



HAL
open science

Human antimicrobial proteins in ear wax

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

► **To cite this version:**

M. Schwaab, A. Gurr, A. Neumann, S. Dazert, A. Minovi. Human antimicrobial proteins in ear wax. *European Journal of Clinical Microbiology and Infectious Diseases*, 2011, 30 (8), pp.997-1004. <10.1007/s10096-011-1185-2>. <hal-00666674>

HAL Id: hal-00666674

<https://hal.science/hal-00666674v1>

Submitted on 6 Feb 2012

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



HAL Authorization

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 characteristics, 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 β -Defensins (hBD)

The family of human β -Defensins (hBD) is named after their beta-sheet structure which is stabilised by intramolecular disulfide bonds. Human β -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 β -Defensin 2 (hBD2) is an inducible (by *Escherichia coli*, *Pseudomonas aeruginosa*, *Helicobacter pylori*, Lipoprotein, *Candida albicans*, Tumor-Necrosis-Factor- α , Interleukin-1 β) 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 β -Defensin 3 (hBD3) can be induced by tumor necrosis factor α and contact with *Pseudomonas aeruginosa* or *Staphylococcus aureus* e. g. in keratinocytes [16]. hBD3 has antimicrobial activity against *Staphylococcus aureus*, including Methicillin resistant *Staphylococcus aureus* (MRSA), *Streptococcus pyogenes*, *Pseudomonas aeruginosa*, *Escherichia coli*, Vancomycin-resistant *Enterococcus faecium*, *Candida albicans* and *Haemophilus influenzae* [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, neutrophils, 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 neutrophils [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 Caucasians and Africans and has a brown or dark colour. The dry type is most common in Asians and Native Americans 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 mortar 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™

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 Pharmaceuticals. 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 β -defensin-1, β -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 β -Defensin 1 (hBD1) and human β -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 neutrophils 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 boric 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.

Acknowledgement:

Part of these data was reported at the annual meeting of the Association of Ear- Nose- and Throat - Physicians of West Germany in Bochum in March 2010.

We thank Dr med. M. D. Pearson for proofreading this manuscript and making corrections in his mother language.

Conflict of interest: The authors have declared that no conflict of interest exists. There was no sponsorship or funding arrangements relating to the research.

Literature

1. Cole AM, Dewan P, Ganz T (1999) Innate antimicrobial activity of nasal secretions. *Infect Immun* 67: 3267-3275.
2. Hiemstra PS (2007) The role of epithelial beta-defensins and cathelicidins in host defense of the lung. *Exp Lung Res* 33(10):537-42.
3. Beisswenger C, Bals R (2005) Antimicrobial peptides in lung inflammation. *Chem Immunol Allerg* 86:55-71.
4. Shai Y, Makovitzky A, Avrahami D (2006) Host defense peptides and lipopeptides: modes of action and potential candidates for the treatment of bacterial and fungal infections. *Curr Protein Pept Sci* 7(6):479-86.
5. Koczulla AR, Bals R (2003) Antimicrobial peptides: current status and therapeutic potential. *Drugs* 63:389-406.
6. Yang D, Biragyn A, Hoover DM, Lubkowski J, Oppenheim JJ (2004) Multiple roles of antimicrobial defensins, cathelicidins, and eosinophil-derived neurotoxin in host defense. *Annu Rev Immunol* 22:181-215.
7. Yang D, Biragyn A, Kwak LW, Oppenheim JJ (2002) Mammalian defensins in immunity: more than just microbicidal. *Trends Immunol* 23:291-296.
8. Bensch K, Raida M, Magert HJ, Schulz-Knappe P, Forssmann WG (1995) hBD-1: a novel β -defensin from human plasma. *FEBS Lett* 368:331-335.
9. Sørensen OE, Cowland JB, Theilgaard-Mönch K, Liu L, Ganz T, Borregaard N (2003) Wound Healing and Expression of Antimicrobial Peptides/Polypeptides in Human Keratinocytes, a Consequence of Common Growth Factors. *J Immunol* 170: 5583-5589
10. Goldman MJ, Anderson GM, Stolzenberg ED, Kari UP, Zasloff M, Wilson JM (1997) Human b-defensin-1 is a salt-sensitive antibiotic in lung that is inactivated in cystic fibrosis. *Cell* 88:553-560.
11. Krisanaprakornkit S, Weinberg A, Perez CN, Dale BA (1998) Expression of the peptide antibiotic human b-defensin 1 in cultured gingival epithelial cells and gingival tissue. *Infection and Immunity* 66(9): 4222-8.
12. Schneider JJ, Unholzer A, Schaller M, Schafer-Korting M, Korting HC (2005) Human defensins. *J Mol Med* 83(8):587-95. Epub 2005 Apr 9.
13. Schröder JM, Harder J. Human beta-defensin-2 (1999) *Int J Biochem Cell Biol* 31(6): 645-651.

14. Sørensen OE, Thapa DR, Rosenthal A, Liu L, Roberts AA, Ganz T (2005) Differential Regulation of α -Defensin Expression in Human Skin by Microbial Stimuli. *J Immunol* 174: 4870–4879.
15. Uehara N, Yagihashi A, Kondoh K, Tsuji N, Fujita T, Hamada H, Watanabe N (2003) Human beta-defensin-2 induction in *Helicobacter pylori*-infected gastric mucosal tissues: antimicrobial effect of overexpression. *J Med Microbiol* 52: 41–45.
16. Harder J, Bartels J, Christophers E, Schröder JM (2001) Isolation and Characterization of Human β -Defensin-3, a Novel Human Inducible Peptide Antibiotic. *J Biol Chem* 276(8): 5707–5713.
17. Hiratsuka T, Mukae H, Iiboshi H, Ashitani J, Nabeshima K, Minematsu T, Chino N, Ihi T, Kohno S, Nakazato M (2003) Increased concentrations of human α -defensins in plasma and bronchoalveolar lavage fluid of patients with diffuse panbronchiolitis. *Thorax* 58:425.
18. Bals R, Wang X, Zasloff M, Wilson JM (1998) The peptide antibiotic LL-37/hCAP-18 is expressed in epithelia of the human lung where it has broad antimicrobial activity at the airway surface. *Proc. Natl. Acad. Sci. USA* 95:9541–9546.
19. Agerberth B, Charo J, Werr J, Olsson B, Idali F, Lindbom L, Kiessling R, Jörnvall H, Wigzell H, Gudmundsson GH (2000) The human antimicrobial and chemotactic peptides LL-37 and α -defensins are expressed by specific lymphocyte and monocyte populations. *Blood* Nov 1;96(9):3086–93.
20. Wang Y, Walter G, Herting E, Agerberth B, Johansson J (2004) Antibacterial activities of the cathelicidins prophenin (residues 62 to 79) and LL-37 in the presence of a lung surfactant preparation. *Antimicrob Agents Chemother.* Jun;48(6):2097–100.
21. Frohm Nilsson M, Sandstedt B, Sørensen O, Weber G, Borregaard N, Stähle-Bäckdahl M (1999) The Human Cationic Antimicrobial Protein (hCAP18), a Peptide Antibiotic, Is Widely Expressed in Human Squamous Epithelia and Colocalizes with Interleukin-6. *Infect Immun* 67(5):2561–2566.
22. Agerberth B, Gunne H, Odeberg J, Kogner P, Boman HG, Gudmundsson GH (1995) FALL-39, a putative human peptide antibiotic, is cysteine-free and expressed in bone marrow and testis. *Proc. Natl. Acad. Sci. USA* 92:195–199.
23. Travis SM, Anderson NN, Forsyth WR, Espiritu C, Conway BD, Greenberg EP, McCray PB Jr, Lehrer RI, Welsh MJ, Tack BF (2000) Bactericidal activity of mammalian cathelicidin-derived peptides. *Infect. Immun.* 68:2748–2755.
24. Koczulla R, von Degenfeld G, Kupatt C, Krötz F, Zahler S, Gloe T, Issbrücker K, Unterberger P, Zaiou M, Lebherz C, Karl A, Raake P, Pfosser A, Boekstegers P, Welsch U, Hiemstra PS, Vogelmeier C,

- Gallo RL, Clauss M, Bals R (2003) An angiogenic role for the human peptide antibiotic LL-37/hCAP-18. *J Clin Invest* 111(11):1665-72.
25. von Haussen J, Koczulla R, Shaykhiev R, Herr C, Pinkenburg O, Reimer D, Wiewrodt R, Biesterfeld S, Aigner A, Czubayko F, Bals R (2008) The host defence peptide LL-37/hCAP-18 is a growth factor for lung cancer cells. *Lung Cancer* Jan;59(1):12-23. Epub 2007 Aug 31.
26. Larrick JW, Hirata M, Balint RF, Lee J, Zhong J, Wright SC (1995) Human CAP18: a novel antimicrobial lipopolysaccharide-binding protein. *Infect. Immun* 63:1291–1297.
27. Abe T, Kobayashi N, Yoshimura K, Trapnell BC, Kim H, Hubbard RC, Brewer MT, Thompson RC, Crystal RG (1991) Expression of the secretory leukoprotease inhibitor gene in epithelial cells. *J Clin Invest* 87(6):2207–2215.
28. Brown A, Farmer K, MacDonald L, Kalsheker N, Pritchard D, Haslett C, Lamb J, Sallenave JM (2003) House dust mite Der p 1 downregulates defenses of the lung by inactivating elastase inhibitors. *Am J Respir Cell Mol Biol* 29(3 Pt 1):381–389.
29. Hiemstra PS, Maassen RJ, Stolk J, Heinzl-Wieland R, Steffens GJ, Dijkman JH (1996) Antibacterial activity of antileukoprotease. *Infect Immun* 64(11):4520–4524.
30. Wahl SM, McNeely TB, Janoff EN, Shugars D, Worley P, Tucker C, Orenstein JM (1997) Secretory leukocyte protease inhibitor (SLPI) in mucosal fluids inhibits HIV-I. *Oral Dis* 3 Suppl 1:64–9.
31. Weiss J, Elsbach P, Olsson I, Odeberg H (1978) Purification and characterization of a potent bactericidal and membrane active protein from the granules of human polymorphonuclear leukocytes. *J Biol Chem* 253:2664–72.
32. Ooi CE, Weiss J, Elsbach P, Frangione B, Mannion B (1987) A 25-kDa NH₂-terminal fragment carries all the antibacterial activities of the human neutrophil 60-kDa bactericidal/permeability-increasing protein. *J Biol Chem* 262:14891–4.
33. Reichel PH, Seemann C, Csernok E, Schroder JM, Muller A, Gross WL, Schultz H (2003) Bactericidal/permeability-increasing protein is expressed by human dermal fibroblasts and upregulated by interleukin 4. *Clin Diagn Lab Immunol* 10:473–5.
34. Peuravuori H, Aho VV, Aho HJ, Collan Y, Saari KM (2006) Bactericidal/permeability-increasing protein in lacrimal gland and in tears of healthy subjects. *Graefes Arch Clin Exp Ophthalmol* 244:143–8.
35. von der Mohlen MA, Kimmings AN, Wedel NI, Mevissen ML, Jansen J, Friedmann N, Lorenz TJ, Nelson BJ, White ML, Bauer R (1995) Inhibition of endotoxin-induced cytokine release and neutrophil

- activation in humans by use of recombinant bactericidal/permeability-increasing protein. *J Infect Dis* 172:144–51.
36. Demetriades D, Smith JS, Jacobson LE, Moncure M, Minei J, Nelson BJ, Scannon PJ (1999) Bactericidal/permeability-increasing protein (rBPI21) in patients with hemorrhage due to trauma: results of a multicenter phase II clinical trial. rBPI21 Acute Hemorrhagic Trauma Study Group. *J Trauma* 46:667–76.
37. Levin M, Quint PA, Goldstein B, Barton P, Bradley JS, Shemie SD, Yeh T, Kim SS, Cafaro DP, Scannon PJ, Giroir BP (2000) Recombinant bactericidal/permeability-increasing protein (rBPI21) as adjunctive treatment for children with severe meningococcal sepsis: a randomised trial. rBPI21 Meningococcal Sepsis Study Group. *Lancet* 356:961–7.
38. Giroir BP, Quint PA, Barton P, Kirsch EA, Kitchen L, Goldstein B, Nelson BJ, Wedel NJ, Carroll SF, Scannon PJ (1997) Preliminary evaluation of recombinant amino-terminal fragment of human bactericidal/permeability-increasing protein in children with severe meningococcal sepsis. *Lancet* 350:1439–43.
39. Stenfors LE, Bye HM, Raisanen S (2002) Immunocytochemical localization of lysozyme and lactoferrin attached to the surface bacteria of the palatine tonsils during infectious mononucleosis. *J Laryngol Otol* 116 (4): 264-268.
40. Yamauchi K, Tomita M, Giehl TJ, Ellison RT III (1993) Antimicrobial activity of lactoferrin and a pepsin-derived lactoferrin peptide fragment. *Infect Immun* 61 (2): 719-728.
41. Otto BR, Verweij-van Vught AM, MacLaren DM (1992) Transferrins and heme-compounds as iron sources for pathogenic bacteria. *Crit Rec Microbiol* 18 (3): 217-233.
42. Ganz T, Selsted ME, Szklarek D, Harwig SS, Daher K, Bainton DF, Lehrer RI (1985) Defensins. Natural peptide antibiotics of human neutrophils. *J. Clin. Invest* 76:1427–1435.
43. Soehnlein O, Kai-Larsen Y, Frithiof R, Sorensen OE, Kenne E, Scharffetter-Kochanek K, Eriksson EE, Herwald H, Agerberth B, Lindbom L (2008) Neutrophil primary granule proteins HBP and HNP1–3 boost bacterial phagocytosis by human and murine macrophages. *J. Clin. Invest* 118:3491–3502.
44. Aarbiou J, Ertmann M, van Wetering S, Van Noort P, Rook D, Rabe KF, Litvinov SV, Van Krieken JH, de Boer WI, Hiemstra PS (2002) Human neutrophil defensins induce lung epithelial cell proliferation in vitro. *J. Leukoc. Biol* 72:167–174.
45. Murphy CJ, Foster BA, Mannis MJ, Selsted ME, Reid TW (1993) Defensins are mitogenic for epithelial cells and fibroblasts. *J. Cell. Physiol* 155:408–413.

46. Alvord LS, Farmer BL (1997) Anatomy and orientation of the human external ear. *J.Am.Acad.Audiol* 8 (6): 383-390.
47. Overfield T (1985) *Biologic Variation in Health and Illness: Race, Age, and Sex Differences*. Menlo Park, CA, USA: Addison-Wesley Publishing.
48. Yoshiura K, Kinoshita A, Ishida T, Ninokata A, Ishikawa T, Kaname T, Bannai M, Tokunaga K, Sonoda S, Komaki R, Ihara M, Saenko VA, Alipov GK, Sekine I, Komatsu K, Takahashi H, Nakashima M, Sosonkina N, Mapendano CK, Ghadami M, Nomura M, Liang DS, Miwa N, Kim DK, Garidkhuu A, Natsume N, Ohta T, Tomita H, Kaneko A, Kikuchi M, Russomando G, Hirayama K, Ishibashi M, Takahashi A, Saitou N, Murray JC, Saito S, Nakamura Y, Niikawa N (2006) A SNP in the ABCC11 gene is the determinant of human earwax type. *Nat Genet* Mar;38(3):324-30. Epub 2006 Jan 29.
49. Bass EJ, Jackson JF (1997) Cerumen types in Eskimos. *Am.J.Phys.Anthropol* 47 (2): 209–210.
50. Ibraimov AI (1991) Cerumen phenotypes in certain populations of Eurasia and Africa. *Am J Phys Anthropol* Feb;84(2):209-11.
51. http://en.wikipedia.org/wiki/Mendelian_inheritance_in_Man. Accessed 01 December 2010
52. Burkhart CN, Kruge MA, Burkhart CG, Black C (2001) Cerumen composition by flash pyrolysis-gas chromatography/mass spectrometry. *Otol Neurotol* 22(6):715-22.
53. Bortz JT, Wertz PW, Downing DT (1990) Composition of cerumen lipids. *J.Am.Acad.Dermatol* 23 (5): 845-849.
54. Okuda I, Bingham B, Stoney P, Hawke M (1991) The organic composition of earwax. *J Otolaryngol* Jun;20(3):212-5.
55. Schwaab M, Hansen S, Gurr A, Schwaab T, Minovi A, Sudhoff H, Dazert S (2009) Protein isolation from ear wax made easy. *Eur Arch Otorhinolaryngol* Nov; 266(11):1699-702. Epub 2009 Apr 4.
56. Roeser RJ, Ballachanda BB (1997) Physiology, pathophysiology, and anthropology/epidemiology of human earcanal secretions. *J Am Acad Audiol* 8:391-400.
57. Creed E, Negus VE (1926) Investigations regarding the function of aural cerumen. *J. Laryngol. Otol* 41: 223-230.
58. Perry EJ, Nichols AC (1956) Studies on the growth of bacteria in the human ear canal. *J. Invest. Dermatol* 27:165-170.
59. Singer DE, Freeman E, Hoffert WR, Keys RJ, Mitchell RB, Hardy AV (1956) Otitis externa: bacteriological and mycological studies. *Ann Otol Rhinol Laryngol.* 61:317-330.

60. Campos A, Betancor L, Arias A, Rodríguez C, Hernández AM, López Aguado D, Sierra A (2000) Influence of human wet cerumen on the growth of common and pathogenic bacteria of the ear. *J Laryngol Otol Dec*;114(12):925-9.
61. Hyslop NE Jr (1971) Earwax and host defense. *N. Engl. J. Med.* 284:1099-1100.
62. Petrakis NL, Doherty M, Lee RE, Smith SC, Page NL (1971) Demonstration and implications of lysozyme and immunoglobulins in human earwax. *Nature* 229:119-120.
63. Chai TJ, Chai TC (1980) Bactericidal activity of cerumen. *Antimicrob Agents Chemother* 18 (4): 638-641.
64. Lum CL, Jeyanthi S, Prepageran N, Vadivelu J, Raman R (2008) Antibacterial and antifungal properties of human cerumen. *J Laryngol Otol Aug* 11:1-4.
65. Pata YS, Ozturk C, Akbas Y, Gorur K, Unal M, Ozcan C (2003) Has cerumen a protective role in recurrent external otitis? *Am J Otolaryngol Jul-Aug*;24(4):209-12.
66. Stone M, Fulghum RS (1984) Bactericidal activity of wet cerumen. *Ann Otol Rhinol Laryngol Mar-Apr*;93(2 Pt 1):183-6.
67. Stoeckelhuber M, Matthias M, Andratschke M, Stoeckelhuber BM, Koehler C, Herzmann S, Sulz A, Welsch U (2006) Human Ceruminous Gland: Ultrastructure and Histochemical Analysis of Antimicrobial and Cytoskeletal Components. *The Anatomical Record Part A* 288A:877–884.
68. Meyer JE, Schwaab M, Beier UH, Görögh T, Buchelt T, Frese K, Maune S (2006) Association between human beta defensin expression and cholesteatoma formation. *Auris Nasus Larynx Jun*;33(2):159-65. Epub 2006 Jan 23.
69. Bøe R, Silvola J, Yang J, Moens U, McCray P, Stenfors LE, Seljfelid R (1999) Human β -Defensin-1 mRNA is transcribed in tympanic membrane and adjacent auditory canal epithelium. *Infection and Immunity* 67(9):4843-4846.
70. Yoon YJ, Jin Woo Park JW, Lee EJ (2008) Presence of hBD-1 and hBD-2 in human cerumen and external auditory canal skin. *Acta Otolaryngol Aug*;128(8):871-5.
71. Jung HH, Chae SW, Jung SK, Kim ST, Lee HM, Hwang SJ (2003) Expression of a Cathelicidin Antimicrobial Peptide Is Augmented in Cholesteatoma. *Laryngoscope* 113:432–435.
72. Lee JK, Chae SW, Cho JG, Lee HM, Hwang SJ, Jung HH (2006) Expression of secretory leukocyte protease inhibitor in middle ear cholesteatoma. *Eur Arch Otorhinolaryngol* 263:1077–1081.
73. Robinson AC, Hawke M, Naiberg J (1990) Impacted cerumen: a disorder of keratinocyte separation in the superficial external ear canal? *J Otolaryngol* 19(2):86-90.

74. Singh PK, Tack BF, McCray PB Jr, Welsh MJ (2000) Synergistic and additive killing by antimicrobial factors found in human airway surface liquid. *Am J Physiol Lung Cell Mol Physiol* 279(5):799-805.
75. Chen X, Niyonsaba F, Ushio H, Okuda D, Nagaoka I, Ikeda S, Okumura K, Ogawa H (2005) Synergistic effect of antibacterial agents human beta-defensins, cathelicidin LL-37 and lysozyme against *Staphylococcus aureus* and *Escherichia coli*. *J Dermatol Sci* 40(2):123-32. Epub 2005 Jun 15
76. Kim JK, Cho JH (2009) Change of external auditory canal pH in acute otitis externa. *Ann Otol Rhinol Laryngol* 118(11):769-72.
77. Weber PC, Roland PS, Hannley M, Friedman R, Manolidis S, Matz G, Owens F, Rybak L, Stewart MG (2004) The development of antibiotic resistant organisms with the use of ototopical medications. *Otolaryngol Head Neck Surg* 130(3 Suppl):89-94.

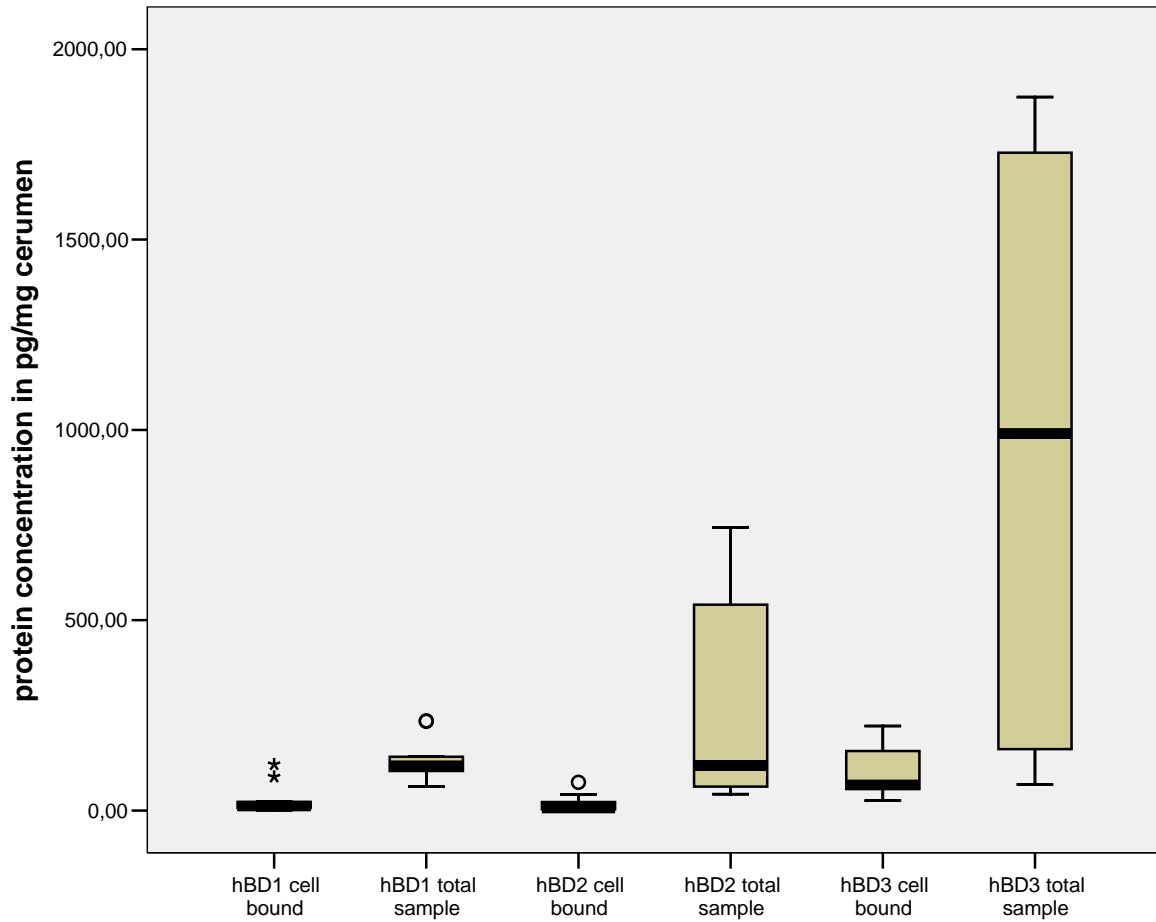


Figure 1 shows the hBD1-3 concentrations in ear wax between the two compared groups (only cell bound fraction and total sample). For all three analyzed human β -Defensins there was a significant difference in the scores between both groups. The concentrations of hBD 1-3 were higher in the total samples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

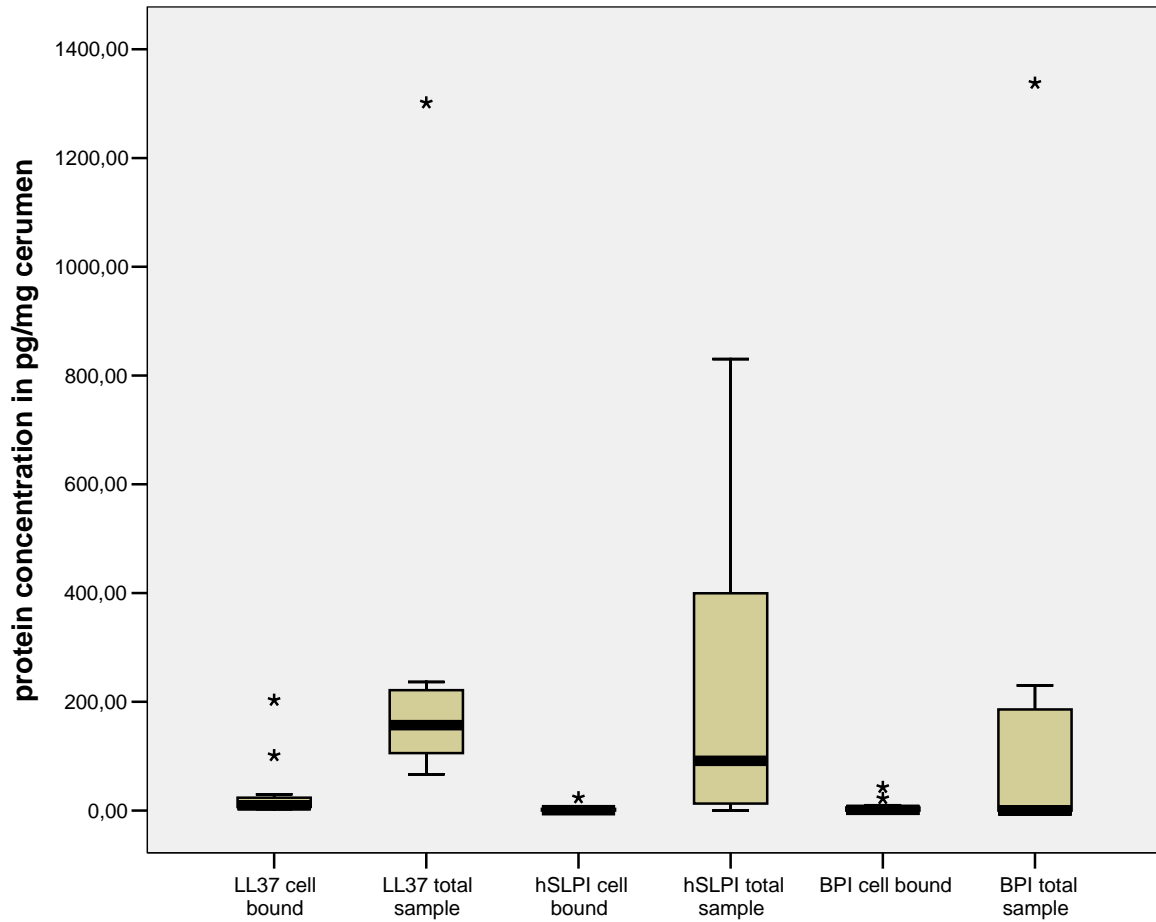


Figure 2 illustrates the concentrations for LL37, hSLPI and BPI in ear wax between the two analysed groups (only cell bound fraction and total sample). For all three proteins there was no significant difference in the scores between both groups.

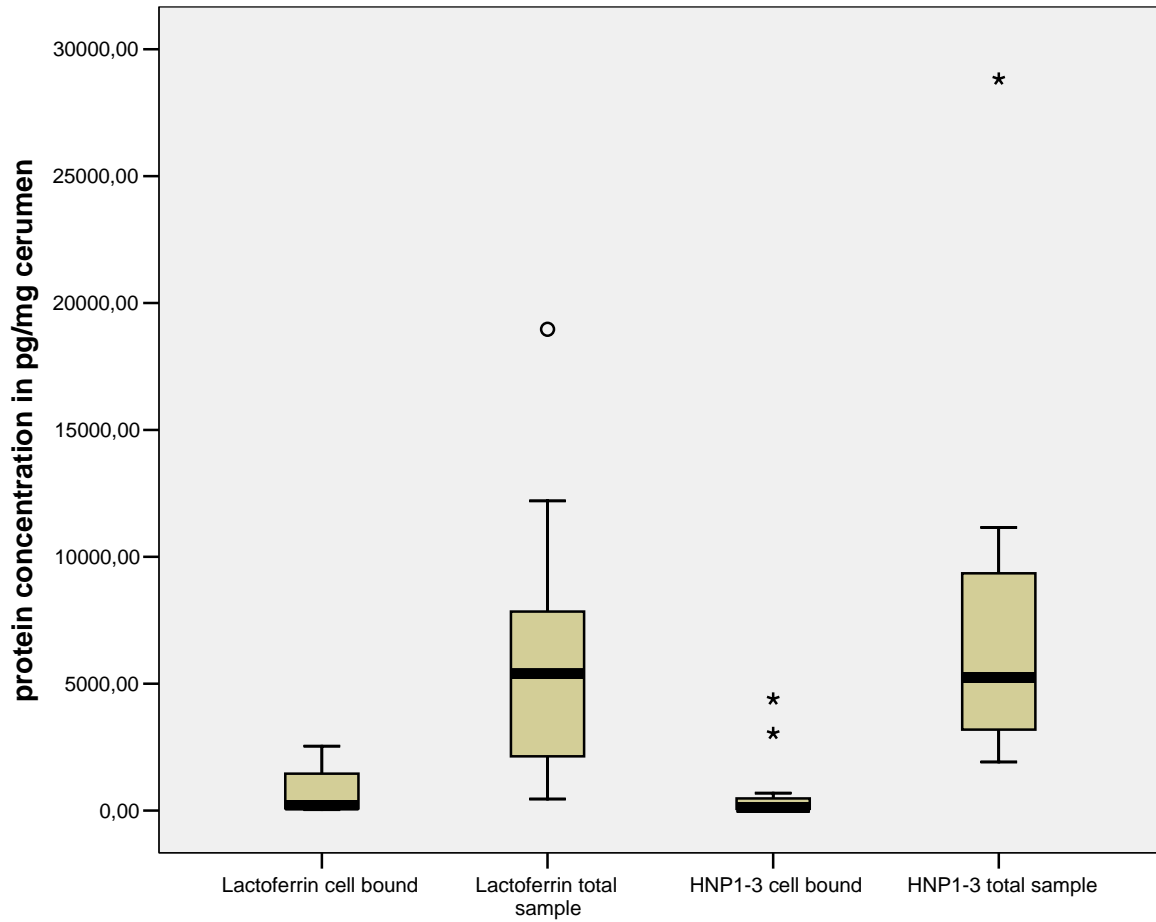


Figure 3 illustrates the concentrations for Lactoferrin and HNP1-3 in ear wax between the two analyzed groups (only cell bound fraction and total sample). For both analysed protein groups there was a significant difference in the scores between both groups. The concentrations were higher in the total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