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Clinical Relevance of Cathelicidin in Infectious Disease

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Abstract

The human body is subjected to constant microbial exposure from both a resident microbiome as well as the surrounding environment. Staying healthy in a world filled with "dangers" as such necessitates gene-encoded tools to enable an immediate and effective immunological shield. Antimicrobial host defense peptides are imperative to an apt innate immune response in which they serve both regulatory as well as executorial roles to eliminate infectious pathogens, control inflammation, and support healing of injured tissue. Cathelicidin peptides were originally isolated from bone marrow and neutrophils, although their pattern of expression is now known to span a broader spectrum. The clinical importance of an effective host defense peptide repertoire is best illustrated by data from patient groups with a deficient expression pattern and increased disease susceptibility, as well as experimental work with relevant animal models. This review paper is focused on the human cathelicidin LL37 and its clinical implications in infectious disease.

Keywords: Host defense peptides; Innate immunity; Antimicrobial; LL-37; hCAP18; Human microbiome; Cationic peptides

Abbreviations: ACES: Azithromycin and Coronary Events Study; AZACS: AZithromycin in Acute Coronary Syndromes; ICAM: Intercellular adhesion molecule-1; IFN: Interferon; MCP-1: Monocyte Chemoattractant Protein 1; MIC: Minimal Inhibitory Concentration; NFkB: Nuclear Factor Kappa Beta; NOD2: Nucleotide-binding Oligomerization Domain (NOD) protein. The NOD-like receptors, NLRs are a group of pattern recognition receptor, PRRs; PROVE-IT: PRavastatin or atorVastatin Evaluation and Infection Therapy; TLRs: Toll-like receptors; WIZARD: Weekly Intervention with Zithromax for Atherosclerosis and its Related Disorders; $1,25(OH)_2D_3$: 1,25-dihydroxy-vitamin D_3

Introduction

Host defense peptides (HDPs) are expressed ubiquitously within an individual organism as well as across different species. The cathelicidin and defensin peptide families constitute the major groups of HDPs in mammals, where they perform a principal role on the stage of the innate immune response [1]. Our focus in this review is directed towards the cathelicidins, which have been documented broadly in nature, including numerous different animal species[2].

Cathelicidins are cationic amphipathic peptides that span a functionally diverse repertoire to enable homeostasis in response to pathogen-, disease- and danger-associated-molecular patterns [3,4]. The group serves as natural antimicrobials against bacterial, fungal, viral, and parasitic pathogens, while also contributing to the orchestration of the immune response, and elimination of abnormal host cells. The peptides' broad pattern of distribution and efficacy make them attractive as templates for development of new antibiotics [5], and of particular interest in context of a species-spanning approach to health and medicine - "Zoobiquity" [6]. In this review we seek to provide an overview of the clinical relevance of the naturally occurring human cathelicidin in context of infectious disease, including the potential application of synthetic analogues as novel biotherapeutics.

Distribution and Biological Characteristics

The term "cathelicidin" was introduced twenty years ago to describe a subset of HDPs that was first isolated from bovine neutrophils[7]. Much research has since been dedicated to the field of innate immunity, and members of the cathelicidin peptide family have been documented in a variety of species across the animal kingdom. In

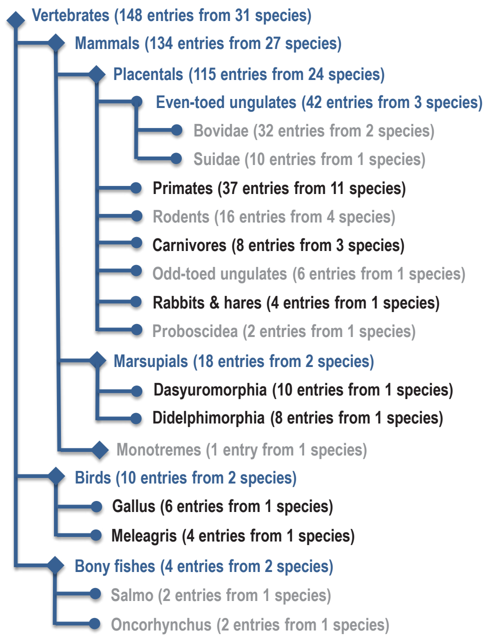

Figure 1: Distribution of Cathelicidin Sequences across Taxonomic Groups

The tree was constructed based on GenBank data (RefSeq search, January 2013). The species listed in the tree are outlined in Table 1, which includes the corresponding cathelicidin peptides.

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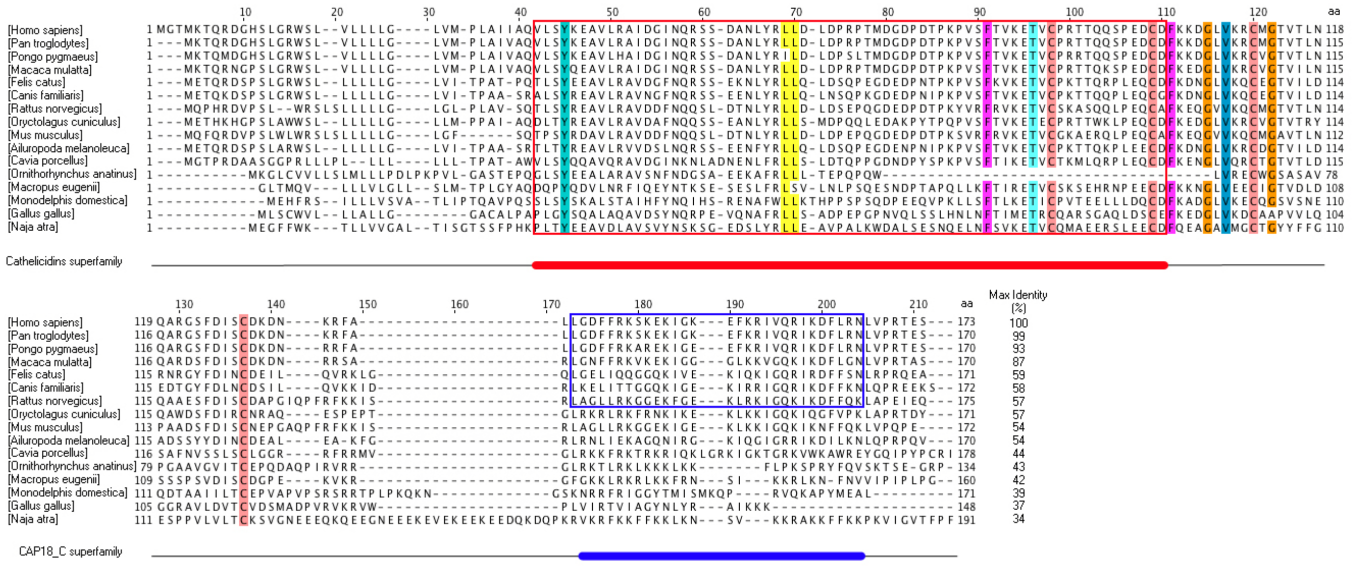

Figure 2: Multiple Sequence Alignment of Cationic Antimicrobial Proteins

Conserved domains are indicated with squares, including the Cathelicidins superfamily (red), and CAP18_C (blue). Amino acid residues that present 100% identity are shown in aquamarine (tyrosine, Y), royal blue (valine, V), and pink (cysteine, C). Residues with 93% identity are shown in yellow (leucine, L), light purple (phenylalanine, F), sky-blue (threonine, T) and orange (glycine, G). Accession numbers were obtained from NCBI GenBank. Sequence alignment and graphic made with Clustal Omega (Ver.1.1.0) and Jalview (Ver.2.8.). Species key: human (*H. sapiens*), chimpanzee (*P. troglodytes*), orangutan (*P. pygmaeus*), rhesus macaque (*M. mulatta*), cat (*F. catus*), dog (*C. familiaris*), rat (*R. norvegicus*), rabbit (*O. cuniculus*), mouse (*M. musculus*), panda (*A. melanoleuca*), guinea pig (*C. porcellus*), platypus (*O. anatinus*), wallaby (*M. eugenii*), opossum (*M. domestica*), chicken (*G. gallus*), cobra (*N. atra*).

example, the antimicrobial sequence database (AMSD) at University of Trieste yields sixty entries in response to the search term “cathelicidin”, while GenBank lists a total of 538 nucleotide entries, including 148 hits using Ref Seq[a].

Examples of cathelicidin peptides identified in animal species examined to date are shown in Figure 1 and Table 1. The sequence homology between cathelicidins from different - closely as well as more distantly related - animal species is shown in Figure 2, while Figure 3 exemplifies the structural similarity between three of the mammalian cathelicidins.

The size and structural characteristics of cathelicidins vary broadly with peptides ranging from a dozen to about a hundred amino acid residues, and spanning both cyclic and linear structures (Figure 4). Cyclic peptides are typically characterized by a loop structure (beta-sheet) stabilized by di-sulphide bridges, while linear peptides tend to form amphipathic alpha helices or structures with repeat motifs of amino acids such as proline or tryptophan [8]. The common feature that ties this heterogeneous peptide family together is a conserved (100 residues) pro-sequence at the N-terminal, the so-called cathelin[b] domain (Figures 2 and 4).

The number of cathelicidins varies from a single peptide in humans to several within artiodactyl species (Table 1), and the potency and functional spectrum of individual cathelicidin peptides therefore also varies [1,9]. Many of the peptides are inducible in response to various molecular patterns (e.g. bacterial fragments or chemical “danger” signals released upon tissue injury), which signal through innate pattern recognition receptors and downstream pathways (such as TLRs and NFkB signaling), so the pattern of HDP expression fluctuates over time [1]. Elite members of the group exhibit broad-spectrum antimicrobial activity at sub-micromolar concentrations, including killing of pathogens that are otherwise resistant to commercially available antibiotics.

Microbial resistance to HDPs is rare in nature because the peptides utilize a fast-acting and relatively non-specific mechanism of action, which relies on the difference in lipid composition between normal eukaryotic versus prokaryotic (or transformed eukaryotic) cell membranes. One of the generally accepted mechanisms of action for HDPs, schematized in the Shai-Matsuzaki-Huang model [10] (Figure 5), involves bacterial killing via peptide-induced pore formation in the cell membrane, membrane destabilization and lysis (Figure 6). Synergism has moreover been observed between different HDPs, as well as between cathelicidins and conventional antibiotics [8,11,12].

Mechanisms of microbial resistance towards HDPs might involve bacterial modification of the (anionic) surface composition to diminish the attraction of the cationic HDPs, peptide removal from the bacterial interior via an efflux pump, as well as down - regulation, neutralization or proteolytic degradation of the cathelicidin peptides [13]. However, unlike commercially available antibiotics – which on an evolutionary scale “only” date back to 1929 with Alexander Fleming’s discovery of penicillin - natural antimicrobial (host defense) peptides are ancient effector molecules of innate immunity [14]. HDPs have enjoyed long-lasting success in nature, and been very effective from an evolutionary perspective. While mutant (bacterial) strains can manipulate surface charge to a point where killing by HDPs is avoided under laboratory

[a]The NCBI Reference Sequence (RefSeq) database is a non-redundant set of reference standards derived from the INSDC databases, while the International Nucleotide Sequence Database Collaboration (INSDC) represents an archival repository of all sequences submitted to GenBank, the European Nucleotide Archive, and the DNA Data Bank of Japan. Sequence data is exchanged daily between collaborators to ensure current worldwide coverage (http://www.ncbi.nlm.nih.gov/books; Bookshelf ID: NBK50679. Accessed 01.09.13).

[b]Homologous to cathelin, which is a cathepsin L protease inhibitor from pig leukocytes (FEBS Lett. 1989 Sep 25;255(2):211-4).

Species (Order, Family or Subfamily)	Common Name	Peptide (s)	Reference (s)
Bos taurus (Artiodactyla/Bovidae)	Cow	BMAP-27. BMAP-28. BMAP-34. Bac5. Bac7. Dodecapeptide. Indolicidin	PMID: 9067433 PMID: 12960280 AMSDb
Ovis aries (Artiodactyla/Bovidae)	Sheep	SMAP-29. SMAP-34. oaBac6. oaBac11. sBAc7.5 sBac5. s-dodecapeptide.	PMID: 7498547 PMID: 12960280 AMSDb
**Capra hircus* (Artiodactyla/Bovidae)	Goat	chMAP-28. chMAP-34. chBac5. chBac7.5	PMID: 10417180 PMID: 12960280
Sus scrofa (Artiodactyla/Suidae)	Pig	PMAP-23. PMAP- 36. PMAP-37. PR-39. Prophenin1, 2. Protegrin1-5	PMID: 1765098 PMID: 16053249 AMSDb
Homo sapiens (Primate)	Human	LL-37 (hCAP-18)	PMID: 7890387
Pan troglodytes (Primate)	Chimpanzee	ptrLL-37	PMID: 16720578
Pan paniscus (Primate)	Bonobo	1 cathelicidin predicted	XM_003818426
Gorilla gorilla gorilla (Primate)	Gorilla	1 cathelicidin predicted	XM_004034062
Pongo pygmaeus (Primate)	Orangutan	ppyLL-37	PMID: 16720578
Papio anubis (Primate)	Baboon	1 cathelicidin predicted	XM_003894490
Macaca mulatta (Primate)	Rhesus macaque	mmuRL-37	PMID: 16720578
Callithrix jacchus (Primate)	Marmoset	cjaRL-37	PMID: 16720578
Saimiri boliviensis boliviensis (Primate)	Squirrel monkey	1 cathelicidin predicted	XM_003926293
Hylobates moloch (Primate)	Gibbon	hmdSL-37	PMID: 16720578
Otolemur garnettii (Primate)	Bush baby	1 cathelicidin predicted	XM_003800549
**Trachypithecus obscurus* (Primate)	Leaf monkey	pobRL-37	PMID: 16720578
Mus musculus (Rodentia)	Mouse	mCRAMP	PMID: 9148921
Rattus norvegicus (Rodentia)	Rat	rCRAMP	PMID: 12737313
Cavia porcellus (Rodentia)	Guinea pig	CAP-11	PMID: 9278433
Cricetulus griseus (Rodentia)	Hamster	1 cathelicidin predicted	XM_003504259
Felis catus (Carnivora)	Cat	feCATH	PMID: 21533281
Canis lupus familiaris (Carnivora)	Dog	K9CATH	PMID: 17462733
Ailuropoda melanoleuca (Carnivora)	Panda	Cathelicidin-AM	PMID: 22101189
Equus caballus (Perissodactyla/ Equidae)	Horse	eCATH-1, 2, 3	PMID: 11181349
Oryctolagus cuniculus (Lagomorpha)	Rabbit	CAP-18	PMID: 1883348
Loxodonta Africana (Proboscidae)	Elephant	1 cathelicidin predicted	XM_003409891
Sarcophilus harrisii (Dasyuromorphia)	Tasmanian devil	1 cathelicidin predicted	XM_003762945
Monodelphis domestica (Didelphimorphia)	Opossum	12 cathelicidin genes	PMID: 17495011
**Macropus eugenii* (Diprotodontia)	Wallaby	14 cathelicidin genes	PMID: 21912615
Ornithorhynchus anatinus (Monotremata)	Platypus	8 cathelicidin genes	PMID: 21912615
Gallus gallus (Galliformes)	Chicken	Fowlicidin-1, 2, 3	PMID: 16326712
Meleagris gallopavo (Meleagridinae)	Turkey	2 cathelicidins predicted	XM_003206909/10
Salmo salar (Salmonidae)	Atlantic salmon	*as*CATH-1, 2	PMID: 16377685
Oncorhynchus mykiss (Salmonidae)	Rainbow trout	*rt*CATH-1, 2	PMID: 16377685
**Ophiophagus Hannah* (Elapidae)	King cobra	OH-CATH	PMID: 18620012

*Indicates species not included in the tree shown in Figure 1

Table 1: An Overview of Cathelicidin Host Defense Peptides in Animal Species.

conditions, such change is likely to also impact bacterial viability and limit existence in nature. The notion of pathogens developing broad-scale resistance against natural HDPs is therefore, relatively speaking, less of a concern [1].

Our current knowledge on cathelicidins stems from a compilation of *in vitro* studies, animal models, and clinical investigations, which combined position these peptides at the core of the mammalian innate immune response. The distinction between maintenance of health versus development of disease depends first and foremost on the aptitude of the host's innate immune response. The following sections serve to outline the association between human infectious disease susceptibility and the pattern of cathelicidin expression.

Human Cathelicidin

The innate immune defense repertoire in humans includes a single cathelicidin, which was first identified as FALL-39 (human counterpart of the proline-rich porcine cathelicidin PR-39) based on cDNA isolated from bone marrow [15,16]. This term was subsequently adjusted to FA-LL-37 [17], where LL-37 is the mature human cathelicidin peptide–highlighting the two first amino acids (leucine) and the overall length (amino acids) of the peptide. Meanwhile, hCAP18 refers to the 18kDa form of the "human cationic antimicrobial protein" [18,19], which maps to chromosome 3p21, involving four exons and three introns [20] (Figure 4).

The full-length hCAP18 (holoprotein) includes a signal peptide (N-terminal), a conserved cathelin-like domain (CLD), and the antimicrobial LL37 peptide domain (C-terminal). The hCAP18 precursor is synthesized in neutrophil progenitor cells in the bone marrow and stored in unprocessed form in specific granules of neutrophils [21]. hCAP18 is moreover expressed in epithelium throughout the body either constitutively or in response to molecular

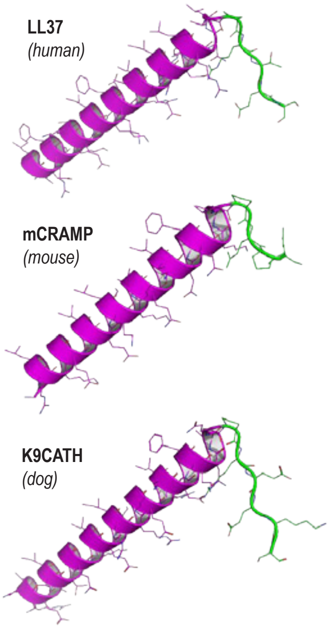

Figure 3: Cathelicidin Structures from Human, Mouse and Dog

Computational modeling of alpha-helical cathelicidin structures from three mammalian species. Ribbon diagrams made with PyMol Molecular Graphics System (Ver.1.5.), and sequence data from *Current Protein* and *Peptide Science*, 2005, 6, 23-34.

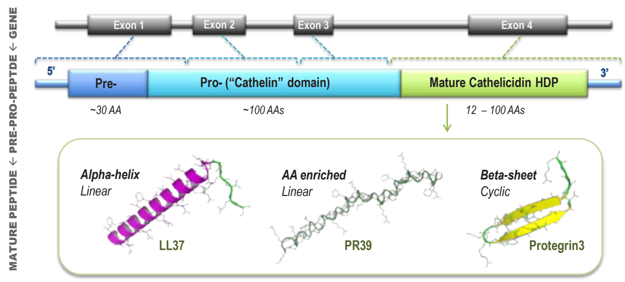

Figure 4: Gene Organization and Major Structural Classes of Cathelicidins

Cathelicidins are ancient gene-encoded (four exons) immune compounds produced in the bone marrow as pre-pro-peptides with a conserved signal- and cathelin - domain, stored as pro-peptides in neutrophil granules, prior to extracellular release and cleavage into mature (variable) cathelicidin HDPs. AA: amino acid. The cathelicidins listed here are human (LL37), and porcine (PR39, Protegrin-3). Figure content also based on: *Scand. J. Infect. Dis*. 35: 670-6, 2003.

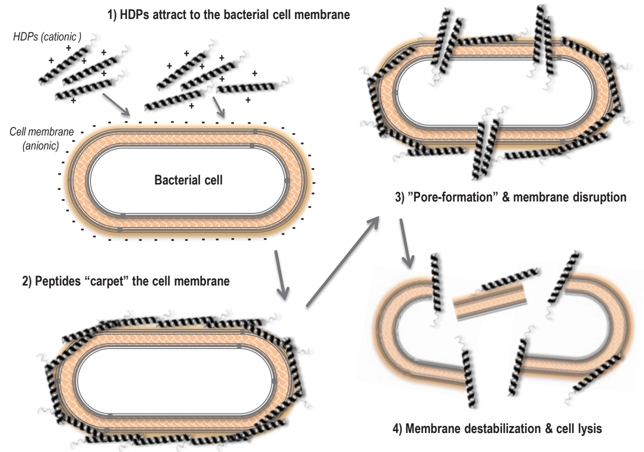

Figure 5: The Shai-Matsuzaki-Huang Model for HDP Antimicrobial Activity

Cationic amphipathic HDPs connect to the bacterial surface via strong electrostatic and hydrophobic interactions made possible by the acidic phospholipid content (anionic) in the prokaryotic cell membrane. This is distinct from normal eukaryotic cell membranes that are electro-neutral consequent to the cholesterol content in their phospholipid bilayer. Figure based on: *Nature* 2002 Jan 24;415(6870):389-95.

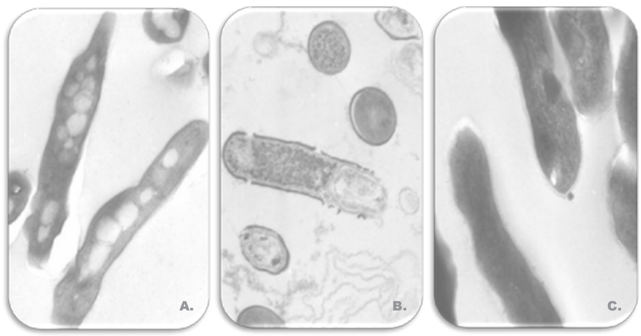

Figure 6: Electron Microscopic Images of *M. tuberculosis* after Cathelicidin Exposure

Canine cathelicidin (K9CATH) exposure creates significant and increasing vacuolization (A.) of the *Mycobacterium tuberculosis* (TB) bacteria, followed by membrane disruption (B), and bacterial lysis – as compared to untreated Control (C). Photos republished (with permission) from: African Journal of Microbiology Research (2012) 6: 38; 6726-29.

patterns from the host or microbial pathogens [20]. Following exocytosis, the holoprotein undergoes extracellular cleavage via protease 3 from azurophil granules to yield the active LL37 peptide (5 kDa) [22].

The peptide's spectrum of biological activity spans broadly, and includes direct pathogen killing, promotion of angiogenesis in wound healing, mast cell histamine release, as well as chemo-attraction of monocytes, neutrophils, and T cells, and modification of dendritic cell differentiation [20,23]. This further highlights that the role of human cathelicidin as such spans beyond the immediate immune response. The mouse cathelicidin (mCRAMP) shares several similarities with the human LL37, and *in vitro* studies as well as infection models in CRAMP-deficient mice have received substantial research attention [24,25]. mCRAMP also regulate B- and T-cell function, which implicates this cathelicidin as a role player within the adaptive immune response [26]. The LL-37/CAP18 molecule has been studied in non-human primates, and the high inter-species homology makes these valuable model organisms for further study of the innate immune response [27].

Factors such as peptide concentration, ion composition and pH influence the biological activity and structural conformation of the LL37 peptide, while antimicrobial activity against Gram-negative and -positive bacteria correlates with factors such as degree of alpha-helicity [28]. The peptide exerts broad antimicrobial activity (*in vitro*) against clinically relevant pathogens such as *Escherichia coli, Listeria monocytogenes, Pseudomonas aeruginosa, Salmonella typhimurium, Staphylococcus aureus, Staph. epidermis,* and Vancomycin-resistant

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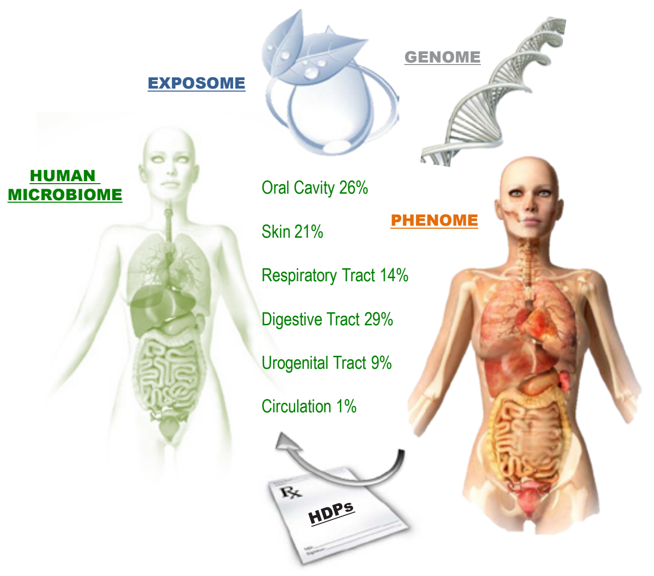

Figure 7: The Human Composition from a Biological Dataset Perspective

The terms genotype and phenotype were introduced in 1903 by Danish geneticist Wilhelm Johannsen, and their interrelationship has classically been conceptualized as: Genotype (G)+Environment (E) → Phenotype (P). Fast-forward in time to the "era of -omics", a plethora of biological datasets is available that also warrant consideration. The ubiquitous microbiota housed in/on the body is here represented by the green "humanoid shadow", while percentages indicate distribution of bacterial species across body sites (Genome Res. 2009;19(12):2317-23). The body enables homeostasis via intrinsic production ("prescription", Rx) of HDPs. Now, the physical traits (phenome) becomes a matrix reflecting the sum of the individual's genetic layout (genome), exposures from the environment (exposome) and the resident microflora (microbiome), as well as the combined interactions. Figure made with images from 123RF.com.

enterococci at MICs<10 μg/ml and in the presence of salt (100mM NaCl) [29].

LL37 can exhibit cytotoxic effects towards eukaryotic cells (*in vitro*) at a concentration of 25 μM, although this effect is attenuated by the presence of human serum [28]. The plasma level of hCAP18 is around 1.2 μg/ml with a significant amount of hCAP18 bound to lipoproteins [22]. Cathelicidin's broad-ranging biological activities are also explained by the peptide's interaction with different cell types in the body. The peptide impacts neutrophil function to increase the cellular uptake of bacteria, and can decrease release of pro-inflammatory mediators (TNF-alpha, IL-1beta, IL-6, and IL-8), thereby preventing a cytokine storm [24]. Other functions are highlighted in context of the examples below.

The Human Microbiome

The microbial collection hosted within the human ecosystem constitutes approximately 10,000 species, which contribute about 8 million (unique) protein-coding gene sequences – in other words, more than 300 times the number of (protein-coding) sequences contained in the human genome[cd]. With trillions of microorganisms present in the body, human cells are outnumbered by a factor 10 at any given time [30]. Fortunately microorganisms stay true to their name and take up relatively little space – although this still translates into 1-3% of our total body weight[d]. Consequently, if a 70 kg adult hosts about 2 kg of microorganisms (commensal species and potential pathogens) the importance of a functional and well-orchestrated immune system becomes self-evident. Figure 7 serves to visualize the key players in this complex act, as well as the distribution (%) of bacterial species in the human microbiome across body sites.

The Human Microbiome Project (HMP), launched by the National Institutes of Health in 2007, provides novel insight on the composition of the microflora in a healthy Western population. Depending on body site, anywhere from 20% to 60% of the human-associated microbiome is uncultivable, which stress the value of new high-throughput technologies [30]. Below, we reference data in context of the five major body sites targeted by the HMP: oral cavity, gastrointestinal tract, skin, respiratory tract and urogenital/vaginal tract [31].

Oral cavity

Swedish pediatrician Rolf Kostmann (1909-1982) was the first to report on infantile genetic agranulocytosis – now known as morbus Kostmann – in a 1956 article in *Acta Paediatrica Scandinavia*. While the mechanism underlying this recessive genetic disorder was still unknown, Kostmann speculated on a missing serum factor [32,33]. The disease renders patients more susceptible to pathogens consequent to abnormal myelopoiesis, and clinical findings are characterized by chronic skin and mucous membrane infections that necessitate repeated treatment with antibiotics. Overall life-quality for patients was improved after introduction of recombinant human granulocyte colony-stimulating factor (G-CSF) in the late 1980s. While G-CSF works to restore neutrophil levels, problems with chronic infections – particularly severe periodontitis and chronic gingivitis – persisted despite treatment [34,35]. In the decades to follow, research in immunology focused increasingly on effector molecules of innate immunity, leading also to evaluation of HDPs in this patient category. In 2002, Hans Boman's group reported that human cathelicidin is absent in plasma and saliva in morbus Kostmann patients, while individuals subjected to bone marrow transplantation have essentially normal LL-37 concentrations [36]. The study as such established accordance between the occurrence of severe periodontal disease in morbus Kostmann patients and deficient LL-37 production.

The role of LL37 in oral immunocompetency has been further investigated, and high LL37 levels in saliva have been correlated with resistance to caries. LL37 is present in gingival crevicular fluid, where it may contribute as a marker for oral health status [37]. Data from children show that the LL37 saliva concentration is higher in girls versus boys, increases with age, and is higher in those with mixed or permanent dentition, while levels are diminished in individuals with high caries activity [38].

Gastrointestinal system

The commensal gut flora creates a biological barrier, which together with the thick mucus layer (mechanical barrier) and secretion of immune defense molecules (chemical barrier) serve to maintain gastrointestinal health [39,40]. Cathelicidin is expressed by macrophages and epithelial cells of the colon to form part of the barrier system pathogens must bypass to invade the intestinal tissue [39]. Breach of this lining enables attachment of Shiga-toxin-producing bacteria such as enterohemorrhagic *Escherichia coli* O157:H7 (EHEC) to epithelial cells and secretion of virulence factors into the circulatory

[c]National Institutes of Health (NIH) Human Microbiome Project, http://www.hmpdacc.org

[d]NIH News, http://www.nih.gov/news/health/jun2012/nhgri-13.htm (retrieved 02.05.13).

system. Hemolytic uremic syndrome with acute renal failure from EHEC infection is a major complication seen in about 15% of clinical cases [39]. Cathelicidin deficiency enhances host susceptibility to pathogenic *E. coli* O157:H7 in a mouse model through thinning of the mucus layer, which facilitate bacterial attachment to the epithelium, and systemic toxin release [39].

Helicobacter pylori can colonize the human gastric epithelium on a chronic basis, and the hCAP18/LL37 levels in gastric epithelial cells and gastric secretions are significantly increased in infected patients, which is not the case with gastric inflammation from other causes such as neoplasia or hyperplastic polyps [11]. Inflammatory bowel diseases, such as ulcerative colitis and Crohn's disease, involve an abnormal immune response to bacterial pathogens [41]. Cathelicidin is upregulated in colonic macrophages and epithelium in a murine colitis model, while mCRAMP knockout mice develop more severe colitis supporting that cathelicidin also plays an important role in induction of colitis [42]. On the other hand, colonic epithelial expression of hCAP18 is not upregulated in patients with Crohn's disease [43].

Cathelicidins exert a broad spectrum of antimicrobial activity against enteropathogenic bacteria, and mice are used extensively to model human disease, although differences exist. In humans, Salmonellosis is caused by *Salmonella enterica* serovar *Typhimurium*, which can also cause a typhoid-like response in mice [44]. However, while mCRAMP can inhibit growth of *S. typhimurium* [45], LL37 does not seem to be a key player in intracellular clearance of this pathogen [44].

Amebiasis is a significant health concern that affects about 10% of the global population, and cause potentially fatal amebic colitis and hepatic abscesses [46]. Studies on human intestinal epithelial cells and a mouse model of amebic colitis have shown that *E. histolytica* trophozoites upregulate intestinal cathelicidin expression, although amebic cysteine proteases cleave the cathelicidin into fragments without antiparasitic activity [46]. This enzymatic processing enables protozoan evasion of the innate immune response, although the peptides' antibacterial activity remains intact [46]. Another recent study found that while the antiparasitic activity of LL37 is limited, shorter analogues (KR-20 and KS-30) are able to adversely affect the growth and integrity of *E. histolytica* trophozoites [47].

Expression of cathelicidin has also been documented in human breast milk, as well as in murine mammary gland tissue [48]. This suggests that cathelicidin serves to protect the mammary epithelium during lactation while also providing the neonatal gut with a natural antibiotic [49]. Importantly, the lactose component in human breast milk induces expression of the cathelicidin gene (*CAMP*) in colonic epithelium, and acts synergistically with other cathelicidin-inducing factors such as butyrate and phenylbutyrate [50].

Like other HDPs, the biological activity of LL37 spans beyond that of a simple antibiotic, and the peptide in example exerts a tumor-suppressing mechanism in colon tumorigenesis [51].

Integumentary system

Normal-weight adults have on average 2 m^2 of body surface area, which is covered by a 2 mm thick layer of skin that effectively constitutes the first line of defense [52,53]. HDPs are expressed constitutively in normal skin, where they create a biochemical shield against external threats, ensure homeostasis with a diverse cutaneous microflora, and help to promote wound healing [54,55]. The principal (stationary) sources of HDPs in human skin are keratinocytes, eccrine glands, sebocytes, and mast cells, while circulatory cells such as neutrophils and NK-cells make significant HDP contributions also [56]. In keratinocytes, cathelicidin is stored in granules (lamellar bodies), and production is increased during times of cell differentiation [57]. Additionally, HDP production (bacteriocins) from the commensal skin flora (e.g. *Staphylococcus epidermidis*) acts synergistically with the host's defense repertoire [58-60]. Imbalance in this expression pattern and collapse of the symbiotic arrangement correspond with clinical diseases such as psoriasis, atopic dermatitis, rosacea, as well as other skin conditions [59]. Cathelicidin protect the cutaneous surface via direct antimicrobial action as well as immunomodulation, including release of cytokines, inflammation, angiogenesis, and re-epithelialization [61].

In addition to its role in bone metabolism and calcium homeostasis, the focus on vitamin D_3 has been directed increasingly towards immunoregulation in context of both the innate and adaptive arms of the system. Vitamin D_3 is central to the cutaneous immune response, and involved in regulation of cathelicidin expression at epithelial surfaces [56,62]. Vitamin D_3 deficiency is common in the US, with an overall prevalence of 42% in a recent study [63], and generally caused by either insufficient intake or a mainly indoor lifestyle. Also, the recommended daily allowance for vitamin D_3 was historically geared towards bone health more so than optimization of the body's immunological aptitude [56]. Keratinocyte development and function rely on the presence of vitamin D_3, and deficiency leads to abnormal skin barrier formation and a deficient immune defense [56].

Atopic dermatitis (AD) is the most prevalent inflammatory skin disorder in humans, affecting up to 25% of children and about 3% of adults in the US [64]. The epidermal elements responsible for maintenance of skin homeostasis (physical, chemical, microbial, and immunological) are compromised to varying extents in this patient category [64]. The disease is associated with suppression of HDP induction, mediated by IL4 and IL13, and a diminished antimicrobial barrier at the cutaneous level [56]. Patients with AD are therefore also more susceptible to bacterial (e.g. *Staphylococcus aureus*) as well as viral, (such as herpes simplex) infections [65-67]. Meanwhile, oral vitamin D3 supplementation administered to AD patients at 4,000 IU/day for 21 days is able to boost the cutaneous cathelicidin expression by more than a factor six [67].

Psoriasis is another highly prevalent inflammatory disease of the skin, which affects between 1% to 5% of the population depending on geographical location and ethnicity [68]. The maturation of keratinocytes is accelerated from the usual 30 days to about 3 days in psoriasis patients, and this dramatically shortened skin turnover leads to accumulation of excess cells and plaque formation on the body surface [68]. The disease is characterized by a genetic predisposition, an autoimmune component, as well as abnormal HDP activity [56]. LL37 from psoriatic skin converts inert self-DNA into a 'danger-signal', leading to aberrant interferon production from plasmacytoid dendritic cells (pDCs), and local T-cell-mediated autoimmune inflammation, which exacerbates the injury to the skin [69]. In normal skin, plasmacytoid dendritic cells sense "danger signals" released from common skin wounds and play a critical role in the early inflammatory response and re-epithelialization of the cutaneous injury [70].

Rosacea is an inflammatory skin disorder with vascular abnormalities that typically involves the central part of the face. Also known as the "curse of the Celts" (due to the higher prevalence in people of Northern European descent) it affects an estimated 16 million adults (predominantly middle-aged) in the US [71,72]. The diagnosis is based solely on clinical manifestations, and subtypes of rosacea are erythematotelangiectatic, papulopustular, phymatous, and ocular, as well as a non-inflammatory (granulomatous) variant of the disease [71]. The skin's innate immune response to environmental factors is exaggerated in affected individuals, where the vascular reactivity and abnormal inflammatory response also inspired the study of patients' cathelicidin expression profiles [71]. Normal post-translational processing generally renders shorter forms of LL37 on the cutaneous surface of healthy individuals [48]. However, rosacea patients have abnormally high levels of unprocessed LL37, and the enzymatically-processed forms are furthermore different (more pro-inflammatory) compared to controls [61,73].

Data from dermatology centers in North America indicate that infections of the nail bed are a significant epidemiological problem, and that the prevalence of onychomycosis is about 14% [74]. The nail environment is isolated from direct cell-mediated immunity due to lack of a vascular supply, however, LL37 is expressed abundantly in epithelium across the nail bed unit. LL37 inhibits growth of *Candida albicans* at 25 μM, which shows that cathelicidin also serves a role in the immune defense against fungi and nail pathogens [74]. The role of LL37 in fungal skin infections, e.g. dermatophytosis (*Epidermophyton, Microsporum*, and *Trichophyton species*) and tinea versicolor (*Malassezia furfur*), has also been explored. Findings support that cathelicidin is key to an efficacious innate immune defense against fungal infections of the cutaneous surface [75].

Cathelicidin is moreover able to limit dissemination of cutaneous Leishmaniasis. The disease (cutaneous, mucocutaneous and visceral forms) affects approximately 12 million people in 88 countries, and is as such a significant health problem globally [76]. Data from a murine model support that cathelicidin is able to kill the parasitic protozoan through both indirect (enhanced parasite uptake by macrophages and neutrophils) as well as direct (membrane disruption) measures. Without cathelicidin (CAMP knockout model), the parasite grows uncontrolled and metastasizes to visceral organs [76].

Respiratory system

The internal surface area of the human lung covers a prodigious 50 square meters, which in context of a total lung volume of approximately 5 liters enable sufficient gas exchange [77]. Effective host defense of the airway system rely on mechanisms such as mucociliary clearance, reflex coughing and sneezing as well as cellular components and different innate immune factors [78]. Airway epithelium, neutrophils and alveolar macrophages impact the efficacy of pathogen clearance in part through secretion of HDPs into the airway surface fluid (ASF) [78].

While the airways historically were considered a sterile environment, more recent data has documented a commensal lung microbiota (consisting primarily of *Proteobacteria, Firmicutes*, and *Bacteroidetes*) in clinically healthy individuals [79]. It has furthermore become evident that the gastrointestinal microbiota impacts that of the lungs, and can tip the balance from lung health to disease [79]. LL37 serves an important role to protect the airway epithelium both through direct antimicrobial activity as well as via activation of epithelial cells [80]. Upregulation of LL37 in human alveolar macrophages utilizes both NLR- (NOD2) as well as TLR-mediated signaling (TLR-2, -4, and -9) [81]. Studies on newborns support that lung mucosal host defense peptides, including LL37, are expressed in both mature and preterm individuals, and that concentrations increase in the presence of systemic or pulmonary infection [82].

Cystic fibrosis (CF) is caused by a genetic defect in the chloride conductance of the airway epithelium, which is associated with chronic airway infection and inflammation [83]. Importantly, the activity of HDPs is influenced to varying extents by the salt concentration in the tissue environment, which in the case of CF patients is outside the normal reference range. Data from a human bronchial xenograft model support that the endogenous LL37 concentration is similar (about 2 μg/ml) in ASF from normal versus CF xenografts. Correction of the defect in CFTR (cystic fibrosis transmembrane conductance regulator) via adenovirus mediated gene transfer to surface epithelial cells is able to correct the defect in bacterial killing, without change in the LL37 concentration [83].

Vitamin D deficiency is a common finding in patients with both non-cystic-fibrosis and CF-associated bronchiectasis [84]. Vitamin D receptors are expressed ubiquitously on epithelial and immune cells, including neutrophils, macrophages and lymphocytes [62,84]. The vitamin is converted to its active form 1,25(OH)2D3 at a cellular level, and helps to support the body's immune competency via facilitation of HDP production and regulation of the inflammatory response [62]. Supplementation with Vitamin D suggests that some level of protection against infection, including development of bronchiectasis, can be achieved at a clinical level [84].

Pneumonia from *Pseudomona aeruginosa* is the second most prevalent type of lung infection in hospital settings, with mortality rates reaching up to 90% in mechanically ventilated patients [85]. In a mouse pneumonia model, compartmentalized pre-exposure to *P. aeruginosa* flagellin can upregulate cathelicidin (via TLR5 mediated signaling) and boost protective mucosal immunity in the lungs [85]. CRAMP is similarly required for an apt lung mucosal immune response in pneumonia due to *Klebsiella pneumonia* [86]. This data may be exploited further to advance treatment options for pneumonia caused by Gram-negative pathogens [85].

Approximately 1/3 of the world's population has latent tuberculosis (TB) caused by the intracellular bacteria *Mycobacterium tuberculosis,* while the mortality from clinical TB in 2011 was 1.4 million globally according to WHO statistics[e]. Although the disease is currently preventable and treatable, the increase in multi-drug resistant TB creates cause for concern. Several studies have therefore also assessed the role of LL37 in killing of mycobacteria, and found that cathelicidin plays a role in controlling this infection. *In vitro* studies support that LL37 and truncated versions can directly kill pathogenic mycobacteria including *M. bovis* and *M. tuberculosis*, as well as *M. smegmatis* (non-pathogenic) [87]. Mycobacteria target host macrophages where they rely on maturation-arrest of the phagosome for their survival. Regardless, LL37 is able to effectively reduce the intracellular survival of bacteria [87]. The thought is that LL37 after endocytosis targets the maturation-arrested mycobacterial phagosome to kill off the bacteria, and subsequently enable fusion with lysosomes to eliminate the intracellular pathogen [87].

TB is causative of 25% of all deaths amongst HIV$^+$ patients[e]. Levels of vitamin D are markedly low in bronchoalveolar lavage fluid from patients with HIV, which results in further impairment of critical immune responses and renders these individuals more susceptible to opportunistic infections [88,89]. Exogenous vitamin D_3 supplementation of alveolar macrophages from HIV$^+$ patients can rescue this impaired (innate) response through TLR-mediated

[e]Tuberculosis, Fact Sheet No 104, http://www.who.int/mediacentre/factsheets/fs104/en/#, World Health Organization (retrieved 02.22.13)

signaling [88]. The active form of vitamin D also induces autophagy to inhibit the replication of HIV, while the availability of cathelicidin is central to both the suppression of HIV replication and inhibition of mycobacterial growth [89].

While the anti-viral capacity of cathelicidin has been studied less as compared to defensins, recent reports support that LL37 is an active player with respect to ridding the body of seasonal influenza. Unlike the antiviral activity exerted by other HDPs, LL37 seemingly interacts directly with the virus to cause surface disruption [90]. The antiviral activity of LL37 against different strains of influenza A virus is dose-dependent, and additive with other innate components like surfactant proteins and human neutrophil defensin peptides (HNPs). The virus neutralizing effect is reduced, however, in the presence of human serum due to LL37's binding with high-density lipoprotein (HDL) - the key serum lipoprotein that binds to cathelicidin [90].

Urogenital system

The HMP has contributed significant new knowledge on the human body's endogenous microbial ecosystem, and more than one clinical dogma has had to face rejection as a result. Urine from clinically normal individuals was historically also thought to be sterile, however, recent high-throughput screening and metaproteomics analysis have since characterized the "healthy urine microbiome", which is dominated by *Corynebacterium* in men and *Lactobacillales* in women [91]. While urinary tract infection (UTI) is a significant health problem [92], the presence of uncultivated bacteria in normal urine of both genders speaks for reassessment of the current use of conventional antibiotics.

Similar to other body system, homeostasis in the urogenital tract correlates with the combined efficacy of mechanical (urine flow & bladder emptying) and biochemical (HDP production and immune cell recruitment) defense measures [39,93]. Acute UTI is often caused by uropathogenic *Escherichia coli* that is also the host's most abundant fecal strain (prevalence hypothesis) [94], which highlights the continuous balancing act that takes place between compartments of the human microbiome and the host's innate defense repertoire. The LL37 peptide is produced constitutively by resident uroepithelial and tubular renal epithelial cells as well as neutrophils, and is measureable in normal urine at a median concentration of 0.3 ng/ml - versus an eight-fold up-concentration in cases with cystitis or pyelonephritis [93]. Cathelicidin kills clinical isolates of uropathogenic *E. coli* at a concentration of about 30 μg/ml (MIC 8 μM) with the greatest efficacy against isolates from lower urinary tract infections (cystitis) [93].

Improved understanding of the genital tract microbiota and local host defense mechanisms will help to better define human reproductive health, and optimize therapeutic strategies in the field. Culture-independent methodologies have enabled mapping of the vaginal microbiota and revealed that *Lactobacillus* species, as anticipated in human females, constitute the predominant finding in most women. However, a diverse microflora dominated by facultative and strict anaerobic species exists in a significant number of clinically normal females [95]. The dynamics in the spectrum of the vaginal flora in light of life span transitions and between different ethnicities has also been elucidated through HMP-funded research [95].

Research focused on the male reproductive tract microbiota is more limited, although a recent review outlines current knowledge in the field [96]. While the upper male genital tract is seemingly germ-free, the lower tract hosts a microbiota dominated by *Actinobacteria, Bacteroidetes, Firmicutes, Fusobacteria,* and *Proteobacteria*. Corynebacterium (*Actinobacteria*) is often identified as a commensal in the male reproductive tract, while it may also be associated with disease [96]. The interaction between the microbiota in the male versus female reproductive tract is similarly understudied, although post-coital increase in coliforms and a shift towards a vaginosis-type microflora has been reported [96].

Human cationic antimicrobial protein expression has been documented in tissues and body fluid from the male and female reproductive tract, including testes, epididymis, spermatozoa, seminal plasma, vagina and cervix [97-99]. The concentration of hCAP18 in seminal plasma is e.g. 70-fold higher than in blood, while 6.6×10^6 hCAP18 molecules are associated with each spermatozoa based on ELISA analysis [99]. When exposed to a pH equivalent to that of the vagina, the prostate-derived protease gastricsin is able to cleave the inactive hCAP18 into a 38 AAs antimicrobial peptide ALL-38, which represents one protective mechanism working to support reproductive tract health [100].

Other Clinical Considerations for Cathelicidin

Circulatory system

Morbidity and mortality from cardiovascular disease, in particular coronary artery disease (CAD), place a major burden on the healthcare system in industrialized countries. Atherosclerosis refers to inflammation of the vessel wall, which involves a network of cellular and molecular components. Well-established cardiovascular risk factors include hypertension, hypercholesterolemia, smoking, and diabetes mellitus, all of which exert potent pro-inflammatory effects on the vasculature [101].

LL37 cathelicidin is expressed at a six-fold increase in atherosclerotic lesions, and mainly by macrophages and endothelial cells. The peptide induces expression of the adhesion molecule ICAM-1 and the MCP-1 chemokine in endothelial cells - suggesting an immunoregulatory role - and may impact neovascularization [102]. In a murine arteriosclerosis model neutrophil-derived cathelicidin (CRAMP) promotes disease via recruitment of inflammatory monocytes [103]. CRAMP released from neutrophils is transported across the endothelium, and promote cell adhesion of classical (but not non-classical)[f] monocytes [104].

Percutaneous coronary intervention with stent implantation is a common treatment option used to dilate arterial narrowing caused by atherosclerotic plaque formation [105]. Drug-eluting stents serve to reduce the occurrence of restenosis through release of anti-inflammatory and anti-proliferative compounds. Interestingly, experimental studies suggest that a biofunctionalized LL37-coated stent can limit neointima formation to reduce in-stent stenosis in mice [105].

Seroprevalence studies suggest that infectious agents such as *Chlamydia pneumonia* (Cpn), *Helicobacter pylori*, enteroviruses, cytomegalovirus (CMV), herpes simplex virus (HSV-1, and -2), and hepatitis A virus are implicated in the atherosclerotic process [106]. The human respiratory pathogen *Chlamydia pneumonia* has received much research attention in an effort to establish a causative link to atherosclerosis. The presence of Cpn-specific DNA in peripheral

[f]Classical monocytes are linked to inflammatory processes, e.g. the early response in infections, as well as sterile inflammatory conditions, including atherosclerosis or myocardial infarction. Non-classical monocytes are involved in phagocytosis, tissue remodeling, and wound healing. Monocyte subsets are divided according to CD14 and CD16 expression profiles (*Circ Res*. 2013 Jan 2. [Epub ahead of print], PMID: 23283724).

monocytes from patients with CAD suggests that this is the pathogen's mode of transportation from the lungs into the systemic circulation [107]. Animal studies indicate that Cpn can start the atherosclerotic process, or accelerate lipid-induced atherosclerosis in ApoE-deficient mice [106].

While experimental studies support the Cpn-CAD link, large-scale clinical trials like WIZARD, AZACS, ACES and PROVE-IT have, however, all been unable to establish a clinical benefit of antibiotics in prevention and treatment of CAD [101,108-110]. Furthermore, Cpn appears unaffected by LL37's antimicrobial action [102].

Indeed, infection as a causative factor in human CAD remains controversial. Nevertheless, neutrophil-derived LL37 is central to host protection in bacterial invasion of the circulatory system, and the peptide's multifunctional properties make it an important template for the development of novel treatment solutions for sepsis [111]. Data from a murine sepsis model suggest that administration of LL37 in combination with G-CSF is the most effective treatment strategy in reducing bacterial growth and lethality in neutropenic sepsis [112].

Conclusion

In many ways, cathelicidins set the stage for the clinical outcome in several infectious disease processes and inflammatory disorders studied to date. Knowledge on the mechanisms that impact cathelicidin expression patterns in the body is important, because it holds the potential of offering new and improved treatment strategies. Meanwhile, the omnipresence of the human microbiota provides food for thought in light of how conventional antibiotics are administered.

The pipeline for research and development of a new drug - antibiotic or otherwise – easily spans a decade or more. Conventional antibiotics have specific targets that define their mechanism of action, while also rendering them more vulnerable to pathogen countermeasures. Antimicrobials with novel *modi operandi* are consequently in high demand in a world faced with an increasing number of multi-drug resistant pathogens, as well as a changing panorama of infections.

Our emphasis in this review has been on the naturally occurring human cathelicidin, and the ways the peptide operate to preserve human health across and within organ systems. Evolution has modified and optimized the biological function of cathelicidins in animal species over time. "Immunological inspiration" attained from domestic and exotic species can likely provide us with superior templates for use in development of new immunomodulating and antimicrobial drugs.

While the One Health movement has placed emphasis on zoonotic/vector-borne infections, the more recent Zoobiquity initiative takes a broader, species-spanning, approach to health and medicine viewed in light of evolution. Molecular solutions utilized by more robust and (evolutionarily-speaking) longer-lived species can also help guide the design of new drugs that are more fit-to-fight challenging infections and possibly other debilitating diseases. It is through this "zoobiquitous" research approach that the search for new drugs is most likely to excel.

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Conflict of Interest

The authors declare no conflicts of interest.

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