Human antimicrobial proteins in ear wax

M. Schwaab, A. Gurr, A. Neumann, S. Dazert, A. Minovi

Introduction

Antimicrobial peptides (AMP)

Generally speaking surfaces like the skin or the oral mucosa are protected by several factors including the adaptive and innate immune systems against invading bacteria and fungi. The antimicrobial mucosal shield of the human airways has been subject of several studies and is well investigated [1-3]. A growing number of different human antimicrobial peptides (AMP) have been described which are produced e. g. by surface epithelial cells or by neutrophil granulocytes. The AMPs have a broad, but not identical spectrum of antimicrobial activity against bacteria, virus or fungi. A synergistic and additive effect of different AMPs has been reported [4]. In addition to their antimicrobial charactics, AMPs play a major part in inflammation, immune activation, and wound healing [5-7]. Some of the well described AMPs are subject to this study and therefore will be further described.

The human ß-Defensins (hBD)

The family of human ß-Defensins (hBD) is named after their beta-sheet structure which is stabilised by intramolecular disulfide bonds. Human ß-Defensin 1 (hBD1) was first isolated from hemofiltrate [8] and is expressed in epithelial cells of the urinary, respiratory tract and in keratinocytes [9-10]. It has a strong antimicrobial effect on Gram-negative bacteria [11-12]. Human ß-Defensin 2 (hBD2) is an inducible (by Escherichia coli, Pseudomonas aeruginosa, Helicobacter pylori, Lipoprotienacid, Candida albicans, Tumor-Necrosis-Factor- α, Interleucine1-ß) peptide [13-15] with a strong antimicrobial effect on Escherichia coli, Pseudomonas aeruginosa and Candida albicans, and a relatively weak effect on Staphylococcus aureus [13]. The human ß-Defensin 3 (hBD3) can be induced by tumor necrosis factor α and contact with Pseudmonas aeruginosa or Staphalyococcus aureus e. g. in keratinocytes [16]. HBD3 has antimicrobial activity against Staphylococcus aureus, including Methicillin restistant Staphylococcus aureus (MRSA), Streptococcus pyogenes, Pseudomonas aeruginosa, Escherichia coli, Vancomycin-resistant Enterococcus faecium, Candida albicans and Haemophilus influenza [9; 12; 17].

Human LL-37 (LL-37)

LL-37 is the 37-amino acid long C-terminus and the antimicrobial active component of the human Cathelicidin antimicrobial peptide 18 (hCAP-18). It is expressed in leukocytes (monocytes, neutrophiles, T-cells, B-cells), epithelial cells (skin, gastrointestinal and respiratory tract) and secreted into wound and airway surface fluid [18-

21]. LL-37 alone and in combination with other AMPs is bactericidal against a broad spectrum of Gram-positive and Gram-negative bacteria [18; 21-23]. Apart from the antimicrobial activity LL-37 plays a role in angiogenesis, cancer development, neutralisation of bacterial lipopolysaccharide and is chemotactic for human monocytes, neutrophils and CD 4 T-lymphocytes [24-26.].

Human Secretory Leucoprotease Inhibitor (hSLPI)

hSLPI is a 11.7-kDa heavy protein which is expressed in macrophages, neutrophils and epithelial cells [27-28.]. The antimicrobial activity against Gram-negative and Gram-positive bacteria lays in the N-terminal domain of the protein [29]. hSLPI inhibits human immunodeficiency virus (HIV) infections by blocking the viral DNA synthesis [30].

Human Bactericidal/permeability-increasing protein (BPI)

The human bactericidal / permeability- increasing protein (BPI) is a 55 kD heavy single-chain cationic protein [31] which can be divided by proteolysis into two fragments with the antibiotic and endotoxin-neutralizing functions in the N-terminal fragment [32]. BPI was mainly found in the granules of neutophiles [31] but was also detected in dermal fibroblasts [33], and the excretory lacrimal gland ducts [34]. BPI has a strong antimicrobial potency and selectivity towards Gram-negative bacteria [31]. Von der Mohlen et al. could show that BPI has a significant protective effect in meningococcal sepsis [35]. Several phase I and II studies have dealt with the pharmacokinetics and possible indications of a recombinant fragment of bactericidal/permeability increasing protein [36-38], but have not yet led to an approved clinical application.

Human Lactoferrin (Lfc)

The human Lactoferrin (Lfc) can be found in tears, saliva, milk, neutrophil granulocytes, salivary glands and nasal mucosa [39]. Its antimicrobial spectrum includes Streptococcus mutans, Vibrio cholerae, Escherichia coli, Actinobacillus actinomycetemcomitans, Legionella pneumophila, Enterobacteriaceae, Candida albicans and Pseudomonas aeruginosa [40-41].

Human Neutrophil Peptides 1-3 (HNP1-3)

The Human Neutrophil Peptides 1-3 (HNP1-3) belong to the α-Defensin family and were originally isolated from the azurophilic granules of neutrophil granulocytes [42]. Apart from a broad-spectrum of antimicrobial activity HNP1-3 play a role in the regulation of inflammatory and immunologic processes influencing

complement activation, cytotoxicity, chemotaxis of immature dendritic cells, T-cells and monocytes, enhancement of immune response and wound repair [43-45].

Ear wax (Cerumen)

Cerumen, colloquial known as ear wax, is a waxy substance which is secreted by 1000-2000 sebaceous glands and modified apocrine sweat glands of the external auditory canal (EAC) [46]. In general two different kinds of cerumen exist: the wet type can be found in Causasians and Africans and has a brown or dark colour. The dry type is most common in Asians and Native Amaricans and is grey in colour [47]. The reason is a change of a single nucleotide in the "ATP-binding cassette C11" gene [48-49]. This genetically determined difference of ear wax was even used to track human migratory patterns [50-51].

Cleaning and lubricating the blind ending EAC and thereby providing mechanical protection from bacteria and fungi are described as the main functions of ear wax [52]. The high lipid content produced by the sebaceous glands prevents desiccation, itching and burning. These characteristics were utilized in the first lip balm which was based on ear wax [53]. The lipids consist of squalene (6.4%), cholesterol esters (9.6%), wax esters (9.3%), triacylglycerols (3.0%), fatty acids (22.7%), cholesterol (20.9%), ceramides (18.6%), cholesterol sulfate (2.0%), and several unidentified polar components (7.5%) [54]. Apart from this merely chemical analysis of ear wax very little is still known about the proteins within the ear wax.

Material and Methods:

This study was approved by the commission of ethics of the Ruhr-University of Bochum (No.: 2949) and the experiments were conducted in accordance with the Helsinki Declaration of 1975, as revised in 1983. Cerumen was collected with a sterile ear-wax-hook under microscopic control from 20 healthy adults consulting the ENT department of the Ruhr-University of Bochum, Germany. All cerumen belonged to the typical wet form. There was no incidence of infection of the EAC. The samples were kept in a sterile bottle at -80°C until further preparation.

Isolation of proteins:

We performed the isolation of proteins from ear wax according to the already published protocol [56]. The ear wax was treated as one pool. After weighing the pool of cerumen was divided into similar parts and pulverized using mortal and pestle which were cooled by liquid nitrogen. The pulverized sample was then transferred into a 2 ml sterile tube. As described previously the isolation of proteins was conducted using the Qproteome^{TM}

Mammalian Protein Prep Kit by Qiagen (Catalog no. 37901). 11 samples were treated according to the protocol with PBS washing before isolation of proteins. The washing reduced the ear wax to the cellular components within the wax. 9 samples were not treated with PBS washing so that the sample was used as a whole. Afterwards the total protein concentrations of all 20 samples were measured using the "Pierce® BCA Protein Assay Kit" by Thermo Scientific (Catalog number: 23225) and the Ultrospec® 2000 UV/visible Spectrophotometer originally supplied by Pharmacia Biotech, now part of GE Healthcare, at 562 nm wave length. The protein concentration of the ear wax samples was determined based on the standard curve. Each sample was than diluted to a concentration of 20 µg/ml.

ELISA:

ELISA were conducted according to the protocols using "Human Beta Defensin 1 ELISA Kit" (ALPHA DIAGNOSTIC INTERNATIONAL; Cat. No.: 100-240-BD1), "Human Beta Defensin 2 ELISA Kit" (ALPHA DIAGNOSTIC INTERNATIONAL, Catalog No.: 100-250-BD2), "Beta-Defensin 3 (Human) ELISA Kit" (Phoenix Pharmaceutics. Inc. U.S.A., Catalog No.: EK-072-38), "Lactoferrin ELISA Kit" (Calbiochem, Catalog No.: 427275), "Human LL-37 ELISA Test Kit" (Hbt Hycukt biotechnology b.v., Catalog No.: Hbt HK321), "Human SLPI ELISA Test Kit" (Hbt Hycukt biotechnology b.v., Catalog No.: Hbt HK316), "Human BPI ELISA Kit" (Hbt Hycult biotechnology b.v., Catalog No.: Hbt HK314), "HNP1-3 ELISA Kit" (Hbt Hycult biotechnology b.v., Catalog No.: Hbt HK317). The specific protein concentrations in pg were calculated and related to the weight of the cerumen in mg.

Statistics:

After checking the results for equality of variances by using the Levene´s test an independent samples t-test was conducted to compare the concentrations of hBD1-3, Lactoferrin, human LL-37, human SLPI, human BPI, HNP1-3 for the preparation with (cellular components of ear wax sample) and without PBS washing (whole ear wax sample).

Results:

For the hBD1 concentration there is a significant difference in the scores for with PBS washing (M=28,17 pg/mg; SD=39,2 pg/mg) and without PBS washing [M=136,6 pg/mg; SD=60,68 pg/mg; t(18)=-4,83; p<0,005; eta square=0,56]. For the hBD2 concentration there is a significant difference in the scores for with PBS washing (M=17,1 pg/mg; SD=23,29 pg/mg) and without PBS washing [M=306,16 pg/mg; SD=287,8 pg/mg; t(8,09)=-

3,0; p=0,017; eta square=0,33]. For the hBD3 concentration there is a significant difference in the scores for with PBS washing (M=102,19 pg/mg; SD=64,42 pg/mg) and without PBS washing [M=947,89 pg/mg; SD=751,53 pg/mg; t(8,1)=-3,37; p=0,01; eta square=0,39]. Diagram 1 shows the concentrations of hBD 1-3. For the human LL-37 concentration there is no significant difference in the scores for with PBS washing (M=36,73 pg/mg; SD=61,87 pg/mg) and without PBS washing [M=279,86 pg/mg; SD=387,29 pg/mg; t(18)=-2,06; p=0,054; eta square=0,19]. We can not find a significant difference for the human SLPI concentration with PBS washing (M=3,48 pg/mg; SD=7,02 pg/mg) and without PBS washing [M=697,26 pg/mg; SD=1429,95 pg/mg; t(8)=-1,46; p=0,184]. For the human BPI concentration there is no significant difference in the scores for with PBS washing (M=7,13; SD=13,65) and without PBS washing [M=201,02 pg/mg; SD=435,38 pg/mg; t(8,01)=-1,34; p=0,22]. Diagram 2 illustrates the scores of LL-37, hSLPI and BPI. For the human Lactoferrin concentration there is a significant difference in the scores for with PBS washing (M=819,95 pg/mg; SD=980,24 pg/mg) and without PBS washing [M=6361,77 pg/mg; SD=5967,51 pg/mg; t(8,35)=-2,76; p=0,024; eta square=0,3]. For the HNP1-3 concentration there is a significant difference in the scores for with PBS washing (M=815,25 pg/mg; SD=1480,53 pg/mg) and without PBS washing [M=11853,31 pg/mg; SD=13507,48 pg/mg; t(8,16)=-2,44; p=0,04; eta Square=0,25]. Diagram 3 illustrates the concentrations of Lactoferrin and HNP 1-3.

Discussion:

We still lack knowledge on a subject with which the ENT doctor is confronted every day: ear wax. After all obturating ear wax is present in 10% of all children and up to 57% of older patients in nursing homes [56]. Contradictory reports about the bactericidal activity of cerumen are published. The argumentation against a bactericidal activity are based on the consideration that the rich nutrients of ear wax enable bacteria and fungi to grow [57-61]. On the other hand there are several reports describing an antimicrobial effect of ear wax with an effect against a wide range of bacteria including Haemophilus influenzae, Staphylococcus aureus, Escherichia coli and fungi [61-66]. Stoeckelhuber et al. reported the detection of the antimicrobial proteins ß-defensin-1, ß-defensin-2, cathelicidin, lysozyme, lactoferrin, MUC1 and the secretory component of IgA in the ceruminous glandular cells by histochemical analysis [67]. It could be shown that not only the glands but also the skin of the EAC produce antimicrobial peptides like human ß-Defensin 1 (hBD1) and human ß-Defensin 2 (hBD2) [68-69]. Yoon et al. already isolated hBD1 and hBD2 in human cerumen in 2008 [70].

Because of the complex chemical composition of ear wax isolation and identification of special proteins was complicated until we developed a protocol for the protein isolation as reported previously [55]. This method in combination with well established ELISA-Kits enabled us not only to prove the existence but also determine the

concentration of ten well defined human antimicrobial peptides in ear wax. Apart from answering the question about the source of the antimicrobial effect of cerumen these data also demonstrate that some proteins are primarily cell bound and some proteins are not. The proteins hLL-37, hSLPI and hBPI had no significant difference in the protein concentration with and without PBS washing**.** After washing the samples with PBS at the beginning of the protein isolation the cell bound fraction only was used in 11 samples for further preparation. Those proteins which were not cell bound were washed away. This indicates that the proteins hLL-37, hSLPI and hBPI are more or less cell bound proteins. Jung et al. reported on the expression of hCAP18 / hLL-37 in the granular and prickle cell layers of the skin of the external ear canal [71] and the expression of SLPI in the stratum granulosum of the skin of the external ear canal [72]. This is the first report on the existence of hBPI in the human ear in general and especially in the EAC.

The proteins hBD 1-3, Lactoferrin and HNP 1-3 have a higher concentration in those samples which were not washed with PBS before protein isolation. These 9 samples consist of cells and not cell bound proteins. The sources for hBD1-2 and lactoferrin have been reported to be in the ceruminous glands, the skin of the external canal and the tympanic membrane [67-69]. HNP 1-3 are released into the extracellular space by exocytosis from the azurophilic granules of neutrophiles in general [42] although this is the first report on the existence of HNP 1-3 as well as hBD3 in the human EAC. The difference in the protein concentrations indicates that hBD 1-3, Lactoferrin and HNP 1-3 must be actively secreted into the ear wax because during the protein isolation the not cell bound proteins were not washed away.

Taking into consideration that up to 60% of a cerumen plug consists of cellular components and that some AMPs are cell bound [73] may explain why some previous studies have failed to prove the antimicrobial effect of ear wax if they did not include the cellular components within the ceruminal plug.

Several studies have proven that each antimicrobial peptide has an antimicrobial effect itself, but more than that they have an even stronger additive and synergistic effect in combination [74]. Singh et al. could show that combinations of lactoferrin and SLPI were synergistic and the combinations involving the human β-defensins, LL-37 and tobramycin had an additive effect [74]. Chen et al. analyzed the individual and synergistic activities of hBD 1-3, LL-37 and lysozyme in different milieus and found that these AMPs exhibited an antibacterial activity against S. aureus and E. coli in a dose-dependent manner in neutral and acidic pH milieus. The antibacterial activity of hBD 1-3 and lysozyme but not LL-37 was even significantly enhanced in acidic milieu (pH 4.6). The synergistic effect of hBDs, LL-37 and lysozyme against S. aureus was significantly enhanced in acidic milieu. The proteins showed to have an additive not synergistic effect on antibacterial activity in acidic milieu against E. coli [75].

Clinical consequences

In healthy individuals the physiological pH of ear wax is around 5,4 [76] and therefore provides optimal conditions for the synergistic and additive effect of AMPs. Kim et al. could show that there is a change of the pH in the EAC in an acute otitis externa with an increase of the pH-value [76] leading to a loss of antimicrobial efficiency of the cerumen. Often acidifying agents such as boratic acid are used to treat an infection of the EAC. These substances help to regain the antimicrobial potency by restoring the physiological pH in the EAC. Another clinical aspect resulting from the knowledge that ear wax has a protective antimicrobial potency is that the fast and often used practice to clean the external ear canal by rinsing the ear with water leads to a complete elimination of cerumen and a change in the physiological pH. This may explain why otitis externa can develop after rinsing the ear with water. In conclusion removing ear wax manually for example with a hook and may be even leaving some ear wax in the ear canal would maintain the physiological antimicrobial potency of the EAC.
Apart from the antimicrobial potency some antimicrobial peptides like HNP1-3 and hLL37 play a role in the regulation of inflammatory and immunologic processes, influencing complement activation, cytotoxicity, chemotaxis, wound repair, angiogenesis or cancer development [43-45; 23-25]. At the moment we are learning to understand the complexity behind the synergistic and additive effects of AMPs. Although Weber et al. could show that no significant antibiotic resistances have developed from the use of ototopical antibiotic treatment [77] gaining a better understanding of the natural possibilities of the external ear canal could lead to new therapeutic strategies for barely understood diseases like otitis externa or otitis necroticans and thereby help to prevent resistant bacteria in the EAC.

It is essential for a blind ending sac like the EAC to have a good protection against invading bacteria and fungi. We could identify 10 out of a variety of different human antimicrobial peptides to be part of the local protection of the external ear canal. Further studies are necessary to decode the components of cerumen and determine possible changes during acute or chronic infections. Understanding the antimicrobial potency of the human body in general may lead to new strategies preventing infections without using a chemical treatment.

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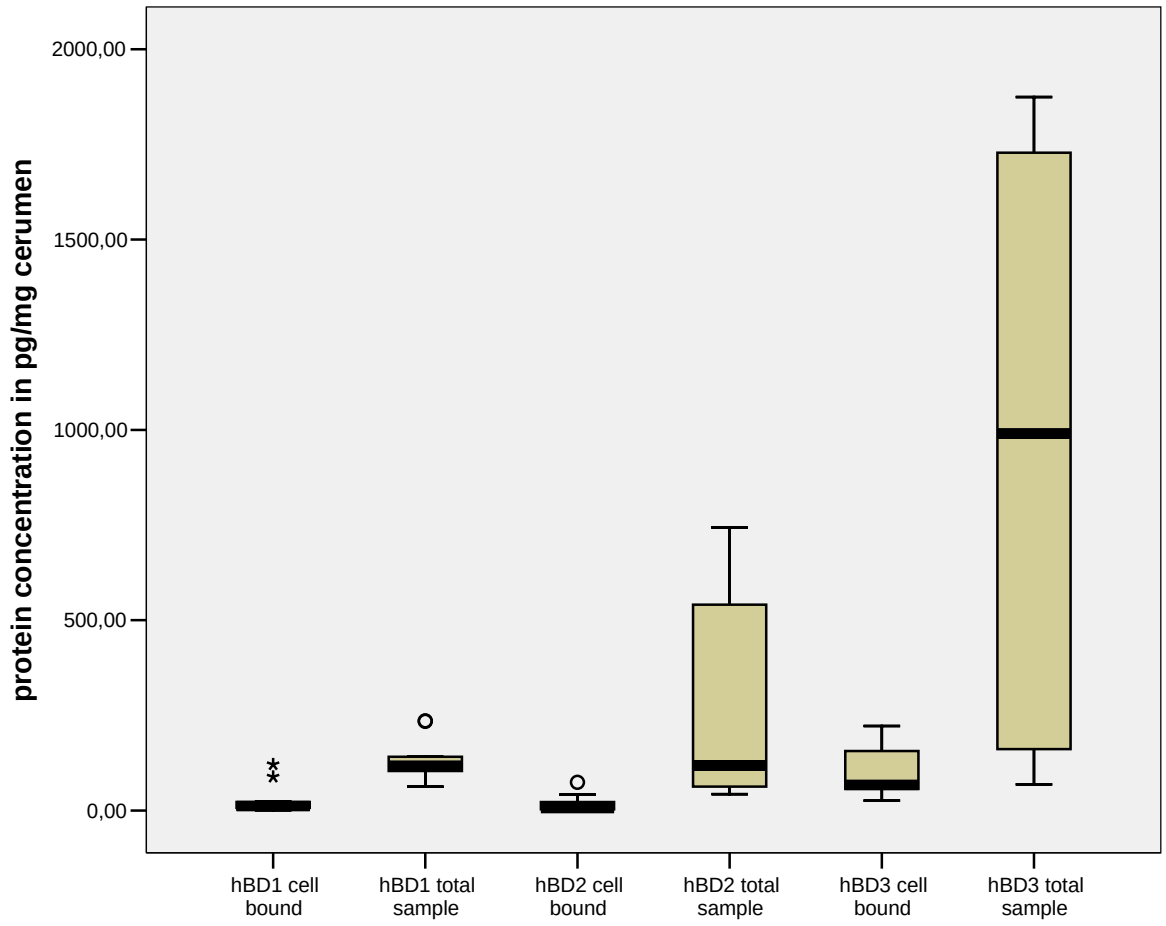

Figure 1 shows the hBD1-3 concentrations in ear wax between the two compared groups (only cell bound fraction and total sample). For all three analyzed human ß-Defensins there was a significant difference in the scores between both groups. The concentrations of hBD 1-3 were higher in the total samples indicating that the proteins are not only cell bound in the ear wax.

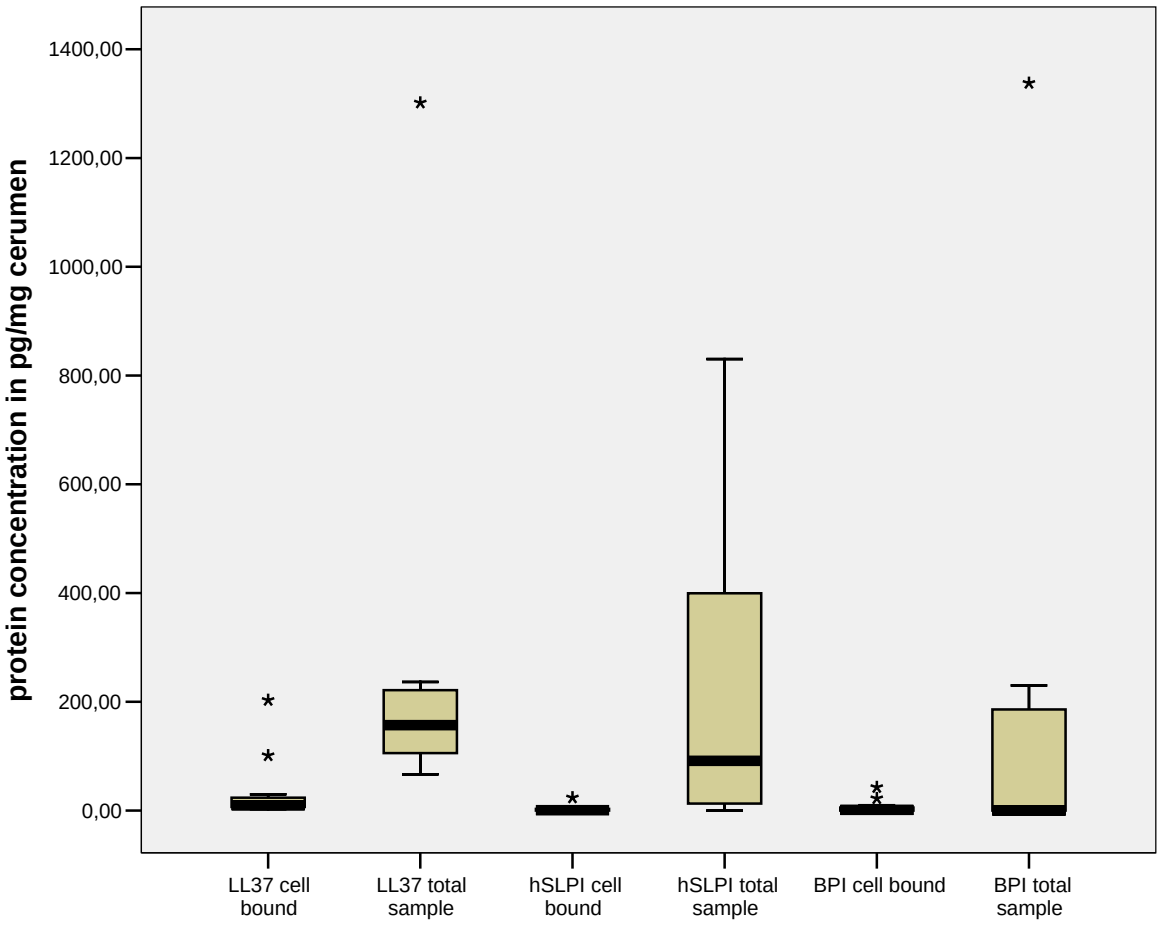

Figure 2 illustrates the concentrations for LL37, hSLPI and BPI in ear wax between the two analysed groups (only cell bound fraction and total sample). For all three proteins there was no significant difference in the scores between both groups.

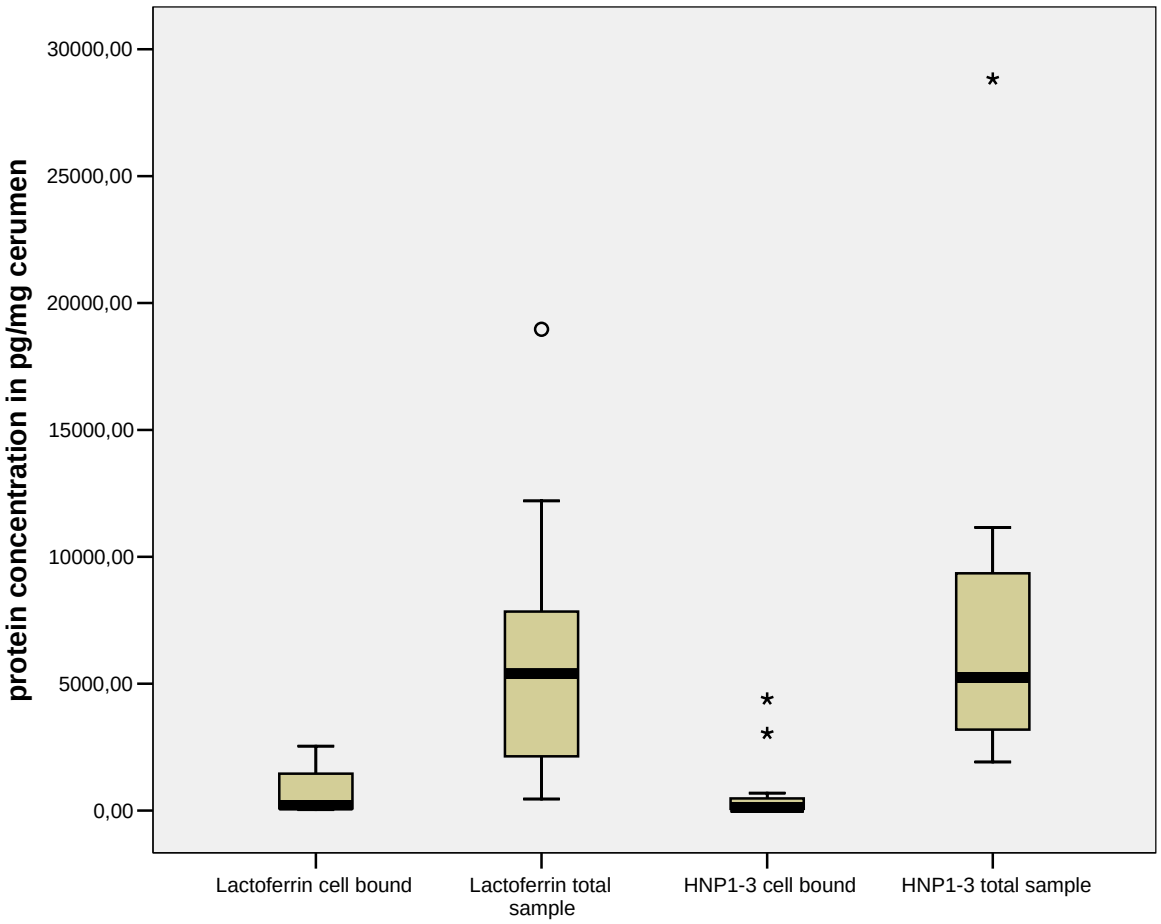

Figure 3 illustrates the concentrations for Lactoferrin and HNP1-3 in ear wax between the two analyzed groups (only cell bound fraction and total sample). For both analysed protein groups there was a significant difference in the scores between both groups. The concentrations were higher in the total samples indicating that the proteins are not only cell bound in the ear wax.