 20. Log-transformation was performed for variables that were not normally distributed. Linear association between audiometric thresholds and WBC were performed for the whole cohort (17-89 years), then for subjects 60 years or more, subjects age 60 to 74, and 75 years or more. Given the evidence that stratification of older people according to WBC tertiles is a useful way of predicting age-related morbidities (Baylis et al., 2013b), subjects were stratified according to WBC tertiles. A one-way analysis of variance (ANOVA) was performed to identify significant differences in audiometric threshold between WBC tertiles for the three age groups. Association between WBC and audiometric threshold was compared between the MRC cohort and the cohort from Verschuur et al. (2012) (HAS) study.

3.3 Results

3.3.1 Demographics

The total number of subjects, based on the criteria used for data extraction discussed in section 2.2.2, was 320 for the MRC cohort. Using similar criteria yielded 636 subjects for the HAS cohort. Although the age range for the MRC cohort was wider, mean age remained close in both cohorts; with similar gender distribution. Mean WBC and audiometric threshold were higher (worse) in the MRC cohort than in the HAS. Demographic characteristics for both MRC and the HAS cohort are shown in table 2.1.

3.3.2 Linear associations between WBC and hearing level

For the whole MRC population from ages 17 to 89 (N = 1134), no associations was found between WBC and pure-tone average threshold ($r = 0.04$; $p = 0.19$). There was however a significant positive correlation found between WBC and pure-tone average threshold in subjects aged 60 years and over ($r = 0.12$; $p < 0.05$) (figure 3.1). Correlation between WBC and low frequency average ($r = 0.105$; $p = 0.06$) or for high frequency average ($r = 0.108$; $p = 0.06$) were not significant. Linear associations between pure-tone average threshold and WBC for the subgroups were tested. A significant linear association was found between pure-tone average and WBC for the older subgroup age ≥ 75 ($N = 51$; $r = 0.44$, $p < 0.01$) (figure 3.2), however, no association was

found between WBC and pure-tone average for the younger subgroup (age 60-74) (N = 269; r = 0.12, p = 0.55).

Characteristics	MRC study	HAS study
Number of subjects	320	636
Age		
Mean (SD)	68.3 (6.2)	67.5 (2.3)
Range	60 - 89	63 - 73
Gender (%)		
Male	56	56
Female	44	44
WBC (x 10^9/L)*		
Mean (SD)	7.3 (1.9)	6.2 (3.8)
Range	2.9 – 15.3	2.8 – 18.4
PTA**		
Mean (SD)	37.3 (20.4)	28.8 (12.2)
Range	2 - 108	6 - 92

*Normal WBC range 4-10 x 10^9/L; **Pure-tone average threshold in worse ear at frequencies 0.5, 1, 2 and 4 kHz.

Table 3.1 Demographic characteristics of MRC and HAS cohorts

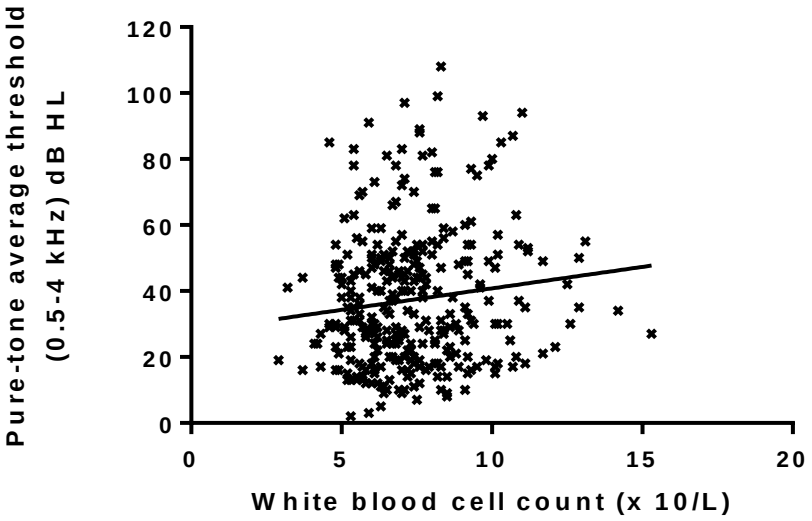

Figure 3.1: Scatter plot for all subjects showing correlation between pure-tone (0.5-4 kHz) average and white blood cell count. Thresholds are for the worse hearing ear, N = 320, R = 0.12, p < 0.05

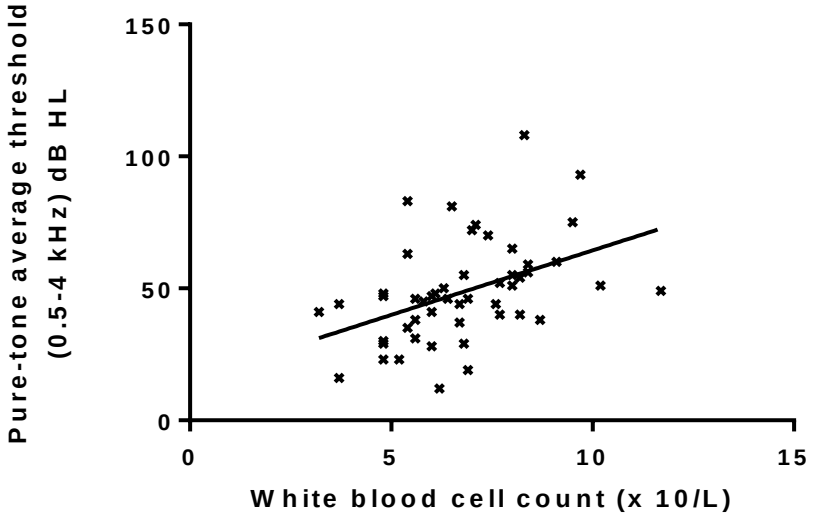

Figure 3.2: Scatter plot showing correlation between pure-tone average (0.5-4 kHz) and white blood cell count for subjects 75 years and over , Threshold are from air conduction measured in the worse hearing ear N = 51, R = 0.44, $p < 0.01$

3.3.3 Hearing level among WBC tertiles

Subjects were stratified into tertiles based on their WBC in order to examine the extent to which WBC predicts hearing level and how this may vary across the different age groups. Univariate ANOVA was performed with WBC tertiles, age and gender as independent variables and pure-tone average threshold the dependent variable.

Results showed a significant association between and pure-tone average threshold and WBC tertiles, $F(2, 205) = 4.14$; $p < 0.05$ and between pure-tone average and gender $F(1, 205) = 4.41$; $p < 0.05$. There were no significant interactions between the variables. The analysis was repeated for subjects 60 to 74 years and for subjects 75 years and older. There was no significant association found in subjects age 60 to 74, $F(2, 263) = 1.04$, $p = 0.36$. However in subjects aged 75 and over, WBC tertile was a significant predictor of pure-tone average threshold $F(2, 45) = 6.09$; $p < 0.01$. Post-hoc analysis using Fisher's LSD test showed significant difference between the low and high WBC tertiles ($p < 0.05$ for 60 years and over; $p < 0.01$ for 75 years and over). The association between WBC tertiles and pure-tone average threshold translated as a difference of 6.5 dB between the first and third tertiles for all subjects 60 years and over, 4.7 dB difference in subjects age 60 to 74, and as much as a 22 dB difference in subjects 75 years and over (figure 3.3).

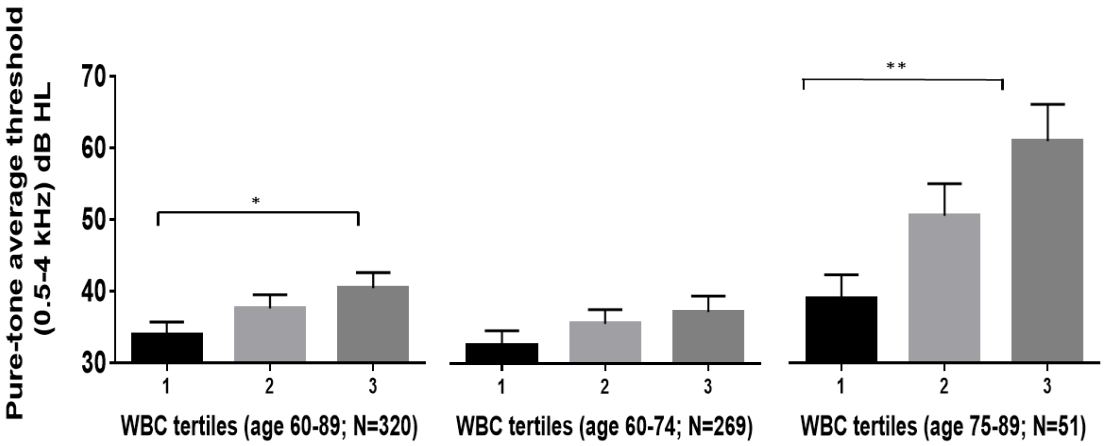

Figure 3.3: Pure-tone average audiometric thresholds of subjects stratified into tertiles based on white blood cell (WBC) count. Thresholds were obtained by air conduction in the worse hearing ear. Error bars indicate standard errors of mean. Subjects were those with both audiometric and WBC data from the MRC cohort subdivided into all subjects 60 years and over, 60-74 or 75 and over as indicated. 1 represents low WBC tertile, 2 intermediate WBC tertile and 3 high WBC tertile.

Univariate ANOVA was also performed using low frequency average threshold and high frequency average threshold as dependent variables and age, gender and WBC tertile as independent variables. Results showed significant association between low frequency average threshold and WBC tertiles $F(2, 204) = 4.94$; $p < 0.01$, independent of gender and age. Post hoc Fisher's LSD test showed the association to be between the low and the high tertile ($p < 0.05$). There were no significant interactions. The analysis was repeated for the two subgroups. For the younger subjects (60-74 years), there was no association between WBC tertiles and low frequency average threshold $F(2, 263) = 1.02$, $p = 0.36$. Again, for older subjects (≥ 75 years) there was an association between WBC tertiles and low frequency average threshold $F(2, 44) = 6.28$, $p < 0.01$. Fisher's LSD post hoc test shows a significant difference between the low and the intermediate tertile, as well as the low and the high tertile. The association between low frequency average threshold and WBC count translated into 6.4 dB difference between low and high tertiles within the whole group (60-89), 3.9 dB in (60 – 74) and 25.7 dB in the oldest group (≥ 75) (figure 3.4).

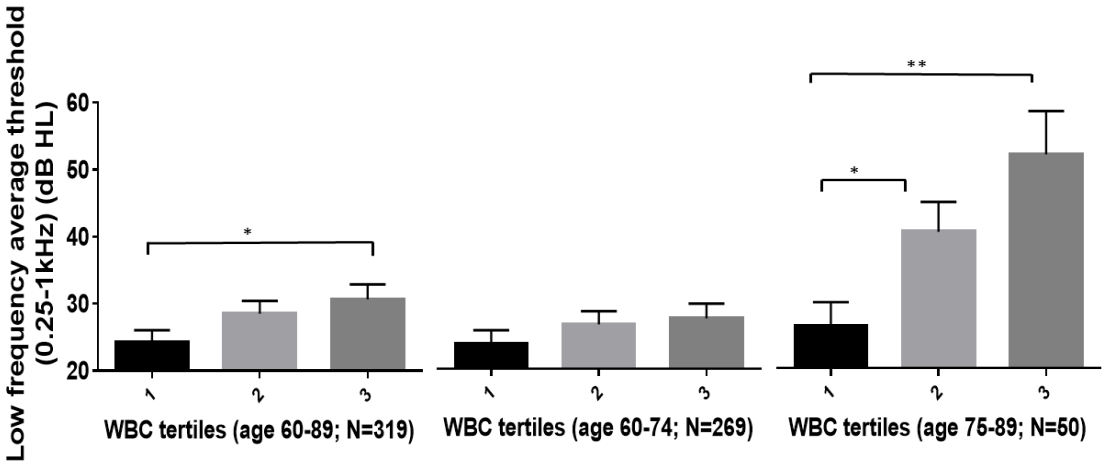

Figure 3.4: Low frequency audiometric thresholds (0.25-1 kHz) of subjects stratified into tertiles based on white blood cell count (WBC). Thresholds were obtained by air conduction in the worse hearing ear and error bars indicate standard errors of mean. Subjects were those with both audiometric and WBC data from the MRC cohort subdivided into all subjects 60 years and over, 60-74 or 75 and over as indicated. 1 represents low WBC tertile, 2 intermediate WBC tertile and 3 high WBC tertile.

High frequency hearing average was significantly associated with gender $F(1, 193) = 10.62$; $p < 0.01$ and age $F(27, 193) = 1.76$; $p < 0.05$. There was no association between high frequency average and WBC tertiles $F(2, 193) = 0.91$; $p = 0.41$. There were no significant interactions between the variables. Since WBC was not associated with high frequency average, further analysis with subgroups was not performed.

3.3.4 Difference between the MRC and HAS cohorts

In order to compare the effect of WBC and hearing level between the two cohorts (MRC and HAS) we used a WBC cut-off point of 6.0×10^9/L, which had been previously used to distinguish between older people who had low-grade chronic inflammation and those who did not (Baylis et al., 2013a). The cut-off value was used in order to be able to make direct comparisons between the two cohorts. A two-way ANOVA was performed with pure-tone average as the dependent variable with cohort (HAS and MRC) and WBC (low and high) as independent variables. The results showed a significant main effect of cohort on pure-tone average threshold $F(1, 947) = 41.33$, $p < 0.001$. There was also a significant effect of WBC on pure-tone average threshold $F(1, 947) = 5.06$, $p = 0.03$. There was no significant interaction between the cohort and WBC $F(1, 947) = 1.16$, $p = 0.28$. This translated in 1.5 dB difference in pure-tone threshold between low and high WBC in the HAS cohort and 4 dB in the MRC cohort (figure 3.5).

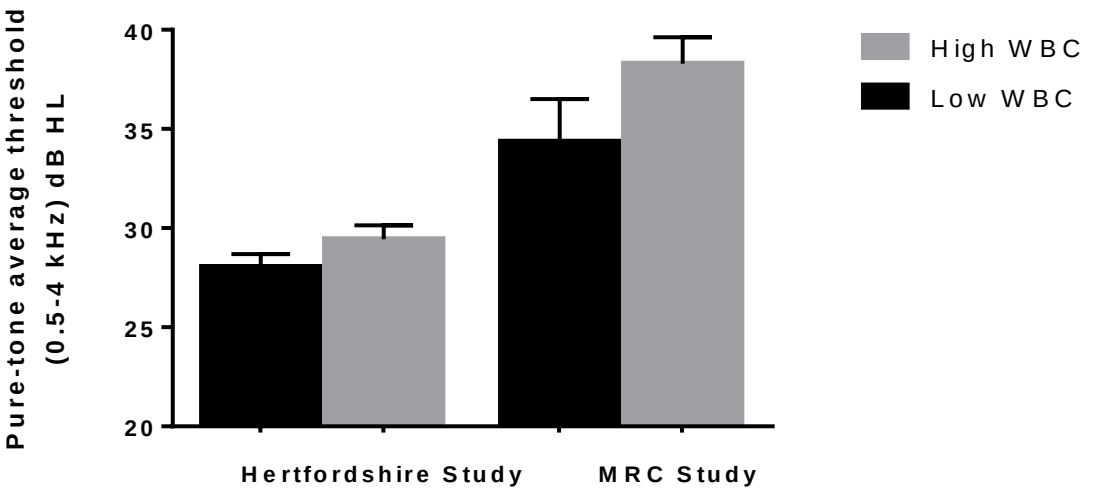

Figure 3.5: Comparison of pure-tone average threshold between MRC and HAS cohorts. Thresholds were by air conduction in the worse hearing ear for subgroups categorised according to WBC cut-off of 6 x 10^9/L between MRC and HAS cohorts. There was a statistical difference between low and high WBC for MRC cohort HAS cohort. MRC cohort low WBC (N = 77), high WBC (N = 243). HAS cohort: low WBC (N = 357), high WBC (N = 274).

3.4 Discussion

The study yielded the following findings: WBC tertiles was associated with both low frequency average threshold and pure-tone average threshold, adjusted against age and gender. The association between WBC tertiles and hearing function was stronger for the older subgroup compared to the younger subgroup. This translated as pure-tone average and low frequency average thresholds being 22 dB and 25.7 dB worse in subjects in the highest WBC tertile compared to subjects in the low tertile. The study has shown similar results to the HAS and has provided evidence to support the link between inflammatory status and ARHL.

Evidence from a number of studies has shown that the ageing immune system is inefficient in regulating immune response and this is a significant contributor to the progression of a number of age-related diseases (Baylis et al., 2013b; Leng et al., 2007). Evidence from dementia studies indicates that systemic challenges results in further deterioration of cognition and disease progression (Perry et al. 2007). Our results give preliminary evidence that inflammation is linked with ARHL. Two studies, the HAS and this study, with different cohort characteristics in terms of age range, location and the audiometric range measured have corroborated each other in their findings, providing stronger evidence of the link between inflammatory status and ARHL. The idea that inflammation is a key contributor to ARHL has important implications. Since there are well-known lifestyle and pharmacological products that can reduce inflammation, it suggests that ARHL may be modifiable. If our hypothesis that inflammatory status contributes to the

progression of ARHL is true, it is possible that factors including chronic disease and smoking contribute to ARHL through inflammation.

One of the aims was to determine whether the association between inflammatory status and hearing level was stronger in the much older subgroup. Among subjects age 75 and over, there was a stronger association between WBC and hearing level. The older subjects (age ≥75) showed the greatest difference in hearing level between the high and low WBC tertile. This suggested that inflammatory status had a greater effect with advancing age. The reason could be that inflammaging was more prevalent among much older group. Alternatively, it could be that the effect of other covariate of hearing loss such as noise exposure and gender become less important in the older cohort and as such highlights the effect of other factors such as inflammatory status.

The more comprehensive audiometric data in the MRC study provided the opportunity to investigate the effect of inflammatory status on the different average hearing frequencies. Similar to the HAS, increased WBC was associated with poor pure-tone hearing threshold. It was hypothesized that low frequency hearing will be associated with high inflammatory status since low frequency hearing loss has been linked with vascular diseases (Eckert et al., 2013). This link was found in our study. According to Dubno et al (2013), humans audiograms fall into are four main patterns: normal old, metabolic, sensory and metabolic-sensory. In the normal old audiometric pattern, thresholds at all the frequencies are within normal range (≤20 dB HL), with the lower frequencies slightly better than the higher frequencies. The metabolic audiogram, which is associated with vascular and chronic diseases, is characterised by a flat low frequency loss, which may be worse than the other types (10-40 dB HL) with a steady sloping loss at the higher frequencies. The sensory type is associated with hair cell loss; therefore, it is seen particularly in ototoxicity and noise exposure. It is characterised by normal low frequency thresholds and a steep sloping high frequency hearing loss. Metabolic-sensory is a combination between metabolic and sensory audiogram (Dubno et al. 2013). There is evidence that low frequency hearing loss is common in females (Eckert et al. 2013), however, in our study, low frequency average threshold was not found to be associated with gender. There was no association found between high frequency hearing and WBC in this study. A possible explanation could be that the effect of other factors including noise exposure was stronger at the high frequencies compared to inflammation. Unfortunately, data on noise exposure was not available for the cohort therefore the effect of noise could not be ascertained. High frequency hearing was found to be associated with age and gender.

The findings provide evidence for the link between inflammatory status and hearing level. Although we do not have evidence to show causal association, the results identify the possibility that inflammation play a role in the mechanism of ARHL.

One time measure of inflammatory marker may not be the ideal method of determining a person's inflammatory status. Therefore the remaining chapters of the thesis discusses a longitudinal study that we have conducted to measure inflammatory biomarkers at multiple time points to estimate the progression of ARHL in subgroups of people. We also explored the use of other biomarkers and their relation with hearing health. Other hearing function tests such as otoacoustic emission was explored as a potential tool for the early detection of ARHL. As part of the thesis, evidence from animal studies that shows link between inflammation and ARHL have been discussed. Animal studies are useful in understanding mechanisms involved in ARHL, since unlike in humans, they can be manipulated in a controlled way. Ultimately, the goal of the experiment in the next two chapters is to have a clearer understanding of the mechanisms involved in ARHL, to be able to stratify people with ARHL and to provide appropriate treatment based on the pathology.

3.4.1 Limitations

- The absence of data on other potential lifestyle and demographic factors such as noise exposure history, which are known to contribute to hearing loss.
- Another limitation was that WBC was the only inflammatory marker. Inclusion of other inflammatory markers would have strengthened the association found between inflammaging and hearing sensitivity.

3.5 Conclusion

- This study has shown in a different cohort, a significant association between WBC and hearing level independent of age and gender. This has corroborated the findings of HAS study.
- The found association between hearing level and WBC becomes stronger with age. This translated as an average elevation of 22 dB for pure-tone average threshold and 25.7 dB for low frequency average threshold in the very old subjects (age 75-89).
- The difference in hearing threshold as a function of WBC was greater for the MRC cohort compared to the HAS cohort. There was a 4 dB difference in pure-tone threshold between high and low WBC (inflammatory load) in the MRC cohort compared to 2 dB in the HAS cohort.

- The effect of WBC is associated with pure-tone average threshold and low frequency average threshold and not high frequency average threshold.

3.6 Publication

Verschuur C, Agyemang-Prempeh A, Newman TA. Inflammation is associated with worsening of presbycusis: Evidence from the MRC national study of hearing. *International Journal of Audiology* 2014; 53(7):469-75. (Appendix A)

Chapter 4: Neopterin, an inflammatory biomarker for age-related diseases

This chapter describes experiments conducted to investigate the variability in inflammatory load among community-dwelling older adults and the link between inflammatory load and the prevalence of chronic diseases. The chapter explores evidence from literature that suggests that neopterin; an inflammatory metabolite produced by activated monocytes/macrophages is a potential biomarker for age-associated inflammation. To investigate this, serial monthly neopterin levels were measured for 12 months, along with cross-sectional measures of other serum inflammatory biomarkers in a cohort of 57 subjects, aged 65-67 years. Multiple regression analysis did not reveal an association between mean neopterin levels and concurrent age-related chronic diseases. However, lifestyle factors known to increase inflammatory state including statin use and smoking was found to be associated with mean neopterin levels. The results demonstrate the variability of inflammatory load in the elderly, and the need for a stratified approach in the treatment of age-related diseases. It also validates the use of urine neopterin as viable biomarker for inflammaging.

4.1 Introduction

With the world's population becoming an aged one, there is increased prevalence of age-related diseases (Vos & et al 2015). Inflammaging, which is a state of low-grade chronic inflammation or immune activation manifested as an upregulation of inflammatory mediators, has been associated with age-related diseases (Franceschi et al. 2000). The concept of inflammaging suggests there is a subset of elderly people who age successfully. Such people demonstrate low amounts of pro-inflammatory markers and a low prevalence of age-related diseases. At the opposite end of the spectrum is a subset that has multiple age-related diseases and high expression of pro-inflammatory molecules (Franceschi et al., 2007).

There has been a lot of interest generated around appropriate biomarkers for inflammaging since the levels of the biomarkers give an insight to the risk of progression of age-related diseases. Studies have shown different serological, cellular and genetic markers to be elevated in inflammaging (De Martinis et al., 2006; Krabbe et al., 2004). However, there is no consensus as to the most appropriate biomarker to use and the cut off limit of biomarker levels for healthy ageing and unhealthy ageing. Upregulation of pro-inflammatory cytokines is thought to be one of the hallmarks of inflammaging (figure 1.3) therefore many studies determine levels of cytokines

including CRP, TNF-α, IL-1β and IL-6 to demonstrate inflammaging in the older population (Bruunsgaard et al, 1999; Franceschi et al., 2007; Krabbe et al., 2004).

Cytokines are small proteins produced mainly by immune cells, but may also be produced by epithelial cells and fibroblasts, that modulate immune responses. The quantification of cytokine concentrations as a method of monitoring immune activation may be let down by several factors. Cytokines may exert their action locally and may not be available to be assayed in circulation (Fuchs et al., 1992). In addition, cytokines are biologically labile and liable to rapid binding to ligands on target cells which may render them immeasurable (Fuchs et al., 1992). Binding of cytokines to target cells and membrane receptors and their interaction with other cytokines may modify their effect (Fuchs et al., 1992). For example, cytokines that have been implicated in ageing including IL-6, and TNF-α have been shown to bind to circulating proteins such as α2-macroglobulin, the concentrations of which is affected by ageing and inflammation (Leng et al. 2008). Changes in α2-macroglobulin concentrations will influence the amount of cytokines available for detection, which may be different from the true levels of the cytokine. Such properties and interactions of cytokines modify the levels of detectable cytokines and limit their use as markers in monitoring immune activation. An alternative way to monitor immune activation is to assay levels of stable metabolites produced or induced by cytokines. One example of such a metabolite is neopterin, a molecule produced by monocytes under stimulation of the cytokine, interferon- gamma (IFN- ɣ).

4.1.1 Neopterin as a marker of inflammaging

Neopterin is a metabolite of guanosine triphosphate (GTP), mainly produced by activated monocytes/macrophages but under stimulation from T-cell produced IFN-ɣ. Therefore neopterin production is influenced by both the innate system and T-cell mediated cellular immune system, which are the main systems contributing to inflammaging (Giunta et al., 2008; Hunt et al., 2010a). Neopterin levels have been found to rise during an acute infection and peak just prior to the formation of specific antibodies and the development of clinical symptoms (Murr et al., 2002). Since neopterin is released unchanged in urine, serum levels have been found to correlate well with urine levels (Nancey et al. 2008). Therefore, neopterin levels can be measured non-invasively in urine. Many of the studies that have explored the use of neopterin as a biological marker have centred on monitoring T-cell mediated chronic inflammation such as Crohn's disease (Nancey et al. 2008), systemic lupus erythematosus (Lim et al., 1994), coronary artery disease (Zouridakis et al., 2004) and monitoring of transplant rejection (Weimer et al. 2006). Some studies have measured neopterin levels in a cross-section of the population with subjects ranging from children to a few older adults, in these studies, neopterin levels have been found to be relatively

high in older people (Fuchs et al., 1992; Hausen et al., 1982; Werner et al., 1987). However, these studies have measured neopterin only at a single time point and very few studies have specifically investigated neopterin in the elderly and how inflammaging and age-related diseases are reflected by neopterin production (Spencer et al. 2010; Parker et al. 2013). There is a gap in the literature regarding the use of neopterin measured at multiple time points as a measure of inflammaging. This study provides normative neopterin data in the older population, as well as longitudinal data that can allow for the determination of an individual's immune profile, which can potentially be used as a stratification tool for the risk of age-related diseases.

4.1.2 Biosynthesis of neopterin

Neopterin [2-amino-6-(1, 2, 3-trihydroxypropyl)-H-pteridin-4-one] belongs to the class of chemicals known as pteridines, derived through the catabolism of GTP (figure 4.1). The enzyme GTP-cyclohydrolase I, promotes the cleavage of GTP to form 7,8-dihydroneopterin triphosphate. In human epithelial cells, fibroblasts and in lower animals, 7,8-dihydroneopterin is further broken down by 6-pyruvoyl-tetrahydrobiopterin synthase to form 5,6,7,8-tetrahydrobiopterin (Biopterin). However, due to the deficiency of 6-pyruvoyl-tetrahydrobiopterinsynthase in human monocytes/macrophages, the intermediate product 7,8-dihydroneopterin triphosphate is converted by phosphatases 7,8-dihydroneopterin and oxidised to neopterin (Murr et al., 2002), making monocytes/macrophages the only true source of neopterin in humans. Interferon-gamma (IFN-ɣ), mainly produced by activated T-cells, is the key stimulus for neopterin production in monocytes/macrophages, by stimulating the activity of GTP- cyclohydrolase I, therefore suggesting a positive association between neopterin and IFN-ɣ (Huber et al., 1984) . Other inducers of monocytes/macrophages including interferon-alpha (IFN-α), interferon-beta (IFN-β) and colony stimulating factor do not stimulate production of significant amounts of neopterin (Huber et al., 1984).

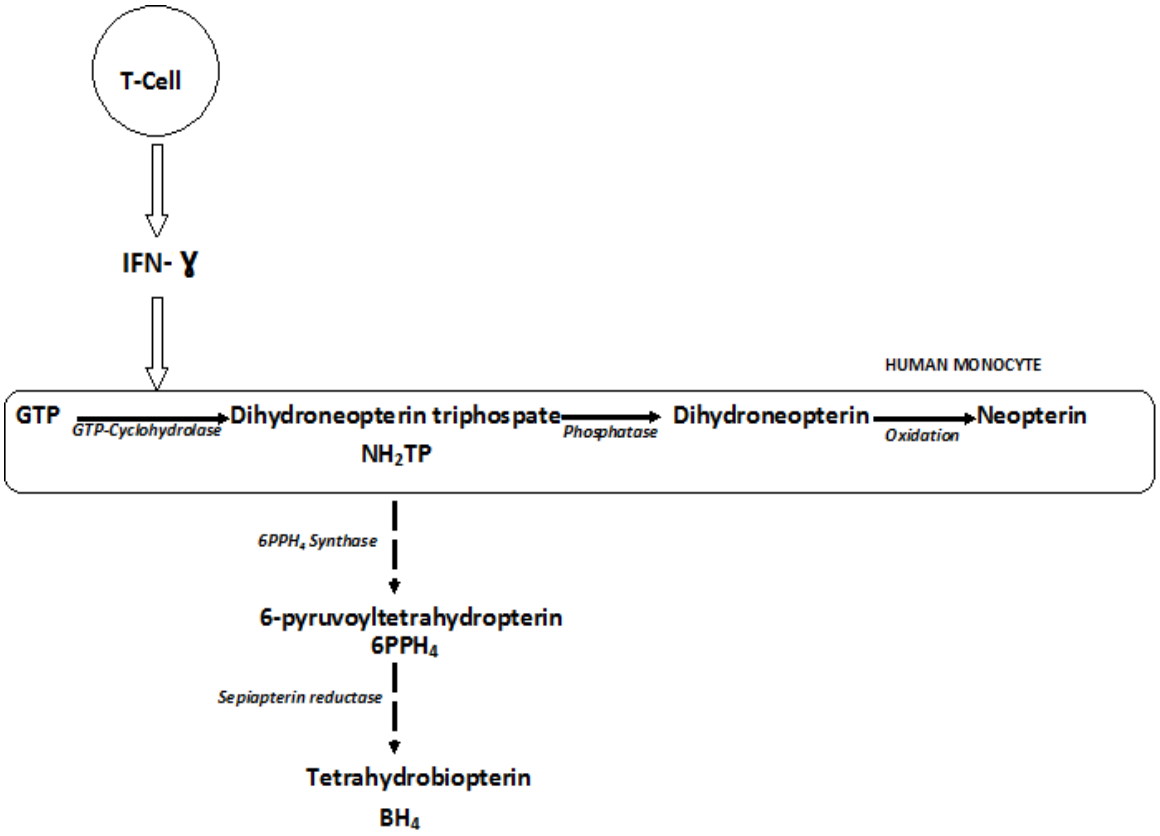

Figure 4.1 Biosynthetic pathway of neopterin in the human monocyte and the production of biopterin in lower animals

4.1.3 Detection of neopterin

Biological markers are cellular, molecular, biochemical or physical characteristics that can be objectively measured to aid in understanding the prediction, diagnosis, progression, treatment and prognosis of a disease (Mayeux 2004). An ideal biomarker is both sensitive and specific, obtained in a non-invasive way, easily detected or measured and is highly reproducible in different laboratories. Finding an ideal biomarker for inflammaging will mean being able to assess the risk of progression of many age-related diseases. Neopterin has a small molecular mass of 253g/mol; it is biologically inert and chemically stable in body fluids, making it an ideal molecule to analyse. In contrast, 7,8-dihydroneopterin, a bi-product of neopterin synthesis, decomposes easily making it a less suitable assay tool. Neopterin is stable for up to three days at room temperature, which means samples can be transported at room temperature without becoming degraded (Fuchs, et al., 1992). Samples may be stored for up to two week at 4^{o}C and for months at -20^{o}C, however neopterin is sensitive to light therefore must be protected from sunlight during sample collection and storage (Fuchs et al., 1992; Fuchs et al., 1994).

Neopterin levels can be assayed in serum, plasma, urine and cerebrospinal fluids (Fuchs et al. 1994). Neopterin is eliminated unchanged in urine therefore when renal function is normal, the

amount of neopterin in urine reflects serum levels (Fuchs et al. 1994). Urine samples could be assayed either from a 24-hour urine collection, or from early morning urine. The amount of neopterin in urine is measured as a ratio of the amount of creatinine. Creatinine is an amino acid by-product derived from the breakdown of creatine in skeletal muscle. Serum levels of creatinine are an important indicator of renal function since it is excreted unchanged in urine. Two percent of the body's creatine is converted to creatinine, resulting in excretion of constant amounts of creatinine. Due to this property of creatinine, levels have been used to standardise the concentrations of other molecules including neopterin whose levels may be affected by state of hydration or physiological variations of urine concentration (Fuchs et al., 1992; Hausen et al., 1982). Urine creatinine levels will decrease when there is advanced renal failure, which has affected more than 50% of nephrons, which will result in an overestimation of urine neopterin (Lhee et al., 2006).

Neopterin levels are measured by three established methods: high-pressure liquid chromatography (HPLC), radioimmunoassay (RIA) or enzyme linked immunosorbent assay (ELISA). High-pressure liquid chromatography is a separation technique in which the sample is passed through a stationary column and the individual components are separated based on physical or chemical interaction between the molecules of the different components and the stationary column. A detector measures the amount of the separated components, based on their individual emission wavelengths. Both neopterin and creatinine have different emission wavelengths of 438nm and 235nm respectively, therefore both molecules can be separated in urine using the same experimental set up (Fuchs et al., 1992; Hausen et al., 1982). Although HPLC is recognised as the gold standard for measuring neopterin, this method is less efficient when running several samples as multiple samples cannot be measured concurrently. In both ELISA and RIA, an unknown amount of antigen in the sample competes with a known amount of antigen for binding sites of a known antibody. In RIA, the known antigen is radiolabelled; therefore, the radioactive complex formed between neopterin in the sample and the antibody is measured. The concentration of neopterin is determined through the production and use of a standard curve derived from known analyte concentration. In ELISA on the other hand, neopterin in the sample binds to an enzyme-linked antibody, which produces a calorimetric colour change. A spectrophotometer is used to measure the optical density of the coloured solution at 450nm. The concentration of neopterin in the sample is determined also using a standard curve derived from known neopterin concentration. Enzyme linked immunosorbent assay (ELISA) has the advantage of being able to measure concentrations of multiple samples using the same set-up, making it a more efficient method (Fuchs et al., 1992).

4.1.4 Neopterin in infectious diseases

During an acute viral infection, neopterin levels rise steadily and reach a peak before antibodies against the virus become detectable, a process which may occur up to two to four weeks before the onset of clinical symptoms (Murr et al. 2002). After specific antibodies are produced, neopterin levels decline to normal as the immune system effectively controls the infection. However, in chronic infections such as Human immunodeficiency virus (HIV) or chronic hepatitis, neopterin levels do not decline to baseline levels after the acute episode (Berdowska & Zwirska-Korczala 2001). Levels stay elevated throughout the quiescent period of diseases and begin to rise again with disease progression. Neopterin production correlates positively with disease progression and this has been used in studies to monitor infection progression (Brown et al., 1990; Fuchs et al., 1989). This property of neopterin can potentially be explored to monitor inflammaging since inflammaging has similar attributes with chronic infection.

Acute bacterial infection does not usually result in neopterin production since bacterial infections elicit a humoral immune response. Neopterin levels can be therefore used as a tool for differential diagnosis between acute viral and bacterial infection (Murr et al. 2002). However, intracellular bacteria such as *Mycobacterium tuberculosis* causes activation of the cellular immune system, therefore increased neopterin production occurs (Fuchs et al. 1984). In septic conditions, neopterin levels have been shown to increase and predict survival (Strohmaier et al., 1987). Parasitic infections show increased production of neopterin since they elicit a T-cell mediated immune response. There is positive correlation between parasitic load and urine and serum neopterin levels; and neopterin levels decline in response to treatment (Brown et al., 1990).

4.1.5 Research gap

Evidence suggests ageing results in a state of immune activation known as inflammaging, which drives many age-related diseases. Studies have examined different ways of characterising inflammaging using varying inflammatory biomarkers yet few studies have investigated the use of neopterin, a product of immune activation. In addition, studies that have measured increased inflammatory markers to show immune activation in older people have done so only at one time point (Bruunsgaard et al., 2000; Rocha et al., 2012). However, given the fluctuations in the levels of inflammatory markers that can occur with acute illnesses or inflammatory episodes such as during a surgical procedure, a single time point measure of an inflammatory biomarker may not be a true reflection of an individual's general inflammatory status. This study undertook serial neopterin measurement in a cohort of community dwellers within a similar age group with the rationale that differences in their neopterin levels will not be attributed to age; instead, it will be a

reflection of their general state of immune activation and their response to inflammation. The longitudinal data allowed changes in neopterin levels over time within individuals and in the cohort to be measured as well as the ability of individuals to resolve inflammation. It was intended to identify individuals who were poor resolvers of inflammation and potentially at a higher risk of developing or of rapidly progressing age-related diseases.

4.1.6 Hypothesis

1. Neopterin is a biomarker of immune activation is associated with age-related diseases, high neopterin levels is associated with concurrent diseases.

4.1.7 Aims

1. To measure serial levels of urine neopterin levels in a group of community dwelling older adults.
2. To measure serum and urine levels of neopterin in the cohort and compare with other serum inflammatory markers including WBC and cytokines.
3. To investigate the association between neopterin levels and demographic, lifestyle and age-related diseases.

4.2 Methods

This study is a three-year longitudinal study to investigate the association between inflammation and age-related hearing loss (ARHL). It is hypothesized that inflammaging is key in the progression of age-related diseases including ARHL therefore assessing levels of inflammatory mediators will help in determining those at risk of ARHL and other age-related diseases. This chapter comprises the first year of the study, which investigates the inflammatory/immune profile of participants and how it relates to the co-occurrence of age-related diseases.

4.2.1 Sample size calculation

Sample size calculation was based on the findings in the MRC study that there was an association between inflammatory markers and hearing loss. Using the G*Power software (version 3.0.10), an expected effect size based on an assumption of a moderate correlation between neopterin and hearing threshold ($r = 0.3$), power of 0.8 and α of 0.05 was computed. Based on G*Power calculation, a two-tailed t-test required a sample size of 82 (N=82). From our previous MRC hearing study, we identified a 6.5 d B difference in hearing threshold between subjects with low WBC and high WBC. Therefore, allowing for 15% attrition rate over the three-year study period,

we estimated that 100 participants would be required to identify 6.5 dB hearing difference between participants with low and high inflammatory load. It is expected that the results of this study would provide evidence for future measurements and sample size calculations. However, we were able to recruit 61 participants and four dropped out. Therefore, the sample size for this study was 57 and the revised power using the new sample size in G*Power software was 0.65 and therefore underpowered.

4.2.2 Participants

Posters advertising for participant for the study were made and posted at large retail shops such as Sainsbury's and ASDA, local libraries, GP surgeries and newsletter of the University of Third Age (U3A). A recruiting advert was posted online on the University of Southampton and U3A websites. We requested and were invited to give talks about the study and the opportunity to recruit participants at the meetings of some elderly groups including 'the over 50s group', Southampton; Age UK lunch programmes, Southampton; and U3A in Southampton, Fair Oak and Lymington. Six participants were recruited through the poster and website advertisement, three from Age UK lunch meetings, four from the over 50's group, 18 from U3A Southampton, 11 from U3A Lymington, seven from U3A Fair Oak and 12 through recommendation of other participants. Recruitment begun from October 2013 to May 2014. By the end of May 2014, 61 participants had been recruited which was short of the original 100 participants required from the sample size calculation. However, since the PhD programme was for a limited period we decided to go ahead with the study with 61 participants, bearing in mind that the study was underpowered.

People who were interested in the study were given information sheets that outlined the study and they were given the opportunity to ask questions. Participants who then decided to take part in the study signed consent forms. One subject who had severe asymmetrical hearing loss was advised to see their GP with a copy of their hearing results provided and was excluded from the study. Three other people withdrew from the study within the first month of the study; therefore, their data was not included in the study. Out of the remaining 57, a further three subjects withdrew one year into the study due to health issues and another person withdrew after the first year, due to personal reasons. Data for the four subjects were included in the analysis since they had agreed to continued use of their data to that point. Data presented is for 57 participants at baseline, 16 males and 41 females and 53 subjects at year 1.

4.2.3 Inclusion and exclusion criteria

The main inclusion criteria was people age, subjects recruited ranged from age 65 to 75. According to the inflammaging hypothesis by Franceschi et al. (2000) the period in which unsuccessful ageing occurs is maximally between ages 60-80 (Franceschi et al. 2000), therefore the age-range was selected to capture a cohort in whom inflammaging had potentially began to occur. Secondly, since our aim was also to measure change in hearing threshold, which we know progresses with age, we chose a relatively younger cohort whose hearing is unlikely to go beyond the audiometric limit. Lastly, a narrow age range was chosen to limit variations that would occur due to a wide age range.

There were a number of exclusion criteria. People with severe to profound hearing loss were excluded to avoid subjects with hearing loss above the audiometric limit. People with other known otological pathology apart from ARHL were excluded. People who were unable to give informed consent such as people with dementia were excluded. People with disease conditions like cancers, autoimmune diseases, or long-term use of immunosuppressive medications were excluded as such conditions may cause high levels of neopterin.

4.2.4 Study design

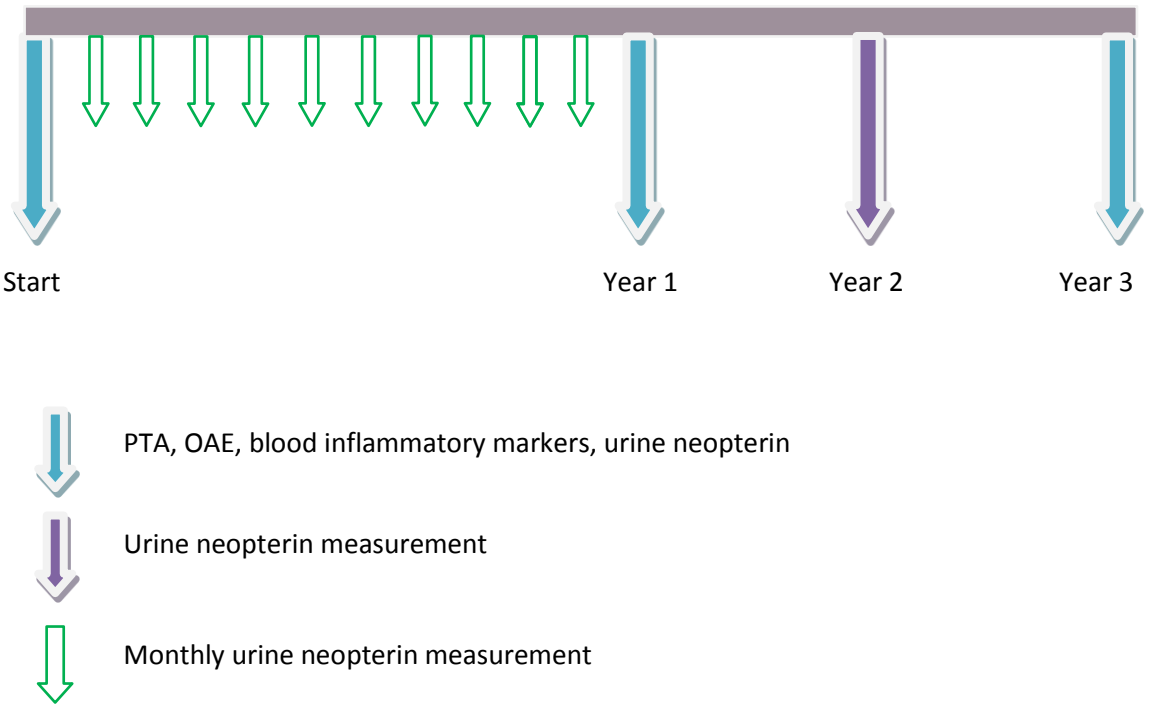

Figure 4.2: Experimental design for neopterin study. Hearing (PTA, OAE) and inflammatory (WBC, urine and serum neopterin and cytokines IL-1β, IL-6, TNFα, IFN-γ) measures collected at the start, year 1 and year 3 of the study. Monthly urine collection within the first year of study and a single time-point urine collection at year 2 for neopterin analysis.

4.2.5 Procedure

4.2.5.1 Questionnaire

Testing took place in the Hearing and Balance Centre of the University of Southampton. After consent had been obtained, participants were given a questionnaire to fill. The questionnaire was a modification of the questionnaire used by Davis et al (1989) to investigate the prevalence of hearing impairment and disability in Great Britain. The questionnaire was groups into four sections:

1. **Demographics**: demographic characteristics including age, gender and occupation, which are known to impact on hearing.

2. **Hearing**: this section explored individual hearing difficulties, if any. Questions included history of childhood ear infections, family history of hearing loss and noise exposure.

3. **Lifestyle**: lifestyle factors that influence hearing such as smoking were inquired about.

4. **Medical History**: this section inquired if subjects had common chronic diseases that are associated with greater hearing loss including diabetes and cardiovascular diseases.

Questionnaire is included in Appendix G

4.2.5.2 Hearing assessment

Hearing was assessed using pure-tone audiometry (PTA) and otoacoustic emission (OAE). The procedure of these tests are discussed in section 4.2.3.1.

4.2.5.3 Blood sampling

All blood samples were taken in the morning (between 9am and 12 noon) to avoid diurnal variations that may occur with blood markers. Under sterile conditions and using a non-touch procedure, venesection was performed. Blood was taken from the median cephalic vein in the cubital fossa. Prior to this, the vein was identified and the skin area cleaned with alcohol wipe. The skin was allowed to air dry, then a venous blood sample was taken. Blood samples were collected into vacutainer EDTA bottles and serum bottles. Blood in the EDTA bottle was used to measure white blood cell count (WBC) immediately. Blood in the serum bottle was allowed to stand for 30-60 minutes in order for a clot to form. Separation of the clot was performed by centrifuging the sample at 2000rpm at a temperature of 4^{o}C for 10 minutes. The supernatant serum obtained was pipetted into 500ul aliquots and stored at -80^{o}C to be used to measure serum neopterin, IFN-ɣ, interleukin-1 beta (IL-1β), tumour necrosis factor- alpha (TNF-α)and

interleukin-6 (IL-6) levels. Blood samples were taken at two time points during the study; at the start of the study and at the end of the first year.

4.2.5.4 Urine sampling

Participants gave morning urine samples once a month for 12 months. At the months 1 and 12 when blood samples had to be collected as well, participants brought in their urine samples to the centre. During the remaining months, participants posted their urine samples by first class mail using urine collection tubes and pre-paid envelopes that were supplied to them. Urine samples received were centrifuged at 2000rpm at a temperature of 4^{o}C for 10 minutes and stored at -20 ^{o}C for analysis of neopterin and creatinine levels.

4.2.6 Measurement of inflammatory markers

4.2.6.1 White blood cell count

The amount of WBC in the blood was measured using an automated machine, HemoCue WBC and differential analyser (Radiometer group). About 100ul of blood that had been previously collected in EDTA tubes was placed onto a microcuvette and inserted in the HemoCue analyser to give an automated WBC and differential reading. The differential count included neutrophils, lymphocytes, monocytes, eosinophils and basophils.

4.2.6.2 Serum cytokines

Cytokine levels have been known for their association with inflammaging. Some of the implicated cytokines that were measured to further investigate their role in inflammaging were IFN-γ, TNF-α, IL-1β and IL-6.

Interferon-γ is a soluble cytokine belonging to the type II class of interferons. It is involved in both the innate and adaptive immunity. Interferon gamma, which is produced by T-helper I cells, cytotoxic T-cells and natural killer cells, is an activator of macrophages and is able to induce the production of major histocompatibility complex two (MHC II). It is involved in inflammatory response against viruses, which includes the induction of macrophages to produce neopterin and acts as a regulator of the immune system.

TNF- α is a cell-signalling protein that is involved in the acute phase response. It is produced mainly by activated macrophages but may also be produced by other cells including mast cells, lymphoid cells and endothelial cells. TNF-α is involved in the regulation of immune system, producing fever, triggering apoptosis and has a role in cachexia. Dysregulation of TNF- α has been implicated in diseases including Alzheimer's disease.

Interleukin 1 beta (IL-1β) is produced by activated macrophage and it is released as a pro-inflammatory mediator of inflammatory response. It is involved in cell proliferation, differentiation and apoptosis. Its production in the CNS is associated with inflammatory pain hypersensitivity. In the CNS, IL-1 β is one of the cytokines produced by activated resident microglia during neurodegeneration.

Interleukin 6 has both pro-inflammatory and anti-inflammatory properties. It is secreted by T-cells and macrophages in response to infection or injury. Interleukin 6 induces fever and is an acute phase response protein that is able to cross the blood brain barrier. It has been found to be involved in the clinical syndrome frailty (Leng et al., 2011) and is thought to be a marker of inflammaging.

The concentrations of IFN-γ, TNF-α, IL-1β, IL-6 were measured using 96-well MSD Multi-spot Pro-inflammatory panel-1 kit. Testing of cytokines level was performed only at one time point, at the start of the study. Only IFN- γ measures showed significant variations in levels between individuals, the rest of the measured cytokines either had levels within normal limits or below detection limits. Therefore, we did not go ahead to measure cytokine levels at the second time point, which was after one year. Another person unrelated to the study in order to blind the investigator did re-labelling of samples.

Each serum sample was thawed on ice, centrifuged at 2000rpm for 4 minutes, diluted 2-fold with MSD diluent, and pipetted into randomly selected two adjacent wells on the ELISA plate. Standards and controls were also Wells contained pre-fixed capture antibodies. Samples were tested in duplicates to ensure repeatability. After incubation and washing stages, MSD detection antibody and then read buffer were added to sample solution. Calibrators and controls for each cytokine were processed the same way as samples. The concentrations the measured cytokines were read on an MSD plate reader according to the curve obtained from the calibrators.

4.2.6.3 Blood and urine neopterin

Neopterin levels were measured using Neopterin ELISA kit from IBL with reference number RE59321. Two different batches of the kit were used for all the neopterin assays, batch number ENO219 was used for assay for the first six months of urine and the first serum neopterin assays. Batch ENO220 was used for the second six months and second serum assays. The protocol of the kit was adhered to. The kit consisted of neopterin standards, assay buffer, neopterin enzyme conjugate, neopterin antiserum, wash buffer, substrate solution and a stop solution.

Thawed samples were centrifuged at 2000rpm for 4 minutes. Each sample, standard and control was placed into two adjacent wells. Enzyme conjugate followed by neopterin antiserum were added to the wells and incubated. After washing, substrate solution followed by a stop buffer were added to the wells. Optical density of the solutions in the wells was measured using spectrophotometer at 450nm. Neopterin concentration in each sample was calculated based on the curve obtained from the standard solutions.

4.2.6.4 Urine creatinine

Creatinine levels of the urine samples were measured to standardise the amount of neopterin. The kit used was Creatinine (urinary) calorimetric assay kit (500701) from Cayman Chemical. Batch 0459442 was used for the first six months assay and batch 472163 used for the second six months.

Alkaline picrate solution was added to samples, standards and controls, which had been placed in wells on a plate. After incubation, the plate was read on a spectrophotometer at 490nm (initial absorbance). After the addition of creatinine acid solution, a final absorbance was read at 490nm. Creatinine concentration was obtained from the standard curve.

4.2.7 Ethical approval

The study was ethically approved by National Research Ethics Service (REC reference number: 13/SC/0507) and the University of Southampton Ethics and Research Governance Online (reference number: 7923.

4.2.8 Statistical analysis

Data was compiled using Microsoft Excel 2013 spreadsheet. Statistical analysis was performed using Graph Pad Prism 6 and IBM SPSS version 24. Normality tests were performed for variables and non-normally distributed variables were transformed using $\log_{10}$ transformation. Non-parametric tests were performed on variables that could not be transformed into normal distribution. Spearman's correlation was performed between measured baseline inflammatory markers (serum and urine neopterin, IFN-γ, TNF-α, IL-6, IL-1β, WBC and differentials). Multiple regression analysis was performed with log of mean urine neopterin level as outcome measure with age, gender, chronic disease, smoking and statin use as independent variables

4.3 Results

4.3.1 Subjects characteristics

Subjects comprised 57 adults between age 65 and 75, comprising 16 males and 41 females, who lived in and around Southampton. Nineteen subjects had been a smoker at some point in their lives; ten out of 19 gave up smoking before age 40. Four common age-related chronic diseases were inquired about in the questionnaire; diabetes, stroke and cardiovascular diseases (including hypertension and coronary artery disease). Hypertension was the most common chronic disease, affecting 35.1% of the subjects, 12.3% had other cardiac diseases, 5.3% had type II diabetes and none of the subjects had stroke. Statin use was quite common among subjects, 38.6% had been prescribed statins.

A majority of subjects (71.9%) had no difficulty in hearing speech in a quiet environment. However, as typically seen in sensorineural hearing loss, 80.7% had between slight to great difficulty hearing speech in noisy environment. Fourteen out of 57 (24.6%) subjects had history of noise exposure, five of whom the noise exposure had lasted for more than 5 years (8.8%). Twelve subjects (21.1%) had been prescribed hearing aids and 18 (31.6%) experienced tinnitus. Subjects who had no history of repeated ear infections was 61.4%. About a third had a positive family history of hearing loss before aged 60. Table 3.1 summarises response to questionnaire.

Characteristics	Number	Percentage (%)
Age	69.4	
Male	16	28.1
Female	41	71.9
Smoking		
No	36	66.7
Yes	21	33.3
Gave up smoking before age 40		
Yes	11	17.5
No	10	15.8
Chronic diseases		
Hypertension		
No	37	64.9
Yes	20	35.1
Heart disease		
No	50	87.7
Yes	7	12.3
Stroke		
No	57	100
Yes	0	0
Diabetes		
No	54	94.7

Characteristics	Number	Percentage (%)
Yes	3	5.3
Statin use		
No	35	61.4
Yes	22	38.6
Difficulty hearing in quiet		
No difficulty	41	71.9
Slight difficulty	11	19.3
Moderate difficulty	5	8.8
Difficulty hearing in noise		
No difficulty	11	19.3
Slight difficulty	21	36.8
Moderate difficulty	18	31.6
Great difficulty	7	12.3
Noise exposure		
No	43	75.4
Yes	14	24.6
Time/Level of noise exposure		
Less than 6 months	1	1.8
6-11 months	0	0
1-5 years	3	5.3
More than 5 years	5	8.8
Fired more than 10 rounds from shotgun/rifle	5	8.8
Hearing aids has been prescribed		
No	45	78.9
Yes	12	21.1
Tinnitus		
No	39	68.4
Yes	18	31.6
Recurrent ear infections		
No	35	61.4
Yes	12	21.1
Unsure	10	17.5
Family history of hearing loss		
No	27	47.4
Yes	19	33.3
Unsure	11	19.3

Table 4.1 Responses to questionnaire for 57 subjects (age 65 – 75).

Results of levels of inflammatory markers measured have been shown in in table 3.2. Apart from serum levels of neopterin at the start of the study, mean levels of all other measured inflammatory markers were within normal limits. On average, levels of inflammatory markers measured at the start of the study were higher than after year one.

Inflammatory Marker	Mean (range)	Normal levels
Serum neopterin 1 (nmol/L)	10.3 (2.8 – 19.7)	≤ 10
Serum neopterin 2 (nmol/L)	6.9 (2.1 – 19)	≤ 10
Average urine neopterin (umol/mol Creatinine)	202.0 (103.5 – 391.9)	≤ 251
TNF-α (pg/ml)	3.3 (1.8 – 5.9)	≤ 32.5
IL-6 (pg/ml)	3.2 (1.6 – 33.3)	≤ 12.7
IFN-γ (pg/ml)	8.6 (0.2 – 134)	≤ 30
IL-1β (pg/ml)	0.05 (0.005 – 0.2)	≤ 4
WBC 1 (x 10^9/L) **Neutrophils** **Lymphocytes** **Monocytes** **Eosinophils** **Basophils**	6.6 (3 – 18) 3.9 (1.7 – 10.3) 2.2 (0.8 – 6) 0.3 (0 – 1.3) 0.3 (0 – 2.3) 0.005 (0 – 0.2)	4 – 10 2.5 – 7.5 1.7 – 3.5 0.2 – 6 0.1 – 0.3 0.04 – 0.1
WBC 2 (x 10^9/L) **Neutrophils** **Lymphocytes** **Monocytes** **Eosinophils** **Basophils**	5.7 (2.8 – 9.3) 3.5 (1.6 – 6.3) 1.7 (0.8 – 2.9) 0.3 (0.1 – 0.6) 0.2 (0 – 0.4) 0	4 – 10 2.5 – 7.5 1.7 – 3.5 0.2 – 6 0.1 – 0.3 0.04 – 0.1

Table 4.2 Levels of inflammatory markers for subjects (N=57, age 65 - 75)

4.3.2 Neopterin levels over twelve-month measuring period

Mean urine neopterin concentration for each month ranged between 140 – 277 umol/molCreatinine. The median level for each month was always within normal limits, that is ≤ 251 umol/molCreatinine. Figure 4.4 shows the distribution of urine neopterin over 12 months.

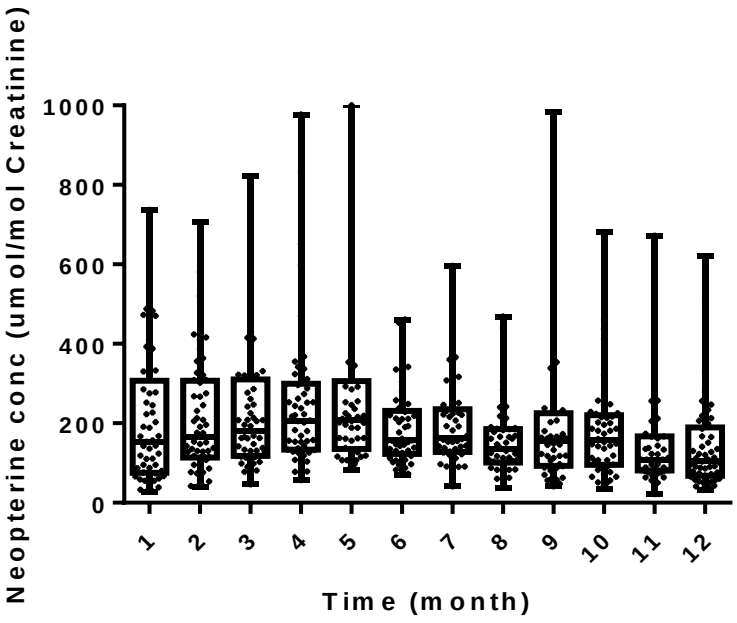

Figure 4.3: Boxplot showing average monthly urine neopterin levels for 12 months with individual subjects' mean monthly neopterin levels. Whiskers represent the minimum and maximum points. The box represent 25th and 75th percentile and the line represents median point. N = 57

From available literature, normative urine neopterin level among people over age 60 is 251 umol/molCreatinine (Fuchs et al., 1992; Hausen et al., 1982; Murr et al., 2002). Levels above this value is considered elevated. Four main patterns of neopterin levels over 12-month period were identified (figure 4.4):

A. Subjects with urine neopterin levels always below normal limit (251 umol/ mol creatinine) (N=7)

B. Subjects with neopterin levels above normal levels less than 25% of the time (N=23)

C. Neopterin levels above normal limit between 25 ≤ 50% of the time (N=22)

D. Subjects with neopterin levels above normal limits equal or greater than 50% of the time (N=5)

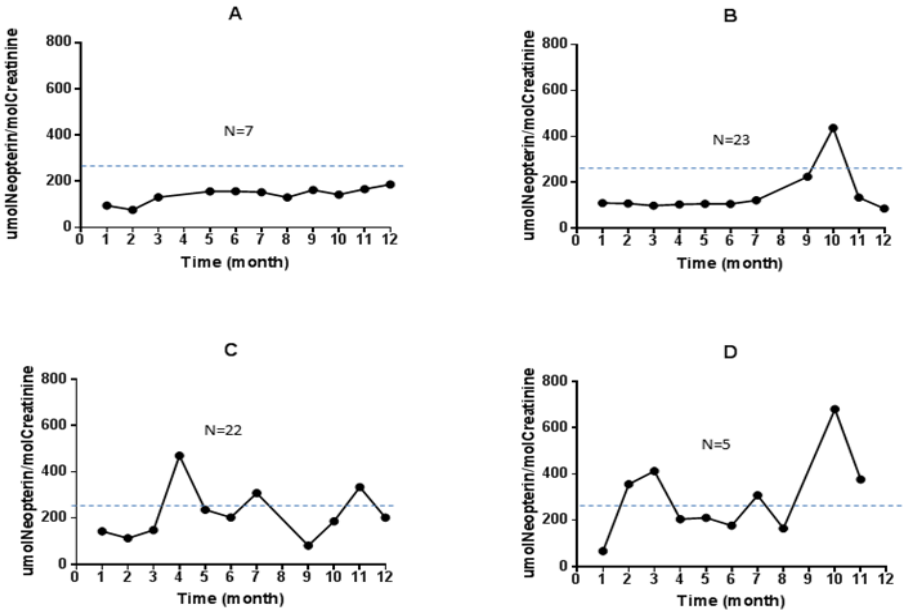

Figure 4.4: Exemplars of urine neopterin pattern over 12-month period. A; monthly neopterin levels always within normal range (N=7), B; neopterin levels above normal levels less than 25% of time (N=23), C; neopterin levels above normal levels 25-50% of time (N=22), D; neopterin levels above normal levels more than 50% of time (N=5). Blue dashed line represent normal limits of urine neopterin (251 umol/molCreatinine).

4.3.3 Linear associations between measured baseline inflammatory markers

Inflammatory markers (urine neopterin, serum neopterin, WBC, neutrophils, lymphocytes, monocytes, eosinophils, basophils, IFN-γ, TNF-α, IL6 and IL1-β) were not normally distributed even after data transformation was performed. Spearman correlation between baseline inflammatory markers showed significant correlations between serum neopterin and urine neopterin ($r = 0.311$, $p = 0.048$), serum neopterin and IFN-γ ($r = 0.394$, $p = 0.007$), IFN-γ and TNF-α ($r = 0.320$, $p = 0.018$). IFN-γ and IL6 ($r = 0.341$, $p = 0.012$), IFN-γ and monocytes ($r = 0.342$, $p = 0.036$), IL6 and TNF-α ($r = 0.468$, $p < 0.001$). Other significant correlations were between WBC and differentials: WBC and neutrophils ($r = 0.936$, $p < 0.001$), WBC and lymphocytes ($r = 0.664$, $p < 0.001$), WBC and monocytes ($r = 0.699$, $p < 0.001$), WBC and eosinophils ($r = 0.474$, $p = 0.002$). Neutrophils and lymphocytes ($r = 0.419$, $p = 0.008$), neutrophils and monocytes ($r = 0.688$, $p < 0.001$), neutrophils and eosinophils ($r = 0.370$, $p = 0.020$), lymphocytes and monocytes ($r = 0.547$, $p < 0.001$) lymphocytes and eosinophils ($r = 0.366$, $p = 0.022$). Table 4.3 shows correlation results for relevant inflammatory markers, the remaining correlation results are in appendix F.

The result highlights that urine neopterin levels correlates with serum levels. Additionally, the significant positive correlation between IFN-γ levels and neopterin and between IFN-γ and monocytes supports the evidence that monocytes under the stimulation of IFN-γ produce neopterin.

	UNeop	SNeop	TNF	IFN	IL6	IL1	WBC	Mono
UNeop(R)	1	**0.311**	-0.008	0.231	-0.139	-0.129	0.165	0.250
p-level		**0.048**	0.954	0.106	0.335	0.420	0.338	0.142
SNeop(R)	**0.311**	1	0.105	**0.394**	0.098	0.136	-0.202	-0.104
p-level	**0.048**		0.494	**0.007**	0.524	0.411	0.294	0.591
TNF (R)	-0.008	0.105	1	**0.320**	**0.468**	0.170	0.020	-0.068
p-level	0.954	0.494		**0.018**	**<0.001**	0.265	0.907	0.687
IFN (R)	0.231	**0.394**	**0.320**	1	**0.341**	-0.047	0.046	**0.342**
p-level	0.106	**0.007**	**0.018**		**0.012**	0.761	0.785	**0.036**
IL6 (R)	-0.139	0.098	**0.468**	0.314	1	0.149	0.180	-0.097
p-level	0.335	0.524	**<0.001**	0.012		0.327	0.279	0.562
IL1 (R)	-0.129	0.136	0.170	-0.047	0.149	1	0.138	0.019
p-level	0.420	0.411	0.265	0.761	0.327		0.453	0.919
WBC (R)	0.165	-0.202	0.020	0.046	0.180	0.138	1	**0.688**
p-level	0.338	0.294	0.907	0.785	0.279	0.453		**<0.001**
Mono (R)	0.250	-0.104	-0.068	**0.342**	-0.097	0.019	**0.688**	1
p-level	0.142	0.591	0.687	**0.036**	0.562	0.919	**<0.001**	

Table 4.3: Table showing Spearman's correlation between inflammatory markers measured for 57 subjects at baseline. UNeop (urine neopterin), SNeop (serum neopterin) WBC (white blood count). Significant correlations are in bold.

4.3.4 Multivariate associations between neopterin and demographic characteristics

Multiple regression analysis was performed with log of mean neopterin as the outcome measure and age, gender, chronic disease, smoking and statin use as independent variables. Age was the only continuous variable the other variables were binary. Gender was coded as 1= male 2 = female; chronic disease as 0 = no chronic disease, 1 = chronic disease; smoking status as 0 = non-smoker, 1 = smoker and statin use as 0 = non-user of statin, 1 = statin-user. Using the enter method, a significant regression equation was found $F(5, 51) = 6.15$, $p < 0.001$, $R^2 = 0.38$. The analysis showed that $\log_{10}$ mean neopterin level is predicted as = 1.74 + 0.11 (gender) + 0.11 (chronic disease) + 0.09 (smoking status) – 0.12 (statin use).

From the results, female gender, being a smoker and having a chronic age-related disease was associated with high neopterin levels; however, the use of statins was associated with lower neopterin levels. Log_{10} mean neopterin data was back transformed in the graphs representations (figures 4.5-4.8) to make the differences more relevant and informative. Age was not a significant

variable in the regression, which could be because subjects were all within a small age range (65 - 75). Table 4.4 shows multiple regression results for all variables.

Variable	Unstandardized coefficient (B)	SE	Standardized coefficient (β)	p-value
Constant	1.74	0.33		**<0.001**
Age	0.01	0.01	0.12	0.34
Gender	0.11	0.03	0.41	**0.001**
Chronic disease	0.11	0.04	0.45	**0.006**
Smoking	0.09	0.04	0.27	**0.03**
Statin use	-0.12	0.04	-0.47	**0.003**

Table 4.4: Association between $\log_{10}$ transformed mean neopterin level, demographic and lifestyle variables. Significant p-values are highlighted in bold. N = 57

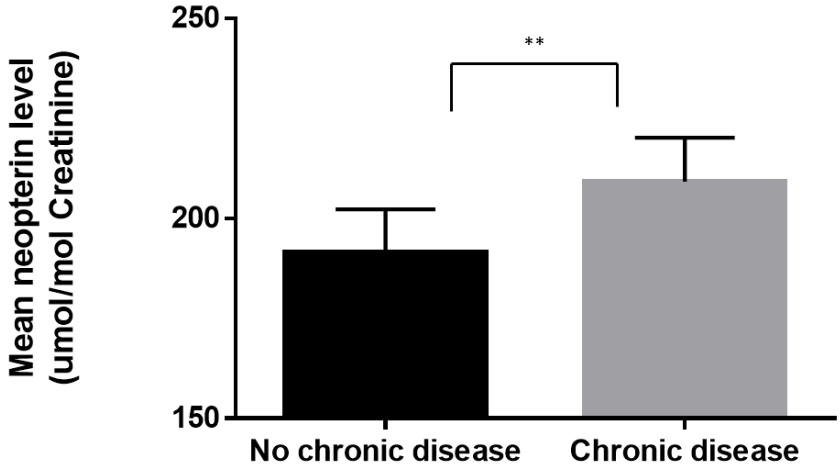

Figure 4.5: Bar chart showing the difference in mean neopterin levels between subjects with no chronic disease and subjects with at least a chronic disease. Error bar represent standard error of mean. No chronic disease (N = 23) Chronic disease (N = 34)

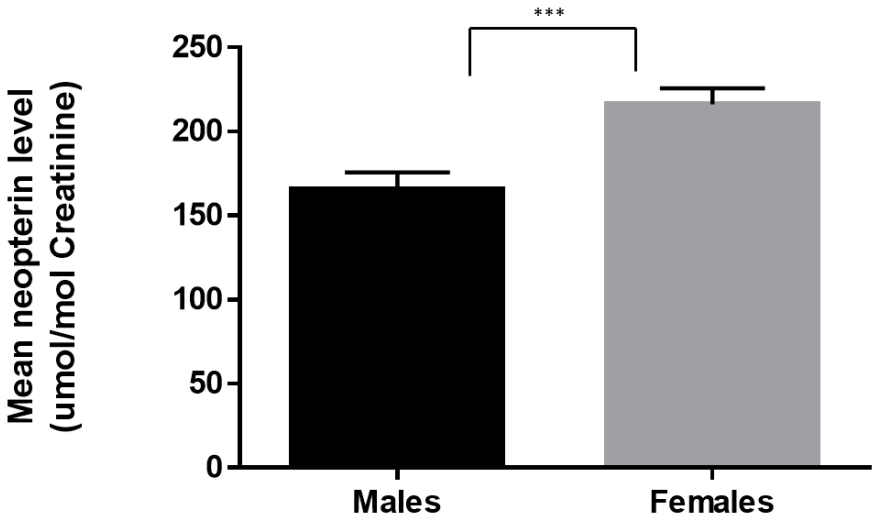

Figure 4.6: Bar chart showing the difference in mean neopterin levels between males and females. Males (N = 16) Females (N = 41).Error bar represents standard error of mean

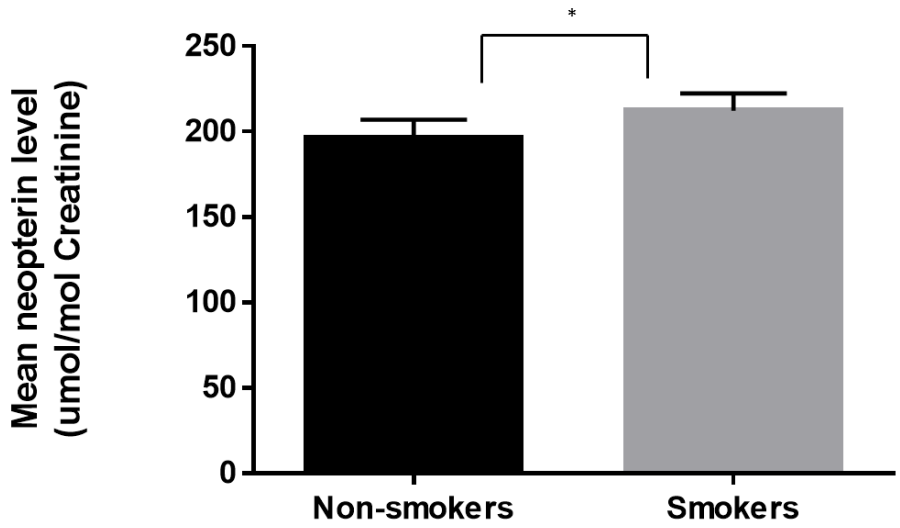

Figure 4.7: Bar chart showing differences in mean neopterin levels between smokers and non-smokers. Non-smokers (N = 36) Smokers (N =21). Error bar represents standard error of mean.

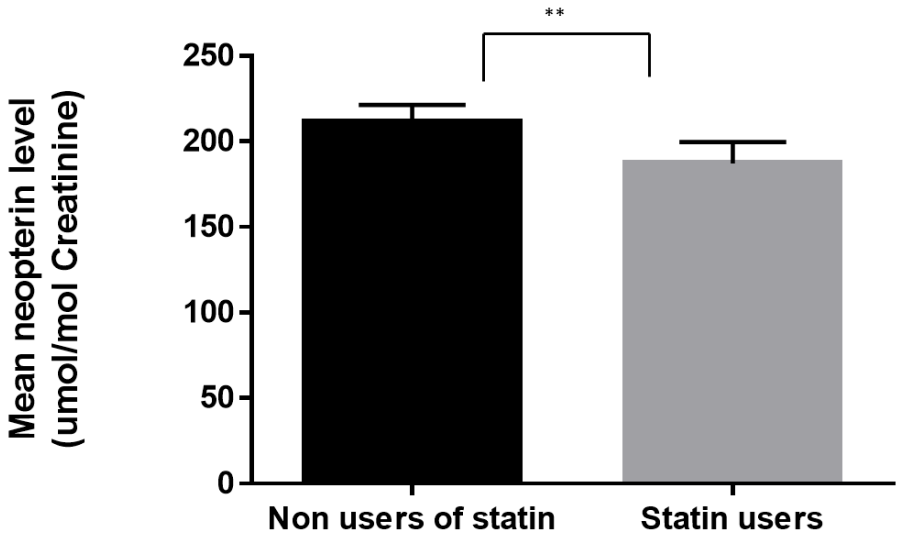

Figure 4.8: Bar chart showing differences in mean neopterin levels between statin users and non-users. Statin users (N = 22) Non-users (35). Error bar represents standard error of mean.

4.4 Discussion

In this study, we have measured serial urine neopterin levels in 57 community dwelling older individuals of ages 65 to 75 to assess their level of inflammatory status, and also to investigate the relationship between their inflammatory status and chronic age-related diseases. There is growing evidence that low-grade chronic inflammation associated with ageing is a key driver of many age-related diseases (Franceschi et al. 2000; Hunt et al. 2010b). This means that control of inflammation may be a viable way of reducing the progression of chronic diseases.

Subjects showed variation in urine neopterin levels both from month to month and generally between individuals. Four neopterin pattern were identified over 12-month period; a group that always had neopterin levels below normal levels, a group with flare-ups in neopterin levels less than 25% of the time, a third group with raised neopterin levels 25 – 50% of the time and a last group with raised levels more than half of the time. The last group can be viewed as the inflammatory group. All samples were taken in the morning (between 9am and 12 noon), to avoid diurnal variations. The design of the study was such that the difference in age between subjects was not more than a decade. The rationale for this was that since decline in the immune system's ability to mount appropriate responses is broadly associated with ageing, having subjects of similar age would reduce the effect of age on their inflammatory status. Variations in neopterin levels is likely to be due to exposure to acute or chronic inflammatory agents and the individual's ability to respond to inflammation. Also, since neopterin levels rise before clinical symptoms,

subclinical infection may also cause elevations in neopterin levels and contribute to the creation of a pro-inflammatory milieu in the absence of actual disease (Hunt et al. 2010a).

Our results confirm that measuring urine neopterin levels is a credible way of assessing immune activation status. From our results, urine neopterin levels are correlates with serum neopterin levels. This supports results from other studies (Werner et al. 1987; Nancey et al. 2008). The use of urine instead of blood samples provides a non-invasive and easy way to obtain samples for monitoring purposes. This provides an opportunity in future to develop this further such that non-medical professionals will be able to administer neopterin tests in a home setting. Serum neopterin levels measured in our cohort also correlated with IFN-γ levels. Interferon-gamma, is known to be a primary stimulator of neopterin (Murr et al. 2002) and the association between serum levels of IFN-γ and neopterin have been shown in studies (Huber et al. 1984).

Although neopterin is produced chiefly by activated monocytes/macrophages, monocyte count was not found to be correlated to neopterin levels. Monocytes perform three main functions when activated; phagocytosis, antigen presentation and cytokine production (Fujiwara & Kobayashi 2005). Therefore, functional measures of monocytes including phagocytic abilities, levels of neopterin or other cytokine levels may provide better assessment of monocyte activation than cell count. In addition, IFN-γ is known to be an inducer of monocyte activation, which explains the association between monocytes and IFN-γ levels instead of between monocytes and neopterin. Activated monocytes leave the blood stream to form tissue macrophages and therefore a count of monocytes in systemic circulation may not reflect an activated state. There is evidence that monocytes are able to produce microparticles (Piccin et al. 2007). Microparticles are vesicles measuring 100 - 1000 nm released into circulation by budding of plasma membrane of cells (Mause & Weber 2010). This is a physiological process; however, it has also been associated with the chronic diseases like atherosclerosis (Mause & Weber 2010). Since microparticles relay biological signals from the parent cells, they are capable of performing like the parent cell, functions including cytokine production, cell signalling, protein synthesis and exchange of biological information (Mause & Weber 2010). It is possible that monocyte-producing microparticles can produce the effects of activated monocytes without increase in actual monocyte cell count.

Neopterin measures have been used in different settings to monitor disease progression (Reibnegger et al. 1987; Weimer et al. 2006; Zouridakis et al. 2004; Nancey et al. 2008). In autoimmune diseases including SLE, Rheumatic arthritis and Crohn's disease, neopterin levels have been found to correlate well with disease activity since the activity of these diseases are modulated by proliferation of T-lymphocyte activity, which induces the production of IFN-ɣ to

activate the neopterin pathway (Altindag et al., 1998; Elwy et al., 2010; Nancey et al., 2008). Neopterin levels have also been used to monitor the progression of certain malignancies and for their prognosis as neopterin production may be induced in certain types of tumours including haematological tumours, breast cancer, lung cancer and pancreatic cancer (Berdowska & Zwirska-Korczala 2001; Murr et al. 2002). These studies provide evidence that neopterin measures can be used as a monitoring tool in diseases that causes immune activation.

Studies have investigated associations between other inflammatory markers and age-related diseases including frailty and Alzheimer's disease (AD) (Holmes et al. 2011; Baylis et al. 2013). Some of the inflammatory markers that have been investigated include TNF-α, CRP and IL-6 (Holmes et al. 2011; Nash et al. 2013). In the Holmes et al (2011) study, both TNF-α and IL-6 was found to be associated with a 2-fold increase in sickness behaviour in 300 AD patients. In their analysis, cut-off points were used to distinguish between low and high inflammatory levels. Other studies have used similar groupings in their investigation of the link between inflammatory markers and chronic age-related diseases (Holmes et al. 2009; Green et al. 2010). In our study, however, since we had up to 12 measures from each subject, mean levels of markers were used for the analysis. Another study that investigated the association between CRP levels and depression in a longitudinal study (Copeland et al. 2012), also used mean levels of CRP assay in a multiple regression analysis. Similarly, other studies, both cross-sectional and longitudinal, have used one-time measured levels or mean levels of inflammatory markers to investigated associations with diseases (Teunissen et al. 2003; Jenny et al. 2012; Baylis et al. 2013). Baylis et al (2013) investigated the association between white blood cell count (WBC) and 10-year risk of frailty. Adjusting for other covariates, the study used regression models to analyse associations between measured WBC and frailty. Evidence from these studies show that categorising inflammatory markers into low and high groups and using the actual levels as a continuous data are both acceptable ways of analysing results within the inflammation literature.

From our previous MRC study (Verschuur, Agyemang-Prempeh, & Newman, 2014) and other cross-sectional studies (Leng et al., 2009; Verschuur et al., 2012) elevated immune markers including WBC measured at a single time point has been associated with age-related diseases. These studies were conducted in subjects who self-reportedly had no acute illness; therefore, it was assumed that their level of inflammatory markers was consistent with their baseline measures. Few studies have investigated associations between neopterin and age-related diseases, one such study which found associations between high neopterin levels and frailty, used serum neopterin measured at a single time point (Leng et al. 2011). To the knowledge of the author, no study has investigated the association between longitudinal measures of urine neopterin and the occurrence of age-related diseases and lifestyle factors in the elderly.

Longitudinal inflammatory measures are more likely to reflect a person's immune status and therefore may be better associated with age-related chronic disease. However, our results did not show an association between neopterin levels and age-related chronic disease. The fact that in a small sized cohort, serial neopterin measures is associated with the prevalence of age-related chronic disease suggests that the association between inflammaging or immune activation and age-related diseases is robust.

Interestingly, we found associations between neopterin levels and demographic factors like female gender and lifestyle factors like smoking. Elevated neopterin levels were associated with female gender (figure 4.6). While similar gender pattern have been shown in some studies (Fuchs et al. 1992b), others have not found this association (Werner et al. 1987). The disparity in the number of male (16) and female (41) subjects may have contributed to this effect. Smoking is known to exert pro-inflammatory effect by direct activation of immune cells and by inducing secretion of pro-inflammatory chemokines and cytokines including IFN-γ (Lee, Taneja, & Vassallo, 2012). From our results, neopterin levels are affected by smoking status and smokers are associated with higher levels of neopterin (figure 4.7).

Statins are lipid-lowering medications that act by inhibiting the enzyme HMG-CoA reductase in the formation of cholesterol. Statins have been shown to reduce the risk for cardiovascular diseases (Antonopoulos et al., 2012). In addition, statins have anti-inflammatory effects. They are known to reduce the levels of pro-inflammatory cytokines, especially c-reactive protein (Albert et al., 2001). Statins are also thought to reduce T-cell activation, macrophage infiltration and vascular inflammation (Antonopoulos et al. 2012). The study reflected the anti-inflammatory properties of statins, as statin use was associated with low neopterin levels (figure 4.8).

Results of this study have demonstrated that serial urine neopterin measures can be used to measure inflammatory status in older adults and that inflammatory status is associated with chronic diseases. This underscores the significance of inflammation in the mechanisms of age-related diseases. If the hypothesis that inflammation plays a role in the progression of age-related diseases is supported, it means controlling inflammation will potentially reduce the progression of age-related diseases.

To further extend this study and to make it relevant for ARHL, the association between neopterin levels and ARHL will be investigated in the next chapter.

4.4.1 Limitations

The results of the study and its interpretation is limited by the fact that the study was underpowered. Therefore, the interpretation of the results, both significant and non-significant, should be done with caution. The study however, provides preliminary data for further investigation on the association between age-related diseases and inflammatory markers including neopterin.

4.5 Conclusion

- Serial urine neopterin levels can be measured in older adults to assess inflammatory status.
- Serum neopterin levels are positively associated with urine neopterin levels, indicating that urine neopterin can be used in place of serum levels to monitor immune activation.
- High neopterin level (inflammatory state) is associated with the co-occurrence of age-related diseases.
- Female gender and smoking is associated with increased levels of neopterin and statin use is associated with reduced levels of neopterin.

Chapter 5: Inflammatory load can predict risk of age-related hearing loss

Chapter 4 explores the use of extended high frequency pure-tone audiometry (PTA) and otoacoustic emissions (OAE) to detect early changes in the progression of ARHL. In addition, the chapter investigates the effect of neopterin in the progression of hearing loss. Audiometric and OAE (TEOAE, DPOAE 65/65 and DPOAE 70/70) measures were obtained from the neopterin cohort (chapter 3) at the start of the study and after one year. Otoacoustic emission measures at some frequencies detected decline in hearing function after year 1, however, neither conventional PTA nor extended high frequency OAE detected significant worsening of hearing. Low frequency average threshold was associated with inflammatory load (mean neopterin levels). This chapter highlights the potential use of a combination of OAE and inflammatory measures for early detection and monitoring of ARHL.

5.1 Introduction

Findings in chapter 3 have demonstrated an association between inflammatory load measured by neopterin volatility and chronic age-related diseases. This provides a potential marker for stratifying older people according to their risk of age-related diseases. In this chapter, the inflammatory tertile groups identified in chapter 3 were used to predict the prevalence as well as the progression of ARHL in the cohort. The long-term objective of the study is to use a combination of inflammatory and hearing measures including traditional pure-tone audiometry (PTA), extended high frequency PTA and otoacoustic emission (OAE) to predict people who may be at greater risk of rapidly progressing age-related hearing loss (ARHL). This suggests that especially in high-risk people, inflammation may play a pivotal role in the mechanism of ARHL, therefore in such people, anti-inflammatory measures that may be lifestyle or pharmacological interventions have the potential to reduce the progression of ARHL. This is an on-going 3-year longitudinal study aimed to determine the association between inflammation and age-related hearing loss (ARHL). Data shown here are for the first year into the study.

5.1.1 Monitoring hearing decline with pure-tone audiometry

A number of tests including pure-tone audiometry, otoacoustic emission and auditory brainstem response can measure hearing function. Pure-tone audiometry is a behavioural test that is used to measure hearing sensitivity. It also gives information about the configuration and type of hearing

loss. Pure-tone audiometry measures the audibility of the quietest tone that a person can respond to at least 50 % of the time (BSA 2011). The conventional frequencies measured are octave intervals from 0.25 to 8 kHz. Pure-tone audiometry is thought to be the gold standard for measuring hearing function (Sindhusake et al. 2001) and it is currently the most common diagnostic hearing test carried out at clinics. The test is believed to assess the whole auditory pathway (Mehrparvar et al. 2014; Büchler et al. 2012). However, PTA is a subjective test, since the person being tested has to respond to the audibility of the sound that is presented. In addition, since PTA thresholds are tested in 5 dB steps, it is less sensitive to changes in hearing threshold that are less than 5 dB. Test-retest reliability of audiometry using insert phones has been found to be 5 dB (Jungmee Lee et al. 2012; Borton et al. 1989), and 10 dB using HDA 200 ear phones (Schmuziger et al. 2004). In order to assess changes in hearing, the increase in threshold should ideally be greater than the test-retest reliability of audiometry.

There is evidence that measuring hearing threshold at frequencies higher than conventional ones (9-20 kHz), extended high frequency (EHF) audiometry, can detect early changes in hearing before conventional frequencies (0.25-8 kHz) (Mehrparvar et al. 2014). This is based on the premise that damage to the organ of Corti typically begins first at the basal region that is responsible for high frequency sounds (Balatsouras et al. 2005). However, since decline in the EHF threshold is thought to begin very early in life, it is difficult to ascertain the cause of the decline. Measuring EHF has some challenges such as the absence of internationally accepted standards and challenges with calibration and individual variability (Balatsouras et al. 2005). Despite these challenges, many studies have successfully used conventional and EHF audiometry to monitor changes in hearing threshold (Büchler et al. 2012; Yu et al. 2014; Campbell et al. 2003).

5.1.1.1 Detecting ototoxicity induced and noise induced hearing loss

Exposure to noise at high intensities have been known to result in changes in hearing which can be temporary, known as temporary threshold shift (TTS) or a permanent threshold shift (PTS). Changes in hearing threshold caused by noise exposure typically manifests on the audiogram as a notch between frequencies 3 and 6 kHz (Cooper & Owen 1976). However, studies have also explored the use of EHF in detecting changes in hearing due to noise exposure and ototoxicity (Mehrparvar et al. 2014; Büchler et al. 2012; Campbell et al. 2003).

Buchler et al (2012) investigated the use of conventional PTA, EHF and otoacoustic emission (OAE) in monitoring hearing loss due to acute noise trauma. Forty-one soldiers (age 19-24) who had acquired hearing loss as a result of rifle-induced acute noise, together with 30 age-matched control subjects with normal hearing (< 25 dB HL for frequencies 0.125 – 8 kHz), participated in the study. All subjects had normal hearing prior to acoustic trauma. Pure-tone audiometry at

frequencies 0.125 – 8 kHz and EHF audiometry at frequencies 9 – 20 kHz was performed. Conventional PTA identified hearing loss at frequencies 3-6 kHz. In addition, EHF audiometry identified hearing loss at 11-14 kHz (Büchler et al. 2012). The findings led to the conclusion that two frequency regions were affected by rifle-induced noise trauma, indicating that both conventional and EHF audiometry were both useful in the monitoring of noise induced hearing loss. Another study (Mehrparvar et al. 2014) had similar results to the Buchler study. The study compared the use of conventional audiometry, EHF audiometry and OAE in the evaluation of noise-induced hearing loss. Subjects included 142 noise exposed ceramic workers and 121 age-matched control who had no other known risk factor to hearing loss. The mean exposure to noise for the exposed group was 91.97 ± 4.15 dB A. Their results showed that conventional PTA as able to detect hearing loss in 29% of subjects at frequencies 4-6 kHz, EHF audiometry detected hearing loss in 69% of subjects at frequencies 14-16 kHz and OAE between 22- 52% of subjects (Mehrparvar et al. 2014). The study suggests that EHF audiometry is the most sensitive test for monitoring noise-induced hearing loss. There are other studies (Balatsouras et al. 2005) that have not found additional benefit for performing EHF audiometry since most of the changes in hearing occurred within 4-8 kHz regions.

Some studies have investigated the best monitoring protocol for hearing loss following ototoxic medication (Yu et al. 2014; Konrad-Martin et al. 2010; Bass & Bhagat 2014). Hearing monitoring following ototoxic medication is important not only for recovery from cochlea damage but also for the patient's well-being and quality of life. For example, in children, timely management of hearing loss is important for language development. The aim of hearing monitoring is to preserve hearing whenever possible, and detect hearing loss early in order to limit adverse consequences to quality of life as a result of hearing loss. Monitoring provides early diagnosis, progression of hearing loss, evidence of dose limits for ototoxic medication, at what point to begin hearing rehabilitation and when to consider alternative treatment or changes to dosage (Jacob et al. 2006). American Speech-Language-Hearing Association (ASHA) have set up some guidelines for monitoring ototoxicity (ASHA, 1994). This involves a four-stage approach:

1. Baseline test – Auditory thresholds prior to the commencement of treatment should be obtained, which should be done latest by 72 hours into treatment with aminoglycoside antibiotics and no later than 24 hours into treatment with cisplatin or similar medication. Baseline test are PTA from 0.25 – 8 kHz and EFH audiometry at 9, 10, 11, 12, 14, 16 18 and 20 kHz.
2. Monitoring tests – Conventional and EHF audiometry should be performed during treatment. This may be dependent of the dosage and the medication being administered,

which should ideally be 2-3 days within treatment with aminoglycosides and 24 hours before each new dose of cisplatin.

3. Immediate post-treatment test – Full audiometric testing should be performed as soon as practicable after completion of treatment.
4. Post-drug follow-up tests - follow-up hearing tests should be performed three and six months after completion of treatment and if hearing loss is found, weekly monitoring should be performed to monitor progression.

Based on ASHA guidelines and evidence from other studies (Yu et al. 2014; Campbell et al. 2003; Büchler et al. 2012), using conventional and EFH audiometry is viable way of monitoring hearing loss in ototoxicity and noise-exposure. Based on these findings the possibility of using similar methods to monitor ARHL have been investigated in some studies.

5.1.1.2 Monitoring ARHL using audiometry

Although many studies have investigated the incidence and prevalence of ARHL (Gates et al. 1990; Mościcki et al. 1985; Cruickshanks et al. 1998; Davis 1989) only a few studies in comparison have explored monitoring the progression of ARHL (Wiley et al. 2008; Cruickshanks et al. 2003). The results of studies that have investigated the progression of ARHL are often difficult to compare with each other because the rate of hearing loss progression differs for different age groups and different studies have used different age groups for their investigation. Some epidemiological studies have investigated the progression of hearing loss using pure-tone audiometry are discussed below.

Cruickshanks et al. (2003) investigated the 5-year incidence and progression of hearing using participants from the Epidemiology of Hearing Loss Study, Beaver Dam, Wisconsin. The study recruited 1636 participants, age 48-92 years without hearing loss and performed baseline tests including otoscopy to examine the ear canal and tympanic membrane, tympanometry to exclude conduction hearing loss and PTA. After five years, tests were repeated for 1085 participants who reported for follow up. Hearing loss was defined as a pure-tone average threshold at frequencies 0.5, 1, 2 and 4 kHz of greater than 25 dB HL in either ear. Progression of hearing loss was defined as a change of more than 5 dB greater than baseline measurement. Among participants with normal hearing, the incidence of hearing loss after five years assessed by PTA was 21.4%. In participants with baseline hearing loss, their average hearing threshold after five years increased (became poorer) from 39.5 to 46.3 dB HL, with a calculated progression rate of 53.3%. The rate of hearing loss progression was found to be increased with advancing age; 33% for age 48-59, 47.9% for age 60-69, 62% for age 70-79 and 75.4% for age 80-92 (Cruickshanks et al. 2003).

Gates and Cooper (1991) studied the progression of hearing loss over six years in 1475 participants in the Framingham Heart study. They performed PTA for the participants at the start of the study and repeated after six years. Changes in hearing were analysed for each frequency (0.25, 0.5, 1, 2, 3, 4, 6 and 8 kHz), average pure-tone thresholds (0.5, 1 and 2 kHz) and high frequency average thresholds (4, 6 and 8 kHz). Changes were compared across age, gender and ear (left and right). Change in hearing after six years ranged 1-8 dB at 0.25-6 kHz and 10-15 dB at 8 kHz. Average pure-tone threshold (0.5-2 kHz) worsened in 8.5-13.5% of participants in at least one ear and 4.1% in both ears. High frequency average threshold worsened in 31-35% of participants in one ear and 18.7% both ears. The rate of decline in hearing acuity increased with age, although age accounted for 9% of the variance, suggesting that other covariates may be involved in hearing loss progression. Wiley et al (2008) investigated threshold changes in 2130 participants aged 48-92 over a 10-year period. They measured pure-tone thresholds (0.25-8 kHz) at baseline, 2.5, 5 and 10 years. From their results, changes in thresholds were more remarkable at low frequencies than high frequencies in the older (70-89) participants but in younger (50-69) participants the reverse was found, changes at high frequency thresholds were greater than changes in low frequencies. In addition, the rate of hearing change was greater for males compared to females (Wiley et al. 2008). Although the study measured hearing thresholds at 2.5 and 5 years, no significant threshold changes were reported at those time points; hearing change reported was found at the 10-year measurement.

From the above epidemiological studies, a number of conclusions can be inferred. Change in hearing in ARHL may take more than five years to detect using PTA, unlike hearing loss due to ototoxic medication or noise trauma. Changes in hearing vary across age group, frequencies and between males and females. The rate of hearing loss progression was lower at high frequencies compared to low frequencies, especially for the elderly participants and for younger participants, high frequency hearing loss progression was observed (Wiley et al. 2008; Gates & Cooper 1991; Brant & Fozard 1990). The reason for these findings could be that older participants had greater baseline hearing loss at high frequencies, which for many had approached the audiometric limit and therefore further change in hearing could not be measured (Wiley et al. 2008). Gates and Cooper (1991) suggested that different pathological processes were responsible for high and low frequency hearing loss. Using evidence from histological studies, they postulated that high frequency loss is due to hair cell loss, hearing loss at 4 kHz is mostly due to environmental noise and low frequency hearing loss was due to strial atrophy.

Human hearing ranges from 20 Hz to 20 kHz from birth, with progressive loss of high frequency hearing occurring through life. Since hearing loss typically starts at the high frequencies; EHF testing could potentially be used to monitor the progression of hearing loss. There is evidence

that more than 50% of people have measurable thresholds up till 14 kHz (Wiley et al. 1998). Some studies have explored the use of EHF in measuring changes in ARHL (Rodríguez Valiente et al. 2014; Wiley et al. 1998; Lee et al. 2005).

Wiley et al (1998) investigated the effects of ageing on high frequency hearing in 3396 participants between ages 48 and 92. Pure-tone thresholds were measured at conventional frequencies (0.25-8 kHz) and EHF (9-20 kHz). From their results, thresholds were significantly higher for older age groups. Thresholds for males were significantly higher than for female at the lower EHF (9-14 kHz) however, no difference was found at the very high frequencies (16-20 kHz). In addition, from their results, EHF thresholds (up to 14 kHz) was measurable for more than 50% of adults up to age 7. A study by Lee et al (2005), measured pure-tone thresholds at conventional and extended high frequencies for 188 older adults aged 60-81 years. The study was longitudinal, spanning 3-11.5 years and EHF measured from 9-18 kHz. The average rate of change of thresholds for EHF was 1.23 kHz, compared to the rates for conventional frequencies, which was 0.7-1.2 dB. The rate of change of thresholds increased with increasing age, with older females showing a faster rate at low frequencies (Lee et al. 2005).

From the above studies, EHF at frequencies 9 -14 kHz is measurable in older adults. Further investigations into the use of EHF and improved international standards for EHF audiometry are needed in other to develop the potential for its use as a screening and monitoring tool for ARHL progression. As part of this study, we will investigate the use of EHF (12 kHz) in the progression of ARHL.

5.1.2 Production of otoacoustic emission

A second test that can potentially detect a decline in hearing function before it becomes detectable with PTA is the OAE test. Otoacoustic emissions (OAE) testing is a form of hearing test that measures sounds that are produced by the inner ear when stimulated. This is an objective test, as it does not require responses from the person being tested. The ear will produce OAE response when hearing is normal but will fail to produce an OAE when hearing loss of 25 dB or greater is present (Kemp 2002). The most common application of OAE is in the neonatal hearing screening programme. Other uses of OAE are testing people who are unable to perform behavioural tests, to aid in the diagnosis of auditory processing disorder, audiological assessment for cochlear implantation and in the monitoring of ototoxicity.

Otoacoustic emissions (OAE) are low intensity sound vibrations produced in the cochlea and transmitted through the middle and outer ears (Kemp 2002). As sound is transmitted through the cochlea, vibrations of the basilar membrane are detected by the outer hair cells (OHCs) causing

the OHCs to respond by contracting and elongating. The motion of the OHCs adds extra energy to amplify the amplitude of the sound wave before it reaches the IHC for activation and onward transmission through the auditory nerve. However, the motion of the OHCs is enough to send vibrations to the basilar membrane in the opposite direction to produce OAEs (figure 4.1). A probe fitted with a microphone placed in the ear canal can pick up sound waves travelling back from the cochlea through the round window to the middle and outer ear (figure 4.2). The cochlear amplifier role of the OHC processes sound non-linearly (Gorga et al., 2007). Low intensity sounds are amplified to enhance detection, as stimulus level increases, cochlear gain reduces by becoming nonlinear, and the response becomes compressive. The non-linear way in which the cochlea processes sound increases the dynamic range of hearing (Gorga et al., 2007). Otoacoustic emissions are produced only when OHC are intact and are thought to be a by-product of the nonlinearity of the cochlea (Kemp 2002). Therefore, OAEs are a reflection of the cochlear function. Although OAEs are unlikely to reveal anything about the function of parts of the auditory pathway beyond the cochlea, it is well correlated to PTA (Gorga et al., 1993).

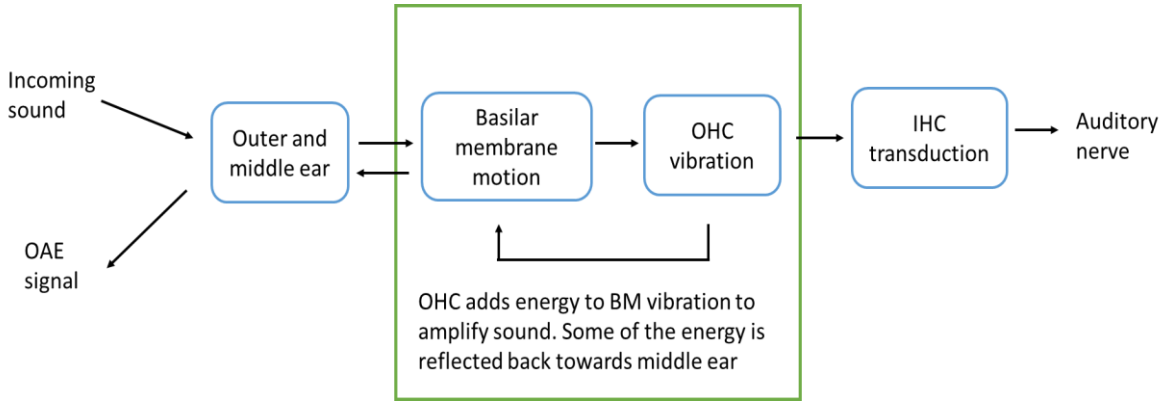

Figure 5.1 Pathway for the production of otoacoustic emission (OAE). Incoming sound presented at the outer ear travels through the middle ear to the cochlea, causing vibrations of the basilar membrane. Outer hair cells (OHC) contracts to add energy to the wave until it is enough to result in an action potential in the inner hair cells (IHC) with release of neurotransmitter. Some of the energy travels towards the round window as OAE, which can be detected.

5.1.3 Types of otoacoustic emissions

Otoacoustic emissions can be either spontaneous or evoked. Spontaneous otoacoustic emissions occur naturally in the ear without applying any additional stimulus and are present in 50% to 70% of normal hearing individuals (Stach 2010). This study focuses on evoked OAEs, which are elicited by presenting a sound. Two types of evoked OAE have been investigated in this study are transient emission otoacoustic emissions (TEOAE) and distortion product otoacoustic emissions (DPOAE).

5.1.3.1 Transient evoked otoacoustic emissions (TEOAE)

Transient evoked otoacoustic emissions are elicited by stimuli such as broadband frequency clicks presented at the external auditory meatus. A probe containing a loudspeaker, which produces the click signal, and a microphone to record OAE response, is fitted into the ear canal (figure 4.2). The clicks stimulate the length of the cochlea and then after recording, present the response in frequency bands. Two waveforms recordings are completed in order to ensure reproducibility. In a normal hearing ear, a click stimulus of about 84 dB SPL peak equivalent (pe) will elicit a response. Responses are strongest at the speech frequencies, 1 – 4 kHz. Unlike other forms of hearing tests like PTA, sound intensity does not need to be at threshold level in order to detect abnormalities. Transient evoked otoacoustic emission are highly sensitive to cochlear damage, a loss greater than 20 dB will result in absence of TEOAE at that frequency (Kemp 2002). Due to its sensitivity, TEOAE has applications in the neonatal hearing screening programme.

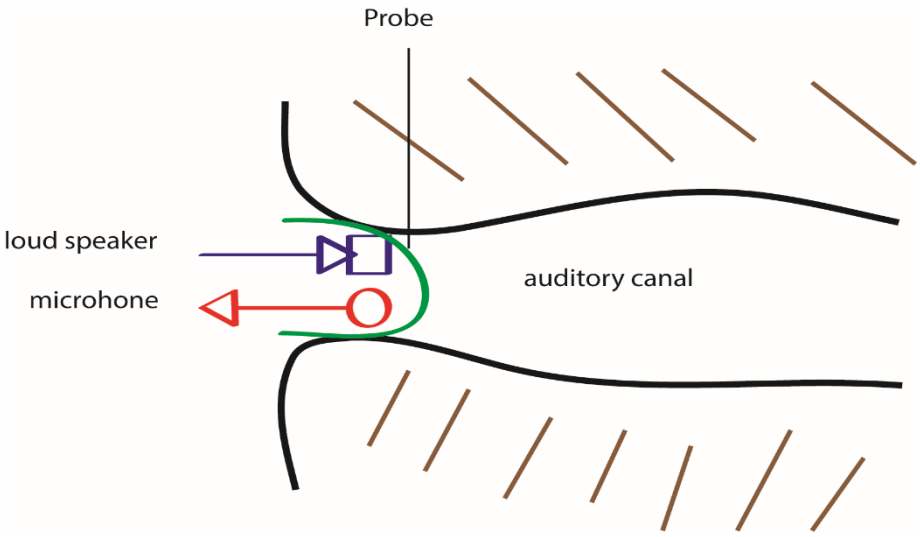

Figure 5.2 Diagram of an OAE probe fitted into the ear canal. Probe consists of loudspeaker that produces the incoming signal and a microphone to pick up OAE signal reflected back from the cochlea

5.1.3.2 Distortion product otoacoustic emissions (DPOAE)

Distortion product otoacoustic emissions (DPOAE) is an evoked OAE response by two pure-tones presented simultaneously. When two tones, f1 and f2, of slightly different frequencies (f1 = 1425 and f2 = 1500 Hz) are presented simultaneously, non-linear intermodulation of the two waves are generated, the largest of which is produced at frequency 2f1-f2 (Kemp 2002). The new generated wave can travel towards the ear canal as OAE, which can be detected by a fitted microphone (figure 4.2). Therefore, the loudspeaker in this case produces two pure-tones of slightly different frequencies. The response elicited from DPOAE typically range from 1 – 6 kHz, offering a broader frequency range compared to TEOAE (Stach 2010). DPOAE can be produced in ears with hearing

loss of up to 50-60 dB (Kemp 2002), making it possible to be used in people with mild to moderate hearing loss, therefore potentially useful in monitoring progression of ARHL.

5.1.4 Application of OAE testing in hearing loss progression

The success of the use of OAE in the new born hearing screening in many countries have led to the consideration that OAE can be potentially used in early detection and monitoring of ARHL. Since OHC degeneration is a prominent feature of ARHL, it implies that an assessment of OHC function by OAE testing can contribute to the assessment of the type and extent of ARHL. A screening programme that allows for early detection of ARHL would mean difficulties in communication and social issues associated with ARHL could be reduced through early intervention. This study therefore investigates the possibility of measuring and monitoring hearing loss in the elderly using OAE.

Many of the studies that have investigated the use of OAE in monitoring hearing loss progression have been for noise-induced (Helleman & Dreschler 2012; Job et al. 2009) or ototoxicity-induced (McMillan et al., 2012; Reavis et al., 2011) hearing loss, since noise and ototoxic medication are known to cause degeneration of the OHC. The effect of ototoxic medication and high intensity noise on hearing can occur within a short time, which allows changes in hearing function to become detectable at an earlier time point.

Job et al (2009) investigated the ability of DPOAE to predict long-term hearing loss in pilots who had been exposed to aircraft engine noise. The study performed PTA and DPOAE measurements for 512 pilots aged 20-40. Normal audiogram was defined as thresholds of up to 10 dB at all frequencies (0.25-8 kHz); 219 subjects had normal audiograms. DPOAE levels for the subjects with normal audiograms were categorised into either low or high DPOAE response based on an index calculated by 69 control subjects. After 3-year follow-up, subjects who at baseline were categorised as having low DPOAE responses had worse audiograms, and a higher risk of early hearing loss compared to the group with high DPOAE responses. They concluded that DPOAE was a risk marker for subsequent hearing loss (Job et al. 2009). The study provides evidence that DPOAE can detect changes in hearing function before they become detectable by PTA. However, control study used in their study was not age or gender-matched, therefore, it was not possible to know if ageing or gender effects contributed the difference in DPOAE.

A study by Lapsley Miller (2006) measured audiometric threshold, TEOAE and DPOAE responses in 338 sailors age 18 – 46, before and after 6 months exposure to noise on an aircraft carrier. Their results showed reduction in OAE amplitudes before they became apparent on PTA. They observed

that low level or absent OAE often resulted in permanent threshold shifts. The study showed TEOAE at 4 kHz to be the best predictor of permanent threshold shifts (Lapsley Miller et al., 2006).

Some studies have compared the ability of conventional PTA, EHF and OAE testing in detecting hearing loss progression (Yu et al. 2014; Mehrparvar et al. 2014; Knight et al. 2007). Yu et al. (2014) investigated the use of PTA, EFH and OAE in monitoring hearing loss due to ototoxicity in ten patients who had undergone treatment with cisplatin. Conventional PTA covered frequencies 0.25-8 kHz, EFH thresholds were measured at frequencies 9-20 kHz and DPOAE from 0.5 to 8 kHz. The patients were tested prior to the start of chemotherapy and again after receiving six cycles of chemotherapy. The results obtained was of a mixed nature. Together, conventional and EHF PTA detected changes in threshold in four out of ten subjects, DPOAE also detected changes in amplitude in four subjects however, only two of the subjects showed changes for both PTA and DPOAE. In one subject, DPOAE changes were detected earlier than PTA changes. Frequencies at which hearing loss occurred were 3-10 kHz (Yu et al. 2014). With only ten subjects, the sample size may have affected the reliability of their results.

Mehrparvar et al. (2014) compared the sensitivity of conventional PTA (0.25-8 kHz), extended high frequency PTA (10, 12, 14 and 16 kHz) and DPOAE (0.5-8 kHz) to detect hearing abnormality in 142 noise exposed workers and 121 control subjects. Subjects were less than 50 years, had no exposure to ototoxic medication, no conductive hearing loss and non-smokers. Their results showed DPOAE amplitude significantly differentiated between subjects and controls at all frequencies. Comparing the three tests ability to detect abnormal findings, EHF detected hearing loss in 69% of subject, DPOAE detected abnormal changes in 52% of subjects and conventional PTA was least able to show hearing loss, as only 29% showed change in threshold (Mehrparvar et al. 2014). In a similar study, PTA, EHF and DPOAE were measured for 32 children receiving chemotherapy. Baseline and serial hearing measurement were performed. In 62.5% of subjects, PTA detected changes in thresholds between baseline and after treatment whiles DPOAE detected reduced amplitudes in 81.3% of patients and EHF identified reduced thresholds in 94.1% (Knight et al. 2007).

These studies shows evidence that OAE testing may be sensitive in the early detection of hearing loss and potentially can be used to monitor progression. Many of the OAE studies have used younger subjects and it is important to investigate whether OAE can be produced in the older population and whether this method can be used for early detection and monitoring of ARHL. Unlike hearing loss due to ototoxicity, the progression of ARHL is generally a slow one. Lee et al (2005) reports an average progression rate of 1 dB per year for people over age 60. Since threshold changes in ARHL are less than the test-retest reliability of PTA, small threshold changes

may not be picked up by either conventional or EHF audiometry. Therefore, OAE presents as an alternative or complimentary test for early detection and monitoring progression of ARHL.

5.1.5 Otoacoustic emission in ARHL

Age-related hearing loss in humans typically begin as loss to high frequency sounds that eventually spread to affect mid and low frequencies. These changes have been primarily attributed to damage to the cochlea structures including atrophy of the stria, loss of outer hair cells and loss of spiral ganglion cells, although there is now evidence to suggest contribution from central auditory system. Otoacoustic emission has been shown to be a measure of the functioning of OHC therefore there is a good correlation between OAEs and the non-linear function of OHC (Kemp 2002; Gorga et al. 2007). Otoacoustic emission testing has been used as an objective substitute in situations where it is difficult to obtain behavioural measures. It has been suggested that OAE may be more sensitive in identifying cochlear damage earlier than will be detectable by PTA. From the studies discussed above (Mehrparvar et al. 2014; Knight et al. 2007), there is evidence that OAE detects reduction in hearing function due to ototoxicity of noise exposure in a greater number of subjects than conventional PTA.

There is the hypothesis that OAEs are affected by age and many studies have investigated this association (Uchida et al. 2008; Oeken et al. 2000; Stover & Norton 1993). A subject under controversy is whether OAE changes is due to purely ageing factors or due to decline in hearing sensitivity independent of ageing. Some researchers have found age to significantly affect OAE independent of hearing thresholds (Abdala & Dhar 2012; Uchida et al. 2008) while others have postulated that the decline of OAE is mainly due to decreased hearing thresholds (Stover & Norton 1993; Oeken et al. 2000). Some of the controversy have come about from differences in methodology, the inclusion of confounding variables and the age group being investigated.

Abdalah and Dhar (2012) investigated how ageing affects DPOAE in 156 ears. Subjects ranged from pre-term new born to older adults. Their results showed that DPOAE levels is highest at 6-8 months old and after that begin to decline with age (Abdala & Dhar 2012). However, only non-infants could be tested using PTA, which meant comparison of DPOAE with PTA results was limited. In addition, pure-tone thresholds were poorer in older subjects, therefore it could not be determined if the decline in OAE for older subjects OAE was due to age or hearing loss.

Stover et al (1993) measured PTA, spontaneous, click-evoked, tone-burst-evoked and DPOAE in 42 subjects from age 20 to 80, grouped into seven decades. Their results showed a decline in OAE measures with age however, further analysis was unable to separate the age effect from the effect of hearing threshold. Although all the subjects had pure-tone average threshold within

normal limits (< 25 dB HL), there was a significant difference between thresholds for the older subjects (60 years and above) and the younger subjects (less than 60 years), especially at frequencies 4-8 kHz. They therefore concluded that the effect of age is intricately related to hearing sensitivity since factors responsible for OAE production are also related to audiometric thresholds (Stover & Norton 1993).

Uchida et al (2008) investigated the effect of ageing on DPOAE response in 331 subjects between age 40 and 80 with normal hearing. To isolate the effect of hearing loss from ageing, all subjects had strict audiometric thresholds of better than 15 dB HL. Analysis was performed separately for males and females. Their results showed significant differences in DPOAE amplitudes among age groups at frequencies 4-6 kHz for males and all measured frequencies except 3 kHz for females. Despite the stringent audiometric criterion, younger males had significantly better audiometric thresholds at 4 kHz compared to younger females, and younger females had significantly better thresholds than older females at all the measured frequencies 0.5-8 kHz. The PTA findings was similar to the DPOAE findings, indicating how inter-related hearing thresholds is with OAE response. However, multivariate analysis showed a negative effect of age on DPOAE (Uchida et al. 2008). The subjects in this study were selected from a larger population-based cohort, offering external validity to the study results.

These studies give evidence that OAE levels decline with age. However, it is difficult to distinguish between pure age-effects from the effects of hearing sensitivity. This is mainly because cochlear damage, particularly loss of OHC, which results in decline of DPOAE, also contribute significantly to decline in PTA threshold. It is possible that OAE is able to detect age-related hearing changes before they become detectable by PTA. Another concern would be whether OAE could detect insidious hearing changes since ARHL progresses slowly. Evidence from DPOAE studies in diabetics have shown this is possible (Ottaviani et al. 2002; Botelho et al. 2014).

Ottaviani et al (2002) investigated OAE changes in 60 patients with type 1 diabetes. Patients' age range was 20-43, with duration of disease between 2 and 39 years. All patients and 58 control subjects had normal hearing. Their results showed that a lower OAE response from diabetes than controls. Mean intensity and reproducibility of TEOAE was significantly lower in diabetics than for control subjects. Whiles every control subject had measurable TEOAE response, 28.3% of diabetic subjects had absent TEOAE response in at least one ear and 10% in both ears. Similarly, DPOAE responses were significantly reduced compared to control subjects. Similar results have been shown in other studies (Botelho et al. 2014; Lisowska et al. 2001). In the Botelho et al (2014) study, whiles 7.7% of diabetic subjects had significantly worse pure-tone compared to controls,

32% of diabetics had poor DPOAE response compared to 3.7% in control subjects. It is possible that DPOAE may have revealed subclinical hearing changes that did not show up in audiometry.

Other studies on the other hand have not found significant OAE differences between diabetics and non-diabetic controls (Erdem et al. 2003). Erdem et al (2003) investigated subclinical auditory dysfunction in 21 subjects with diabetes, 21 with high levels of triglyceride and 15 with high cholesterol. None of the subjects had a combination of the diseases. From their results, apart from DPOAE at 4 kHz, there was no difference in TEOAE response and the other DPOAE frequencies between the diabetic subjects and non-diabetic control subjects. The study combined people with high cholesterol, high triglycerides and diabetes in the same sample pool, which could have confounded the results. In addition, the study did not exclude or account for subjects with other risk factors for hearing loss including noise exposure and use of ototoxic medication, which could have accounted for the findings at 4 kHz.

From the above studies, there is evidence from ototoxicity, noise exposure and diabetes studies that OAE can potentially detect subclinical hearing loss.

5.1.5.1 Otoacoustic emission test for screening and monitoring ARHL

Older adults would benefit from hearing screening programmes since hearing loss is most prevalent in this group. Screening programmes often involve PTA or hearing handicap scales. Since older adults are often limited by cognitive function, mobility and other medical conditions, objective tests including OAEs would be beneficial screening test since they are fast and do not require response from the person being tested.

Jupiter (2009) investigated the use of DPOAE as a screening tool and compared the outcome with PTA and hearing handicap inventory for the elderly. The study subjects were 106 elderly adults age 65-91. The screening protocols used were pure-tone average ≤ 30 dB HL at 1, 2 and 3 kHz, ≤ 40 dB HL at 1, 2 and 3 kHz, screening version of Hearing handicap Inventory for the elderly (HHIE-S) and DPOAE. The DPOAE test performed was a screening type and a pass was obtained if a response is obtained in three out of four frequencies (2, 2.5, 3.2 and 4 kHz). The findings showed a significant positive correlation between PTA and DPOAE results, pure-tone average ≤ 40 dB HL at 1, 2 and 3 kHz having the strongest correlation with DPOAE. The study concluded that DPOAE was comparable to PTA as a screening tool for elderly adults, with sensitivity of 98%.

Scudder et al (2003) found DPOAE screening to have good test-retest reliability of 80%, however, it could not predict actual hearing loss in older adults 67% of the time, when compared to ASHA standard of hearing loss, (pure-tone average > 25 dB HL). These results were obtained from a study that was aimed at evaluating the ability screening for hearing loss in adults using hearing

screening tools including screening PTA, screening DPOAE, otoscopy, self-assessment of communication and case history screening. The study did not exclude people with conductive hearing loss which could have accounted for the weak correlation between DPOAE and PTA, as the presence of conductive loss interferes with OAE production (Kemp 2002). Other studies have however shown a higher correlation between DPOAE and PTA, particularly at high frequencies (Boege & Janssen 2002; Gorga et al. 2003).

Although some studies have investigated screening hearing loss using OAE, there is lack of evidence for using OAE measures to monitor ARHL. In this chapter, we first investigated the link between inflammation and ARHL by assessing associations between mean neopterin levels and hearing function using both PTA and OAE measures. Secondly, we investigated the ability of DPOAE, TEOAE and EHF audiometry to detect ARHL earlier than it becomes detectable by conventional PTA. In addition, we explored the use of TEOAE and DPOAE in monitoring ARHL in a longitudinal setting.

5.1.6 Hypotheses

1. Inflammation is associated with ARHL. Therefore, older adults with high neopterin (inflammatory) levels will have poorer hearing compared to subjects with lower neopterin levels.

2. Extended high frequency audiometry, TEOAE and DPOAE measures are sensitive to detect change in hearing function within one year.

3. High inflammation (neopterin) levels is associated with the progression of hearing loss.

5.1.7 Aims

1. To measure changes in hearing thresholds within one year using PTA and extended high frequency PTA.
2. To measure OAE responses of subjects at two time points, one year apart using TEOAE, DPOAE at f1/f2 levels of 65/55 and 70/70.
3. To investigate the association between neopterin levels and hearing sensitivity and hearing loss progression.

5.2 Methods

5.2.1 Participants

Recruitment of subjects is as described in section 4.2.2. All 57 subjects had their hearing measured at the start of the study, which comprised PTA, TEOAE and DPOAE. Four subjects however, did not have their hearing measured at the end of the first year; therefore the data showing hearing change was for 53 subjects.

5.2.2 Equipment

5.2.2.1 Pure-tone audiometry

Pure-tone audiometry testing was performed using GSI G1 Clinical Audiometer, which was calibrated according to ISO-389-1: 2000 standards. Tones of frequencies 0.5 - 8 kHz were presented through TDH39 supra-aural earphones. Extended high frequency PTA was measured only at 12 kHz and tones were presented through HDC200 supra-aural earphones. The RadioEar B71 bone vibrator was used for measuring bone conduction thresholds.

5.2.2.2 Otoacoustic emission testing

Measurement of OAE was performed using Otodynamics ILO 292 equipment and EZ-Screen software (Otodynamics, ILO V6 Module) connected to a laptop computer (figure 11). The ILO 292 equipment is designed to measure both Transient evoked otoacoustic emission (TEOAE), Distortion product otoacoustic emission (DPOAE). A standard adult probe was used for delivery of the stimulus into the ear.

5.2.3 Procedure

Hearing measurement took place in a soundproof booth. The ambient noise in the booth, measured with a sound level meter was always below 35 dB A. Otoscopy was performed in each subject to exclude the presence of wax, and other ear abnormalities including perforated tympanic membrane and middle ear infections. Subjects with occluding wax were requested to have them removed by their GP. Subjects completed the questionnaire covered in section 3.2.3.1

5.2.3.1 4.2.3.2 Pure-tone audiometry

Pure-tone audiometry (PTA) was performed according to the British Society of Audiology (BSA) recommended procedure (BSA 2011). Pure-tone audiometry air-conduction threshold was

conducted at frequencies 0.25, 5, 1, 2, 3, 4, 6, 8, and 12 kHz for both ears. TDH39 earphones were swapped for HDC200 at 12 kHz since it had the appropriate calibration to measure thresholds at 12 kHz. Thresholds for bone conduction were determined at 0.5, 1, 2 and 4 kHz. Thresholds were determined at 5 dB intervals. The limit of the audiometer was 110 dB HL, when threshold was beyond the limit of the audiometer it was assigned a threshold of 120 d B HL. Where the air-conduction threshold between at a frequency left and right ears was more than 40 dB or the bone-conduction thresholds more than 10 dB, masking was performed. Pure-tone average threshold was calculated with the threshold at frequencies 0.5, 1, 2 and 4 kHz for each ear. Low frequency average (0.25, .5 and 1 kHz) and high frequency averages (6, 8 and 12 kHz) were also calculated for each ear. Asymmetry between ears was defined as more than 20 dB difference between left and right ears; subjects with asymmetry between the ears were excluded from the study since it was likely that other pathologies such as acoustic neuromas and not ARHL may account for it. One subject who had asymmetrical hearing was withdrawn from the study and was given a copy of his audiogram and advised to make an appointment with his GP. Full PTA procedure is included in appendix B

5.2.3.2 Otoacoustic emission

TEOAE, DPOAE and input-output functions were recorded from subjects comfortably seated in a treated sound booth. Stimuli were transmitted to the ear via a standard adult probe-microphone system. TEOAE recordings were made at 1, 1.5, 2, 3 and 4 kHz using Quickscreen mode. The stimulus was a conventional non-linear click train presented as a set of four clicks at rate of 50 clicks/s, each set comprising one at a maximum of 90 dB peSPL and three at 80 dB peSPL.

DPOAE recordings were generated from two pure-tone stimuli, f1 and f2, and presented simultaneously at two different sound levels:

- f1= 65 dB and f2 = 55 dB
- f1 = 70 dB and f2 = 70 dB

The ratio of the presented pure-tone frequencies was f1/f2 = 1.22. DPOAEs were recorded at four frequencies per octave for 1, 2 and 4 kHz, and two frequencies per octave at 8 kHz, resulting in responses at the following frequencies: 841, 1000, 1189, 1414, 1682, 2000, 2378, 2828, 3364, 4000, 4757, 5657, 6727 and 8000 Hz. The criteria for accepting DPOAE as present was SNR > 3 dB (Abdala & Visser-Dumont, 2001; Dorn et al., 2001). Distortion product (DP) growth function was recorded at 2000 and 4000 Hz using L2 levels 45 dB to 75 dB, in 5 dB steps. L1 levels were based on the formula L1 = 0.4L2 + 39 dB (L1 level corrected to the nearest 5 dB) (Boege & Janssen 2002). Protocol for OAE is included in appendix C.

5.2.4 Statistical analysis

Statistical analysis was completed using Graph Pad Prism version 6 and IBM SPSS version 24. Three audiometric averages were calculated from individual frequency threshold to create workable outcome measures, since individual frequencies were not normally distributed. Pure-tone average (PTAv) was average threshold of 0.5, 1, 2 and 4 kHz, LFA 0.25, 0.5 and 1 kHz and HFA 6, 8 and 12 kHz. The analysis was performed on the worse ear, which was the ear with the highest PTAv. The worse ear was chosen in order to create consistency with our previous MRC study in chapter 3 and to enable comparisons to be made with other studies (Lin et al. 2012; Verschuur et al. 2012; Helzner et al. 2005). In addition, since data with asymmetric hearing loss had been excluded, it was unlikely that the choice of ear will greatly affect the results. Multiple regression was performed using baseline PTAv, LFA and HFA as outcome measures with independent variables as mean neopterin level, average WBC and other known risk factors of hearing loss including age, gender, noise exposure, chronic disease and smoking status. Multiple regression analysis was repeated with SNR at TEOAE (1, 1.5, 2, 3 and 4 kHz), DPOAE 65/55 (1, 2, 4 and 8 kHz) and DPOAE 70/70 (1, 2, 4 and 8 kHz). T-test was performed for hearing outcome at baseline and year 1 to investigate significant change in hearing. Where significant change in hearing was found, linear regression was performed to determine the effect of neopterin level on the change in hearing. Data on DP growth function was not analysed in this report.

Ethics approval was covered in section 3.2.6

5.3 Results

5.3.1 Descriptive data

5.3.1.1 Pure-tone audiometry

Pure-tone audiometry configuration compiled for the cohort using data at the start of the study showed a bilateral, high frequency sloping hearing loss (figure 4.3). Hearing loss was observed at frequencies above 1 kHz (figure 4.3 and tables 4.1). The cohort comprised 16 males and 41 females with average ages of 69.3 and 69.2 years respectively. Males had worse hearing compared to females, particularly at the mid to high frequencies (figure 4.4). Conventionally, hearing threshold levels ≤ 20dB is said to be within normal limits (BSA, 2011); the percentage of subjects within normal limits declined from 80% at 0.25 kHz to zero at 12 kHz. Five subjects had hearing thresholds within normal limits at all the conventional audiometric frequencies (250 Hz to 8 kHz) in at least one ear. Forty-six subjects (80.7%) had measureable thresholds at 12 kHz, ranging from 50 to 100 dB HL. Degree of hearing loss, determined by the average threshold at

frequencies 0.5, 1, 2 and 4 kHz was calculated for subjects; 40.3% had normal hearing, 50.9% with mild hearing loss and 8.8% with moderate hearing loss (table 4.1).

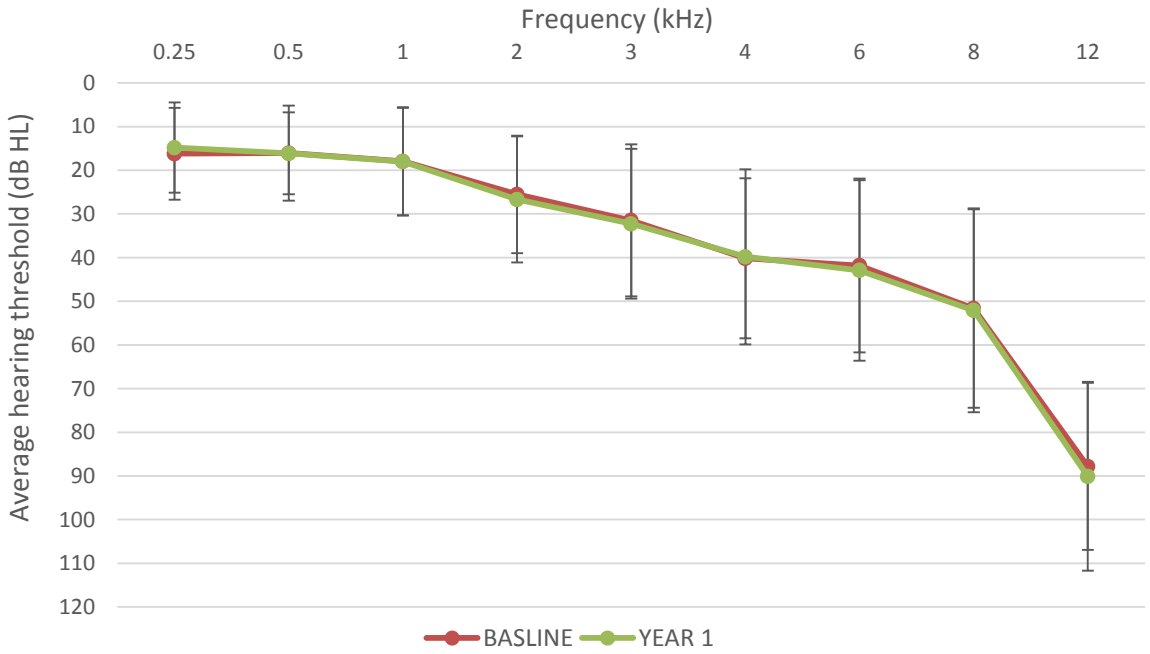

Figure 5.3 Average pure-tone audiometric thresholds for subjects. Thresholds from the worse hearing ear by air conduction across frequencies 0.25-12 kHz. Thresholds at the start of study and after one year. Error bars represent standard deviations. Thresholds show a high frequency sloping hearing loss. (N = 57) Year 1 (N = 53)

Degree of hearing loss	*Pure-tone average (dB HL)*	*Number (percentage %)*
Normal hearing	≤ 20	23 (40.3)
Mild hearing loss	21 - 40	29 (50.9)
Moderate hearing loss	41 - 70	5 (8.8)

Table 5.1 Proportion of subjects with hearing within normal limits, mild or moderate hearing loss. Based on pure-tone average thresholds (0.5, 1, 2and 4 kHz) at the start of the study (N = 57).

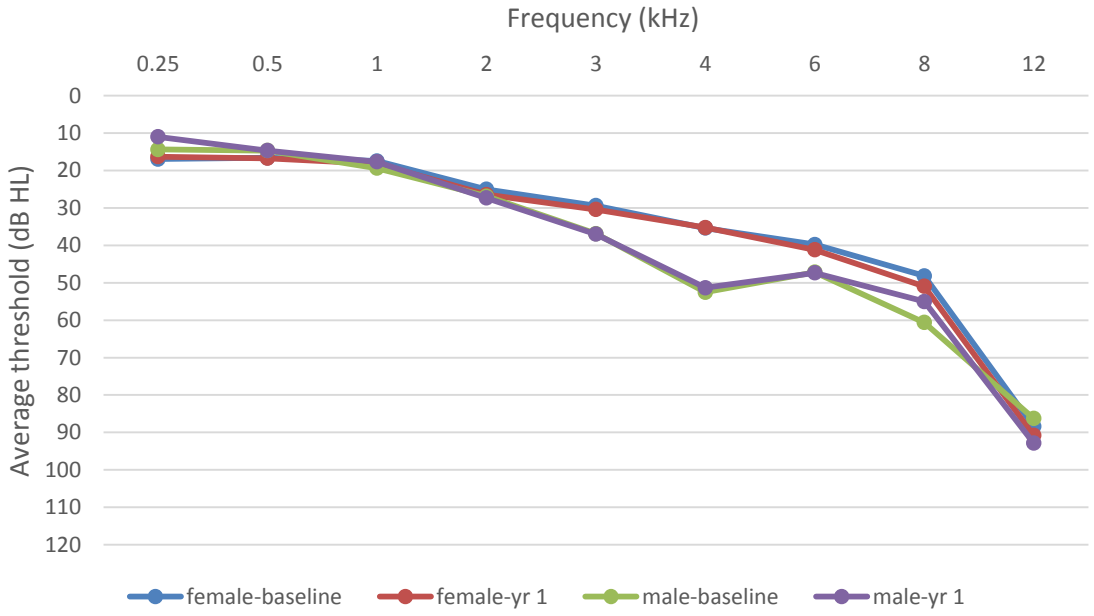

Figure 5.4: Average audiometric thresholds of worse hearing ear for male and female subjects at the start of study and after one year. Females (N = 41), males (N = 16)

5.3.1.2 Otoacoustic emission

Otoacoustic emission responses were measured as a signal to noise ratio (SNR), therefore they were measured against noise floor. Some of the responses were below the noise floor, which may have been caused by low emission due to cochlea damage, high noise levels or both. Different studies have used different criteria for accepting OAE responses. Some studies have used a criterion of SNR ≥ 6 dB (Botelho et al. 2014) to signify true response, while others have used lower SNR criteria (Dorn et al. 2001; Helleman & Dreschler 2012). The conservative approach would have been the use an SNR ≥ 6 dB as criteria for a true OAE response. However, given that we were investigating ARHL, we expected some of subjects to have some level of cochlea damage and therefore exhibit low OAE emission. We therefore decided to use all data points measured in order to include all subjects, with the expectation that the noise level will cancel out since measurements were performed by the same person and under the same experimental conditions. Secondly, the use of a high SNR criterion of ≥ 6 dB could potentially eliminate some true responses. On the other hand, accepting all responses could potentially introduce a large number of false positive responses (noise) into the data, however, we expect the noise to be relatively constant in all subjects and therefore cancel out.

Responses for TEOAE, DPOAE at f1/f2 levels of 65/55 and 70/70 were measured at baseline and year 1 (figure 5.5 -5.7). Generally, average SNR for all types of OAEs were higher (better) than SNRs at year 1. TEOAE responses were highest at 1.5 and 2 kHz and lowest at 4 kHz. DPOAE

responses were lowest at the high frequencies, particularly at 8 kHz. TEOAE produced the highest SNR and DPOAE 65/55 had the least response (figure 5.5-5.7). In all three types of OAE, females generally had better SNRs than males (figures 5.8 – 5.10).

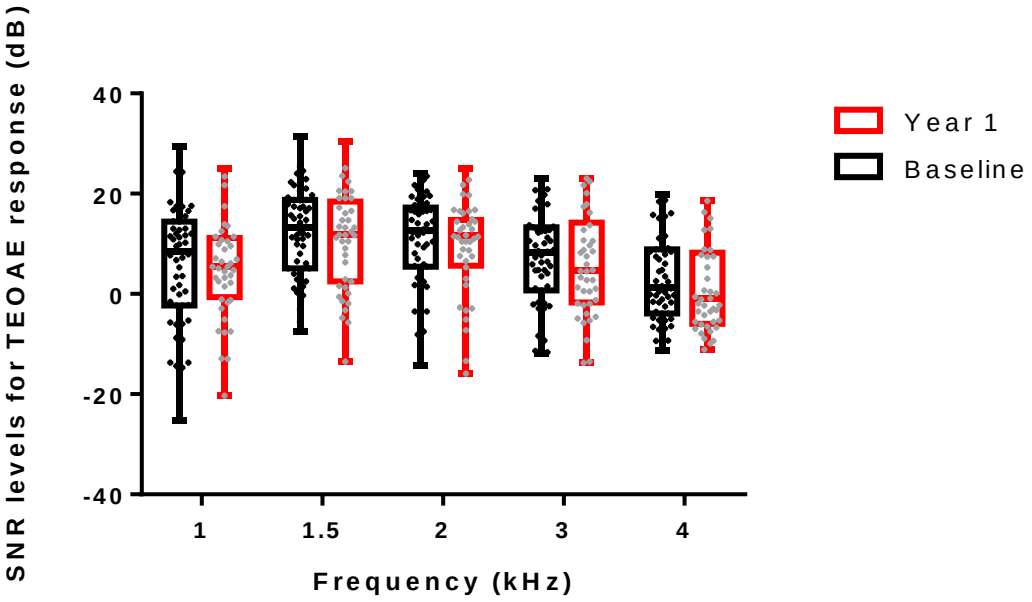

Figure 5.5: TEOAE response of subjects at baseline and year 1. Box represents 25th aand 75th quartile, line represents the median SNR, whiskers represent the minimum and maximum SNR and each point represents individual SNR for each subject. Baseline (N = 57) Year 1 (N = 53)

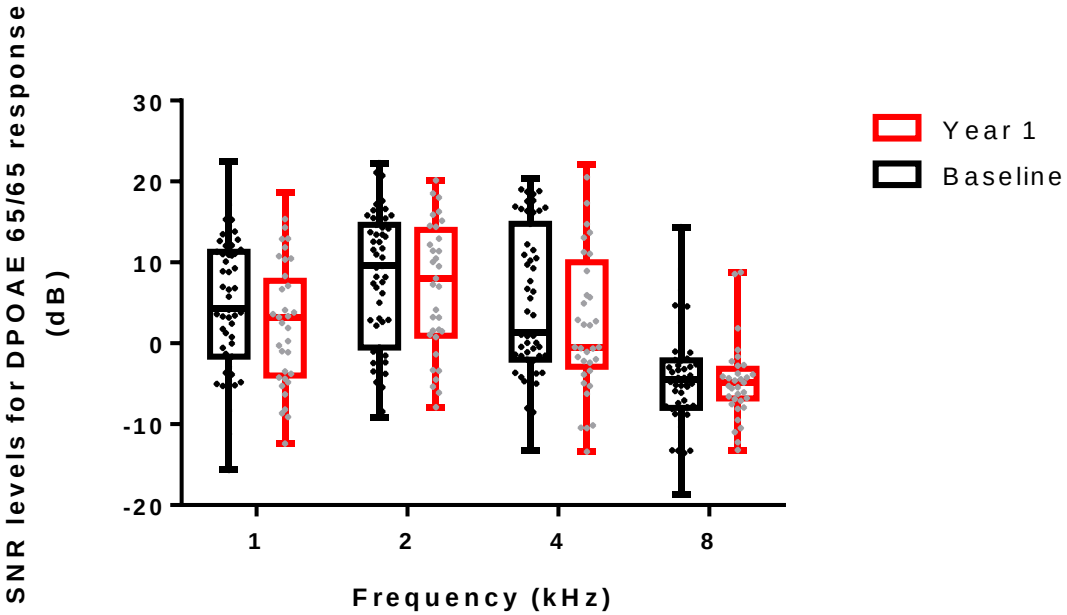

Figure 5.6: DPOAE 65/55 response for subjects at baseline and year 1. Box represents 25th and 75th quartile, line represents the median SNR, whiskers represent the minimum and maximum SNR and each point represents individual SNR for each subject. (N = 57) Year 1 (N = 53)

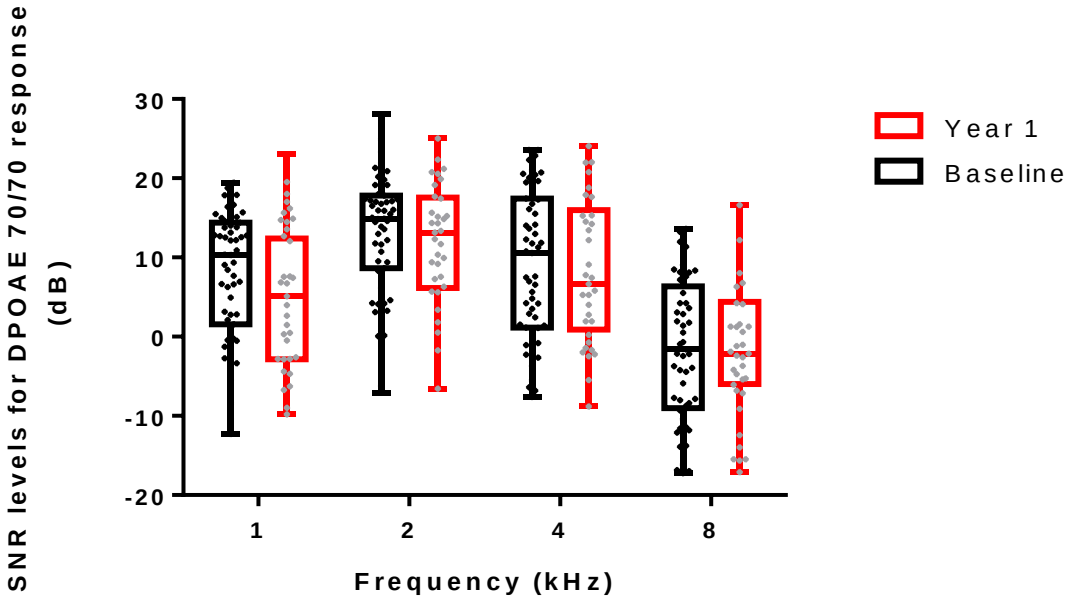

Figure 5.7: DPOAE 70/70 response for subjects at baseline and year 1. Box represents 25th and 75th quartile, line represents the median SNR, whiskers represent the minimum and maximum SNR and each point represents individual SNR for subjects. (N = 57) Year 1 (N = 53)

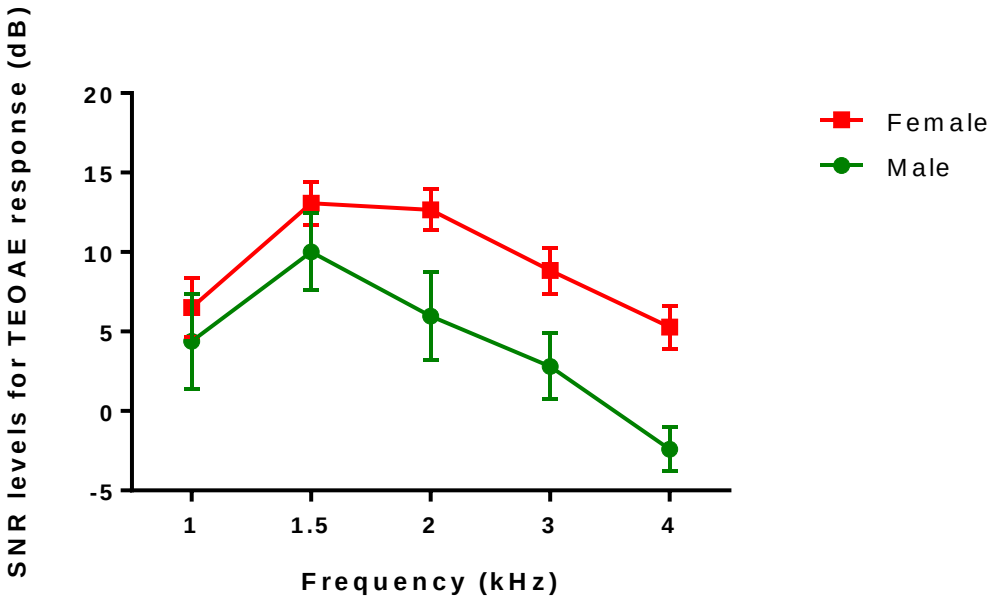

Figure 5.8: Mean TEOAE responses at frequencies for males and females. Error bars represent standard error of mean. Baseline N (males = 16, females = 41) Year 1 (males = 14, females = 39).

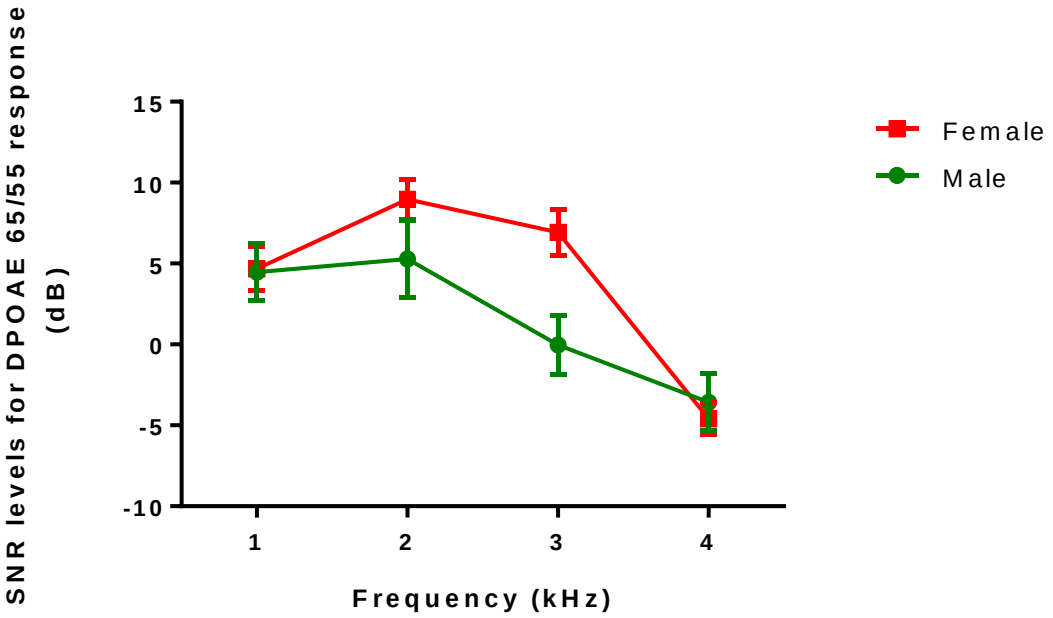

Figure 5.9: Mean DPOAE 65/55 responses for males and females. Error bars represent standard error of mean. Baseline N (males = 16, females = 41) Year 1 (males = 14, females = 39).

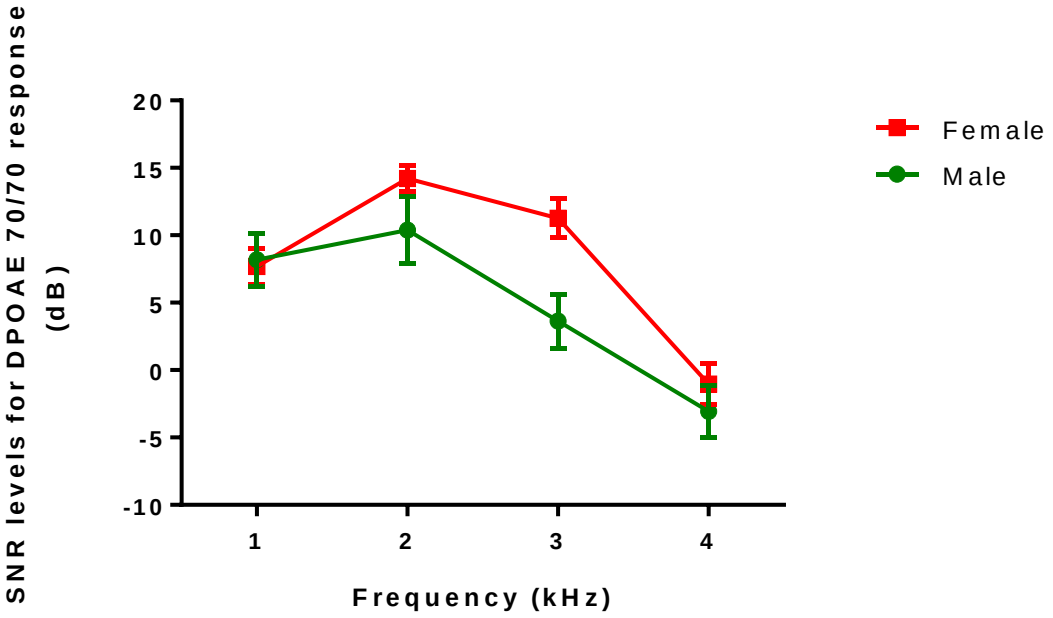

Figure 5.10: Mean DPOAE 70/70 responses for males and females. Error bars represent standard error of mean. Baseline N (males = 16, females = 41) Year 1 (males = 14, females = 39).

5.3.2 The relationship between neopterin levels and hearing outcome

The relationship between inflammation, using mean neopterin and WBC levels, and hearing outcome (PTA and OAE) was analysed in a multiple regression together with known covariates of hearing (age, gender, noise exposure, smoking and chronic disease). Neopterin and age were continuous variables and gender, noise, smoking and chronic were binary variables.

Using the enter method, there was a significant multiple regression between PTAv as the outcome measure and the independent variables $F(7, 55) = 4.01$, $p = 0.002$, $R^2 = 0.37$. Therefore, 37% of the variability in PTAv was explained by the variables analysed. However, none of the variables analysed was significant (table 5.2). Neopterin levels were not associated with PTAv (figure 5.11). Similar analysis was undertaken for LFA and HFA. Multiple regression analysis showed a significant association between LFA and the variables $F(7, 55) = 2.40$, $p = 0.03$, $R^2 = 0.26$. Significant variables were mean neopterin level and noise exposure (table 5.2), therefore the regression equation was predicted as LFA = -14.96 + 0.07 (mean neopterin level) + 7.99 (noise exposure). High neopterin levels were significantly associated with high (poor) LFA threshold (figure 5.12). Noise exposure was a binary variable where 0 represents no noise exposure and 1 represents positive noise exposure, subjects with noise-exposure history had significantly poorer LFA threshold compared to subjects with no noise exposure (figure 5.13). Noise exposure is known to destroy hair cells and therefore affect the ability to produce OAE. The analysis between HFA and the variable did not yield significant results, $F(7, 55) = 1.66$, $p = 0.14$, $R^2 = 0.20$. From the analysis, mean neopterin level is associated with poor low frequency hearing.

Variable	PTAv		LFA		HFA	
	B (SE)	p-value	B (SE)	p-value	B (95%CI)	p-value
Constant	-39.08 (31.77)		-14.96 (31.56)		-73.07 (58.32)	
Mean neopterin	0.05 (0.03)	0.07	-0.07 (0.03)	**0.01**	0.01 (0.05)	0.84
Mean WBC	-0.46 (0.69)	0.51	-0.17 (0.69)	0.81	-0.88 (1.27)	0.49
Age	0.80 (0.46)	0.09	0.17 (0.46)	0.71	2.07 (0.85)	0.02
Gender	-2.19 (3.44)	0.53	1.49 (3.42)	0.67	-4.71 (6.31)	0.50
Noise exposure	7.99 (3.52)	0.11	7.99 (3.52)	**0.03**	-1.12 (6.51)	0.87
Chronic disease	4.85 (3.09)	0.12	2.10 (3.07)	0.50	2.31 (5.68)	0.69
Smoking	4.55 (3.54)	0.20	0.66 (3.51)	0.85	3.12 (6.49)	0.63

Table 5.2: Multiple regression results between pure-tone audiometry averages and independent variables showing regression co-efficient (B) with the standard error of mean (SE). Significant p-levels are highlighted in bold. Pure-tone average (PTAv), low frequency averages (LFA) and high frequency average (HFA). N = 57

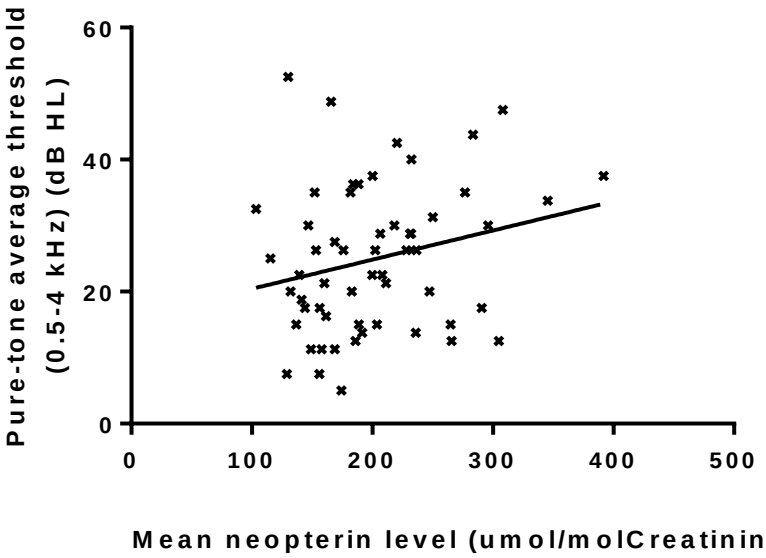

Figure 5.11: Scatter plot showing the relation between mean neopterin level and pure-tone average (0.5- 4 kHz). R= 0.22. N = 57

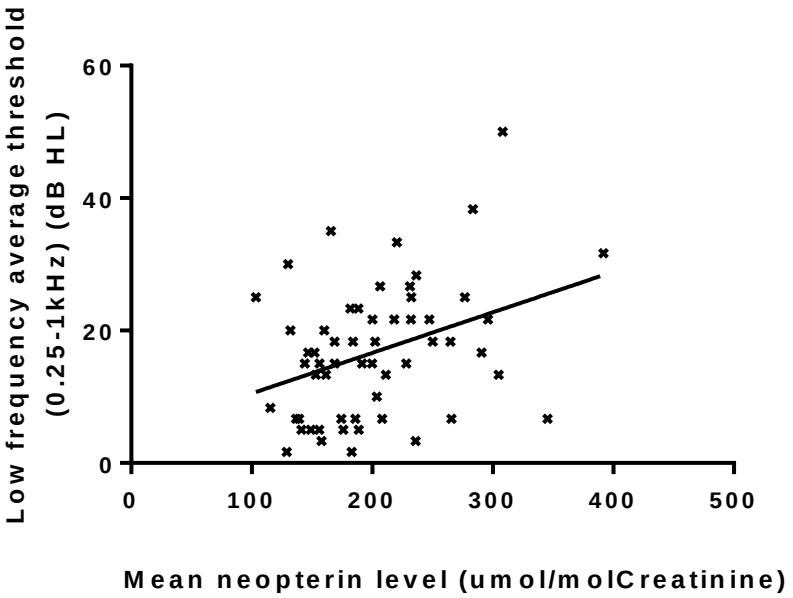

Figure 5.12: Scatter plot showing the association between mean neopterin level and low frequency average threshold, LFA (0.25-1 kHz). R = 0.35, N = 57

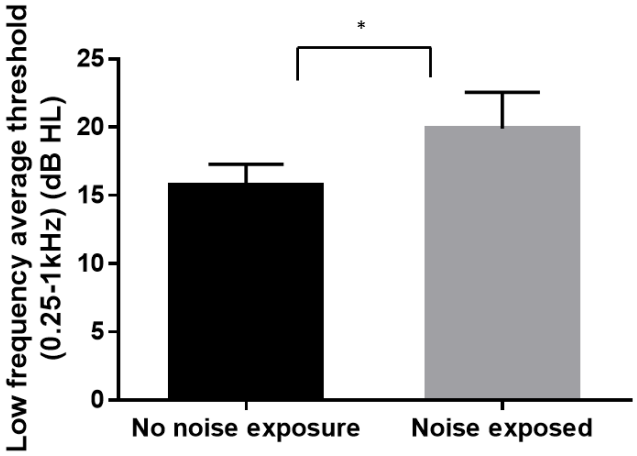

Figure 5.13: Low frequency average threshold between noise-exposed subjects and subjects with no noise exposure history, N (No noise exposure = 43, noise exposed = 14)

5.3.2.1 Relation between neopterin levels and OAE measures

The relationship between OAE measures, inflammatory measures and other covariates of hearing loss were assessed using multiple regression analysis.

It was expected that high mean neopterin levels would be associated with low (poor) TEOAE SNR; rather, high neopterin levels were associated with better TEOAE response. Interestingly, age, smoking and noise exposure showed significant associations with TEOAE response. There was no significant associations at low frequencies 1 kHz, F (7, 53) = 1.02, p = 0.43, R^2 = 0.13 and at 1.5 kHz F (7, 53) = 1.37, p = 0.24, R^2 = 0.17. There was however, significant multiple regression result for TEOAE at 2 kHz F (7, 53) = 2.67, p = 0.02, R^2 = 0.29 (table 5.3), with age being the significant predictor (p = 0.01) (figure 5.14). Significant association was also found for TEOAE at frequency 3 kHz F (7, 53) = 2.99, p = 0.01, R^2 = 0.31 and 4 kHz F (7, 53) = 3.83. p = 0.002, R^2 = 0.37. Mean neopterin (p = 0.01) (figure 5.15) and smoking (p = 0.03) were the significant variables for TEOAE at 3 kHz (table 5.3). Smoking was a binary variable with 0 and 1 representing non-smokers and smokers respectively. The significant variables for TEOAE at 4 kHz were mean neopterin (p = 0.04) (figure 5.16) and chronic disease (p = 0.04)(5.17).

Variable	**TEOAE- 2kHz**		**TEOAE-3 kHz**		**TEOAE- 4 kHz**	
	B (SE)	p-value	B (SE)	p-value	B (95%CI)	p-value
Constant	79.51 (29.71)	**0.01**	43.40 (29.03)	0.15	31.28 (25.38)	0.22
Mean neopterin	0.02 (0.02)	0.50	0.05 (0.02)	**0.03**	0.04 (0.02)	**0.04**
Mean WBC	0.37 (0.62)	0.55	0.96 (0.61)	0.12	0.77 (0.53)	0.15
Age	-1.10 (0.43)	**0.01**	-0.69 (0.42)	0.10	-0.62 (0.36)	0.10
Gender	2.11 (3.34)	0.53	-0.72 (3.27)	0.83	2.68 (2.85)	0.35
Noise exposure	-4.80 (3.32)	0.16	-1.41 (3.25)	0.67	0.32 (2.84)	0.91
Chronic disease	0.11 (2.81)	0.97	-3.54 (2.75)	0.20	-5.04 (2.40)	**0.04**
Smoking	-3.54 (3.20)	0.27	-6.95 (3.12)	**0.03**	-2.95 (2.73)	0.29

Table 5.3: Results of multiple regression analysis between TEOAE at measured frequencies and independent variables, showing regression co-efficients (B), standard error of mean (SE) and significant p-levels highlighted in bold. N = 57

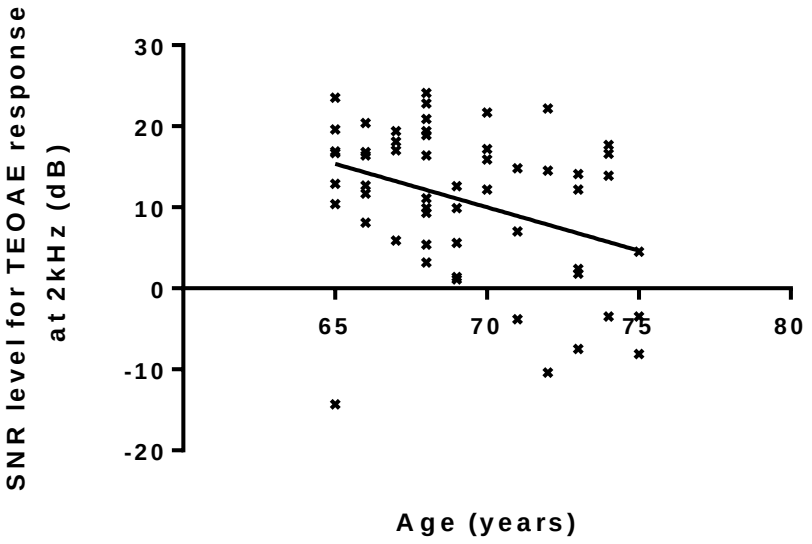

Figure 5.14: Scatter plot showing the association between TEOAE response at 2 kHz and age. SNR levels of TEOAE at 2 kHz declined with advancing age. R = -0.35, N = 57

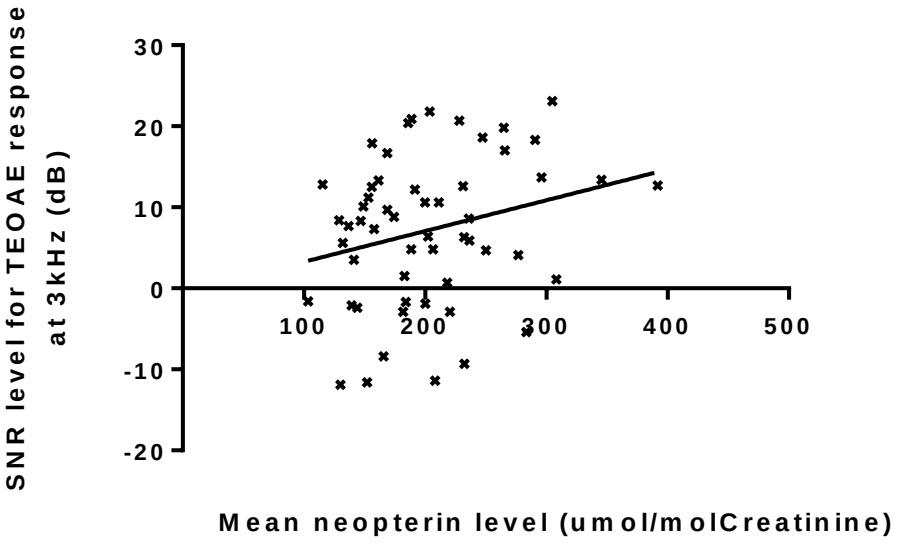

Figure 5.15: Scatter plot showing association between mean neopterin level and TEOAE response at 3 kHz High neopterin levels was associated with better SNR. R = 0.26, N = 57

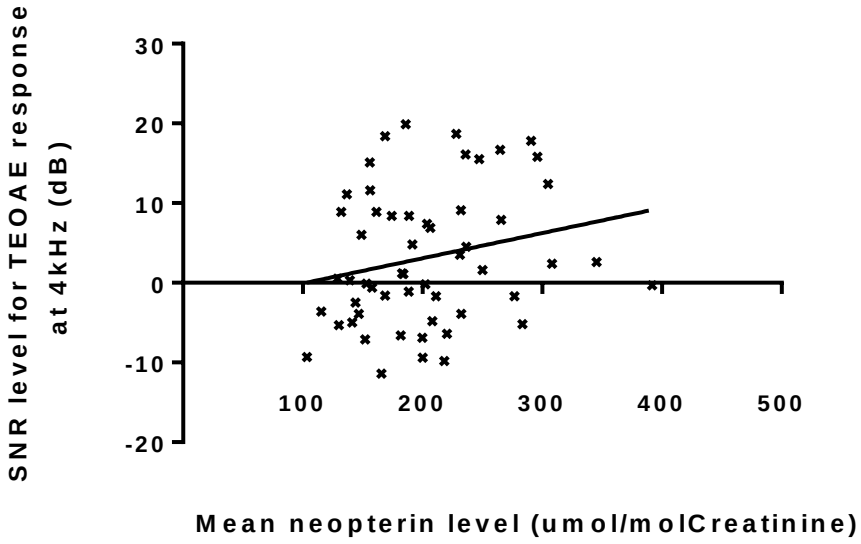

Figure 5.16: Scatter plot showing association between mean neopterin and TEOAE response at 4 kHz High neopterin levels was associated with better SNR. R = 0.33, N = 57

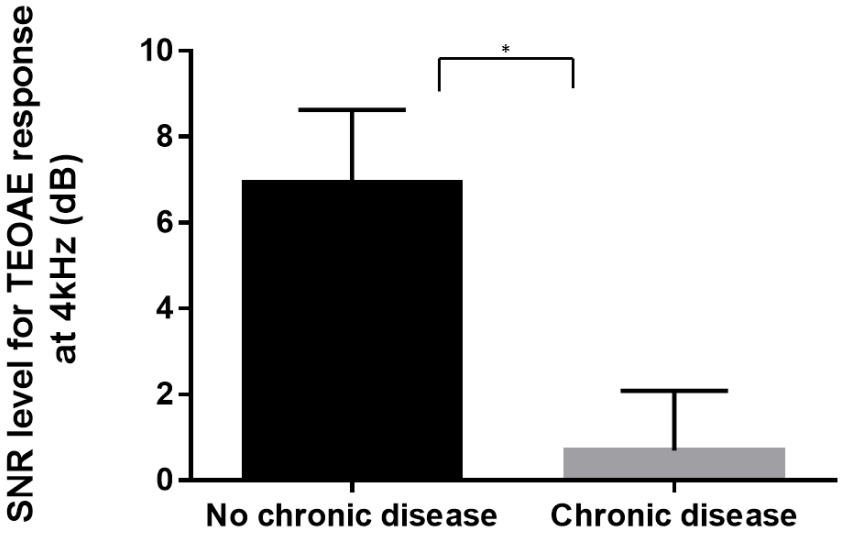

Figure 5.17: Bar chart showing the difference in TEOAE 4 kHz response between subjects with chronic diseases and subject with no chronic disease. N (Chronic disease = 34 No chronic disease = 23)

There was no association between DPOAE measured at f1/f1 of 65/55 (DPOAE-65) and mean neopterin levels (table 5.4). Multiple regression analysis between DPOAE 65/55 and the variables yielded significant results at 2 kHz F (7, 53) = 2.23, p = 0.048, R^2 = 0.25, however, none of the analysed variables reached significance. Significant multiple regression results were obtained at 4 kHz F (7, 53) = 3.68, p = 0.003, R^2 = 0.36, significant variable was age (p = 0.02) (figure 5.18). There

was no significant association at 1 kHz F (7, 53) = 1.31, p = 0.27, R^2 = 0.17; and 8 kHz F (7, 51) = 0.44, p = 0.87, R^2 = 0.07.

There was also no association between DPOAE 70/70 and mean neopterin levels (table 5.5). Significant multiple regression equations were obtained at 2 and 4 kHz; F (7, 53) = 2.84, p = 0.02, R^2 = 0.30 and F (7, 53) = 4.20, p = 0.001, R^2 = 0.39, respectively. Significant variables for 2 kHz were noise exposure (0.049) and smoking (p = 0.02) (figure 5.19). Smoking (p = 0.03) and mean WBC (p = 0.02) were the significant variables at 4 kHz (figure 5.20). Regression equations were not significant at 1 kHz F (7, 53) = 0.86, p = 0.55, R^2 = 0.12 and 8 kHz F (7, 53) = 1.93, p = 0.09, R^2 = 0.24. Table 5.5 shows the results of the regressions.

Variables	DPOAE-65 (1 kHz)		DPOAE-65 (2 kHz)		DPOAE-65 (4 kHz)		DPOAE-65 (8 kHz)	
	B	p-level	B	p-level	B	p-level	B	p-level
Constant	24.98 (28.08)	0.38	59.54 (27.22)	**0.03**	58.85 (27.17)	**0.04**	15.36 (21.82)	0.49
Mean Neopterin	-0.01 (0.02)	0.53	-0.01 (0.02)	0.82	0.01 (0.02)	0.68	0.004 (0.02)	0.80
Mean WBC	0.04 (0.59)	0.95	0.21 (0.57)	0.71	1.04 (0.58)	0.07	0.15 (0.50)	0.76
Age	-0.11 (0.40)	0.80	-0.68 (0.39)	0.09	-0.93 (0.39)	**0.02**	-0.24 (0.31)	0.59
Gender	-3.88 (3.16)	0.23	-0.82 (3.06)	0.79	3.08 (3.06)	0.32	-2.96 (2.45)	0.23
Noise exposure	-6.53 (3.14)	0.04	-5.67 (3.05)	0.07	-1.12 (3.04)	0.71	-2.45 (2.45)	0.32
Chronic disease	-3.13 (2.66)	0.25	-2.11 (2.58)	0.42	-3.30 (2.57)	0.21	1.31 (2.10)	0.54
Smoking	-2.33 (3.02)	0.44	-4.33 (2.93)	0.15	-4.42 (2.93)	0.14	-1.99 (2.36)	0.40

Table 5.4: Multiple regression analysis results for DPOAE 65/55 responses at measured frequencies. B represents regression co-efficient, significant levels are highlighted in bold; SE is standard error of mean, N = 57

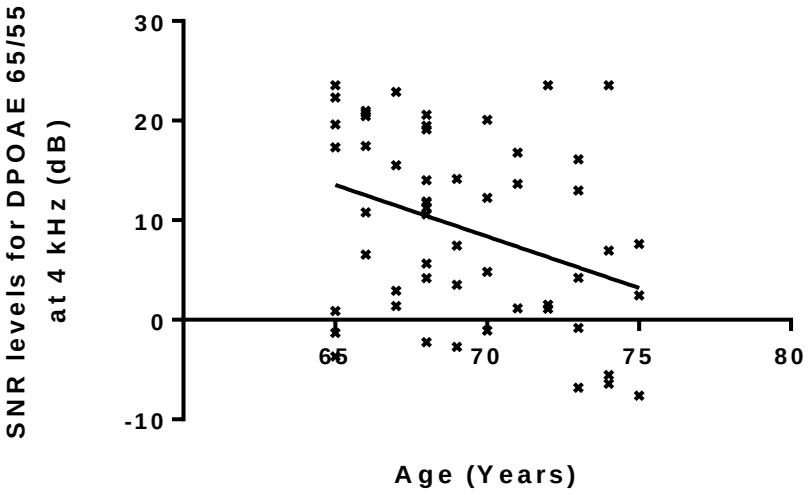

Figure 5.18: Scatter plot showing the association between TEOAE response at 4 kHz and age. TEOAE response declined with advancing age. R = -0.31, N = 57

Variables	DPOAE-70 (1 kHz)		DPOAE-70 (2 kHz)		DPOAE-70 (4 kHz)		DPOAE-70 (8 kHz)	
	B	p-level	B	p-level	B	p-level	B	p-level
Constant	15.97 (29.04)	0.59	54.01 (23.73)	**0.03**	50.46 (27.61)	0.07	30.25 (29.28)	0.31
Mean Neopterin	-0.01 (0.02)	0.65	0.02 (0.02)	0.27	0.03 (0.02)	0.26	-0.01 (0.02)	0.80
Mean WBC	-0.27 (0.61)	0.66	0.05 (0.50)	0.92	1.48 (0.58)	**0.02**	2.00 (0.66)	0.004
Age	0.07 (0.42)	0.86	-0.56 (0.34)	0.10	-0.79 (0.40)	0.051	-0.66 (0.42)	0.12
Gender	-3.36 (3.27)	0.31	-1.91 (2.67)	0.48	1.99 (3.11)	0.52	1.75 (3.29)	0.60
Noise exposure	-4.29 (3.25)	0.19	-5.37 (2.66)	**0.049**	-1.46 (3.09)	0.64	0.89 (3.28)	0.79
Chronic disease	-4.01 (2.75)	0.15	-1.20 (2.25)	0.60	-3.52 (2.62)	0.19	1.12 (2.82)	0.69
Smoking	-0.90 (3.13)	0.66	-6.24 (2.56)	**0.02**	-6.90 (2.97)	**0.03**	-2.10 (3.17)	0.51

Table 5.5: Multiple regression analysis results for DPOAE 70/70 at measured frequencies. Table shows regression coefficients (B) of measured the variables and their standard error of mean (SE), significant levels are highlighted in bold. N=57

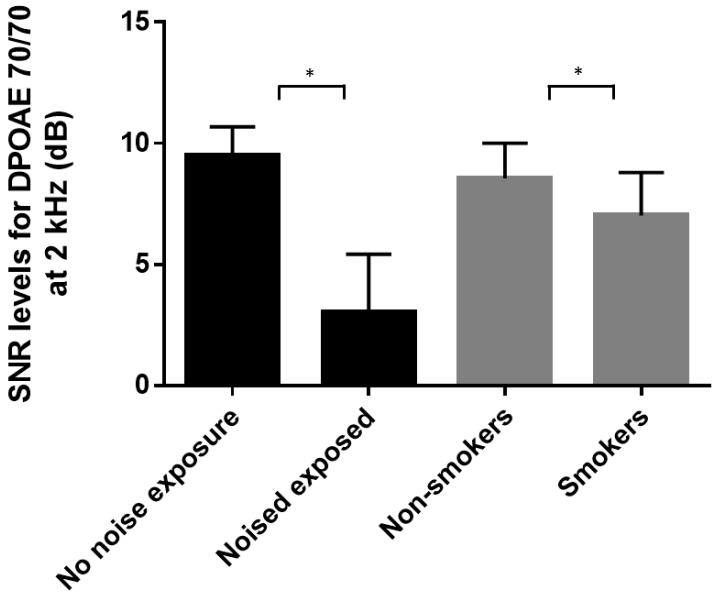

Figure 5.19: Bar chart showing the differences in DPOAE 70/70 response at 2 kHz between subjects' history of noise exposure and smoking N (No noise exposure = 14, noise exposure = 43, non-smokers =36, smokers = 21)

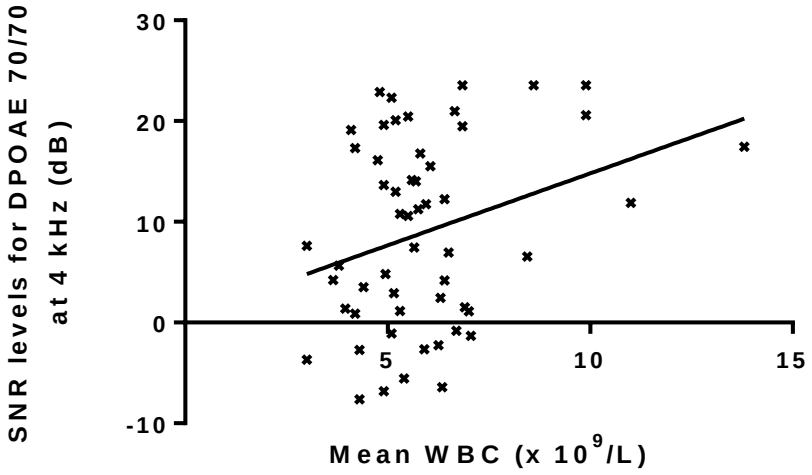

Figure 5.20: Association between white blood cell count and DPOAE 70/70 at 4 kHz. High DPOAE response was associated with high WBC level. R = 0.30, N = 57.

5.3.3 Detecting change in hearing after one year using pure-tone audiometry

Pure-tone audiometry (PTA) was repeated after one year to determine change in hearing. However, paired t-test showed no significant difference between baseline and year one for all three audiometric threshold averages. PTAv baseline (M = 24.65, SE = 1.49) and year 1 (M = 25.16,

SE = 1.58), t (52) = -1.28, p = 0.21. LFA baseline (M = 16.16, SE = 1.27) (M = 16.32, SE = 1.28) and year 1 t (52) = -0.31, p = 0.76. HFA baseline (M = 60.35, SE = 2.48) and year 1 (M = 61.46, SE = 2.49), t (52) = -0.94, p = 0.35 (figure 5.21).

In order to determine whether extended high frequency PTA could detect changes in thresholds between the two time points, we performed Wilcoxon matched-pairs signed-ranks test for thresholds at 12 kHz. There was also no significant difference between 12 kHz thresholds at baseline (mean (M) = 88.6, standard error (SE) = 2.6) and year 1 (M = 91.4, SE = 2.6), Z = -1.64, p = 0.10

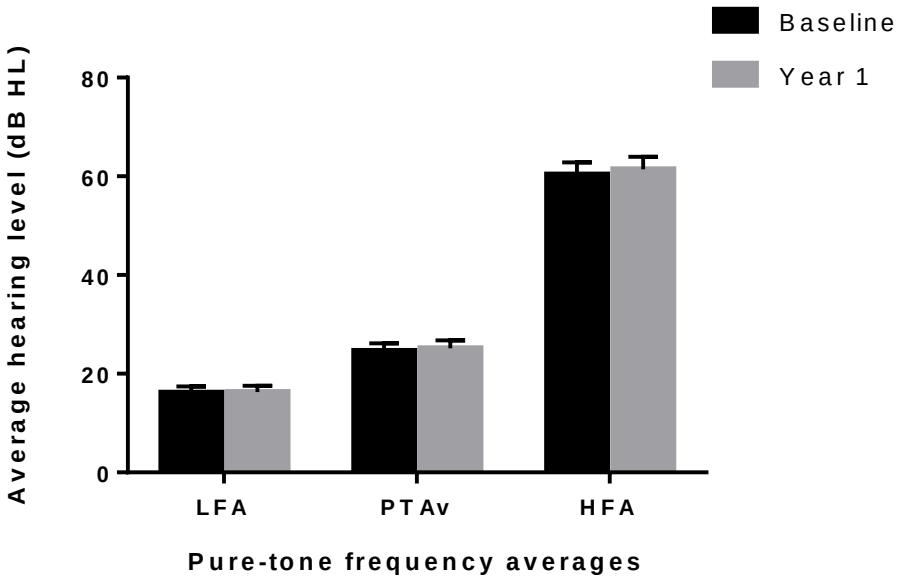

Figure 5.21: Average audiometric thresholds for low frequency (LFA), pure-tone (PTAv) and high frequency averages (HFA). Thresholds obtained from air-conduction of the worse hearing ear (N = 53). Error bars represent standard errors of mean.

5.3.4 Detecting change in hearing using otoacoustic emission

Since PTA did not show changes in hearing function, the ability of TEOAE and DPOAE to detect changes in hearing function within one year was investigated. Results from t-test of TEOAE responses showed a significant difference in OAE response at 4 kHz. Baseline (M = 2.94, SE = 1.26), year 1 (M = 0.77, SE = 1.20), t (45) = 4.04, p < 0.001. Figure 5.22 shows responses at all frequencies at both time points.

DPOAE at 65/65 showed a significant difference between baseline and year 1 SNRs at 1, 2 and 4 kHz. 1 kHz baseline (M = 4.22, SE = 1.33) year 1 (M = 1.85, SE = 1.10), t (42) =2.44, p = 0.02; 2 kHz baseline (M = 8.02, se = 1.27) year 1 (M = 6.70, SE = 1.27), t (42) = 2.56, p = 0.01; 4 kHz baseline (M = 5.11, SE = 1.41) year 1 (M = 2.93, SE = 1.35), T (42) = 3.17, p = 0.003 (figure 5.23).

Analysis of SNR for DPOAE 70/70 revealed a significant difference between baseline (M = 7.49, SE = 1.33) and year 1 (M = 4.44, SE = 1.27) results at 1 kHz, t (42) = 2.98, p = 0.005 (figure 5.24).

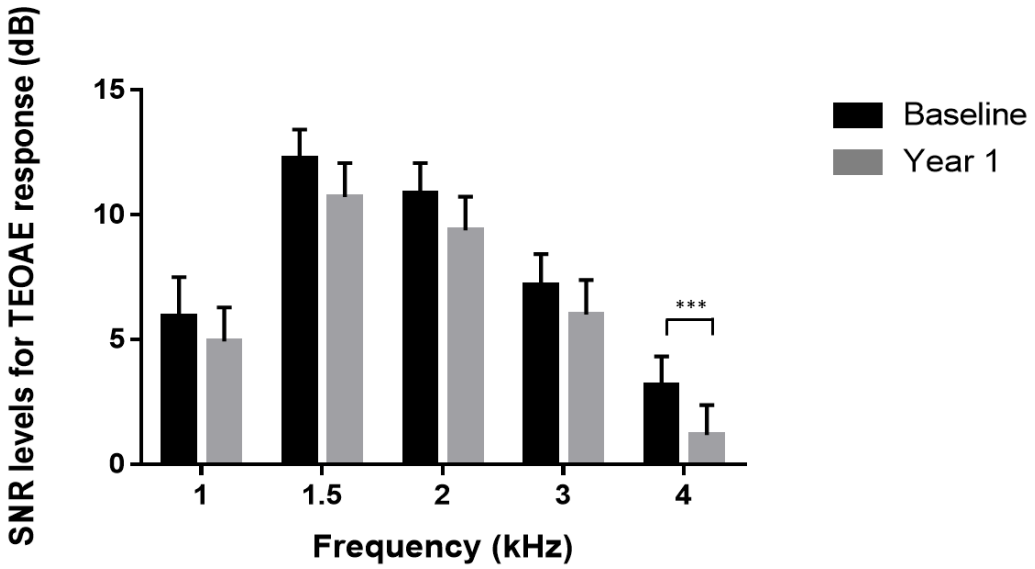

Figure 5.22: Bar chart showing SNRs for TEOAE response (1 - 4 kHz) of subjects at baseline and year 1. Significant difference between the two time points was at 4 kHz, $p < 0.001$.

Error bars represent standard errors of mean. N = 53

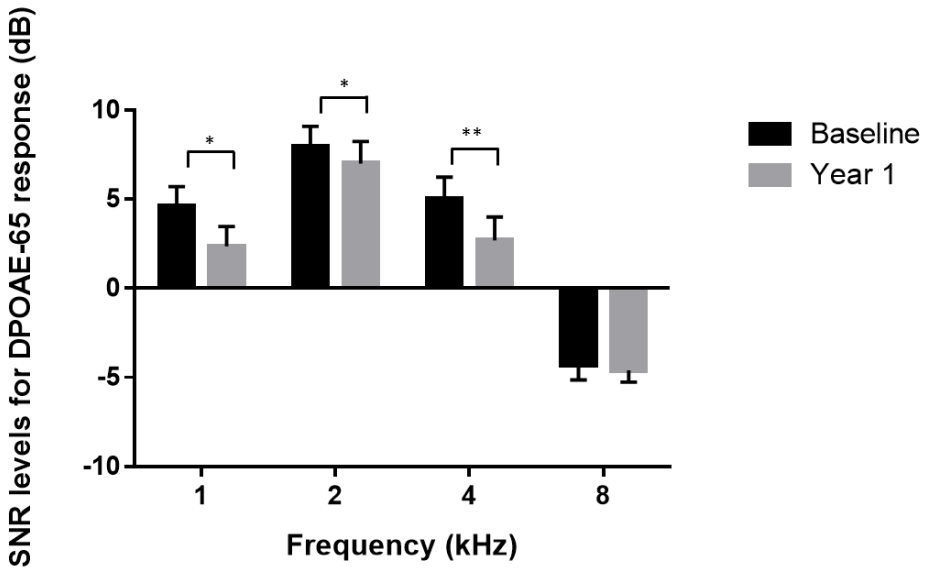

Figure 5.23: Bar chart showing SNRs for DPOAE 65/55 response (1 - 8 kHz) of subjects at baseline and year 1. There were significant differences between time points at 1 kHz (p =0.02), 2 kHz (p = 0.01) and 4 kHz (p = 0.003), N = 53

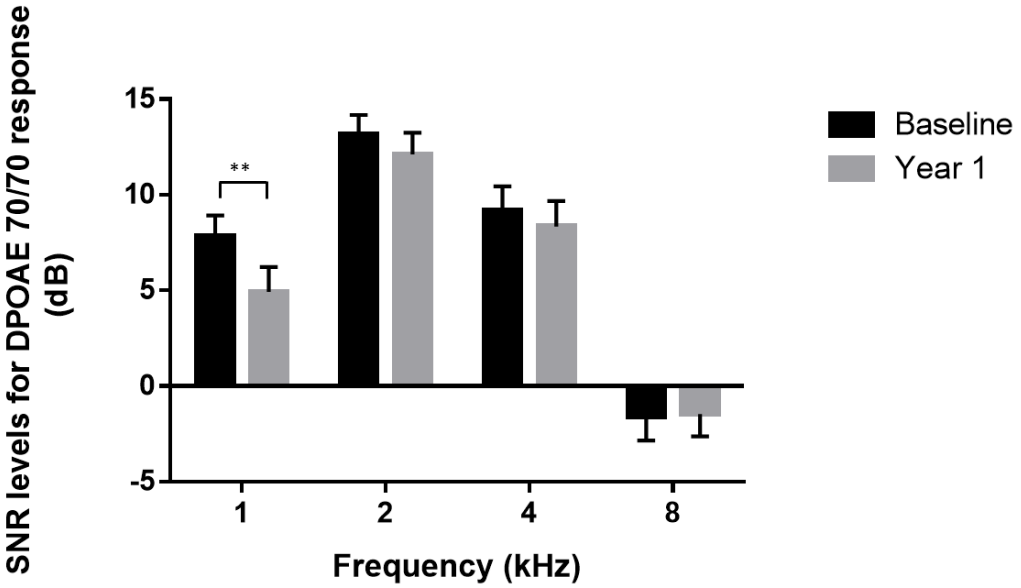

Figure 5.24: Bar chart showing SNRs for DPOAE 70/70 response (1 - 8 kHz) of subjects at baseline and year 1. Significant difference between time points at 1 kHz (p = 0.005), N = 53

5.3.5 Neopterin levels and progression of hearing loss

One of the aims of the study was to determine the effect of neopterin levels in the progression of ARHL. Outcome measures that showed significant change between baseline and year 1 measures, TEOAE at 4 kHz, DPOAE 65/55 at 1, 2 and 4 kHz and DPOAE 70/70 at 1 kHz were used in the analysis. We investigated the association between mean neopterin levels on the progression of hearing loss at these five hearing outcome measures. Progression in hearing loss was defined as a reduction in SNR from baseline to year one. Linear regression analysis was performed between mean neopterin level and change in SNR (baseline SNR minus year 1 SNR). Results showed a significant association between mean neopterin level and change in SNR at TEOAE 4 kHz $F(1, 52) = 4.84$, $p = 0.03$, $R = 0.28$. Significant associations were not found with the other SNRs. Table 5.6 shows results of linear regressions.

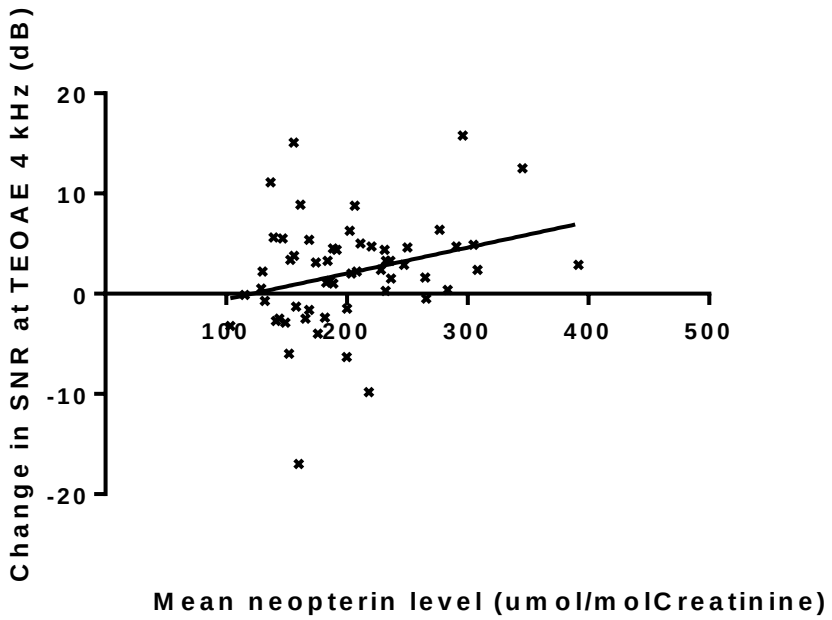

Figure 5.25: Scatter plot showing the association between mean neopterin level and mean change in TEOAE response at 4 kHz within one year , Results show that higher mean levels of neopterin is associated with a greater change in TEOAE response indicating a greater hearing loss progression $R = 0.28$, $N = 53$.

Hearing measure	F-Statistics	R square	p-level
TEOAE (4 kHz)	4.84	0.08	**0.03**
DPOAE 65/65 (1 kHz)	0.00	0.00	0.99
DPOAE 65/55 (2 kHz)	0.26	0.01	0.61
DPOAE 65/55 (4 kHz)	1.90	0.03	0.17
DPOAE 70/70 (1 kHz)	0.37	0.01	0.55

Table 5.6: Linear regression results between mean neopterin and change in OAE SNR after one year. Significant association (highlighted in bold) found between mean neopterin levels and TEOAE at 4 kHz, N = 53

5.3.6 Summary of results

The results showed that 60% of the cohort had some degree of hearing loss consistent with ARHL. Majority of subjects (80%) had measureable PTA threshold at 12 kHz. There was significant association between mean neopterin levels over 12 months and low frequency average thresholds. The results from the analysis of the association between neopterin levels and OAE measures were unexpected, with TEOAE at 3 and 4 kHz showing an inverse association where high neopterin (inflammation) levels were associated with better SNR. However, other covariates including increasing age, smoking and noise exposure were significantly associated with poor OAE SNR.

Pure-tone audiometry did not show differences in thresholds after one year both at the conventional frequencies and at 12 kHz. However, there was significant changes in SNR (progression of hearing loss) after one year in TEOAE response at 4 kHz, DPOAE 65/55 response at 1, 2 and 4 kHz and DPOAE 70/70 response at 1 kHz. The progression of hearing loss (change in OAE response) was associated with neopterin level only for TEOAE at 4 kHz.

5.4 Discussion

The objective of the study was to investigate if older adults with high inflammatory load have poorer hearing as well as worse progression of hearing loss and if so, whether the deterioration in

auditory function could be detected with OAE measurement and extended high frequency audiometry before it becomes detectable with conventional PTA.

From the results, average audiogram for subjects had a configuration of bilateral high frequency sloping hearing loss, which is typical of ARHL as has been found in other studies (Cruickshanks et al. 1998). Similar to results in other studies (Cruickshanks et al. 1998; Helzner et al. 2005), males had poorer hearing compared to females. The difference, which was more pronounced in the high frequencies (3-8 kHz), could be reflective of the fact that, from the responses to the questionnaire, more than 56% of the males had a history of noise exposure (mainly occupational), compared to 12.2% of female. However, the disparity between the genders could also be due to other risk factors of hearing loss such as smoking, or chronic disease being more prevalent in males (Cruickshanks et al. 1998; Cruickshanks et al. 1998b).

From the results of this study, about 60% of subjects had hearing loss (table 5.1). Prevalence rates between 44 and 71% have been found in other studies within age range 60 - 80 (Davis 1989; Cruickshanks et al. 1998). Pure-tone audiometry did not identify significant changes in hearing within one year even at the high frequency average. Age-related hearing loss is thought to progress at a rate of about 1 dB per year (Jungmee Lee et al. 2012), and could explain why PTA could not detect changes within one year unlike the loss seen due to ototoxicity which could cause threshold changes of up to 20 dB (Knight et al, 2007) . At the rate of 1 dB/year, it will take about five years for PTA to detect changes in hearing threshold, given the test-retest reliability of 5 dB (Jungmee Lee et al. 2012). Many studies have used high frequency audiometry to measure changes in hearing function resulting from acute insults to the auditory system such as noise exposure (Somma et al. 2008) and ototoxic medication (Yu et al. 2014). Results from such studies have demonstrated the sensitivity of extended high frequency measures in detecting changes in hearing. Few studies have investigated the use of extended high frequency audiometry for monitoring ARHL. Therefore in this study, we investigated the ability of extended high frequency PTA to investigate ARHL which is a gradual loss rather than acute. Interestingly, more than 80% of subjects had measureable 12 kHz threshold, with the best thresholds at 50 dB HL at start of the study. A year on, a further 5% of thresholds at 12 kHz became immeasurable. However, the difference between baseline and year 1 thresholds were not significant. One possible explanation for the non-significant finding is that 12 kHz was the only extended frequency measured. In the studies that detected threshold changes, four to seven frequencies ranging from 9 - 20 kHz had been measured (Yu et al. 2014; Mehrparvar et al. 2014). Unfortunately, we were mindful of over-burdening elderly subjects with a prolonged extended high frequency measurement instead, and anticipated that thresholds measured at only 12 kHz will capture significant changes. A second

reason is that many of the subjects had reached audiometric limit at 12 kHz, perhaps making further deterioration undetectable.

Otoacoustic emission response is thought to decrease with ageing (Uchida et al. 2008). While the argument could be made that the decline in OAE response is associated with the hearing loss that comes with ageing, other researchers are of the view that the decline in OAE response with ageing occurs even with normal hearing (Uchida et al. 2008). The fact that subjects had measurable OAE responses may have implications for the use of OAE measures in older adults at clinics and in research. Otoacoustic emission could potentially act as a screening tool for the early detection of ARHL, so that appropriate interventions including hearing aid provision, could be instituted early. This would potentially avoid some of the problems such as communication difficulties and social isolation and depression that may become associated with hearing loss (Meister et al., 2015). Secondly, OAE can be beneficial as a quick yearly monitoring tool that could determine the rate of progression of hearing loss. Such monitoring of ARHL progression will stratify people based on their progression in order to provide interventions based on the particular needs of subgroups of people. Yearly OAE and neopterin results could help in the drive towards a state of reduced inflammation and reduced progression of ARHL.

After one year, TEOAE detected differences in SNR at 4 kHz. For people with relatively good hearing, TEOAE at 4 kHz may be the measurement of choice for monitoring changes in hearing function over time. For people with greater hearing loss, DPOAE can be used detect changes in hearing since DPOAE may be elicited in people with greater hearing loss (Kemp 2002). A greater response was elicited with DPOAE 70/70 compared to DPOAE 65/65, as OAE response has been found to increase linearly with input level up to 75 dB (Dorn et al. 2001). This means that many older people can have reliable DPOAE measures through the use of a slightly higher input level of 70/70, which is adequate to stimulate the OHC if they are present. From our results, DPOAE 65/55 frequencies at which hearing change within one year was detectable were at 1, 2 and 4 kHz and for DPOAE 70/70, 1 kHz. This are results for only the first year of the study and it is expected that there will be greater change in OAE response levels at the end of three years of the study.

The relationship between inflammation and hearing loss was investigated by looking at the cross-sectional association between mean neopterin levels and hearing threshold. Data for PTA showed that there was an association between inflammation and low frequency average threshold however, not with pure-tone average threshold and high frequency average thresholds (table 5.2, figures 5.12). There is evidence that suggests inflammation may have an effect on the stria vascularis, which is the region of the cochlea essential for energy generation through the production of the endocochlear potential (Nakamoto et al. 2012). The stria is enriched with blood

vessels essential for the high metabolic activity of the cochlea which may render it susceptible to inflammatory and age-related degeneration (Dubno et al. 2013). These blood vessels have a reduced distribution at the apex of the cochlea (Mom et al., 1997) therefore damage to them is more likely to result in reduced blood flow in the apical to middle regions compared to the basal regions, which may explain the link between vascular diseases and lower frequency hearing loss(Eckert et al. 2013). The lack of significant association between neopterin levels and other frequency averages, PTAv and HFA suggests that other factors such as noise exposure may be more relevant at higher frequencies, although per our results; noise exposure was not significantly associated with high frequency average. Due to limits of audiometry, losses that were greater than 120 dB HL could not be measured at the high frequencies, which could have contributed to the lack of significant results. Since PTA did not show a significant change in hearing after one year, further investigations into the relationship between neopterin levels and change in hearing threshold (progression of hearing loss) were not carried out. However, since the study is due to run over three years, at the end of the third year study, it is possible an association may be shown between inflammatory load and change in threshold.

The relation between neopterin levels on OAE responses was also investigated. Otoacoustic measures showed a significant association with neopterin levels at TEOAE 3 and 4 kHz, which showed an inverse relation to what was expected. That is, subjects with high mean neopterin levels rather had high (better) SNR at 3 and 4 kHz. A possible explanation of the lack of positive association between OAE measures and neopterin could be that inflammation has greater influence on the stria and neural synapses. The stria and the neural synapses are rich in CNS immune cells, microglia, compared to the OHCs, which are responsible for OAE production. This may be the reason why PTA, which is indicative of the functioning of the entire auditory system, reflects differences in inflammatory load compared with OAE, which essentially represents OHC function. However, OAE measures showed significant decline in response within one year at TEOAE 4 kHz, DPOAE 65/55 at 1, 2 and 4 kHz and DPOAE 70/70 at 1 kHz. This suggests OAE measures at specific frequencies could potentially be used for monitoring changes in hearing in the elderly population.

Regular monitoring of hearing loss is essential, especially for people with rapidly advancing hearing loss. Therefore, the use of a combination of OAE and inflammatory measures (monthly neopterin) can be a monitoring tool to identify people with rapidly progressing ARHL.

5.4.1 Limitations

- The study being underpowered may affect the reliability of the findings, which should be considered when interpretations of the results are being made. The study is however, a pilot to provide needed preliminary data and information for a larger study.
- As part of the exclusion criteria of the study, people with severe or profound hearing loss could not participate, therefore the findings may not apply in people with more severe hearing loss.
- Using all OAE SNRs without a cut-off point may have added a noise into the data and as a result may have diluted our results. However, this approach was undertaken in order to include all subject and make it relevant for people with ARHL.

5.5 Conclusion

- Inflammation, measured by mean neopterin levels is associated with low frequency average thresholds. People with high neopterin levels have high worse low frequency hearing.
- Pure-tone audiometry cannot detect age-related hearing changes within one year, however both TEOAE and DPOAE detected decline in SNR at different frequencies after one year.
- Neopterin level was associated with progression of hearing function at TEOAE at 4 kHz

Chapter 6: General discussion

This study set out to investigate the link between inflammation and age-related hearing loss (ARHL). Findings from previous studies and experiments conducted in this study provide evidence that high inflammatory load in the elderly is associated with poor hearing level. It is postulated that such high inflammatory load will be associated with an increased progression of hearing loss. The association between inflammation and ARHL has been shown in our initial analysis of a cross-sectional study and has been further explored in the longitudinal study. The use of urine neopterin as a biomarker of inflammaging has been investigated in this thesis. The novel finding has been that high neopterin over 12-month period is associated with hearing function. The thesis also provides preliminary evidence that OAE measures can detect early hearing change in people with ARHL. These findings contribute to the understanding of the inflammatory effect of chronic diseases and lifestyle on ARHL. There is also the potential outcome of using inflammation-reduction strategies to reduce the progression of age-related diseases including ARHL. However, further investigation is required to help understand the mechanisms involved and to further extend the findings of the thesis.

6.1 The link between inflammation and ARHL

There is mounting evidence that ageing is associated with a state of low-grade systemic chronic inflammation known as inflammaging, which has been linked with the progression of many age-related diseases (Franceschi et al. 2000). Inflammaging, which is evidenced by up-regulation of inflammatory markers (Krabbe et al. 2004) is variably expressed in older individuals and has been shown to be key in the progression of age-related diseases including cardiovascular disease (Licastro et al. 2005), frailty (Leng et al. 2011), and dementia (Akiyama et al. 2000). This has generated interest in investigating whether inflammaging contributes to the progression of ARHL, since it provides a possible way by which ARHL may be modulated.

Studies that have investigated the link between inflammaging and ARHL in different cohorts, have done so using varied inflammatory markers (Nash et al., 2014; Verschuur et al., 2012). This section focuses on studies that have been performed during different eras and in different cohorts but have shown similar findings of an association between inflammaging and ARHL. The MRC study and the neopterin study, which have been reported in chapters 3, 4 and 5; and Hertfordshire Ageing Study (HAS) (Verschuur et al., 2012) are examples of studies that have investigated the role of inflammation on ARHL in humans. These studies were distinct on a number of key levels.

Firstly, the era in which subjects in the three different cohorts lived and the possible impact it had on their health. Participants in the MRC study were recruited in 1980, which meant that subjects we examined (age 60-89) had been born between 1890s and 1920s. Having been born prior to the first and second world wars presupposes that many of the men, who would have been in the army, could have been exposed to noise exposure from gunshots. Considering the low life expectancy during that era (47 for men and 50 for women in 1900), it suggests that subjects we analysed represented some of the oldest and presumably, healthiest subset of their generation. The environment in which the participants lived would have influenced their health, and affected their level of inflammation. Noise exposure would be an important aetiological factor for their hearing function. From the results, their average WBC, which was 7.3 X 10^9/L and average hearing level of 37.3 dB HL were quite high. Participants of the HAS, on the other hand, were all born in the 1920s, their hearing and inflammation measures were collected in the 1990s. This cohort was slightly younger than the MRC group and unlike the MRC participants who were resident in cities, all the HAS participants were rural dwellers, who had lived in Hertfordshire most of their lives, with many probably working in the agricultural sector. Average WBC for this group was 6.3 X 10^9/L, lower than levels found in the MRC cohort and their hearing was averagely better, 28.8 dB HL. It has been suggested that living in cities and industrialized regions may be detrimental to hearing as opposed to living in rural settings (Goycoolea et al. 1986), and this may have also contributed to the better hearing in the rural-dwelling HAS cohort. Subjects in the neopterin study on the other hand, were born after the commencement of the National Health Service therefore have had access to good healthcare, and have a high life expectancy of 81 years. Most of the participants live in and around Southampton, and many have had professional careers. Average hearing threshold was 24.9 dB HL, and average WBC of 5.7 X 10^9/L, even better than the HAS cohort. There appear to be a link between better access to healthcare, better socio-economic status and inflammatory status, although factors including genetics and lifestyle may also contribute. Findings from these three studies indicate that no matter the health status of the population, there is some association between inflammation and hearing function. In these three studies, the lower the average WBC count, the better the average hearing level. Secondly, the fact that an association between inflammatory markers and hearing level have been shown in two fairly large studies and the results have been repeatable in a small neopterin cohort of 57, shows that our findings are robust and that inflammaging plays a key role in ARHL. Furthermore, other inflammatory markers including CRP, IL-6 and neopterin that have been associated with inflammaging and age-related diseases (Leng et al. 2011; Lencel & Magne 2011), have also been found to be associated with ARHL, indicating that inflammaging is a key factor in ARHL.

Longitudinal studies in the investigation of the link between inflammation and ARHL have been important particularly in order to eliminate the confounding effect of acute inflammatory episodes at the time of sample collection on the results. Nash et al (2014) show that long-term CRP levels, particularly in people less than 60, is associated with a 10-year increased incidence of hearing loss. This suggests that not only does inflammation correlate with hearing level, but it may also identify the risk of progression of ARHL. The neopterin study aimed to investigate the effect of inflammatory state on the progression of hearing loss, however, one year into the study this effect has not been observed for PTA thresholds. However, OAE measures have shown changes in hearing function within one year, creating the potential for further investigations with OAE especially at the frequencies that showed sensitivity and later application in a screening and monitoring programme for ARHL. Although OAE has not shown particular sensitivity to mean neopterin levels, it is possible the small sample size could have contributed to this. Therefore, further investigation with a larger sample size must be performed to ascertain the findings. One year into the study, an interesting association have been found between neopterin and low frequency average thresholds. Based on our findings, it is expected that at the end of the third year, greater associations between neopterin levels and progression of hearing loss would be found.

6.2 Factors that contribute to the variability of ARHL

Ageing is the most important risk factor in ARHL since two out of three people over age 65 have some decline in their hearing. Literature identifies other contributors including noise exposure, chronic diseases, genetics, male gender and smoking have been identified. Results from our studies, show that not all elderly people have raised inflammatory markers. There is a subset of the older population with normal levels of inflammatory markers, no known age-related disease and in apparently good health. This suggests that the extent to which inflammaging drives age-related diseases is variable and complete understanding of factors involved in inflammaging remains inconclusive. Some factors that drive inflammaging are preventable, therefore lifestyle measures like exercise and cessation of smoking and pharmacological products that reduce inflammation including statins (Albert et al. 2001) can potentially have an effect on inflammaging and age-related diseases.

Atherosclerosis and other risk factors of cardiovascular diseases increases with age therefore the use of lipid-lowering drugs like statins are common in the elderly. Apart from lipid-lowering effects, statins reduce cardiovascular risk by improving endothelial function, maintaining plaque stability and reducing inflammatory response. In randomised trials, statins have been shown to reduce CRP levels by almost 17% (Albert et al., 2001). In large scale population studies, subjects

who have never used statins were three times as likely to develop ARHL (Nash et al. 2014). Our results in chapter 3 showed that low mean neopterin level was associated with statin use.

There have been conflicting results about the effect of smoking on the auditory system. While some studies have shown an association between smoking and hearing loss (Cruickshanks et al., 1998; Nakanishi, Okamoto, & Nakamura, 2000), others have not observed such association (Gates et al., 1993). In some studies that have shown an association, smoking has shown a dose-dependent effect in high frequency hearing loss (Nakanishi et al. 2000). Nicotine has been implicated as an ototoxic substance and like other ototoxic drugs including gentamycin, it is thought to destroy the hair cells, particularly in the basal region of the cochlea. Smoking may also affect hearing through other mechanisms including ischaemia, vasospasm and increased blood viscosity (Powell 1998). The cochlear artery is devoid of collaterals therefore ischemia will starve delicate sensory cells of oxygen and nourishment. Smoking also promotes atherosclerosis, which is a risk factor of cardiovascular diseases. Cardiovascular diseases and other vascular diseases have been implicated in low frequency hearing loss. In the neopterin study, smoking was associated with high neopterin levels, suggesting that smoking is associated with high inflammatory load. Smoking was also associated with low DPOAE 70/70 response compared to non-smokers at 3 kHz.

Vascular diseases and cardiovascular disease have been associated with ARHL, particularly low frequency hearing loss (Eckert et al. 2013). This is supported by evidence that gerbil raised in quiet environments with no exposure to ototoxic substances which show the strial type of ARHL (Schmiedt et al., 2002). Histological evidence from the cochlea of the gerbils are atrophy of the stria vascularis, loss of stria capillaries and reduction in the endocochlear potential (Willott & Schacht 2010). Also, degeneration of capillaries of the stria has been observed to occur more at the apex than the base of the cochlea (Mom et al. 1997), linking strial ARHL with a more prominent low frequency hearing loss compared to the other two forms of ARHL (sensory and neural). Interestingly, the association between vascular diseases and low frequency hearing loss is particularly found in females (Eckert et al., 2013; Gates et al., 1993). In women, a history of hypertension or coronary artery diseases almost doubles the likelihood of low frequency hearing loss (Gates et al., 1993). Females have been found to have smaller and more resistive vertebral artery, the parent artery of the cochlear artery, making it more prone to blockage (Hansen et al., 1995). Interestingly, evidence from gerbils show that branches of the cochlear artery are supplied with nitric oxide in male gerbils but not females, making the stria of the female more susceptible to ischemia and damage (Reimann et al., 2011). In the neopterin study, 72% of the subjects were females, which may explain why there was an association between mean neopterin level and low frequency hearing loss.

Males generally have worse hearing compared to females particularly, at the high frequencies. In addition, Pearson et al (1995) have found high progression of ARHL in males than in females. Many researchers have attributed this to occupational and lifestyle differences between males and females (Helzner et al. 2005). However, in studies that have eliminated the effect of noise exposure, the incidence and progression of hearing loss remained higher in males (Pearson & Morrell 1995). This suggests there may be other factors contributing to the gender disparity in ARHL. Hormonal influences may play a role in hearing loss. Evidence shows that there are oestrogen receptors in the cochlea that may protect hearing, although the mechanisms are unknown (Stenberg et al. 2002). This is further supported by the fact that by age 80, a female advantage in hearing loss progression seen earlier in life rarely exists (Pearson & Morrell 1995), although it could be argued that this may be rather due to ceiling effect of threshold estimation. Shaw & Piercy (1962) postulated measurement artefacts as the reason for gender differences in low frequency hearing. They postulate that physiological noise, such as heartbeat and breathing, produced in the ear canal when earphones are tightly attached to the ear, is inversely proportional to signal frequencies below 1000 Hz. In addition, the sound pressure of physiological noise varies inversely with the ear canal volume. They argue that since generally, females have smaller ear canals and smaller ear canal volume, when ear phones are used to measure hearing threshold, physiological masking noise levels in females becomes greater than in males, and suggests this is why females have a slightly higher low frequency threshold compared to men (Shaw & Piercy, 1962). If this assertion is true, it suggests that the artefacts could be eliminated by using insert phones or sound field measurements in the determination of hearing threshold. However, in studies that have performed some of the PTA threshold estimation using insert phones, females were still found to have better hearing (Cruickshanks et al., 1998), which suggests that measurement artefacts do not contribute much to gender differences in hearing.

The variability in the incidence, distribution and progression of ARHL may be attributable to different factors, which combine in different ways to affect ARHL. Inflammatory state affect several of these factors to offer protection or promote progression of ARHL.

6.3 Understanding of mechanisms in ARHL using murine model

Preliminary murine work have been based on the hypothesis that microglia in the auditory system become primed by age-related hearing pathology to become highly responsive to systemic inflammatory events, resulting in damaging effect and contributing to the progression of hearing loss.

Findings from the laboratory groups' unpublished work on the central auditory pathway of C57BL/6J mice have showed increase microglia density in the cochlear nucleus of older mice with ARHL. Additionally, the microglia had showed increased soma size and branching compared to younger mice. Increase in microglia density is not associated with normal ageing (Tremblay et al., 2012), therefore it suggests that the microglia were responding to neurodegeneration in the auditory pathway and not normal ageing. Moreover, the changes in microglia observed in the central auditory pathway were found to be significantly more than microglia in non-auditory areas of the brain like the dentate gyrus in similar aged mice. In addition, microglia in the central auditory pathway of older mice also showed increased expression of CD68, CD11c and CD11c, which are markers that show primed or activated microglia. Hearing loss pathology in the C57BL/6J mice is predominantly a peripheral one, characterised by hair cell loss resulting in high frequency hearing loss. Interestingly, the basal regions of the spiral ganglion, which corresponds to high frequency regions (area with the greatest hearing loss) have shown increased microglia density compared to apical regions, which have less hearing loss (Causon, PhD thesis 2016). Therefore, microglia density correlated positively with the amount of hearing loss pathology.

It is evident from mice studies that the inner ear is capable of mounting an inflammatory response. Secondly, the immune response is considerably greater in aged mice than in young mice. Although the microglia did not respond to the LPS challenge, the evidence of priming in the auditory system suggests that the microglia are responding to hearing pathology and therefore involved in ARHL.

Brain dysfunction is not modulated by large scale loss of neurons, however, it is modulated by subtle neuronal degeneration at specific regions (Tremblay et al., 2012). Neurodegeneration that occurs as a result of ARHL does not often involve outright death of neurons. Evidence from both the visual and auditory system suggests loss of sensory stimulation adversely affects the normal morphology and functioning of microglia. It has been suggested that some of the early changes occur in ARHL, takes place at the level of the synapse (Stamataki et al., 2006). Murine studies have shown that loss of presynaptic IHC ribbons and postsynaptic glutamate receptors of peripheral axons of the spiroganglion cells occur early and steadily, long before hair cell loss or death of spiroganglion cells (Sergeyenko et al. 2013). Microglia constantly survey their microenvironment, make contacts with synapses and respond to changes in the brain. From the visual system, microglia have been observed to make contact with synapses once every hour with each contact lasting about five minutes. These contacts are activity-driven, as contacts are diminished during sensory loss (Tremblay, Lowery, & Majewska, 2010). Changes in the synapses results in change in neurological information. Receptors for neurotransmitters and neuromodulators on the microglia become activated and can result in the removal of injured synapses, known as synaptic stripping

(Kettenmann et al. 2013). Ongoing changes in the synapse, as a result of sensory loss in the case of ARHL, can result in reduced regulation of microglia, such that it is possible that microglia may inadvertently phagocytize normal synapses. In the Alzheimer's disease model, the microglia respond to misfolded proteins and neurodegeneration by assuming a primed state. Subsequent exposure to systemic inflammation results in microglia activation. Similarly, primed microglia in the auditory pathway due to neurodegeneration can respond in an exaggerated manner when challenged by systemic inflammation and result in further auditory damage.

6.4 From systemic to inner ear inflammation

Despite the protective role of the blood brain barrier (BBB) in preventing microbial agents into the brain, the brain is not completely immunologically isolated. The peripheral immune system cross-talk with the brain through interaction of cytokines with receptors at the cerebral endothelia, vagal nerve stimulation, or via circumventricular organs (CNS organs that lack BBB) (Cunningham 2013). Microglia, which are the main immunocompetent cells of the brain, play a central role in the cross-talk between peripheral and central immune systems and have been involved in the progression of CNS diseases including Alzheimer's and Multiple Sclerosis. In similar regard, the cochlea is not considered to be immune privileged, as it is able to generate immune response (Harris & Ryan 1984). Autoimmune reactions have been implicated in conditions like Idiopathic Sudden Sensorineural Hearing Loss (ISSHL), suggesting the ability of the cochlea to mount immune response. In addition, the inner ear is known to produce cytokines in response to acoustic injury (Nakamoto et al. 2012). Moreover, there have been studies that have shown antibody production in the cochlea following direct inoculation of Keyhole limpet hemocyanin into the inner ear (Harris 1984). Although these pieces of evidence show cochlea response to direct stimulation, it is hypothesized that systemic inflammation can induce an immune response in the cochlea without breach of the BBB.

The murine models of ARHL may not be exactly translatable to the human study, especially since mice have a greater tolerance for LPS compared to humans (Nomura et al., 2000). Moreover, unlike the mice used in the study, humans do not live in a controlled environment and they are influenced by multiple factors. Therefore, the hypothesized link between inflammation and ARHL was tested by measuring inflammatory biomarkers and performing hearing function tests in elderly humans. The results showed a significant association between biomarkers, WBC and neopterin, and hearing level in both cross-sectional and longitudinal experiments. People with raised inflammatory load have poorer hearing levels compared with people with lower inflammatory load. The involvement of inflammation in sensorineural hearing loss pathologies is evident in the use of intra-tympanic steroid instillation in cases of ISSHL to improve outcome

(Kopke et al., 2001) and intra-operative administration of steroid during cochlear implant surgery (De Ceulaer et al. 2003). New unpublished evidence from studies my laboratory group is involved in has also shown that in cochlear implant patients, systemic IFN-γ is significantly associated with clinical hearing outcome measures such as low frequency hearing preservation and Bamford-Kowal-Bench (BKB) tests. This implies that the hypothesized link between systemic inflammation and ARHL exists and suggests new avenues in the management of ARHL and possibly, other forms of hearing loss.

6.5 Neopterin, a biomarker for inflammaging and ARHL

Many studies have used cytokines as biomarkers for inflammaging (Bruunsgaard et al. 1999; Mcnerlan et al. 2002). In this thesis, the immune biomarker neopterin, a by-product of catabolism of GTP produced by activated macrophages has been used as a measure of inflammaging. Unlike most cytokines, neopterin is biologically stable, inert and its levels respond to inflammation. From the results of our neopterin study and from other studies (Nancey et al. 2008; Murr et al. 2002), serum neopterin levels correlates with urine levels, which makes it ideal for frequent monitoring of inflammatory state.

Factors that contribute to inflammaging including dysregulated immune system, chronic diseases and reduced hormonal influences result a state of systemic chronic inflammation. In addition, inflammaging creates increased susceptibility to infections. Particularly implicated are cytomegalovirus and Epstein Barr virus which frequently cause subclinical infections (Hunt et al. 2010a). Changes in the immune state means that commensals from the gut or oral mucosa which hitherto did not cause disease become a source of inflammation (Medzhitov 2008). Therefore, baseline chronic inflammation is further complicated by acute inflammatory events. The immune system may initially fight to bring the system back to homeostasis, however, prolonged periods of inflammaging causes maladaptive processes such as changes in homeostatic set points and tissue injury (Medzhitov 2008). People with ongoing inflammaging are also prone to having repeated episodes of inflammation. Initially, inflammatory episodes may be fully resolved within a short time; however, as inflammaging progresses resolution of inflammation become may become prolonged and inflammatory markers stay at elevated levels for longer.

From the evidence discussed concerning synaptic changes that occur in the auditory system, we can postulate that neurodegeneration is a feature of ARHL. Like other neurodegenerative diseases, how the immune system responds to challenges affects the progression of disease. In ARHL and other forms of sensorineural hearing loss, frequent or prolonged systemic immune challenges

results in corresponding immune response in the auditory pathway, that result in cumulative tissue injury that may drive progression of hearing loss.

6.6 Clinical implications

Pure-tone audiometry is the main clinical tool for determining hearing thresholds in clinical practice. It is thought to assess the entire auditory pathway (Büchler et al. 2012). However due to the slow progress of ARHL, an average decline of 1 dB per year, PTA with a test-retest reliability of 5 dB is unable to be used in yearly monitoring of progression of ARHL. Results from the study in chapter 5 have shown that OAE can detect auditory function change within an early time point of one year. The use of OAE for hearing screening is not a new concept, as TEOAE has been used for new-born hearing screening for decades. It is a fast and easy way of screening for hearing. This concept can be extended to elderly people

Stratified medicine describes the use of peculiar tools or characteristics such as risk factors or mechanism of disease, or the likelihood of responding to certain management strategies, to subdivide a cohort for optimum management of their disease (UK Trade & Investment 2013). In an era where patient care is moving towards stratified medicine, it is imperative for the management of ARHL, the most common sensory deficit of the elderly, to be stratified. Based on our results, inflammation has been found to affect ARHL, particularly low frequency thresholds. Such individuals tend to have a flat or gently sloping audiogram which suggests strial or metabolic form of ARHL (Dubno et al. 2013). A more holistic approach for people with this type of ARHL, in addition to hearing rehabilitation would be to liaise with GPs in the management of any chronic disease and to engage in lifestyle activities like exercise that reduces inflammation. Neopterin levels can potentially show individuals who are likely to have a more accelerated progression of ARHL.

Current management of ARHL focuses on amplification of speech sounds, for improvement of everyday function. This is achieved through the provision of hearing aids, assistive hearing devices, lip reading and cochlear implants. A new area of focus will be to reduce the progression of hearing loss; however, this can only be done if the mechanisms involved in each case are understood.

6.7 Ongoing and future work

From previous murine studies, ageing and hearing pathology contribute to microglial priming in the auditory pathway. Although, LPS challenge did not result in the expression of pro-inflammatory cytokines, the microglial priming observed suggests that microglia can be

susceptible to systemic challenges. It is possible that LPS dose was not high enough to produce a pro-inflammatory response, therefore in future work, higher concentrations of intraperitoneal LPS could be used.

Other methods of assessing changes in inflammatory response should be explored. Acute phase reactant mRNA expression in the auditory pathway could indicate a response towards pro-inflammatory state. Immunohistochemistry did not show production of pro-inflammatory cytokine, however it is possible that challenged animals may show expression of cytokine gene transcripts.

Astrocytes are in close proximity to microglia and it is possible that changes in microglia density and morphology are associated with changes in astrocytes. There is ongoing work to investigate astrocytic changes in ARHL. Also, since there is evidence that synaptic changes are some of the first that occur in ARHL, it may be useful to examine inflammatory changes at the synapses following systemic challenge.

Murine work can be further extended to measure the effect of systemic inflammation on the functional ability of the auditory system. This can be done by performing hearing tests on the animals after systemic challenge using electrophysiology tests or auditory brainstem response (ABR) tests. A combination of histological and functional evidence for the damaging effect of inflammation on the auditory system will strongly suggest that the progression of ARHL can be reduced by controlling infections and inflammation.

The ARHL pathology in the C57BL/6J mice is known to be a genetic predisposition to ARHL by hair cells loss, which suggests a sensory form of ARHL. From our study, it has been shown that inflammation is more likely to impact on low frequency hearing, strial form of ARHL. Therefore, it may be worth investigating the effect of systemic inflammation on ARHL in animals that show more strial ARHL like the gerbil, which is believed to mimic ARHL in humans. In this context of simulating ARHL in humans, ongoing ARHL work in the group is being done with non-human primates.

The human study in this thesis has served as a pilot study for a potentially large scale population study that can investigate the effect of systemic inflammation in the progression of ARHL. In a large-scale study, it will be possible to separate subjects with different the hearing loss mechanisms discussed, sensory, strial and neuronal. This will enable for investigations on the effect of inflammation and lifestyle factors on the different types of ARHL.

The study can also be broadened to include more extended frequency hearing. In our study, 12 kHz was the only extended frequency testing that was performed. However, studies that

performed extended frequency testing to investigate change in hearing due to ototoxicity or noise exposure, have tested at least three frequencies. The effects of inflammation or other risk factors on hearing threshold at single frequencies have not yielded much positive results. Therefore, in further studies, other high frequencies including 14 and 16 kHz could be measured, as this has shown positive outcomes in some studies (Mehrparvar et al. 2014).

The use of other auditory function tests including auditory brainstem responses (ABR) to determine detect changes in hearing within varying inflammatory groups can be investigated. From our results, OAE was not sensitive to neopterin levels. The reason for this may be that OAE, which measures the function of the OHC, may not be influenced by inflammation. It is possible that ABR, which measures the brain electrical activity from the cochlea to the brainstem (including synaptic activities of the IHC), may capture changes due to inflammatory.

The human study is ongoing for 3 years. It should be further extended to 5 – 10 years with recruitment of more subjects to power the study in order to get a clearer picture on the long-term effect of inflammation on ARHL.

Chapter 7: Conclusions

The body of work from this thesis has provided evidence using both cross-sectional and longitudinal studies, that inflammation is associated with age-related hearing loss.

- Results from the MRC study provided preliminary evidence for a link between inflammatory status, measured by WBC count and worsening age-related hearing loss, corroborating other cross-sectional studies that have found this association. The findings also suggested that the effect of inflammation was likely to impact on low and mid frequency hearing.
- The research has provided normative values for levels of WBC, IL-6, IL-1β, TNF-α, IFN-γ, urine and serum neopterin levels for a cohort of community dwelling elderly people.
- Increased neopterin levels can indicate a high state of immune activation and is associated with increased risk of age-related chronic diseases including ARHL
- High inflammatory state measured from longitudinal measures of neopterin levels has been shown to be associated with worse ARHL, providing a stronger evidence of the association between inflammation and ARHL. Although evidence for increased progression of ARHL has not been shown at this stage, it is expected that by the end of the third year of the study, increased progression of ARHL can be shown for subjects with high inflammatory state.
- The research has provided evidence for the use of OAE tests to detect early changes in hearing function in people with ARHL suggesting the possibility of using OAEs to monitor the progression of ARHL.

There are a number of recommendations from the findings:

- Routine monitoring of inflammation in the elderly and the promotion of anti-inflammatory activities such as exercise to reduce systemic inflammation should be encouraged.
- To further investigate the use of OAE as a hearing screening test, starting from middle age, to detect ARHL earlier in life and to monitor progression of hearing loss.
- To use a stratified medicine approach in the management of age-related hearing loss that takes into account individual aetiology and risk factors and for hearing loss.
- There is a need for further investigation into the biological mechanisms involved in age-related hearing loss.
- This data can be used as a pilot study for a large-scale multi-centered study that will further define the association between inflammation and age-related hearing loss.

Appendix A Inflammation is associated with a worsening of presbycusis: Evidence from the MRC national study of hearing

Appendix A

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Original Article

Inflammation is associated with a worsening of presbycusis: Evidence from the MRC national study of hearing

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The International Society of Audiology
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The Nordic Audiological Society
www.nas.dk

Abstract

Objective: Inflammaging, a state of chronic inflammation in the elderly, is now thought to be a key element of the ageing process and contributor to age-related disease. In a previously published study, we identified a significant association between inflammation levels and severity of presbycusis among individuals aged 63 to 73 ("younger old") within an available audiometric range 0.5 to 4 kHz. Our aim was to see if this association would be identified among participants in the MRC national study of hearing, and whether the strength of the association would increase with greater age, or for very low or very high audiometric frequencies. *Design:* Cross-sectional analysis of cohort data. *Study sample:* Three hundred and sixty community-dwelling adults age 60 years and over, representing all those with white blood cell count and audiometric data available. *Results:* A significant independent association between (higher) WBC and (worse) hearing level was identified. This effect increased with age. The strongest association was among those over 75, for whom average hearing threshold levels among those with lower WBC was 17 dB better than those with higher WBC. *Conclusions:* The current findings support an association between inflammaging (a condition potentially amenable to pharmacological treatment or lifestyle management) and presbycusis.

Key Words: Presbycusis; age-related hearing loss; inflammation; bio-markers

The biological mechanism underlying presbycusis is still poorly understood, despite the fact that it is the most common form of hearing loss in adults, with widespread effects on quality of life, participation in economic activity, and well-being in the older population (Huang & Tang, 2010). A key aspect of presbycusis is that it is variably expressed, with large inter-individual differences in hearing threshold level and hearing disability among older adults (Davis, 1989). Interestingly, a significant proportion of older adults do not become hearing-impaired with age, suggesting that the mechanisms driving the condition are not inevitable. Variability is due to a combination of environmental and genetic factors (Uchida et al, 2011) but is further complicated by the association between presbycusis and other forms of age-related morbidity including hypertension (Brant et al, 1996), cardiovascular disease (Hutchinson et al, 2010; Karpa et al, 2010), and dementia (Lin et al, 2011).

There is some evidence that lifestyle factors and chronic diseases contribute to presbycusis. Dietary factors such as folic acid and vitamin B12 which contribute to homocysteine levels have been associated with presbycusis (Houston et al, 1999; Gopinath et al, 2010). There is evidence that cardiovascular disease and diet both have an effect on presbycusis (Hutchinson et al, 2010; Spankovich & Le Prell, 2013). Elevated homocysteine levels promote the inflammatory processes associated with atherosclerosis which may lead to other cardiovascular diseases (Oudi et al, 2010). Dietary glycaemic load (Gopinath et al, 2010a) and saturated fats (Gopinath et al, 2011) have both been shown to be a predictor of presbycusis and may explain certain aspects of the relationship between type 2 diabetes, cardiovascular diseases, and presbycusis. Other lifestyle factors, in particular smoking (Cruickshanks, 1998), contribute to presbycusis through reduced vascular supply, and anti-oxidants effect. At the same time, recent research in gerontology has shown that inflammaging, a state of chronic systemic inflammation associated with age but variable in its expression between older individuals, is a key factor in the ageing process and in development of age-related disease

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Abbreviations

HAS	Hertfordshire ageing study
MRC	Medical Research Council
NSH	National study on hearing
WBC	White blood cell count

and disabilities (Capri et al, 2006; Larbi et al, 2008) and is linked to many of these medical and lifestyle factors which are themselves linked with presbycusis. If inflammaging is found to be a contributor to presbycusis, this could provide some explanation for the variability in hearing outcomes with age and would point to a possible mechanism by which factors such as dementia, hypertension, and smoking are associated with presbycusis. The aim of the present study was therefore to establish whether a relationship between inflammaging and presbycusis could be identified through analysis of cross-sectional epidemiological data from the MRC national study of hearing and to compare findings with another recently published study in this area.

Inflammaging is a consequence of immunosenescence, the ageing of the immune system (Capri et al, 2006). As people age, their immune system becomes less adept at down-regulating on-going production of inflammatory proteins after acute inflammatory events, leading to a chronically raised inflammatory state which can be measured through a number of biomarkers of inflammation, including relative cytokine expression and white blood cell count (Hunt et al, 2010). Inflammaging has been shown to be a factor in age-related conditions including frailty (Baylis et al, 2013), atherosclerosis (Hunt et al, 2010), cardiovascular disease, peripheral arterial disease, type II diabetes, and Parkinson's disease (Larbi et al, 2008). Many of the same factors associated with inflammaging have also been identified as being linked with presbycusis severity (Frisina et al, 2006; Gates et al, 1993). Cochlear inflammation can occur locally due to factors such as noise or infection (Harris & Ryan, 1984; Satoh et al, 2003). However, it is also likely that systemic inflammation leads to altered inflammatory changes within the cochlea via a similar mechanism by which it has been shown to influence the central nervous system, despite the "blood-brain barrier" (Holmes et al, 2009). In this paradigm inflammatory events within the body, but outside the nervous system, communicate by a combination of neural and molecular signalling to sites of inflammation in the central nervous system. More direct evidence comes from the finding that spiral ganglion cell damage can be caused by immune system changes (Iwai et al, 2003, 2008) while damage to the stria vascularis may be mediated by vascular and metabolic changes (Saitoh et al, 1995; Ohlemiller, 2009; Fetoni et al, 2011) which themselves cause inflammatory damage (although the distinction between vascular and inflammatory mechanisms may be difficult to distinguish). Lasisi et al (2011) found an association between serum immunoglobulin G (IgG) and hearing loss in a cross-sectional study of a cohort of 126 individuals over 60 years of age. It is highly plausible that inflammaging could play a role in causing or accelerating presbycusis.

In a recently published study (Verschuur et al, 2012), we examined data from the Hertfordshire ageing study (HAS), a large birth cohort of individuals born in Hertfordshire, UK, between 1911 and 1948 (Ashfield et al, 2010; Robinson et al, 2009), in order to examine the hypothesis that inflammaging is linked to presbycusis. Data were analysed to determine the degree of independent association between inflammatory markers and hearing status, with additional lifestyle and demographic factors (e.g. gender, age, smoking, occupational and noise exposure history) taken into account. After adjustment for lifestyle factors which were themselves predictive of hearing status, we found that measures of inflammatory status were significantly associated with hearing threshold, namely white blood cell count (henceforth WBC), neutrophil count, IL-6 and C-reactive protein, e.g. for these inflammatory measures, higher serum levels were associated with worse hearing among this group of older people. Findings were consistent with a hypothesized role of inflammaging as a causative factor in presbycusis and also suggested a link between inflammatory markers measured via serum analysis and intra-cochlear inflammatory status. However, there were some limitations in the data available. In particular, audiometric data were limited to frequencies between 0.5 and 4 kHz. This meant that high frequencies (above 4 kHz), which might serve as a particularly useful early indicator of some forms of presbycusis, were not present, nor was testing undertaken at lower frequencies which may be more predictive of more rapidly deteriorating forms of presbycusis (Lee et al, 2005). More crucially, only a relatively narrow age range of participants was available (63 to 73), which meant that it was not possible to establish the extent of association with the "older old" population.

The aim of the present study was to determine if the identified cross-sectional association between presbycusis and inflammatory measures identified in the HAS data set would be found in a different population cohort. A key motivation was to see if the finding from the HAS data would be replicated, but also to consider any differences in the magnitude or pattern of the association that might be due to different population characteristics across the two studies. We therefore examined data from the Medical Research Council national study of hearing (MRC NSH), which took place in the 1980s. The aim of the study, which has been extensively reported elsewhere (Davis, 1989) was to assess the prevalence and distribution of hearing impairment and disability in Great Britain, with data collected across four centres in the UK. Blood markers of various types were available for a sub-set of participants in the study. White blood cell count (WBC), which was shown to be the strongest predictor of presbycusis in the HAS cohort among available inflammatory markers, and shown to be a valid measure of inflammaging in other work (Baylis et al, 2013) was available in a subset of MRC NSH participants, along with detailed hearing data. Baylis et al (2013) showed that WBC can be used as a method of stratifying the population into different risk categories for frailty; therefore, we took a similar approach to the assessment of presbycusis severity.

Our aim was not only to see if the finding in the HAS data analysis would be replicated in a different population cohort, but also to evaluate other aspects not possible to determine from the HAS data. We hypothesized that the association between inflammaging (as measured by WBC) and hearing loss would become stronger with increased age. This was motivated by other work showing that the contribution of inflammaging to age-related morbidity becomes greater with increased age (Hunt et al, 2010). Additionally, hearing data were available at a greater range of frequencies in the MRC data set compared to the HAS data set so we sought to evaluate the relationship between lower and higher frequency hearing with inflammatory status, in particular the possibility that inflammaging might be disproportionately implicated in low frequency presbycusis. Some previous evidence has suggested that low-frequency hearing thresholds may be implicated in vascular or metabolic deterioration with ageing (Dubno et al, 2013), thus raising the possibility that low-frequency thresholds may be more sensitive to inflammaging

Appendix A

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and disabilities (Capri et al, 2006; Larbi et al, 2008) and is linked to many of these medical and lifestyle factors which are themselves linked with presbycusis. If inflammaging is found to be a contributor to presbycusis, this could provide some explanation for the variability in hearing outcomes with age and would point to a possible mechanism by which factors such as dementia, hypertension, and smoking are associated with presbycusis. The aim of the present study was therefore to establish whether a relationship between inflammaging and presbycusis could be identified through analysis of cross-sectional epidemiological data from the MRC national study of hearing and to compare findings with another recently published study in this area.

Inflammaging is a consequence of immunosenescence, the ageing of the immune system (Capri et al, 2006). As people age, their immune system becomes less adept at down-regulating on-going production of inflammatory proteins after acute inflammatory events, leading to a chronically raised inflammatory state which can be measured through a number of biomarkers of inflammation, including relative cytokine expression and white blood cell count (Hunt et al, 2010). Inflammaging has been shown to be a factor in age-related conditions including frailty (Baylis et al, 2013), atherosclerosis (Hunt et al, 2010), cardiovascular disease, peripheral arterial disease, type II diabetes, and Parkinson's disease (Larbi et al, 2008). Many of the same factors associated with inflammaging have also been identified as being linked with presbycusis severity (Frisina et al, 2006; Gates et al, 1993). Cochlear inflammation can occur locally due to factors such as noise or infection (Harris & Ryan, 1984; Satoh et al, 2003). However, it is also likely that systemic inflammation leads to altered inflammatory changes within the cochlea via a similar mechanism by which it has been shown to influence the central nervous system, despite the "blood-brain barrier" (Holmes et al, 2009). In this paradigm inflammatory events within the body, but outside the nervous system, communicate by a combination of neural and molecular signalling to sites of inflammation in the central nervous system. More direct evidence comes from the finding that spiral ganglion cell damage can be caused by immune system changes (Iwai et al, 2003, 2008) while damage to the stria vascularis may be mediated by vascular and metabolic changes (Saitoh et al, 1995; Ohlemiller, 2009; Fetoni et al, 2011) which themselves cause inflammatory damage (although the distinction between vascular and inflammatory mechanisms may be difficult to distinguish). Lasisi et al (2011) found an association between serum immunoglobulin G (IgG) and hearing loss in a cross-sectional study of a cohort of 126 individuals over 60 years of age. It is highly plausible that inflammaging could play a role in causing or accelerating presbycusis.

In a recently published study (Verschuur et al, 2012), we examined data from the Hertfordshire ageing study (HAS), a large birth cohort of individuals born in Hertfordshire, UK, between 1911 and 1948 (Ashfield et al, 2010; Robinson et al, 2009), in order to examine the hypothesis that inflammaging is linked to presbycusis. Data were analysed to determine the degree of independent association between inflammatory markers and hearing status, with additional lifestyle and demographic factors (e.g. gender, age, smoking, occupational and noise exposure history) taken into account. After adjustment for lifestyle factors which were themselves predictive of hearing status, we found that measures of inflammatory status were significantly associated with hearing threshold, namely white blood cell count (henceforth WBC), neutrophil count, IL-6 and C-reactive protein, e.g. for these inflammatory measures, higher serum levels were associated with worse hearing among this group of older people. Findings were consistent with a hypothesized role of inflammaging as a causative factor in presbycusis and also suggested a link between inflammatory markers measured via serum analysis and intra-cochlear inflammatory status. However, there were some limitations in the data available. In particular, audiometric data were limited to frequencies between 0.5 and 4 kHz. This meant that high frequencies (above 4 kHz), which might serve as a particularly useful early indicator of some forms of presbycusis, were not present, nor was testing undertaken at lower frequencies which may be more predictive of more rapidly deteriorating forms of presbycusis (Lee et al, 2005). More crucially, only a relatively narrow age range of participants was available (63 to 73), which meant that it was not possible to establish the extent of association with the "older old" population.

The aim of the present study was to determine if the identified cross-sectional association between presbycusis and inflammatory measures identified in the HAS data set would be found in a different population cohort. A key motivation was to see if the finding from the HAS data would be replicated, but also to consider any differences in the magnitude or pattern of the association that might be due to different population characteristics across the two studies. We therefore examined data from the Medical Research Council national study of hearing (MRC NSH), which took place in the 1980s. The aim of the study, which has been extensively reported elsewhere (Davis, 1989) was to assess the prevalence and distribution of hearing impairment and disability in Great Britain, with data collected across four centres in the UK. Blood markers of various types were available for a sub-set of participants in the study. White blood cell count (WBC), which was shown to be the strongest predictor of presbycusis in the HAS cohort among available inflammatory markers, and shown to be a valid measure of inflammaging in other work (Baylis et al, 2013) was available in a subset of MRC NSH participants, along with detailed hearing data. Baylis et al (2013) showed that WBC can be used as a method of stratifying the population into different risk categories for frailty; therefore, we took a similar approach to the assessment of presbycusis severity.

Our aim was not only to see if the finding in the HAS data analysis would be replicated in a different population cohort, but also to evaluate other aspects not possible to determine from the HAS data. We hypothesized that the association between inflammaging (as measured by WBC) and hearing loss would become stronger with increased age. This was motivated by other work showing that the contribution of inflammaging to age-related morbidity becomes greater with increased age (Hunt et al, 2010). Additionally, hearing data were available at a greater range of frequencies in the MRC data set compared to the HAS data set so we sought to evaluate the relationship between lower and higher frequency hearing with inflammatory status, in particular the possibility that inflammaging might be disproportionately implicated in low frequency presbycusis. Some previous evidence has suggested that low-frequency hearing thresholds may be implicated in vascular or metabolic deterioration with ageing (Dubno et al, 2013), thus raising the possibility that low-frequency thresholds may be more sensitive to inflammaging

effects given the relationship between inflammaging and cardiovascular disease(Ashfield et al, 2010).

Methods

The aims of data analysis were (1) to determine if there was a significant association between presbycusis and WBC in this cohort, (2) to determine if there were significant differences in presbycusis severity among sub-groups stratified according to WBC category, (3) to evaluate an older age group than was possible in the HAS data set to determine if the hypothesized association, if found, would be greater with increased age, and (4) to compare effects with age-equivalent data from the HAS data set. To do this, data from the MRC national study of hearing were processed and interrogated as described below.

The MRC national study of hearing

The Medical Research Council (MRC) Institute of Hearing Research (IHR) undertook the national study on hearing (NSH) from 1980 to 1986 in order to assess the prevalence and distribution of hearing impairment and disability in Great Britain. The study has been extensively described elsewhere (Davis, 1989) but key details are noted here. The study was conducted in two stages across four UK cities. In stage one, questionnaires were sent out to 48 313 people. Two thousand, nine hundred and ten (2910) people were drawn from respondents of the questionnaire to participate in the second stage. Stage two comprised clinical interview and examination, and audiological assessment. Audiometric assessment in sound-treated booths was performed for 2910 subjects. Air conduction thresholds were measured at 0.25, 0.5, 1, 2, 3, 4, 6, and 8 kHz. Bone conduction measurements were done at 0.5, 1, and 2 kHz.

Blood measurements were obtained from 1692 subjects. Blood measurements undertaken included WBC, red blood cell count, haemoglobin, haematocrit, mean corpuscular volume, mean corpuscular haemoglobin, mean corpuscular haemoglobin concentration, plasma glucose and thyroxin. Unfortunately, longitudinal hearing or biomarker data were not available among those with available blood data.

Participants, variables, and exclusion criteria

The number of subjects with both audiometric and blood investigation data was 1134, with subject ages ranging from 17 to 89 at time of testing. In order to compare against our analysis of HAS data and to explore the link between inflammaging and presbycusis we analysed data from only those 60 years and over at time of testing (N = 355) and excluded those with asymmetrical hearing in order to exclude pathologies such as acoustic neuroma or Ménière's disease. Asymmetry between ears was defined as more than 20 dB difference between average hearing threshold (0.25 to 8 kHz by air-conduction) of left and right ear. This yielded a total subject pool of 320 (age range 60 to 89) with a mean age of 68 at time of testing, similar to the mean age of 67 for the HAS cohort, although the age range was wider than for the HAS data set population, who had all been recruited within a relatively short time window around birth.

Table 1 shows contrasting demographic characteristic of the two groups, e.g. the sub-group of MRC participants described above and the subjects from the HAS study previously reported (Verschuur et al, 2012) along with the sub-set of MRC cohort participants falling into the same age limits as for the HAS cohort. This shows

Table 1. Key demographic characteristics of participants in the MRC and HAS population cohorts. Total N was the number of participants in each study for whom both WBC and hearing data were available, excluding subjects with inter-aural asymmetries of greater than 20 dB.

	MRC study (age 60–89)	*MRC (age 63–73)*	*HAS study (age 63–73)*
N	320	195	636
Male/Female %	56/44	57/43	56/44
Age			
Mean (SD)	68.3 (6.2)	67.8 (2.9)	67.5 (2.3)
Age range	60–89	63–73	63–73
WBC*			
Mean (SD)	73 (19.4)	74 (19.6)	62 (3.8)
Range	29–153	29–153	28–184
PTA**			
Mean (SD)	37.3 (20.4)	35.6 (20.0)	28.8 (12.2)
Range	2–108	2–99	6–92

*in 10^9/dl; **Pure-tone average hearing threshold by air conduction in worse-hearing ear averaged across 0.5, 1, 2, and 4 kHz.

that both hearing levels and WBC count values were worse/higher among the MRC cohort compared to the HAS data despite a similar mean age and gender distribution; this was true even when limiting data to the MRC participants in the same age range as the HAS participants. It should be noted that the total subject number for the HAS data reported in the present study (N = 636) is slightly different than reported in Verschuur et al (2012) (N = 611) as the latter also excluded subjects in which otoscopy could not be visualized; in the current analysis we were unable to definitively exclude individuals from the MRC data set. Consequently, these have not been excluded here in order to show as close a comparison as possible. (It is worth noting, in this context, that the key findings of the HAS study, e.g. a significant independent association between a number of bio-markers of inflammation and hearing loss severity, were replicated irrespective of key exclusions, e.g. with or without asymmetric hearing loss cases excluded).In addition to analysis of the over-60 population based on the hypothesis that inflammaging is specifically linked with hearing loss (as it is with other conditions), we also analysed the full data set to determine if any relationship existed between inflammatory level and hearing across the whole group with blood and audiometric data available.

Outcome measures in the present study were composite air-conduction pure-tone thresholds, averaged across a range of audiometric frequencies in the worse hearing ear. This allowed for a clearer comparison with the HAS data analysis and also a more appropriate data distribution for analysis than was possible for single frequency thresholds. The first outcome measure used was the average of 0.5, 1, 2, and 4 kHz air-conduction thresholds (pure-tone average threshold, PTA). Given the availability of audiometric data across a wider range of frequencies than was available in the HAS data, two other audiometric outcome variables were also used: low frequency pure-tone average threshold (mean air conduction hearing threshold at 0.25, 0.5, and 1 kHz) and high frequency pure-tone average threshold (mean air-conduction hearing threshold at 4, 6, and 8 k Hz).

Statistical analysis

Data were tabulated using Excel 2010 and PASW SPSS Statistics version 18. Variables found to be inconsistent with a normal

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distribution were log-transformed. Linear associations between WBC and audiometric variables were first established, both across the whole cohort and restricted to ages 60 and over, 70 and over, or 75 and over. WBC was expressed in terms of total cell $\times 10^9$ count per deci-litre (10^9/dl). Given the absence of associations between WBC and either high frequency or low frequency hearing level measures (see below), further analyses were restricted to pure-tone average threshold only. Stratification of the older population into tertiles according to inflammatory markers, including WBC, has previously been shown to be a useful means of predicting frailty and other age-related morbidity in the community-dwelling older population (Baylis et al, 2013). We therefore used one-way analysis of variance (ANOVA) to identify any significant difference in pure-tone average threshold as a function of inflammatory group stratified according to WBC tertiles. The same analysis was repeated for different age sub-groups, as with the linear associations. Effects of WBC on hearing level across the HAS and MRC population cohorts were also compared directly using the same cut-off WBC value.

Results

There was no significant association between WBC and pure-tone average threshold for the entire cohort above ($r = 0.04$; $p = 0.19$). However, there was a significant positive association between WBC and pure-tone average threshold among the cohort aged 60 and over ($r = 0.13$; $p < 0.05$). However, there was no significant association between WBC and either high frequency pure-tone average ($r = 0.08$; $p = 0.12$) or low frequency pure-tone average ($r = 0.23$; $p = 0.67$) among those aged 60 and over. The linear association between log-transformed WBC and pure-tone average threshold among those between 60 and 69 ($N = 196$; $r = 0.11$) and those between 70 and 74 ($N = 73$; $r = 0.1$) showed similar regression coefficients to the value for all subjects aged 60 and over ($r = 0.13$). However, for those age 75 and over only ($N = 51$), the positive association was markedly greater ($r = 0.44$).

In order to explore the extent to which WBC was an independent predictor of hearing level, and how this might vary across age groups, we undertook a series of within-subject repeated measure analyses of variance (ANOVA) with WBC tertile (thirds of the distribution), age, and gender as independent variables, and pure-tone average threshold as the single dependent variable in each case. The same analyses were undertaken for all subjects aged 60 and separately for age groups 60 to 69, 70 to 74, and 75 and over. For all subjects of 60 and over, WBC tertile ($F(2, 205) = 4.14$) and gender ($F(1, 205) = 4.41$) had significant independent effects on pure-tone average threshold ($p < 0.05$ in each case) but there were no significant interactions between factors. WBC tertile was also an independent predictor of pure-tone average threshold for those 75 and over ($F(2, 48) = 6.89$; $p < 0.005$). *Post-hoc* analysis using Fisher's least significant difference test showed that the comparison between lowest and highest third was statistically significant ($p < 0.05$ for 60 and over, $p < 0.01$ for 75 and over). However, WBC tertile was not found to have a significant independent effect on hearing level among the 60 to 69 or 70 to 74 age groups. Figure 1 shows the magnitude of the difference in mean pure-tone threshold (highest WBC third − lowest WBC third) was 6.5 dB across all subjects 60 and over (see Figure 1, A), 5 dB across subjects 60 to 69 (Figure 1, B), 3.5 dB for subjects in the age range 70 to 74 (Figure 1, C), and 17 dB for subjects 75 and over (Figure 1, D), e.g. in each case pure-tone thresholds were correspondingly worse/less acute for the highest compared to the lowest sub-group stratified according to WBC tertile.

A further question was the extent to which the different population cohorts would show a similar direction and magnitude of effect (of WBC on hearing). In order to make a direct comparison between cohorts, we determined a cut-off WBC level that has been shown to be clinically significant in published studies of inflammaging ($\geq 6 \times 10^9$; see Baylis et al, 2013) and compared pure-tone average threshold between sub-groups defined in this way for both cohorts, as shown in Figure 2. The difference between the two sub-groups ranked according to this WBC cut-off is less than 2 dB for the

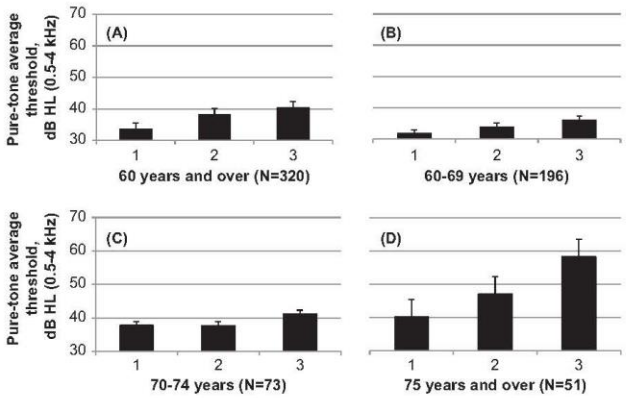

Figure 1. Pure-tone average audiometric thresholds by air-conduction in the worse hearing ear for sub-groups stratified into tertiles according to white blood cell count (WBC). Error bars indicate standard errors of the mean. Subjects were those with both audiometric and WBC data available from the MRC NSH cohort but restricted to all those 60 and over, 60–69, 70–74, or 75 and over as indicated.

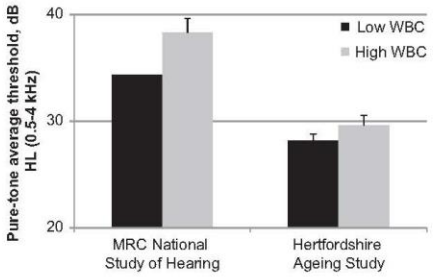

Figure 2. Pure-tone average audiometric thresholds by air-conduction in the worse hearing ear for sub-groups identified according to WBC (cut-off ≥60 defined as high WBC). MRC cohort: low WBC (N = 77; average age = 69.1), high WBC (N = 243; average age = 68.1). HAS cohort: low WBC (N = 359; average age = 67.4), high WBC (N = 277, average age = 67.5).

HAS data set and 4 dB for the MRC data set (again, worse hearing amongst the higher WBC group in both cases).

Discussion

Our findings provide further evidence to support a link between inflammatory status and degree of presbycusis. There is now considerable evidence that inflammaging, caused by the inability of the ageing immune system to adequately regulate responses to immune challenges, is an important factor in a range of age-related diseases (Leng et al, 2011; Capri et al, 2006; Hunt et al, 2010; Baylis et al, 2013) and is thought to be a key driver of the ageing process itself. Identification of a link between inflammaging and presbycusis has wide-spread implications, as it suggests that factors that affect the health of the immune system with age may be important in the severity and rate of progression of presbycusis and, conversely, may provide new avenues to reduce the progression of this condition, including potential pharmacological as well as lifestyle interventions. It also suggests a mechanism underlying the effect of certain lifestyle factors such as smoking, known to lead to higher levels of chronic inflammation, on presbycusis.

Our overriding aim was to determine if the association between inflammatory status and hearing previously identified in a large population cohort, would be replicated in a different cohort of older people with somewhat different characteristics. The MRC national study of hearing provided a data set with which to test this possibility. Although blood markers related to inflammation were limited to WBC only, both our own previous work and that of others has identified this measure as a very useful index of chronic inflammation or inflammaging (Leng et al, 2005; Baylis et al, 2013). We found the hypothesized association between WBC and hearing level in individuals in the MRC cohort over 60 years of age, although the main reason for this appeared to be the very strong association among participants age 75 and over. Interestingly, the magnitude of the effect was greater than that identified in the HAS data set cohort, with additional compelling evidence that the association becomes greater with age. Although the two cohorts were similar in mean age and gender profile, both pure-tone average threshold and WBC were greater for the MRC group whether for all participants over 60 or age-limited to equate to the HAS cohort (see Table 1). This may be due to differences in subject recruitment and characteristics, e.g. the MRC participants were recruited from urban areas and the HAS participants from primarily rural ones. However, elucidation of the reasons for this difference would require further, more controlled comparisons than are possible with a direct comparison of these two different cohorts. One advantage of the MRC data set compared to the HAS data was the availability of more comprehensive audiometric data, including very low and very high frequencies. There is some evidence that vascular and metabolic disorders are linked to low-frequency hearing loss with age, (Eckert et al, 2013) and it is conceivable that this link is mediated by inflammation. It is also possible that higher frequencies which are more prone to presbycusis might have been more tightly associated with inflammatory state. However, neither of these possibilities was borne out, in that mean threshold measures that included very high or low frequencies were in fact not associated with WBC in the MRC data set; rather, mean threshold across key speech frequencies 0.5 to 4 kHz was most sensitive to effects of interest. It should be noted that there was also a non-significant trend for an association with high- or low-frequency threshold; it may be that the fact that low/high frequency threshold measures were a composite of fewer data points (three rather than four audiometric frequencies) reduced statistical power, or that lower and higher frequency thresholds (above 4 kHz and below 0.5 kHz) are more susceptible to other factors, e.g. noise exposure for very high frequencies. Further work is necessary to elucidate the relationship between inflammatory status and audiometric pattern.

There were also some disadvantages of the MRC data set for the purposes set out in the present study. In particular, it was not possible to cross-reference available data consistently against the full range of confounding lifestyle and demographic factors that are known to have an effect on hearing, e.g. noise exposure in particular, in order to explore more fully the extent to which the identified association was independent of other factors (although it did contribute independently from the key predictors of age and gender) as compared with the HAS data analysis, where WBC and other inflammatory markers were shown to contribute independently from noise exposure also. Nevertheless, the similarity in findings across the two cohorts, at least in terms of the presence and direction of the hypothesized association, despite the fact that they were collected entirely independently, for different reasons, and with some different characteristics, is a strong indicator that the effect is likely to be robust. For those over 75, there was a mean difference of 17 dB in pure-tone average threshold between those with highest and lowest inflammatory loads when divided into tertiles according to WBC. Although this is a simple effect that was not adjusted for the range of confounding factors (as was the case with the HAS data), it is a marked effect consistent with the idea that the link between inflammaging and hearing loss does become greater with age. The proportion of the population 75 and over is increasing rapidly, thus making this association particularly important to understand.

A potential benefit of identifying a link between WBC and presbycusis is the possibility of risk stratification for presbycusis, an area which has received relatively no attention in the context of adult hearing loss, but which is becoming increasingly important in the individualized management of other long-term conditions (Yu et al, 2013). The present study shows that the highest third of individuals ranked according to white blood cell count have significantly worse hearing than those in the lowest, a promising method to help define risk of presbycusis in older adults. In order to translate this finding into a risk profile, longitudinal data would need to be gathered to determine if such stratification also provided a prediction of

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longer-term development of presbycusis. However, this represents evidence of a potential bio-marker that can be useful in population risk stratification for hearing loss, adding to recent evidence that bio-markers may be predictive of hearing loss development in older individuals (Gopinath et al, 2010a,b; Lasisi et al, 2011). Further work, particularly via longitudinal data analysis which was not possible from either the MRC or HAS cohort, is now clearly needed to tease out the extent of the causal link between inflammaging and presbycusis, the extent to which inflammatory markers may be used as a method of risk stratification for presbycusis, and to develop novel approaches to slowing down the progression of the condition via management of immune and inflammatory status. There are large potential benefits for the older population.

Conclusion

Analysis of data from the MRC national study of hearing has shown a significant association between white blood cell count (WBC) and hearing level that is independent of age and gender. Moreover, the association becomes stronger with age. The difference in hearing level as a function of WBC status among those over 60 is greater in magnitude than that obtained from analysis of a different population cohort, the Hertfordshire aging study (around 2 dB) and equates to 6.5 dB worse hearing for those with higher WBC (inflammatory load) compared to those with lower inflammatory load among the population over 60, and as much as 17 dB difference for those over 75. Findings from the MRC study therefore provide corroborating evidence that inflammaging, the age-associated chronic increase in inflammatory status, is implicated in presbycusis. This has important implications for the management and prevention of this condition and suggests that lifestyle and pharmacological interventions that improve immune status in the elderly have the potential to reduce the progression of hearing loss with age. It also provides a potential method of risk stratification of presbycusis based on routinely collected bio-marker data. There is a need for further research in this area to better understand the mechanism by which chronic inflammation might accelerate presbycusis.

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Appendix B Protocol for Pure-tone audiometry (air-conduction and bone-conduction) with and without masking

Equipment:

- Otoscope
- Audiometer (GSI G1 clinical audiometer)
- TDH39 supra-aural earphones
- HD200 earphones
- RadioEar B71 bone conductor
- Audiogram sheet

Procedure:

1. Explain procedure to subject
2. Examine the ear for abnormalities using an otoscope
3. Give subject the following instructions *'I am going to measure the quietest sounds you can hear by playing some sounds through the earphone. Press the button when you hear the sound, keep it pressed for as long as you hear it, no matter which ear you hear it or how loud or soft the sound is. Release the button when you no longer hear the sound.'*
4. Start testing at the better ear in the frequency order 1000, 2000, 3000, 4000, 6000, 8000, 500 and 250 Hz. Retest 1000Hz for the first ear.
5. Place TDH39 earphones on subject's ear and play a 1000Hz tone at 40 dB or at a level that can be comfortably heard by subject.
6. Following a positive response, reduce tone levels at 10 dB steps until there is no response, and then increase level in 5 dB steps until a positive response.
7. Repeat step 6 until subject responds at the same level 50% of the time. This is the threshold at the test frequency.
8. Swap TDH39 earphones with HD200 earphones and test threshold at 12000Hz.
9. Place bone conductor on the mastoid prominence of the test ear and measure bone conduction threshold at 500, 1000, 2000 and 4000Hz using the same procedure.
10. Plot the thresholds on an audiogram
11. If there is more than 40 dB difference in air-conduction threshold between left and right ear or more than 10 dB in bone-conduction threshold at a particular frequency, there is a chance of cross-hearing therefore masking is performed for that frequency.
12. Give subject additional masking instruction *'for some time, you will hear a rushing noise, ignore it and press the button only when you hear the tones'*
13. Re-establish threshold for the test ear
14. Introduce masking noise to the non-test ear at an initial level equal to its threshold (effective masking level).
15. Re-establish threshold in the presence of masking noise as described in step 6.

16. Increase masking level by 10 dB steps and re-measure threshold until at least 4 thresholds are obtained with 3 consecutive thresholds within 5 dB of each other (plateau). The hearing level at the plateau is considered the true threshold.

Appendix C Protocol for otoacoustic testing

Equipment:

- Otodynamics ILO 292 OAE equipment
- EZ-Screen software (Otodynamics, ILO V6 Module)
- Probe tips

Procedure:

1. Sit subject comfortably without moving, talking, or responding to played sound.
2. Select appropriate probe tip and insert probe into test ear.

TEOAE recording

3. Measure TEOAE response using the following settings:

- File > Options > Start/Stop/Score tab > select General Diagnostic
- File > Options > Stims tab > Timeout (sweeps) = 260
- Noise reject level = 6
- Target level = 80 dB
- Stim type = Quickscreen

4. Stop manually if a good trace is obtained as defined by all the following together:

- SNR in ALL 5 bands >6 dB
- Number of sweeps at least 100
- Reproducibility >85%

DPgram recording

5. Measure DP-gram response at f1/f2 levels of 70/70 and 65/55 using the following settings:
- File > Options > Stims tab

- FI level = 70 dB (65)
- F2 level = 70 dB (55)
- FI/F2 ratio = 1.22
- Points/octave = 4
- Timeout = 4
- Select = Extended frequency range
- Presentation = Cycle up

6. Record 80 seconds on “auto” and another 80 seconds on selected frequencies to achieve SNR > 6 d B at frequencies 1, 1.4, 2, 2.8, 4, 5.6 and 6 kHz using manual selection.

DP growth function recording

7. Record DP Growth function at 2 and 4 kHz at 5 dB steps using the following settings:

Test >DP Growth > Select DP growth parameters

- Choose frequency = 2 (4) kHz
- Step size = 5 dB
- Initial L2 level = 75 dB
- Select = Formula mode
- Formula: L1 = 0.4L2 + 39 dB

8. Run DP Growth test: Test > Start a DP Growth test

- Record first 80 seconds on “auto” and another 80 seconds on selected levels to achieve DP equal to at least twice the noise level

Appendix D Protocol for neopterin ELISA

Things to be done the day before experiment

1. Make a plate layout
2. Arrange required samples in a separate box and keep in freezer
3. Fill boxes with pipette tips
4. Label tubes
5. Pipette 300ul of assay buffer into tube
6. Get samples out of the freezer and thaw on ice (1 hour to thaw)
7. Dilute samples in assay buffer (1:101). Mix 3ul of sample with 300ul of assay buffer and vortex the diluted samples (30 mins)

On the day of experiment

1. Take neopterin box from the fridge and bring to room temperature.
2. Pipette 20ul of standards, control and diluted samples into wells (45 min)
3. Pipette 100ul of enzyme conjugate into each well (30 min)
4. Pipette 50ul of neopterin antiserum into each well (30 min)
5. Cover plate with adhesive foil and wrap in foil to keep in the dark
6. Put on an orbital shaker (150rpm) and incubate for 90 min
7. Prepare wash buffer whiles samples are incubating. Add 15 ml of wash buffer to 285ml of distilled water.
8. Discard the solution in wells and wash with 300ul of diluted wash buffer 4 times, use multi-channel pipette (20mins)
9. Add 150ul of substrate solution into wells. Use multi- channel pipette. Do reverse pipetting and avoid bubbles (5 min)
10. Incubate on work top for 10 min at room temp
11. Pipette 150 ml of stop solution into each well (5min). Use multi- channel pipette. Do reverse pipetting and avoid bubbles (5 min)
12. Briefly mix contents by gently shaking the plate
13. Measure optical density with a photometer at 450 nm within 15 min (reference wavelength (600-650nm).
14. Obtain OD of the standards (y-axis, linear) against concentration (x-axis, logarithmic) on 4-parameter logistics fit.

Appendix E Protocol for creatinine ELISA

Preparation before day of experiment

1. Make a plate layout
2. Arrange required samples in a separate box and keep in freezer
3. Fill boxes with pipette tips
4. Label tubes
5. Get samples out of the freezer and thaw on ice
6. Pipette 95ul of distilled water into tube
7. Dilute 5ul of sample in 100ul of distilled water

On the day of experiment

1. Prepare alkaline picrate solution: 2ml Creatinine Sodium Borate + 6ml Creatinine Surfactant + 10ml Creatinine Colour Reagent + 3.6ml Creatinine NaOH
2. Store in the dark (wrap with foil)
3. Prepare standards
4. Pipette 15ul of standards and samples into wells
5. Pipette 150ul Alkaline Picrate solution into wells
6. Cover plate with plate cover and incubate on a shaker for 10mmins at room temp
7. Read absorbance 490-500nm (Initial absorbance)
8. Add 5ul of acid solution
9. Cover plate and incubate on shaker for 20 mins at room temp
10. Read absorbance at 490-500 nm (Final absorbance)

Appendix F Spearman's correlations between measured inflammatory markers

	Neutrophils	Lymphocytes	Eosinophils	Basophils
Urine Neopterin (R) **p-level**	0.084 0.627	0.242 0.156	0.090 0.603	0.155 0.368
Serum Neopterin (R) **p-level**	-0.125 0.520	-0.226 0.238	-0.125 0.518	-0.237 0.215
TNF (R) **p-level**	-0.070 0.676	-.032 0.848	0.210 0.205	-0.142 0.394
IFN (R) **p-level**	0.082 0.625	-.053 0.750	-0.101 0.545	-0.o67 0.687
IL6 (R) **p-level**	0.196 0.239	-0.054 0.749	0.284 0.084	0.037 0.823
IL1 (R) **p-level**	0.216 0.235	-0.039 0.834	0.065 0.724	0.243 0.180
WBC (R) **p-level**	**0.936** **<0.001**	**0.664** **<0.001**	**0.474** **0.002**	0.274 0.091
Mono (R) **p-level**	**0.646** **<0.001**	**0.547** **<0.001**	0.300 0.064	0.256 0.116
Neutrophils (R) **p-level**	1	**0.419** **0.008**	**0.370** **0.030**	0.260 0.110
Lymphocytes (R) **p-level**	**0.419** **0.008**	1	**0.366** **0.022**	0.297 0.066
Eosinophils (R) **p-level**	**0.370** **0.020**	**0.366** **0.022**	1	0.297 0.066
Basophils (R) **p-level**	0.260 0.110	0.260 0.110	0.297 0.066	1

Appendix G Subject Questionnaire

University of Southampton

Version 1

Date: 22/07/2013

Project Title: THE EFFECT OF INFLAMMATION ON THE PROGRESSION OF AGE-RELATED HEARING LOSS

Short title: InflamHear

Study questionnaire

Please tick the answer that best describes your situation

DEMOGRAPHICS

Participant code (to be completed by researcher): .....

Age: .....

Gender: .....

Have you lived most of your adult life in the city or rural setting?

City ☐ Rural ☐

What is/was your principal occupation? .....

HEARING (without wearing hearing aids)

a. How well can you follow a conversation with one other person when there is no background noise?

1. Not at all
2. With great difficulty
3. With moderate difficulty
4. With slight difficulty
5. With no difficulty

b. How well can you follow a conversation in a noisy location such as a shop, restaurant or busy street?

1. Not at all
2. With great difficulty
3. With moderate difficulty
4. With slight difficulty
5. With no difficulty

c. How long altogether have you worked in noisy places where you had to shout to be heard?

1. Never
2. For less than 6 months
3. For 6 to 11 months
4. For 1 to 5 years
5. For more than 5 years

d. During your lifetime have you fired more than a total of 10 rounds from a shotgun or military rifle (not counting a .22 rifle)?

Yes ☐ No ☐

e. Do you get noises in your heard or ears such as ringing, buzzing or whistling, that usually lasts for more than 5 minutes and occurs **not** only after loud sounds?

Yes ☐ No ☐

f. Did any of your close relatives (parents, siblings, grandparents, children) have difficulty with their hearing before age 65 years?

Yes ☐ No ☐ Unsure ☐

g. Did you suffer from ear infections as a child?

Yes ☐ No ☐ Unsure ☐

h. Do you use or have you been prescribed a hearing aid?

Yes ☐ No ☐

LIFESTYLE

i. Have you ever smoked regularly (i.e. at least once a day for a year or more)?

Yes ☐ No ☐

j. If you no longer smoke, did you give it up before the age of 40?

Yes ☐ No ☐

MEDICAL

k. Have you been ever prescribed medication for high blood pressure?

Yes ☐ No ☐

l. Have you ever been prescribed Statins?

Yes ☐ No ☐

m. Have you ever been told by a doctor that you have heart failure?

Yes ☐ No ☐

n. Have you ever been told by a doctor that you have problems with your heart valves?

Yes ☐ No ☐

o. Have you ever had a heart attack or angina?

Yes ☐ No ☐

p. Have you ever had have a stroke?

Yes ☐ No ☐

q. Do you have diabetes?

Yes ☐ No ☐

Appendix H National Research Ethics Service Approval

NHS
Health Research Authority
NRES Committee South Central - Hampshire A
Bristol Research Ethics Committee Centre
Level 3, Block B
Whitefriars
Lewins Mead
Bristol
BS1 2NT

Telephone: 0117 342 1381

18 November 2013

Mrs Akosua Agyemang-Pempreh
Full-time PhD student
B85, University of Southampton
SO17 1BJ

Dear Mrs Agyemang-Pempreh

Study title:	**The effect of inflammation on the progression of age-related hearing loss.**
REC reference:	**13/SC/0507**
IRAS project ID:	**138418**

Thank you for your letter of 28 October 2013, responding to the Committee's request for further information on the above research and submitting revised documentation.

The further information was considered in correspondence by a sub-committee of the REC. A list of the sub-committee members is attached.

We plan to publish your research summary wording for the above study on the NRES website, together with your contact details, unless you expressly withhold permission to do so. Publication will be no earlier than three months from the date of this favourable opinion letter. Should you wish to provide a substitute contact point, require further information, or wish to withhold permission to publish, please contact the Co-ordinator Mrs Vicky Canfield-Duthie, nrescommittee.southcentral-hampshirea@nhs.net.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised, subject to the conditions specified below.

Ethical review of research sites

NHS sites

The favourable opinion applies to all NHS sites taking part in the study, subject to management

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