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Antimicrobial Defence in Human Body Fluids – High Antimicrobial Peptide Expression in Postoperative Pleural Fluid and Stressed Mononuclear Cells

Doctoral thesis at the Medical University of Vienna

for obtaining the academic degree

Doctor of Philosophy

Submitted by

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Vienna, 02/2020

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“There are no incurable diseases - only the lack of will. There are no worthless herbs - only the lack of knowledge.”

Avicenna

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Acknowledgements

I would like to thank Hendrik Jan Ankersmit for giving me the opportunity to carry out this thesis and for inspiring me scientifically and personally.

I further wish to express my deep gratitude to my family, in particular my mother, my Father, my sisters, and especially to my beloved wife Zohre, for their unconditional love and support.

Last but not the least, my sincere thanks also go to Shahrokh Taghavi who provided me the opportunity and gave access to the research facilities. His continues guidance helped me in all the time of research.

The results of this thesis have been published in the “Annals of Thoracic Surgery” and the “European Journal of Clinical Investigation” journal and are given in the results section. For the published manuscripts as well as for all graphs, permission was obtained or graphs were taken from open source databases.

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Declaration

This scientific work was carried out at the Department of Thoracic Surgery of the Medical University of Vienna under supervision of Hendrik Jan Ankersmit in cooperation with the Departmen of Dermatology, Medical University of Vienna; Christian Doppler Laboratory for Cardiac and Thoracic Diagnosis and Regeneration; Christian Doppler Laboratory for Biotechnology of Skin Aging (Medical University of Vienna); Department of Biomedical Imaging and Image-guided Therapy (Medical University of Vienna); Institute of the Red Cross Blood Bank for Transfusion Service of Upper Austria, Linz, and the Department of Translational Inflammation Research of Otto von Guericke University Magdeburg, Germany. Animal experiments were performed at the Centre of Biomedical Research of the Medical University of Vienna. All secretomes used for research and therapeutic applications were produced in cooperation with the Red Cross Blood Bank in Linz, under the supervision of Prim. Christian Gabriel. The *in vitro* experiments and Image Flow Cytometry were performed in cooperation with Michael Mildner (Department of Dermatology). Evaluation of data and interpretation of results, as well as manuscript preparation, was performed by Mohammad Mahdi Kasiri (Department of Thoracic Surgery, Medical University of Vienna), under the supervision of Hendrik Jan Ankersmit (Department of Thoracic Surgery, Medical University of Vienna), with the support of the mentor Michael Mildner. (Department of Dermatology). The use of all published parts and figures of the Manuscript is permitted by the respective journals.

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Abstract

Antimicrobial peptides are evolutionarily conserved members of the immune system and are part of the first line defence of all multicellular organisms. They link innate with adaptive immunity, and are involved in a variety of immunological processes which protect the organism from harmful pathogens. Specific unique characteristics, such as potent antimicrobial activity, together with less bacterial resistance, make them an attractive consideration as alternatives to common antibiotics.

The main goal of this thesis was to investigate the expression and antimicrobial activity of antimicrobial peptides in the human body fluids. We have for the first time identified a high abundance of different antimicrobial peptides in pleural fluid and provide evidence for the protective role of these peptides against invading pathogens, especially in patients who had undergone major thoracic surgery. We have also investigated the expression pattern of several antimicrobial peptides and their antimicrobial activity in the supernatant of irradiation induced apoptotic blood mononuclear cells ("APOSEC®", abbreviation for Apoptotic Secretome). We provide compelling evidence for antimicrobial activity of APOSEC® against different gram negative and gram positive bacteria. Furthermore, we chose a xenogenic approach to evaluate immunomodulatory properties of APOSEC® *in vivo.*

Based on our results, the favourable immunomodulatory property of APOSEC® along with its direct antimicrobial activity could be taken advantage of in the treatment of chronic non-healing wounds, such as diabetic foot ulcers, which are predisposed to persistent infection.

Zusammenfassung

Antimikrobielle Peptide sind evolutionär konservierte Vertreter des Immunsystems. Diese sind Bestandteile der ersten Verteidigungslinie des Immunsystems in allen multizellulären Organismen.

Diese Peptide sind ein wichtiges Bindeglied zwischen dem angeborenen und erworbenen Immunsystem und sind in einer Vielzahl von immunologischen Prozessen involviert. Sie beschützen den Organismus vor Pathogenen.

Einzigartige Merkmale, insbesondere die potente antimikrobielle Aktivität, zusammen mit einer geringeren bakteriellen Resistenz, lassen die antimikrobiellen Peptide zu einer attraktiven Alternative zu herkömmlichen Antibiotika werden.

Das Hauptziel dieser Dissertation war, die Expression und die antimikrobielle Aktivität dieser Peptide in menschlichen Körperflüssigkeiten zu erforschen.

Zum ersten Mal beobachteten wir das Vorhandensein von verschiedenen antimikrobiellen Peptiden in hoher Konzentration in pleuraler Flüssigkeit. Wir liefern Evidenz für die protektive Rolle dieser Peptide gegen Pathogene, vor allen in PatientInnen nach einem großen thoraxchirurgischen Eingriff.

Zudem untersuchten wir das Expressionsmuster und die antimikrobielle Aktivität von antimikrobiellen Peptiden, die aus dem Überstand von - durch Bestrahlung induzierte – apoptotischen mononukleären Blutzellen gewonnen wurden. (APOSEC®)

Basierend auf unseren Ergebnissen, könnten die günstigen immunmodulatorischen Eigenschaften von APOSEC® in der Behandlung von chronisch infizierten Wunden, wie z.B. diabetische Ulcera, nützlich sein, die zu persistenten Infektionen neigen. Die Ergebnisse dieser These könnten als Grundlage für klinische Studien dienen.

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Publications Arising from this Thesis

Antimicrobial peptides are highly abundant and active in postoperative pleural drainage fluids. Hoetzenecker K, Hochdaninger M, Traxler D, Gschwandtner M, **Kasiri MM**, Mitterbauer A, Schweiger T, Hegedus B, Klepetko W, Tschachler E, Ankersmit HJ, Mildner M, The Annals of Thoracic Surgery, 2014 Sep;98(3):1042-50.

Dying blood mononuclear cell secretome exerts antimicrobial activity. **Kasiri MM**, Beer L, Nemec L, Gruber F, Pietkiewicz S, Haider T, Simader EM, Traxler D, Schweiger T, Janik S, Taghavi S, Gabriel C, Mildner M, Ankersmit HJ, European Journal of Clinical Investigation. 2016 Oct;46(10):853-863.

Abbreviations

AIS … Adaptive Immune System

AMP… Antimicrobial Peptide

APC… Antigen Presenting Cells

APOSEC … Apoptotic Secretome

ASC … adult or somatic stem cells

ASMA … alpha smooth muscle Actin

B cell … Bone Marrow derived Lymphocyte

BDs ... β-defensins

CAMP … cathelicidin antimicrobial peptide

CMC … carboxymethyl cellulose

DDS-colitis … Dextran Sulfate Sodium induced Colitis

DEFB … Defensin Beta

DFI … Diabetic Foot Infection

DFU … Diabetic Foot Ulcer

E. coli … Escherischia coli

ECM … Extra Cellular Matrix

ECP … Eosinophil cationic protein

EGF … Epidermal Growth Factor

EGFR … Epidermal Growth Factor Receptor

ESBL … Extended-Spectrum-Betalaktamase

ESC … embryonic stem cells

FGF … Fibroblast Growth Factor

GMP … Good Manufacturing Practice

hBD … Human β-defensin

HGF … Hepatocyte Growth Gactor

HNP… Human Neutrophil Peptide

HSCs … Haematopoietic stem cells

(ICAM)-1 … intercellular adhesion molecule-1

IFN-γ … interferon-γ

IGF … Insulin-like Growth Factor

IIS … Innate Immune System

IL … Interleukin

iNOS … Inducible nitric oxide synthase

I.P… Intraperitoneally

iPSCs … induced pluripotent stem cells

LPS … lipopolysaccharide

mBD … Murine β-defensin

MCP … monocyte chemotactic protein

MDRO … Multi Drug Resistant Organism

MMP … Matrix metalloproteinase

MNC … Mononuclear Cells

MRSA … Methicillin-resistenter Staphylococcus aureus

MSC … Mesenchymal Stem Cell

NK cells … Natural Killer Cells

NO … Nitric Oxide

NP … normal pleura

P. aeruginosa … Pseudomonas aeruginosa

PAMPs … Pathogen-Associated Molecular Patterns

PBMC … Peripheral Blood Mononuclear Cells

p-eNOS … phospho- endothelial Constitutive Nitric Oxide Synthase

PGI … Prostaglandin I

PNP … Polyneuropathy

RNase … Ribonuclease

ROS … Reactive Oxygen Species

S. aureus … Staphylococcus aureus

S. pyogenes … Streptococcus pyogenes

T cell … Thymus derived Lymphocyte

TGF … Transforming Growth Factor

TLRs … Toll-like Receptors

TNF … Tumor Necrosis Factor

VASP … Vasodilator-Stimulated Phosphoprotein

VEGF … Vascular Endothelial Growth Factor

VRE … Vancomycin-resistente Enterokokken

CHAPTER ONE: INTRODUCTION

1.1 General Introduction

1.1.1 Innate and Adaptive Immune System

The term ‘Immune System’ refers to the host’s whole defence mechanism including tissues, cells, molecules and other factors. It comprises two major subsets known as the innate (IIS) and adaptive immune system (AIS). While the IIS is essential for the AIS, some of its components act independently from the latter. However, they both account for the immune response against infection and are indispensable for recognition and neutralization of invading pathogens.

The innate or non-specific immune system contains basic immune mechanisms which probably evolved in ancient plants and invertebrates.(1) It includes anatomical features which function as barriers, such as skin, internal epithelial layers, the movement of the intestines and the oscillation of broncho-pulmonary cilia, as well as chemical- and microbial barriers. In addition, the innate immune system includes secretory molecules and cellular components that are important for phagocytosis and inflammation. Moreover, it includes natural killer cells, dendritic cells, and circulating plasma proteins, such as the complement system and antimicrobial peptides (AMPs), which are believed to be the most ancient mechanism of immunity.(2) One unique characteristic of the innate immune system is that these components are constitutively present in a standby state, which facilitates rapid response times in cases of pathogen exposure; thus, it represents the host’s initial defence against invading microbes. Furthermore, an effective antigen-unspecific discrimination between self and non-self, along with early availability in the host’s lifetime, is a further important characteristic of the IIS. Skin, also known as the epithelial surface, represents the first physical barrier pathogens need to cross in order to invade the host’s body. It contains chemical barriers including, AMPs, proteins with a broad-spectrum antimicrobial activity, serving as highly developed natural defence mechanism against invading pathogens, and the acidic mantle, with pH values below 5, essential for inhibiting the growth of pathogens and for

attaching commensal microbiota to the skin, thus providing the skin's microbial barrier.(3, 4). Circulating plasma proteins, constituents of the IIS, such as albumin, transferrin, haptoglobin, components of the blood coagulation system (fibrinogen, prothrombin), components of the complement system(5) and antimicrobial peptides serve many different purposes, including maintaining osmotic blood pressure, increasing blood viscosity, promoting inflammation, and attacking and killing pathogens. The complement system, a subcomponent of plasma proteins, targets invading pathogens by coating the microbial surface after binding to the specific receptors, a process known as opsonisation. This leads to phagocytosis of opsonized microbes(6) by neutrophils, macrophages and dendritic cells, all cellular components of the IIS. Moreover, these cellular components have the ability to secrete antimicrobial peptides.(7)

NK cells originate from the same progenitor bone marrow cells as T and B lymphocytes; however, they are still classified as a part of the IIS due to the limited number of receptors, indicating a reduced antigen specificity. Their receptors bind to a general class of pathogens and eliminate them by releasing cytotoxic proteins(8). In addition, among the phagocytes mentioned above, macrophages and dendritic cells form an important connection between the IIS and AIS by acting as antigen-presenting cells (APCs).

Advanced organisms, including humans, have developed a more intelligent defence mechanism, called the adaptive or specific immune system. This system is necessary, because the innate immune system may not be sufficient enough to eliminate all pathogens. One distinguishing feature of the adaptive immune system is that it develops a permanent immunological memory by adapting to specific pathogens after an initial response, which leads to pathogen specificity. This results in a significantly shorter response time in the case of a subsequent encounter with the same pathogens, and efficient and precise elimination of the microbe. While IIS serves to contain infections within hours, the adaptive immune response takes as much as several days to activate its pathways and clear the pathogens. Two major cellular components of AIS are B and T lymphocytes, also known as B cells for "bone marrow derived lymphocytes" and T cells for "Thymus derived lymphocytes". However,

like all cells of the immune system, both B and T cells originate in the bone marrow. These cells are quiescent in the host's body until their activation, which normally occurs after the first pathogen encounter, triggering a cascade of signalling events, called the adaptive immune response, and resulting in neutralization or eliminating of the invaders. One specific characterization of B cells is producing antibodies making them responsible for humoral immunity. They partly differentiate into plasma cells following stimulation by microbial antigens and produce antibodies which either neutralize or prepare microbes for phagocytosis. T cells, in contrast, as a central element of AIS, facilitate the cell mediated immune response. After the first encounter with their antigen, processed by APC, naïve T cells proliferate into effector "CD4 T helper and CD8 T cytotoxic cells". Cytotoxic CD8 T cells directly attack their infected host cells, bearing the specific antigen, and kill them, whereas CD4 T helper cells either produce cytokines that act cytotoxically or stimulate other effector T and B cells. T helper cells can thus selectively stimulate and activate a sub-population of B cells and induce the rest to differentiate into plasma cells. After an immunological response memory B and T cells persist, and create an immunological memory allowing them to remember eliminated pathogens and fight the subsequent infection with an enhanced response.(9)

1.1.2 Antimicrobial Peptides

The innate immune system produces a vital group of components, including peptides and proteins, with antimicrobial activity (AMPs). These components protect against a broad spectrum of micro-organisms, including gram-negative and gram-positive bacteria, yeasts, fungi, viruses, and even cancer cells.(7) These molecules serve as a constitutively expressed, ever present first-line defence, which can be mobilized and activated upon inflammation and/or injury. Furthermore, they act beyond the first-line defence system by interacting with the adaptive immune system via their immunoregulatory properties, acting as chemokines, or inducing chemokine production.

Up until now, more than 2500 AMPs of different source have been detected (http://aps.unmc.edu/AP/main.php). Most of them share certain common features, such as a small size of 10–50 amino acids, bearing a positive charge and an amphipathic structure. Relying on their secondary structure AMPs can be differentiated into groups with: α-helical, β-sheet; and extended structures. An example for α-helical AMPs is the human cathelicidin and an example for a β-sheet structure is the human defensin, which is usually characterized by two or more β-sheets.(10)

Most AMP expression in the human body has been shown on the skin, intestinal epithelium, oral mucosa and saliva, lung, eye and lacrimal fluid, together with the reproductive tract and vaginal fluids. However, the expression pattern differs depending on the cells and tissue type, and in most cases AMPs are co-expressed as groups that act together. For example, over 20 antimicrobial peptides have been identified in skin.(11) Epidermal keratinocytes secrete a variety of AMPs following exposure to external pathogens. Among them, expression of human β-defensin 1 (hBD1) and RNase7 is primarily constitutive, whereas hBD2 and hBD3 are inducible by injuries or infection.(7) Moreover, induction of human α- and β-defensin has been shown in various epithelia, including human gastrointestinal tract and respiratory tracts.(12) Paneth cells, for instance, as one of the high specific cells of the small intestine inner lining are an important source of AMPs in the small intestine. They are specialized epithelial secretory cells producing AMPs such as, α-defensin, lysozyme C and angiogenin.(13) In addition, the expression of cathelicidin has been shown in several cell types in skin, such as keratinocytes, eccrine sweat glands and sebocytes, and bone-marrow-derived cells within the skin, including neutrophils, mast cells and dendritic cells. Furthermore, Ling-juan Zhang *et al*. showed abundant of cathelicidin in differentiating adipocytes during Staphylococcus aureus infections of skin.(14)

However, emerging data support the view that alterations in expression of specific AMPs predispose individuals to a pathological outcome. On the other hand, the particular bacterial composition of the intestinal microbiota is essential for maintaining physiological and homeostatic functions of the immune system,

since a disruption of this important ecosystem can result in devastating disorders. Consequently, accumulated data suggest that inflammatory immune response induction occurs following attenuation of intestinal AMP activity, indicating the important role of AMPs for balancing and maintaining the intestinal microbiota and delineates them as an essential factor for the host defence system.(15) For example, inefficient production of defensins has been seen in epithelial cells of patients suffering from Crohn's disease. Along with weakening antimicrobial properties of the respective mucosa this deficiency results in bacterial colonisation and imbalance of the microbiota composition promoting an immune response and ultimately chronic inflammation. Interestingly Crohn's disease of the ileum has been shown to be associated with a deficiency in paneth cell production of α-defensins and Crohn's disease of the colon has been associated with low transcription of the β-defensin genes, leading to reduced production of β-defensin in the colonic mucosa. Similarly, reduced expression of particular AMPs, such as cathelicidin or hBD2, together with Staphylococcus aureus colonies, has been detected on the skin of patients suffering from atopic dermatitis, indicating the important role of those AMPs in skin health.(16) On the other hand, overproduction of AMPs can also initiate inflammatory diseases. Psoriasis, an autoimmune skin disease is characterized by excessive expression of different AMPs, including psoriasin (S100A7), cathelicidin, hBD2, hBD3 and calprotectin in the affected skin areas.(17) Similarly, abnormal epidermal expression and enhanced proteolytic activity of AMPs, such as cathelicidin has been seen in rosacea, another autoimmune skin disease, mainly affecting the central part of the facial skin.(18)

Together with direct antimicrobial activity, AMPs also possess immunological and immunomodulatory activity, assisting the host's defence system and modulating the host's immunity at the cellular level. They protect their host by modulating a range of different mechanisms such as cytotoxicity, wound healing, and chemotaxis,(19) and by shaping the composition of the microbiome. In addition, they confer chemotactic activity, for instance on leukocytes, modulate the host-cell responsiveness to TLR ligands, stimulate

angiogenesis, enhance leukocyte/monocyte activation and differentiation, and modulate the expression of pro-inflammatory cytokines.

Of the many different AMPs discovered so far, cathelicidins and defensins are of the oldest, and best studied. Their antimicrobial properties led to their discovery. The cathelicidin family was named after its precursor domain, cathelin. It is the product of the CAMP gene (cathelicidin antimicrobial peptide), an α-helical type AMP expressed in leucocytes such as neutrophils, NK cells, monocytes, T and B cells, and in epithelia such as skin, testis, gastrointestinal and respiratory tract. It has also been isolated from plasma, airway surface fluid and wound secretions.(20) In humans, the first isolated mature peptide from that family was LL-37, thus named because of the two leucine residues and 37 amino acid length of the peptide.(2) The field of activity of cathelicidins is broad and includes direct pathogen killing by non-enzymatic disruption of the membrane. After binding to a pathogenic cell membrane through electrostatic interactions between the cationic part of the peptide and negatively charged phospholipids of the membrane, cathelicidins destroy the membrane integrity and promote lysis of the targeted microbes by forming pores in the cell wall, causing cellular contents to leak out, and resulting in the cell's death.(21, 22) Further biologically relevant activities described for this peptide include immunomodulatory properties such as leukocyte recruitment, leukocyte activation (neutrophils, monocytes, mast cells, T cells), chemoattraction, promotion of cell proliferation, facilitation of cell migration and survival, angiogenesis, promotion of wound healing, suppressing TLR signaling and suppressing pathogen-induced inflammation.(7, 23-25)

High amounts of cathelicidin in healing wounds of healthy people indicate its important role in re-epithelialisation and wound healing.(26) Conversely, its deficiency in chronic wounds, especially in DFU, most likely accounts for the susceptibility to infectious diseases of this patient population, resulting in poor healing success.(27) For example, Rivas-Santiago *et al*. investigated the expression pattern of several AMPs in wound biopsies obtained from diabetic patients and noted compromised secretion of cathelicidin with decreased or even absent gene expression in DFU compared to healthy controls.(27)

Interestingly, compromised skin expression of cathelicidin has also been shown in patients suffering from atopic dermatitis, a possible explanation for increased susceptibility to infectious diseases in those patient populations.(20) Cathelicidin efficiently stimulates wound healing *in vitro*(25) and *in vivo*(28) by enhancing re-epithelialization and granulation tissue formation,(29) angiogenesis, keratinocyte migration and of course by its antimicrobial capacity. Increasing evidence indicates broad antimicrobial activity of cathelicidin against various types of pathogens including bacteria, enveloped viruses and fungi and even parasites. For instance, antibacterial activity against gram-negative organisms such as E. coli, P. aeruginosa, Klebsiella pneumoniae, Acinetobacter baumanii, Salmonella typhimurium, Stenotrophomonas maltophilia, Proteus vulgaris, Neisseria gonorrhoeae, and Gram-positive organisms including Staphylococcus spp., Streptococcus spp., Enterococcus spp., Bacillus spp., Lactobacillus acidophilus, Lysteria monocytogenes and Propionibacterium acnes has been observed. Cathelicidin is also effective against mycobacteria species including Mycobacterium smegmatis, Mycobacterium bovis and Mycobacterium tuberculosis.(30) Furthermore, based on an analysis conducted by Benincasa *et al*. there is evidence for fungicidal activity of cathelicidin against Candida albicans *in vitro*.(31)

According to Koczulla *et al.* induction of angiogenesis by cathelicidin is mediated by formyl peptide receptor-like 1 signaling, a seven-transmembrane, G-protein-coupled cell receptor expressed on endothelial cells, macrophages, neutrophils and lymphocytes. Koczulla showed elevated angiogenesis following cathelicidin treatment *in vitro* and *in vivo*, in both physiologic and pathologic models of angiogenesis, as a result of direct activation of endothelial cells leading to enhanced proliferation and formation of new vessels.(20) Moreover, in line with Tokumaru *et al.,* wound healing enhancement of cathelicidin is in part mediated by its capacity to induce keratinocyte migration, which occurs via heparin-binding-EGF-mediated transactivation of EGFR.(28)

In addition, amongst all the pleiotropic roles of cathelicidin, it has recently been noted that this peptide also has a functional role in islet cell hormone secretion and glucoregulation by enhancing intra-islet communication. β-cells of the islets

of Langerhans of the pancreas have been proposed as a novel expression site for cathelicidin in humans. This compelling evidence, conducted by Pound *et al*., revealed, above all, a positive impact of cathelicidin on islet cell function and regeneration. Moreover, previous studies have implicated cathelicidins beneficial role in a variety of human diseases and have suggested positive impact on colon cancer, promotion of the healing of gastric ulcers, and contributing to a better chemotherapeutic outcome multidrug-resistant cancer cell treatment.(32)

Defensins, one of the most prominent AMP families, are subdivided into two main subfamilies, the α- and the β-defensins. Similar to cathelicidins, they are cationic peptides and are included in the membrane-disrupting AMP group, since they have the capacity to directly kill pathogens. They possess broad-spectrum antimicrobial activity along with various immunomodulatory properties, such as chemoattraction for human neutrophils, and contribute to the induction of the adaptive immune response by recruiting dendritic cells and T cells to the site of infection. They are expressed in leukocytes and epithelial layers in response to various pathogenic stimuli. However, of the 4 well-characterized human β-defensins (BDs,) predominant expression has been shown in the epithelial layers of the human body. The hBD-1 are constitutively expressed, while the expressions of hBD-2, - hBD-3, and, hBD-4 are inducible.(33) Similar to cathelicidin, decreased expression of hBD-2 has been observed in diabetic ulcers and peripheral blood cells of patients suffering from non-insulin dependent diabetes mellitus, suggesting higher susceptibility to the development of infections. In addition induction of hBD-2 expression has been achieved following 1,25-dihydroxyvitamin D3 treatment in those patient population.(25)

Other AMPs also have the ability to disrupt bacterial cell walls by enzymatic digestion. One famous example is lysozyme, the first ever AMP reported in the year 1922 by Alexander Fleming.(7)

Psoriasin or S100A7, is a member of S100 calcium-binding protein family involved in different cellular processes. It was initially identified in psoriatic

keratinocytes for its overexpression in the skin lesions of psoriatic patients.(34) However, various non-specific functions had been attributed to psoriasin prior to its identification as an antimicrobial peptide. Moreover, antimicrobial activity against various pathogens have been proposed for psoriasin including gram negative E. coli and P. aeruginosa and gram-positive S. aureus.(35) In addition to skin keratinocytes, it is expressed on oral mucosal surface in lingual epithelial cells.(36, 37)

Calprotectin is a heterodimeric AMP consisting of two protein monomers, S100A8 and S100A9. It was first described in the 1980s as an AMP with a broad spectrum antimicrobial activity against different bacteria and fungi.(38, 39) Calprotectin has diverse functions; it is involved in different processes of the immune system, including cell signalling, cell cycle regulation, cell growth, and cell differentiation. Elevated calprotectin expression in the intestinal mucosa has been found in patients with inflammatory diseases, such as psoriasis, and in patients with Crohn's disease or ulcerative colitis. Up to 10-fold elevations in faecal calprotectin levels have been detected in patients with inflammatory bowel diseases. Those findings indicated that neutrophils migrated into the intestinal mucosa in response to intestinal inflammation. Accordingly, the faecal calprotectin level is one of the most commonly used biomarkers for monitoring intestinal inflammatory activity in those patient populations.(40-42)

Ribonucleases (RNase) are another family of constitutively expressed hydrolytic enzymes involved in the innate immune system. Since their first discovery in the 1960s, many different members of the RNase family have been identified.(43) Amongst other activities, these proteins show cytotoxicity induced by RNA cleavage, leading to cell apoptosis. Furthermore, RNases play important roles in angiogenesis and antibacterial and antiviral defences; hence, they are considered a first line of defence against RNA viruses. RNase 3 or Eosinophil cationic protein (ECP) is a component of eosinophil granules in humans, with broad antimicrobial activity against viruses and helminths, but also exhibits cytotoxic activity.(44) RNase 5 or angiogenin is a key stimulator of angiogenesis with antimicrobial properties. Increasing evidence has indicated that angiogenin is involved in the innate immune system. Angiogenin is induced or activated

upon infection or inflammation. It has been detected in tears, where it serves as an antimicrobial component of the ocular system, and it was also found in the Paneth cells of intestinal mucosa. Additionally, further antiviral activities against HIV, Hep B and Hep C have been demonstrated for this protein.(45, 46) Angiogenin also acts as a neurotropic and neuroprotective factor. Angiogenin mutations were shown to be associated with specific neurodegenerative diseases, such as amyotrophic lateral sclerosis and Parkinson's disease.(47) RNase 7 is an AMP that has been well investigated, with potent broad spectrum antimicrobial properties against gram-negative and gram-positive bacteria.(48) Moreover, evidence is emerging that, along with expression in epidermis, this AMP is also significantly expressed in the human kidney and urinary tract, playing an essential role in maintaining tract sterility.(49)

Many AMPs also have the ability to combat MDR bacteria and even inhibit biofilm formation. Furthermore, they have the ability to disrupt an existing biofilm.(50) This specific characteristic is of particular interest in view of growing bacterial resistance against common antibiotics. For example, the antibiofilm properties of human cathelicidin, which have been well analysed, down-regulate genes required for biofilm development and hence decrease the adhesive capacity of bacteria.(51) In addition some other AMPs have the ability to bind lipopolysacharids or endotoxins on the bacterial cell wall and neutralize them, preventing potential consequences such as septic shock.(52) Yet other AMPs have cytotoxic and other anticancer properties. For instance, induction of tumour cell apoptosis has been shown for hBD-1, suggesting it to be a potential tumour suppressor in renal cell cancer.(53) Moreover, as mentioned above, some AMPs can recruit leukocytes and/or induce the expression of cytokines and chemokines at the site of inflammation. For example, human α-defensins (HNP-1 and HNP-2) have been shown to play a role in recruiting monocytes to the site of inflammation.(54)

1.1.3 Idea of Secretome Based Therapy

In recent years, numerous investigators have studied stem cells derived from various sources in their quest for a potential therapy for DFU. Eventually, the main focus of that research shifted to MSCs, due to their specific characteristics. In brief, MSCs are multipotent progenitor cells with the ability to differentiate into mesenchymal tissue, which synthesizes growth factors and cytokines. Thus, MSCs can contribute to the repair and regeneration of dysfunctional wound tissue. However, existing literature indicates that stem cells from DFU patients or other populations suffering from chronic wounds are impaired and defective.(55-57) Hence to overcome this deficiency and achieve wound healing success, those patients require delivery of healthy functional MSCs. Stem cell therapy, when applied to a non-healing wound, promotes healing through upregulation of various anti-inflammatory cytokines and growth factors, upregulating angiogenesis, modulating MMP balance and differentiation of various tissues.(58)

Early investigators believed that the beneficial results of stem cell therapy were due to the differentiation and engrafting capacity of the cells. However, subsequent data indicate that the implantation rate of MSCs is often too low to be effective. For instance, MSC survival has been reported to be below 1% a week after transplantation, suggesting that the observed effects are achieved by paracrine factors.(59) This gave rise to the idea of using conditioned media therapeutically, and in 2005 Gnecchi *et a*l. were able to demonstrate promotion of tissue regeneration solely by paracrine factors released from MSCs, without the presence of any cells(60) In addition, antimicrobial, angiogenic and wound healing capacities have also been demonstrated for the MSC secretome.(61-63) These data laid the cornerstone for a novel therapeutic approach, namely secretome based therapy, leading to several investigations on different cell types. In brief, ‘secretome’ is the term given to the mixture of compounds secreted into the extracellular space by conditioned cells. It consists of soluble

proteins, free nucleic acids, lipids and extracellular vesicles subdivided into apoptotic bodies, microparticles and exosomes. All these substances are necessary for conditioned cells, and finally, the organism, to survive.

Amongst others, the secretome of peripheral blood mononuclear cells (PBMC) has been demonstrated to be only moderately different from that of stem cells.(64) Subsequently, Ankersmit and his team conducted several pre-clinical and clinical investigations, and could demonstrate tissue protection and regenerative properties of conditioned culture medium derived from blood mononuclear cells (MNC). Their most exciting findings are summarized below: (i) recovery of infarcted area after acute myocardial infarction in an animal model;(65) (ii) attenuation of microvascular obstruction after heart attack;(66) (iii) protection against autoimmune myocarditis;(67) (iv) reduction of the infarction area after an acute ischemic injury in a rat stroke model;(68) (v) enhancement of wound healing together with accelerated re-epithelialization and neo-angiogenesis, and up-regulation of pro-survival signaling pathways in a mouse model of wound healing;(69) (vi) augmentation of wound healing along with neo-angiogenesis and decreased numbers of mast cells in a porcine burn wound model;(70) (vii) improved neurological function following spinal cord trauma;(71) safety and tolerability of the their product (APOSEC®) have been investigated in a clinical Phase I study in human subjects.(72) Further effects have also been reported, including regenerative, pro-angiogenic and inflammation-modulating capacities, as well as cell mobility-enhancing properties. Furthermore, conditioned culture medium derived from stressed MNCs, augments cytoprotective and anti-apoptotic gene products in primary cultured human cells along with suppression of CD4+ T cell proliferation.(73)

1.1.4 APOSEC®

APOSEC®, an acronym for “apoptotic secretome”, and is the secretome released by cultured, dying PBMCs. Human MNCs on in other words peripheral blood mononuclear cells (PBMCs) all have a round nucleus, and comprise

several immune cell types: lymphocytes (B cells (~15 %), T cells (~70 %)), monocytes (~5 %), natural killer cells (NK cells) (~10 %) and dendritic cells (1-2%).(74-76) These blood cells are critical components of the immune system, necessary for the elimination of pathogens. They are able to adapt to invaders. In fact, APOSEC® consists of a multitude of different key factors secreted from stressed PBMCs which are essential for their survival. It comprises numerous different soluble factors, including proteins such as, among others, cytokines and growth factors, as well as diverse regenerative and pro-angiogenic substances, lipids, free nucleic acids and extracellular vesicles, such as exosomes, microparticles and above all, antimicrobial peptides.(73, 77) To achieve the ultimate release of the secretome and to increase the secretory output of PBMCs, apoptosis was induced via ionizing radiation at 60 Gy. Furthermore, to avoid transmission of infections, a two-step viral inactivation and pathogen reduction essay, including photodynamic treatment with methylene blue (MB) plus visible light and gamma radiation, has been instituted. The advantages of APOSEC® include easy access to the source cells and acellular content of the product, preventing the risk of an unwanted cell dependent immune reaction.

The production of APOSEC® has been described in detail in our previous studies.(68, 78, 79) Briefly, the following steps delineate the preparation process: human PBMCs are obtained from healthy volunteers by venous blood draw. Anticoagulated blood specimens are diluted 1:2 in Hanks balanced salt solution, followed by cell separation via Ficoll-Paque density gradient centrifugation. The tubes are centrifuged for 15 minutes at 800 ×g at room temperature without braking, and buffy coats with MNCs are obtained. Cells are washed in HBSS and resuspended in CellGro serum-free medium. Cell concentrations are determined on a Sysmex automated cell counter. Apoptosis of PBMCs is induced by Cesium-137 irradiation at 60 Gy. Supernatant of PBMCs are collected after 24 incubation and serve as experimental entities.

1.1.5 Mechanism of Cell Death

Cell death is a highly organized biological process and an essential part of tissue homeostasis. In the physiologic context it serves many diverse purposes including, targeted elimination of irreversibly damaged cells, or neutralisation of cells potentially harmful to the organism such as infected cells. Generally speaking cell death can occur passively, for example due to external forces, or actively by the cell itself committing suicide, for example in certain stages of the embryonic development. Furthermore cell death can occur accidentally, as a consequence of physical and chemical damages, like extreme altering temperature or pH value, or cell death induction can happen in a regulated manner by activation of death signals. However each form of cell death displays distinctive morphological and biochemical manifestation. Based on these characteristics different forms of cell death have been classified.

Apoptosis, for example, is a well known mode of programmed or regulated cell death and follows a controlled, predictable process. It occurs when cells are under stress, for instance when they undergo irradiation. It's an important option for a normal development and functioning of the immune system, embryonic development and many other processes in the organism. It takes place as a consequence of caspase activation. It is characterized by several morphological and biochemical changes, including the membrane blebbing and formation of apoptotic bodies containing nucleus and cytoplasm fragments, which are presented to phagocytic cells, or hypercondensation (pyknosis) and fragmentation of the cell nucleus (karyorrhexis) in order to minimize damage to the surrounding tissue.(80)

Necrosis is strictly non-programmed or unregulated passive from of cell death, characterized by loss of membrane integrity, cell swelling, and release of intracellular contents into the extracellular space, thereby activating the immune response. Necrosis is usually not associated with caspase activation.(80) It occurs when a cell is damaged, usually by an external force, such as ischemia or mechanical injuries, leading to inflammation that can cause further injuries within the organism.

Necroptosis is a kind of regulated and programmed inflammatory mode of cell death driven by particular molecular pathways in response to, for example,

chemical exposure, ionizing radiation, ultraviolet light, reactive oxygen species. and endogenous or pathological factors.(81) Despite several common elements shared with apoptosis, necroptosis is caspase independent and can be initiated by death receptor activation such as TNFR1. Moreover the kinase activity of RIPK3 plays an essential role in TNF-induced necroptosis, and the kinase activity of RIPK1 also mediates the induction of necroptosis in some certain settings. The morphological changes include swelling of cells and organelles because of inflow of extracellular fluid into the cells as a consequence of hyperpermeabilized membrane. This, on the other hand, results in membrane disintegrity and leakage of the cell content into the extracellular space. One further distinguishing feature of the necroptosis is the formation of non-fragmented enlarged nuclei.(82) Along with serving as a line of defence against intracellular infection(83), necroptosis is implicated in a wide range of disease states including myocardial infarction(84), stroke, atherosclerosis(85), ischemia-reperfusion injury(86), pancreatitis(82), inflammatory bowel disease(87) and rheumatoid arthritis(88).

1.2 Pleura

The pleura is a double layered membrane lining the thoracic cavity on the parietal side (parietal pleura), and encapsulating the lungs on the visceral side (visceral pleura). It is derived from mesoderm during the early stage of human embryological development.(89) The pleura is important for the functioning of the lung since its anatomical shape imparts flexibility during inspiration and expiration or as the organ grows. The space between the two layers, which is called the pleural cavity, is a closed space filled with about 1 ml of fluid containing 1–2 g protein/100 mL. It serves as lubricant, facilitating the sliding motion of the two pleural layers against each other. The turnover of this fluid is estimated to be about 200-250 mL/day with a variable flow rate dependent on fluid production.(90, 91)

Pleural fluid is produced and reabsorbed by parietal pleura in response to differing stimuli. For instance, the flow rate can be upregulated by inflammation.

Imbalance between production of pleural fluid and filtration capacity results in accumulation of that liquid in the pleural space, forming excessive pleural effusions containing diverse immune cells. This immunological response of the mediastinum is a prominent manifestation of an underlying disease process of different origins, triggered by variety of inflammatory conditions.

The major underlying mechanisms are listed below:

- Infectious conditions or malignancies leading to increased capillary permeability
- inflammatory state, malignancies, traumata, thromboembolic events, resulting in increased permeability of the pleural membrane partly, due to decreased lymphatic drainage capacity
- Atelectasis of the lung, mucus-ridden bronchia or lung fibrosis causing decreased pressure in the pleural space

It is common knowledge that the monolayered pleural mesothelium, developed from embryonic mesoderm, coats the pleural inner surfaces. Those mesothelial cells are the predominant cell types of the pleura, and play a number of vital roles. For example, they prevent membrane adhesion by producing and releasing the lubricant fluid between the layers. The existence of pluripotent progenitor-like mesothelial cells has been indicated by a limited number of data, which show that they respond to their microenvironment and environmental stimuli by high plasticity, and differentiate into several different cell types, including adipocytes, endothelial cells, and osteoblasts. (92) They respond to injuries by proliferation and migration to the injured lesion, thereby initiating regeneration and tissue repair. Moreover, as a central component of the pleural immune system, pleural mesothelial cells play a key role in initiating an immune reaction when exposed to pathogenic stimuli. In addition to acting as a mechanical barrier against invading pathogens, mesothelial cells can present antigens to initiate inflammation, and they recruit diverse inflammatory cells into the infected pleural space by releasing several cytokines, such as tumour necrosis factor-α (TNF-α), interleukin-1 (IL-1), interleukin-6 (IL-6), interleukin-8 (IL-8) and antimicrobial peptides. They consequently activate adaptive immune

responses to recruit further anti-inflammatory cells.(92) Mesothelial cells also respond to inflammatory stimuli by releasing various mediators such as plasminogen activation inhibitor, macrophage inflammatory protein, monocyte chemotactic peptide among others. For example, in response to an invasion of Pseudomonas aeruginosa, they release fibronectin, which prevents adherence of those microbes. In addition, high amounts of interferon-γ (IFN-γ), an important pro-inflammatory cytokine, have been found in the pleural effusions of patients with tuberculous pleurisy.(93) In animal models, after activation by a staphylococcal peptidoglycan, mesothelial cells were shown to release a specific AMP, human beta defensin 2 (hBD2).(92) hBD2 enhances the immune response by recruiting other inflammatory cells to sites of inflammation, including dendritic cells, T lymphocytes, and neutrophil granulocytes.(94, 95) Previous data also indicate that after exposure to specific cytokines such as TNF-α or IFN-γ pleural mesothelial cells express an adhesive glycoprotein, “intercellular adhesion molecule (ICAM)-1”, on their surface, enabling the adherence of those cells to the immune cells. This facilitates the migration of the arrested immune cells through the intercellular spaces into the pleural compartment. These recruited leukocytes, which include, amongst others, neutrophils and MNCs, account partly for the elevated protein content of the pleural effusion, especially augmented amounts of AMPs. Indeed, according to a growing body of evidence, both recruiting leukocytes and the pleural epithelium play important role for the elevated concentration of immune factors secreted into the pleural effusion. For example, IL-8 has been reported to play an important role in the development of acute pleural inflammation. Moreover, IL-8 was shown to be expressed in stimulated mesothelial cells in response to pro-inflammatory cytokines, such as IL-1β, TNF-α, and lipopolysaccharide (LPS).(96)

Additionally, when pleural mesothelial cells are triggered by toll-like receptors, they can induce an immune response. TLRs are a group of transmembrane receptors, that are expressed mainly on the surfaces of leukocytes, but also on the surfaces of a view of epithelial cells, including the pleural mesothelium. They recognize various components of pathogens by the process of pattern

recognition.(97) Pattern recognition takes place at the extra-membrane ligand-binding site of TLRs, where pathogen ligands, with their specific structural shapes, called “pathogen-associated molecular patterns (PAMPs), bind to the TLRs of the host. This binding enables host cells to interact with the cellular environment, which results in enhanced antigen presentation, the activation of the innate immune response, and consequently, the activation of antigen-specific adaptive immune responses.(98)

Altogether, the pleura and its mesothelium are essential components of the immunology and metabolism of the lung. They provide a mechanical barrier and they lubricate the pleural cavity by producing pleural fluid. Additionally, they provide protection by activating TLRs, which leads to the induction of inflammatory cytokines. Subsequently, these cytokines initiate leukocyte recruitment, predominantly mononuclear cells, which lead to increased secretion of diverse immune factors, including AMPs. Pleura and the pleural mesothelial cells thus take an active part in immunological processes of the mediastinum. Elevated protein content of the pleural effusion is one prominent marker of pleural involvement in disease processes.(99)

1.3 Diabetic Foot Ulcer

1.3.1 Background

A wound is considered to be any kind of injury or disruption of the skin, leading to activation of cascades of events, including cell migration, inflammation, formation of granulation tissue, innervation, angiogenesis…. Wound healing is a complex process consisting of several highly regulated overlapping processes working in concert, including: homeostasis, inflammation, proliferation/repair and remodelling.(100) Many factors, including comorbidities, medication, nutrition, and lifestyle, play significant roles in this process. Based on the healing time, wounds can be distinguished between acute and chronic. Acute wounds are defined as skin defects not lasting longer than several weeks. For example, a superficial injury such as a small cut needs a few days to heal,

whereas wounds after abdominal injuries sometimes take up to several weeks to heal completely.

Immediately after an acute injury occurs, coordinated cellular and molecular responses begin to initiate the wound regeneration. The first step, called “primary homeostasis” is the adherence of blood thrombocytes to each other and to the site of injury forming a thrombo-aggregate. This aggregation results in the formation of a platelet plug. This process is followed by “secondary homeostasis”, which refers to the formation of a cross-linked fibrin network by activated coagulation factors, and serves to strengthen and stabilize the plug.(101) Among the important roles of the clot is the production of chemotactic factors released by activated platelets. These attract immune cells, including neutrophils, macrophages and monocytes to the wound site, initiating the second step of wound healing, called inflammation. Its primary goal is to clear pathogens from the wound site and to restrict the spread of damage. The vast majority of the recruited immune cells during this step of wound healing are neutrophils.(102) They mainly clean the wound of debris and bacteria within minutes following injury by engulfing and killing pathogens, with the aid of antimicrobial factors.(103) Neutrophils also provide signals that activate surrounding fibroblasts and keratinocytes, initiating the proliferation phase of wound healing.(104) However, their activity decreases with time and they die after a few days. Concomitantly, pro-inflammatory signals induce monocytes which have migrated to the wound, to become activated and to mature into macrophages. They undertake the neutrophils’ tasks, including phagocytosing pathogens, debris and dead neutrophils.

Macrophages are an important member of inflammatory cells. Their presence is dominant, between days 3 to 5 of the wound healing process.(105) They orchestrate the healing process by continually synthesizing and secreting chemokines into the wound environment. Over 40 chemokines, their function, and expression patterns have been investigated so far, and their complex role in leukocyte recruitment has been well documented by others.(106) Activated by degranulating platelets and neutrophils, they maintain wound repair signals, leading to the next phase of wound healing, called cell proliferation and tissue

reconstruction. In this phase, activated fibroblasts proliferate and migrate into the wound site to form granulation tissue. Granulation tissue fills the wound from the ground and provides a new connective tissue during the healing process. Next, fibroblasts synthesize, secrete, and modify extracellular matrix components, such as collagens, proteoglycans, and fibronectin; thus, fibroblasts are considered to be the primary source of extracellular matrix. Some fibroblasts differentiate into myofibroblasts and acquire contractile and secretory properties. By assisting the contraction of the wound edges, myofibroblasts control tissue architecture, leading to better wound closure.(107) Granulation tissue formation usually occurs around day 4 after the wound healing process begins and persists up to weeks depending on wound size, depth, type and localization. A crucial function of granulation tissue is to support the ingrowth of new blood vessels into the wound tissue, which provide nutrition and oxygen to the growing tissue; this process is termed angiogenesis. Angiogenesis is induced by growth factors derived from macrophages and damaged endothelial cells, and it plays a pivotal role in the wound healing process. Subsequently, keratinocytes migrate through the basal into the upper skin layers to cover the denuded wound surface. This process, called re-epithelialization, is important in maintaining the upper skin layer, or the epidermis. Re-epithelialization restores the barrier function of skin after an injury and leads to effective wound healing.(108) Finally, the final phase of the wound healing process is maturation and remodelling, characterized by a gradual loss of existing extracellular matrix. This process is assisted by proteolytic enzymes (proteases), such as matrix metalloproteinases, which is a family of enzymes responsible for cleaving specific proteins in the ECM.(109) This process allows contraction and reorganization of the wound tissue in order to regain tissue strength by replacing early collagen by a denser, stronger form of collagen-rich ECM. Moreover, degradation of collagens consequently leads to reduced myofibroblast activity which, in turn, causes the cells to undergo apoptosis at the end of the wound healing process.(110, 111) At the same time, endothelial cells involved in angiogenesis also undergo apoptosis, resulting in regression of capillaries during the maturation of the wound tissue. Depending on the wound

size, depth and severity of the injury, this last step can take up to several years to complete.(112)

Thus, wound healing involves several highly regulated stages. Disruption at any point can lead to delay or even cessation of the whole process, resulting in chronic, non-healing wounds. Chronic wounds, in contrast to acute wounds are by definition ulcerative skin defects, which have not healed within 3 months after injury.(113) Chronic wounds develop due to deviations from the previously described pathways. Examples of chronic wounds include DFUs, venous leg ulcers, and pressure ulcers. Interestingly, they share specific common characteristics, despite etiological differences at the molecular level. These characteristics, known as hallmarks of chronic wounds, include persistent and prolonged inflammation, diminished angiogenesis, attenuated epithelialisation and increased hyperkeratosis, persistent and in part therapy-resistant infection(114).

During the past few years, huge improvements have been achieved understanding the biology of wound healing. Numerous investigations have identified distinct cell subpopulations with different healing capacities in chronic wounds compared to acute wounds. For instance, absence of fibronectin and abundance of collagen, reduced vessel sprouting and few myofibroblasts has been demonstrated by Herrik *et al.* in a histological analysis on chronic leg vein ulcers. Furthermore, he described a robust infiltration of inflammatory cells, consisting mostly of neutrophils differing in their phenotypical characterization to their equivalents in acute wounds.(115) In addition, Brem *et al.* found fibroblasts with different morphological features and a pathogenic phenotype (clumped, misshapen and inflated cells with enlarged nuclei and impaired migration capacity).(116) Finally, Stojadinovic *et al.* found hyperkeratosis in the biopsies from non-healing edges of venous ulcers as a result of proliferative activation of cell-cycle genes with suppression of checkpoint regulators.(117) Furthermore microbial infection, as a result of repeated tissue injury, causes permanent recruitment of immune cells into the wound area, leading to an elevated and prolonged inflammatory response. This concomitantly elevates the host's protease expression in the wound tissue, along with augmenting bacterial

protease production, worsening the chronification of the wound. In contrast to acute wounds, where the level of proteases is precisely regulated by their respective inhibitors, this cascade is disrupted in chronic wounds such that accumulation of proteases in the wound tissue results in destruction of the ECM. Together with preventing the wound from healing appropriately, proteolytic destruction of the ECM also contributes to amplifying inflammation by attracting more inflammatory cells.(118, 119)

Another factor affecting wound healing is angiogenesis. By definition, angiogenesis is the ingrowth of new blood vessels from pre-existing vessels into newly build tissue. The new vessels maintain efficient tissue perfusion, and ensure an adequate supply of nutrients and oxygen.(120) Adequate wound tissue oxygenation is essential for proper wound healing as exemplified by people performing regular exercises, who tend to have faster wound healing due to better oxygenation, because of better angiogenesis.(121) For instance, oxygen is required by proliferating cells(122), or to maintain the neutrophils' respiratory burst, which generates antimicrobial ROS, that eliminate internalized particles during phagocytosis of microbial intruders.(123, 124) While low ROS concentrations provide defence against invading pathogens, a hypoxic and inflamed wound environment can lead to increased oxidative stress, and results in significant ECM damage and even cell damage, worsening the inflammatory state of a chronic wound.(125) It has been noted that the minimum oxygen pressure, required for appropriate wound healing under physiological conditions, is about 20mm Hg.(126) In contrast, oxygen tension values of about 5mm Hg have been measured in chronic wounds (127, 128), with decreased angiogenesis and few sprouting vessels in those wounds. Thus decreased oxygen supply will not only result in increased necrotic debris in the wound bed, facilitating bacterial growth, but also in compromising the elimination of those invading pathogens.

Another indicator that is typically used to determine wound healing success is re-epithelialization, an essential concluding step in the wound healing process. Re-epithelialization is compromised in chronic wounds. Growing evidence has demonstrated that chronic wounds harbour senescent populations of

keratinocytes and fibroblasts with impaired proliferative and secretory capacities. Those findings suggested that deficient cell populations might contribute to inadequate re-epithelialization.(116, 129-133) The development of the senescent phenotype in these cells is probably due to the oxidative stress in chronic wounds, with DNA damage following ROS attacks.(114) This senescence of keratinocytes also leads to development of hyperkeratosis and failure to achieve complete re-epithelialisation of wound surface.(108) A wound which cannot be considered as healed provides a portal for pathogen invaders, leading to exacerbation of infection, along with wound re-occurrence as a consequence of barrier malfunction, delineating yet another clinical problem.

Since aberrant wound healing is rarely seen in healthy subjects, the development of a chronic wound is mostly associated with an underlying health problem. A well-known example for chronic wounds is the diabetic foot ulcer usually found as a complication of diabetes mellitus. As the name suggests, DFUs occur mostly on the feet. Other chronic complications of diabetes include retinopathy(134), nephropathy(135), and peripheral vascular disease, which results in peripheral neuropathy and ischemia,(136) These latter complications, and particularly a combination of the two, neuroischemia, are noted to be the major causes for DFU.(136) (137) Peripheral neuropathy is a general term indicating disorders of the peripheral nervous system. It is a common neurological disorder, and higher prevalence has been seen among diabetic patients. Impairment of pain, loss of temperature sensation and autonomic symptoms indicate neuropathy in poorly or unmyelinated small nerves, a condition termed small fibre neuropathy. In contrast, sensory ataxia and loss of position and vibration sense are suggestive of injury to larger myelinated nerves, and is called large fibre neuropathy.(138)

Diabetic neuropathy, accountable for about 60% of DFU in diabetic patients, is defined as signs and symptoms of peripheral nerve dysfunction, and is a result of hyperglycaemia-induced metabolic abnormalities. Metabolic disturbances in nerve cells were shown to result from the intracellular accumulation of glucose products. This accumulation reduces the ability of cells to neutralize reactive oxygen species, which leads to exaggerated oxidative stress. This further

decreases the synthesis of vasodilatory factors, resulting in ischemia with subsequent peripheral nerve injury leading to cell death.(139) Most susceptible nerve cells of the body are those with long axons. The most distal parts of the axons are vulnerable, due to difficulties in providing sufficient nutritional support to the end of the axon and the limited blood supply to the fibres; this might explain the common occurrence of diabetic wounds that are located distally. In diabetic patients, all three components of the nervous system: “sensory, motoric and autonomic”, can be affected by the neuropathy. Yet it is the sensory component that is predominantly affected, with impairment in glove and stocking distribution.(140) The neuropathy increases the risk of neglecting physical and thermal traumas, aggravating DFUs due to lack of alerting pain, and loss of protective sensation on the distal areas of the extremities.(141-143) In contrast, distal neuropathic pain occurs only in a minority of diabetic patients at the onset of diagnosis.(144) The most common subtype of diabetic polyneuropathy is the sensorimotor polyneuropathy ranging from slowly progressive distal sensory dysfunction to distal weakness, starting in the lower extremities.(145) Other mild to moderate symptoms are paraesthesia, burning pain and numbness. Distal motor nerve involvement is less prominent and induces foot deformities as a consequence of imbalance between distal flexor and extensor muscles of feet, leading to excessive plantar pressure.(146) This results in skin rupture as a consequence of uneven distribution of pressure, providing a basis for wound development with potential for worsening into a non-healing ulcer. The most common pressure sites are the plantar surface of the toes, the plantar forefoot and midfoot, as well as heels and dorsal surface of the toes.(143) Furthermore, diabetic neuropathy can also have associated autonomic symptoms, termed diabetic autonomic neuropathy, and can affect cardiovascular, gastrointestinal and urinary systems, resulting in metabolic disturbances such as orthostatic hypotension and gastroparesis.(140) This accounts for nearly half of the deaths in diabetic patients because of silent myocardial infarction.(147, 148), another dramatic consequence of diabetes resulting from myocardial ischemia in the absence of characteristic infarction symptoms.(149)

Peripheral vascular disease is a manifestation of microvascular and macrovascular complications of diabetes. It is defined as vascular insufficiency, particularly in the lower extremities, due to atherosclerotic occlusion. Thus, peripheral vascular disease is a potential risk factor and a major cause of DFU development. In most cases, it is also accompanied by cardiovascular and cerebrovascular events.(150) Peripheral arterial disease refers to atherothrombotic events leading to obstruction of peripheral arteries, resulting in ischemia. The ischemia mostly affects vessels in the lower extremities, which suggests that these vessels play an important role in the development of DFU.(150)

Moreover, the consequences of diabetes are also associated with endothelial cell dysfunction, vascular smooth muscle cell dysfunction, and platelet dysfunction. Loss of nitric oxide homeostasis in diabetic patients contributes to deterioration of NO dependent vasodilation, leukocyte-vascular wall interactions, and platelet aggregation.(151) Furthermore, hyperglycaemia contributes to elevated production of ROS, and subsequently increased oxidative stress, resulting in vascular inflammation and decreased vascular smooth cell migration, compromising the regenerative ability of those cells with respect to injured lesions.(152, 153) All these factors together facilitate the formation of atherosclerotic lesions, leading to arterial obstruction, with ischemic consequences, making this disease entity a leading cause of foot ulcers and its respective consequences, including lower limb amputations, one of the most feared complications of diabetes.

Understanding the morphology of an ischemic wound is essential for clinical assessment and classification of DFU. Important factors regarding vascularity and perfusion of wounds include the degree of granular wound bed bleeding during a debridement, an indication of a healthy, well perfused wound. Moreover, the amount of fibrotic and necrotic tissue, along with a desiccated appearance of the wound, indicates impairment in blood supply, necessitating surgical revascularisation prior to local wound treatment. An example is the plantar ulcer, which develops as a result of repetitive mini traumas to the insensate diabetic foot, resulting in mechanical damage leading to an ischemic

ulcer due to vascular insufficiency. Approximately 25% of patients suffering from diabetes develop DFU over their lifetime.(154) Of that population, up to 24% require limb amputation due to wound-healing disturbances, which result in severe wound infection or osteomyelitis, peripheral arterial occlusion, and gangrene.(155) On the whole, sooner or later, all types of DFUs are accompanied with high morbidity and mortality; thus, patients with DFUs have a poor prognosis for survival. Existing literature indicates the 5 year mortality rates of those patient population to be about 55%(156, 157) This rate is aggravated among amputated patients, approaching 33% within 3 Years.(158)

Growing evidence has argued that persisting, prolonged inflammation in chronic wounds, characterized by an amplified inflammatory cycle, along with delayed healing is probably due to microbial infection. Infection is a further aspect of chronic wounds and is a serious health issue, particularly in patients with diabetes. Along with contribution to delayed wound healing, infections are accountable for considerable morbidity, which leads to limb amputations and reduced quality of life in physical, psychological, and social arenas. These infections represent a significant burden, to both the patient and the health system. It is currently known that all chronic wounds have some kind of bacterial contamination, however only half have a clinically relevant infection.(159, 160) Curently, the presence of at least 10^5 bacteria was determined as the critical threshold for differentiating between a bacterial colonization and an infection.(122, 161) However, according to the literature, up to 50% of all diabetic ulcers will become infected, which in turn increases the risk of an amputation by 50% compared to DFU patients without infection.(162, 163) Elderly, as well as immunocompromised patients are predominantly at risk. Without appropriate care and management, a local infection can progress into a life-threatening systemic infection, such as systemic inflammatory response disease, multiple organ dysfunction syndrome, sepsis and death. Thus the holistic wound assessment as well as individual host characteristics are important indicators of infection in chronic wounds.(159) Understanding the causative organisms accounting for diabetic foot infection and their antibiotic sensitivity is essential for selecting the appropriate antibiotic therapy. DFUs are

mostly contaminated with multiple pathogenic bacteria. Based on the analysis of Diamantopoulos EJ *et al.,* approximately 83% of all DFI are of polymicrobial origin.(164)

The progression of polymicrobial infections and presence of biofilm building microorganisms can lead to formation of biofilms, a survival strategy sustained by diverse groups of microorganisms existing in a multicellular environment.(165) In effect, microbs form a biofilm by adhering to each other and to the wound surface, and they are sheltered in a self-produced ECM.(160) This protects those microbs against external threats, and it results in prolonged inflammation, due to the inability of antibiotics and human immune system to access the site of infection.(166, 167) In addition to biofilm building microorganisms, other multidrug resistant bacteria can colonize the wound area, independently aggravating the situation. However, multidrug resistant organisms appear to be more sensitive to antibiotic therapies than biofilm-building bacteria.(168) These findings have contributed to a better understanding of prolonged inflammation in chronic wounds, and they partly explain the difficult challenges associated with their treatment.

The most common bacteria found in DFUs include *Staphylococcus aureus* and streptococcus species from gram-positive organisms. Other bacteria in DFUs include *Pseudomonas aeruginosa*, *Escherichia coli*, *Enterococcus*, *Klebsiella* species, *Proteus* species, and so on, from gram-negative organisms.(169-172)

P. aeruginosa, a gram-negative bacteria, is responsible for severe chronic and acute infections that are highly medically important. For example, it is involved in hospital-acquired infections, such as nosocomial pneumonia, and various septic syndromes, including catheter-related sepsis. An opportunistic pathogen, *P. aeruginosa* has a strong tendency to form biofilms; it is the cause of over 80% of lung infections in patients with cystic fibrosis. In addition to these patients, *P. aeruginosa* particularly affects patients with acquired immune deficiency syndrome and burn wounds. Indeed, P. aeruginosa has been reported as the second most prominent biofilm former amongst DFU colonizing pathogens.(173) Another challenge concerning this pathogen is the increasing prevalence of multidrug resistant strains as a consequence of frequent

insufficient use of antibiotics.(174) Certain AMPs have been proposed as alternatives for use against P. aeruginosa infections, promising candidates being cathelicidin,(175), and RNAse3, which has strong antimicrobial activity against this gram-negative pathogen.(176)

E. Coli is another important gram-negative bacterium, and a common resident of the human gut, being part of the normal microbiota in a symbiotic relationship with the host. It is one of the well-studied bacteria, and comprises many different strains, most of which are beneficial without causing any diseases. Nevertheless some have gained specific virulence attributes, associating them with a broad spectrum of human illnesses ranging from gastroenteritis, haemorrhagic colitis, Morbus Crohn, haemolytic-uremic syndrome, peritonitis, respiratory infections, wound colonisation, especially of DFUs, to urinary tract infections and septicaemia, for which it is known to be the most frequent causative agent among gram-negative bacteria.(173, 177) The major transmission route is faecal-oral. Amongst the pathogens isolated from DFU, E. coli is the second leading colonising organism, with considerable biofilm building behaviour.(173, 178)

S. aureus is one important gram-positive bacterial pathogen, with over 30 different sub-species. A highly special characteristic of some of these strains is the ability to produce coagulase, an enzyme causing blood coagulation.(179) *S. aureus* is currently known to be the leading cause of bacteraemia. It is also associated with a broad variety of human diseases, including skin and soft tissue infections, respiratory tract infections, endocarditis, and toxic shock syndrome.(180) Patients treated intravenously, for example, by central venous catheters are at special risk. In addition, immunocompromised patients, such as those being treated for malignant diseases, as well as diabetics are particularly predisposed.(181) Another feared aspect of S. aureus infection is the phenomenon of “Methicillin Resistencia” resulting from the frequent but insufficient use of antibiotics. Methicillin-resistant *S. aureus* (MRSA) strains are resistant to a wide range of currently available antibiotics. MRSA strains are treated with approved antibiotics, including vancomycin, daptomycin, clindamycin, and linezolid, as last resort therapeutics. Nevertheless, some

MRSA strains have evolved to resist even these drugs. Thus, current antibiotic treatment strategies are confronted with frustrating outcomes and increasing mortality.

S. pyogenes, another gram-positive bacteria, has been recorded historically for over two thousand years. *S. pyogenes* is one of the most common infectious agents in humans. Amongst all bacterial pathogens, it is responsible for the widest range of human illnesses. *S. pyogenes* is associated with various diseases, including impetigo, scarlet fever, cellulitis, pharyngitis, necrotizing fasciitis, toxic shock syndrome, and glomerulonephritis.(182) According to the literature, amongst the common bacteria isolated from DFUs, S. pyogenes constitutes about 10% of the isolates and accounts for a considerable part within mixed growth pathogens together with S. aureus.(183) Despite nearly unaffected susceptibility of S. pyogenes to common antibiotic therapies, considerable numbers of unsuccessful antibiotic treatments have been reported during the past years.(182) This can be explained by the fact that the antibiotic resistance mechanism of co-colonizing bacteria plays a protective role for S. pyogenes, and supports its colonization. Intraepithelial and intracellular bacterial persistence, even after an antibiotic therapy, may be due to poor penetration capability of the respective antibiotics, and may explain some of the frustrating outcomes observed.

Banu *et al.* investigated 100 samples from patients with DFU for antibiotic sensitivity and biofilm formation. She recorded a rate of over 90% of multi-drug resistance and 46,3% of biofilm formation among the isolated bacteria. S. aureus was detected as a predominant biofilm former, followed by P. aeruginosa. Furthermore, E. coli has been shown to have the leading percentage of extended spectrum beta lactamase (ESBL) production.(173) In addition Xiaoyan *et al.* investigated 157 patients with DFU for multidrug resistance. In total he isolated 78 bacterial species from different multidrug resistant strains, among them Staphylococcus species, Enterobacter species and Pseudomonas species were the most common. Moreover, besides bacterial infections, persons with diabetes have a higher risk for onychomycosis and toe-web tinea infections with the risk for skin disruption.(154)

The issue of growing multidrug resistance and biofilm formation is pushing our currently available antibiotic therapies toward ineffectiveness, and is becoming a serious dilemma to the public health. Patients suffering from diabetes are at particular risk, due to their diabetes related conditions such as vascular insufficiency, hypoxia and excessive production of ROS, resulting in tissue damage with decreased phagocytic and elimination capacity on the part of the hosts' immune cells, leading to an immunocompromised state compared to a healthy human individual. The immunocompromised condition favours the formation of bacterial biofilms, which in turn, potentiates the ineffectiveness of current antibiotics.

1.3.2 Wound Therapy

Medical wound treatment, especially of chronic wounds, has become a major challenge during the last decades, as chronic modern diseases such as diabetes, obesity or vascular disorders increase all over the world, concomitantly with the increasing population age. Insufficient wound healing after a certain period of treatment should be re-evaluated for the need for advanced therapeutic agents, and the underlying pathology should be reassessed. However, selecting the appropriate treatment plan and continual follow-ups are a prerequisite for a successful therapy outcome.

Therefore a careful wound evaluation especially in patient population suffering from non-healing diabetic wounds is essential for choosing the correct treatment plan. Several DFU classifications have been proposed so far, yet still there is no standardized, uniformly accepted score for assessment of DFU. Wagner's classification(184) is mainly based upon depth and size of the wounds. It consists of six wound grades and the ulcer assessment is as follow: grade 0 (intact skin with pre-ulcerative area), grade 1 (superficial ulcer), grade 2 (deep ulcer to tendon, bone, or joint), grade 3 (deep ulcer with abscess or osteomyelitis), grade 4 (localized gangrene), and grade 5 (whole foot gangrene). The classification system of the University of Texas Health Science Centre, San Antonio(185) grades the DFUs first by depth, followed by

subdividing each group into presence of infection or/and ischemia. Grade 0 represents no open lesion with a pre- or post-ulcerative area. Grade 1: superficial wounds penetrating epidermis or/and dermis, with intact tendon, capsule, or bone. Grade 2: wounds penetrating tendon or capsule, omitting the bone and joints. Grade 3: wounds penetrating deep into bone or joint. Each grade in this system is subdivided into four further classes: clean (A), non-ischemic infected (B), ischemic (C), and infected ischemic wounds (D).

Hence, along with wound size and depth, further factors needing a clinician's attention are: presence of sinus tracts to deeper parts such as bone, extent of granulation tissue, amount of fibrotic, ischemic or necrotic tissue, type and amount of drainage, maceration and hyperkeratotic wound margins, and signs of infection such as erythema, edema, odor.(186)

Proper wound management, particularly in patients with DFU, requires a multidisciplinary approach, including early detection and efficient treatment of an ulcer, initiated by the patient him/herself. Above all, in line with existing literature, correct glycaemic control can significantly improve and enhance wound healing in patients with DFU.(187) Furthermore, self-assessment and regular foot inspection, especially in patients with diabetic polyneuropathy, is the first step towards ulcer prevention. This can prevent the progression of complications and avoid possible amputation. Further approaches comprise surgical interventions for revascularization in the case of wounds with vascular impairment, such as DFUs with ischemic components. This is also an important step towards wound healing, as correct oxygen supply and oxygen pressure of the wound bed are vital for physiologic tissue regeneration. Debridement is a well known technique for the removal of devitalized tissue from chronic wounds, thereby decreasing bacterial contamination. A critical component of wound care, debridement contributes to accelerated wound healing and furthers wound granulation by removing necrotic, infected and non-viable tissue.(188) Moreover, debridement stimulates the wound edges and hereby contributed to better contraction and epithelialization of the wound bed promoting the release of growth factors, which facilitates the wound healing process.(189) Furthermore, it practically converts the chronic state of a wound into a, more or

less, acute wound healing situation.(190) Among the several available methods, like autolytic, chemical, mechanical, and biologic, the surgical method is generally well-accepted und preferably used. Its advantages lie in the simple evaluation of the wound size, depth and severity, as well as easy tissue obtainment for further investigation. It is considered as the gold standard method for treating DFU.(186)

In contrast, pressure offloading is the standard therapy for the treatment of plantar ulcers, since high-pressure areas are predilection sites for ulceration. The relevance of this therapeutic approach to wound closure is demonstrated by the increased reoccurrence after insufficient offloading, even in the case of healed ulcers. In addition, increasing evidence indicates that inadequate offloading contributes to delayed wound healing.(191-193) Pressure relief devices are designed to relieve the pressure on an affected part of the body, thus preventing wound or ulcer formation and are classified into low-tech and high-tech devices. Low-tech devices provide conforming support surfaces that help distribute body weight evenly. These include gel-filled, fluid-filled, fibre-filled, or air-filled mattresses/overlays, standard foam mattresses, and alternative foam mattresses. High-tech pressure relief devices use air-filled sacs that use an electric air pump to inflate and deflate the body surface and relieve pressure. The non-removable total-contact cast is considered as the gold standard primarily for forefoot and midfoot ulcers. The functional principle is to distribute the pressure from fore- and midfoot into the heels, thereby avoiding a permanent ulcer stress, whilst allowing a complete rest of the forefoot.(194) Furthermore, there are also numbers of removable cast walkers and half shoes, short leg walkers and felted foam dressings, which provide only a limited pressure relief compared to non-removable total-contact casts.

Further therapeutic approaches include hyperbaric oxygen therapy where accelerated wound healing is achieved by administration of oxygen at high concentrations.(195) Insufficient oxygen supply at the wound site results in a hypoxic environment, which hampers fibroblast proliferation, capillary angiogenesis, and collagen production. Hypoxia leads to bacterial growth at the wound site, further slowing the healing process. Hyperbaric oxygen therapy

provides sufficient tissue oxygenation, thus aiding faster recovery. This therapy is mostly applicable for wounds with less blood perfusion, such as arterial ulcers, diabetic ulcers, and pressure ulcers. When the patient is placed in an oxygen chamber, 100% oxygen is given under pressure. When haemoglobin becomes saturated with oxygen, the blood continues to be hyper-oxygenated by dissolving oxygen within the plasma. Negative-pressure wound therapy, or vacuum-assisted closure uses intermittent or continuous sub-atmospheric pressure to collect wound discharges and by this means promotes wound closure.(196) The mechanism of action includes stimulating the granulation tissue by lowering edema and increasing blood circulation. One of the most frequently performed therapies amongst physicians for treating chronic wounds is electrical intervention. This therapy has been broadly used for stage III and stage IV pressure ulcers, diabetic arterial ulcers, and venous stasis ulcers. Electrical stimulation is considered as an adjunctive therapy and uses electric currents to transfer energy for wound healing. The current is applied through electrodes placed on either the wound or the skin surrounding the wound. This improves blood flow, stimulates protein and DNA synthesis in fibroblasts, and promotes healing. It also helps in reducing edema and bacterial growth.

In addition to the advanced wound care therapy modalities mentioned above, scientists are working on novel therapeutic method to promote wound healing. All of these advanced products, developed in response to extended knowledge of the impaired wound healing mechanism, address particular deficiencies. Hence the ability to identify and target responsive cells to wound healing stimuli is one crucial step towards developing novel therapeutic modalities.(116)

More specifically, wound care methods are subcategorized into traditional and advanced methods, each designed to enhance the healing process in specific types of wounds. Traditional wound care includes basic products that aim to treat mild superficial wounds, in contrast to advanced wound care that facilitates wound healing by keeping the wound environment moist and it is employed on the more severe wounds. It includes treatment for advanced wounds such as chronic ulcers, diabetic foot ulcers, burns, traumatic wounds, and surgical wounds. There are various advanced products available in this market, which

makes the treatment of these wounds simpler. These products are designed to promote and enhance healing by maintaining wound hydration. Furthermore, a good deal of research has proposed an improved therapy modality, namely “active wound care”, consisting of skin substitutes and growth factor products, facilitating debridement of dead or infected tissues, formation of new tissues, and infection control, which are all the highly potent components of advanced wound care. Active wound care is useful for patients suffering from burns, severe trauma, and third-degree pressure and diabetic ulcers. Grafts used in this approach replace burnt or damaged skin and accelerate the recovery of severe wounds. For instance, topical application of growth factors and platelet-rich plasma on uninfected DFUs was shown to improve wound healing.(197, 198) The use of Matrix Metalloproteinase modulators is increasing, based on preliminary evidence of previous clinical studies, where down regulation of MMP-2 expression may improve the healing process in chronic wounds, since an overexpression of MMP-2 in fibroblasts and endothelial cells has been believed to be responsible for the delayed healing.(199, 200) Wound dressings providing clean and moist wound environment, prevent tissue dehydration and accelerate the wound healing process.(201) Different antimicrobial dressings such as Metronidazole gel against anaerobic bacteria,(202) Sisomycin and acetic acid against Pseudomonas, gram-negative bacilli and beta haemolytic streptococci, Neomycin, Gentamycin, and Mupirocin have shown good local antibacterial effects.(203) Further integrative antimicrobial dressings are betaine, chitin, or polyhexamethylene biguanide.(122) Silver ions have also proven effective against several bacteria, including methicillin resistant S. aureus, vancomycin-resistant enterococci, and extended-spectrum β-lactamase producers. Some rare resistance cases have been reported until now but, more interestingly, despite low adverse effects, silver may be effective against biofilm.(204, 205)

Now that polymicrobial infection and the issue of MDRO and biofilm-formation have been realized as important risk factors for treatment failure in the management of DFU, special care must be taken with adapted antimicrobial treatment, based on the bacterial culture and antimicrobial susceptibility. Of the

numerous topical antimicrobial products developed so far, the beneficial effects in treatment of DFUs are still arguable. Aside from the only real indication for the topical application of antibiotic ointments, namely a clinically infected wound, the advantages of their routine usage are moderate due to possible development of antibiotic resistance and further adverse events.(122) Assessing the severity of DFI is one crucial step toward selecting the appropriate systemic antibiotic therapy, since the empirical choice of antibiotics is initially based on clinical features, disease severity and microbial resistance. Generally speaking, narrow-spectrum oral antibiotics are suggested for mild infections and broad-spectrum parenteral antibiotics for severe infections.(206)

Treatment using active wound products has led to advancements in advanced wound care, which in turn has driven the adoption of sophisticated wound dressings, biologics, and devices. The products under this segment include film, foam, collagen, alginate, hydrocolloid, and hydrogel dressings. Some products are now impregnated with anti-infective substances, such as silver or honey, for rapid wound healing and preventing infections.

Foam dressings are absorbent dressings made of hydrophilic polyurethane foam, available in the form of pads, sheets, and cavity dressings. These dressings create a moist environment and provide thermal insulation for wounds. They also help manage wound fluids, preventing them from leaking from the wound, and inhibit external infections and contamination. In order to avert bacterial infections, most foam dressings are impregnated with antibacterial or silver coatings and hydrocolloids.

Hydrocolloid dressings are made of gelling agents such as gelatine, pectin, and carboxymethyl cellulose. These are combined with adhesives and applied to polyurethane sheets to create self-adhesive and absorbent films. The occlusive nature of these dressings promotes a moist environment, which is a major requirement for optimal healing. These dressings also stimulate angiogenesis, increase the number of dermal fibroblasts, and aid the production of granular tissue.

Film dressings are flexible polymer sheets with an acrylic adhesive coating. The most commonly used polymer for manufacturing films is polyurethane. This transparent and elastic dressing comes in various sizes and thickness and has strong adhesive properties. Film dressings provide increased ventilation to the wound, maintain a moist environment, and promote tissue granulation and tissue formation.

Alginates are refined from seaweed and processed into soft woven fibres. They are generally derived from the sodium salts of alginic acid present in seaweed. Alginate dressings are usually manufactured in the form of sheets, ribbons, or ropes. Alginate sheets are used for treating superficial wounds, while the rope and ribbon forms are used to treat cavity wounds. Alginate dressings do not adhere to the wound during removal, and hence prevent debridement of the wound. These dressings can be left on the wound for three to four days, depending on exudation frequency. They are best suited for pressure and diabetic foot ulcers, venous leg ulcers, postoperative wounds, cavity wounds, and traumatic wounds.

Collagen is a key protein involved in the scar formation process of wound healing. Collagen dressings are available in the form of sheets, gels, pastes, polymers, and oxidized regenerated cellulose. These dressings contribute to inflammation control, protein synthesis, and also growth factor release, which promote rapid wound healing. Collagen dressings are well suited for treatment of full-thickness acute and chronic wounds, such as pressure ulcers, venous ulcers, diabetic ulcers, and surgical wounds.

Up until today, growing evidence have focused on tissue-engineered skin substitutes of both allogeneic and autologous lineages, including cellular and acellular matrices, which have been suggested as adjunct therapies to the standard management of DFUs.(207) Artificial skin grafts are synthetically produced in laboratories and used in a process called artificial skin grafting, when large wounds have resulted in significant injury to the deep layers of the skin. An artificial skin graft comprises synthetic epidermis and a collagen-based dermis with fibres organized in a lattice. Collagen promotes the generation of new tissues such as fibroblast blood vessels, neuronal fibres, and lymphatic

vessels. Skin grafts can minimize scars and loss of fluid, stimulate the healing process, reconstruct defective skin, and help prevent infections. The most-preferred conditions for skin grafting include non-healing diabetic ulcers, venous or pressure ulcers, skin tumours, or deep burns. The major risks involved in skin grafting procedures include the rejection of skin grafts, formation of hematoma and necrotic tissue, hyperpigmentation, pain, and ulceration. Artificial skin and skin substitutes are further categorized into growth factors, composite and biologic skin grafts, autografts, allografts, and xenografts. The Human Skin Equivalent Apligraf® is an example for bi-layered cellular wound matrix consisting of keratinocytes and fibroblasts.(208) Integra® is a bilayered acellular wound matrix consisting of bovine collagen, glycosaminoglycans, and silicone as an example for an acellular therapy layer.(209, 210) The latter decellularized biological products act as tissue substitute for cell migration and provide a scaffold for cellular ingrowth. They assist the formation of granulation tissue and promote tissue regeneration, resulting in replacement of defective ECM.(211)

Moreover, increasing evidence have investigated stem cells derived from various sources as a potential alternative therapying DFUs. Stem cells are biologically undifferentiated cells that can differentiate into different other specialized cell types. This could be taken advantage of in repairing compromised tissue such as DFU. The two main types of stem cells widely used in research are embryonic stem cells and adult, somatic, or tissue-specific stem cells. ESCs are pluripotent cells isolated from blastocysts and can differentiate into almost every tissue type. In contrast, ASC are also undifferentiated but more specialized cells encompassed by other differentiated cells in their environment, and can be isolated from many different tissue types. Adult stem cells are also pluripotent cells that generate cells for the specific tissue in which they reside. They remain quiescent, or in other words, non-dividing in their respective tissue throughout the organism’s life until they are activated by disease, injury or normal need. This process is called “tissue homeostasis”. Tissue homeostasis serves as a front-line repair system and provides a sort of protective backup system to prevent tissue loss and hereby maintains the respective tissue of origin.(212) Bone marrow provides a rich

source for ASC, together with brain, heart, blood vessels, skeletal muscle, skin, teeth, gut, liver, ovarian epithelium, testis and peripheral blood. Currently, numerous examples of ASC differentiation into specialized cell types have been noted. For instance, hematopoietic stem cells can generate all types of blood cells including B and T lymphocytes, natural killer cells and neutrophils. Mesenchymal stem cells, which have been isolated from many different tissues generate bone, cartilage, and fat cells. These are also called bone marrow stromal or skeletal stem cells. However, there are certain dissimilarities between MSC from different sources with regard to their differentiation and cell generation capacity. Neuronal stem cells, found in the central nervous system, can differentiate into different cell types that are specific to the central nervous system. Other examples of stem cells include epithelial stem cells, which reside in the epithelial layer of different organs, including the epidermis, cornea, and gastrointestinal, respiratory, and urogenital tracts. Stem cells from different epithelial sources have different homeostatic replacement capacities. For instance, the skin harbours epidermal stem cells that give rise to keratinocytes, which form a protective dermal layer. Skin also harbours follicular stem cells, which give rise to hair follicles. Shinya Yamanaka *et al.* recently demonstrated that adult somatic stem cells can be reverted back into non-specific pluripotent stem cells with laboratory reprogramming processes that induce the expression of embryonic genes. Stem cells generated in this way are called “induced pluripotent stem cells” (iPSCs).(213, 214)

In recent years, research has been focused mainly on MSCs. In brief, these are multipotent progenitor cells that can differentiate into mesenchymal tissues with paracrine functions, like the synthesis of growth factors and cytokines. Additionally, they facilitate cell migration and proliferation, which promotes the repair and regeneration of compromised wound tissue. However, existing literature indicates that stem cells from DFU patients or other populations suffering from chronic wounds are impaired and defective.(55-57) Hence to overcome this deficiency, and achieve successful wound healing, these patients require delivery of healthy functional MSCs. In summary, stem cell therapy promotes healing through upregulating of various anti-inflammatory cytokines

and growth factors, upregulating angiogenesis, and modulating MMP balance and differentiation into various tissues, when applied to a non-healing wound.(58)

1.4 Aims of this Thesis

Antimicrobial peptides are fundamental members of the IIS and are involved in a wide range of immunological processes. Some unique characteristics including, leukocyte attraction, angiogenesis, wound healing, make them attractive potential alternatives to common therapy modalities in different fields medicine. The main goal of the present PhD thesis was to gain further evidence regarding the expression and activity of AMPs in the human body fluids.

Continuing on this line, as the most antimicrobial peptide expression in the human body has been reported at sites with the most pathogen exposure, such as skin and lung, we intended to investigate whether AMPs are present in human pleural fluid, and if so, to which extent they exert antimicrobial activity. This, along with identification of the cellular source for AMPs and the evaluation of the extent of expression, delineate the aim of the first part of this thesis.

In the second part of this thesis we aimed to investigate whether AMPs are also expressed by MNCs and are present in their secretome, and to which extent AMPs exert antimicrobial activity. We were furthermore interested to know if the expression of AMPs, and their antimicrobial activity, augments responding to ionizing radiation.

More specifically, after we have shown upregulated secretory activity in response to ionizing radiation, we were highly interested to know the cell death modalities involved in the whole process. In addition, an animal model was used to investigate the *in vivo* immunomodulatory property of MNC Secretome by evaluating the endogenous stimulation of AMPs.

Since advanced wound care products have been developed in response to improved understanding of the impaired wound healing situation of DFU, we

were encouraged to pursue the next aim of this thesis, namely if MNC secretome (APOSEC®) could serve as a potential therapeutic entity for DFU.

CHAPTER TWO: RESULTS

2.1 Prologue

Considering low incidence of postoperative infectious complications following operations of the pleural cavity, we were encouraged to search for the underlying mechanism. The human lung has been demonstrated to be non-sterile(215) and consequently all operations in this field take place in a non-sterile environment. However, compared to major operations of other organs, the risk of developing postoperative infections are not increased. Increasing evidence point to the crucial involvement of AMPs in human immunity especially in the innate immunity of the lung. Among numerous strategies used by the lung to fight inhaled pathogens production of AMPs have been shown by epithelial cells of the respiratory tract.

That information led us to the hypothesis that pleural fluid also contains antimicrobial components that protect the host against invading lung pathogens. We thus analysed postoperative pleural effusions for the presence and activity of antimicrobial peptides, evaluating the proinflammatory impact on their activity.

The results described in the report added below, are published in the “Annals of Thoracic Surgery” journal.

The author of this thesis contributed to the findings of the following manuscript by investigating the presence of AMPs in the postoperative pleural drainage fluid at protein level and analysed the concentration of those peptides using commercially available ELISA systems. He furthermore processed the generated data and performed the computational analysis.

2.1.1 First paper

Antimicrobial Peptides Are Highly Abundant and Active in Postoperative Pleural Drainage Fluids

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GENERAL THORACIC

Background. The human lung is considered a nonsterile organ, and surgical interventions therefore take place in a more or less contaminated operating field. Nevertheless, infectious complications of the pleural cavity are low after major lung resections. Antimicrobial peptides (AMPs) are part of the innate immunity and display a broad capacity to kill pathogens. We hypothesized that the pleural space must have a high natural antimicrobial barrier and that AMPs might effectively protect the pleural cavity.

Methods. Pleural effusions were collected after lung operations. Antimicrobial activity of the fluids against gram-positive and gram-negative pathogens was analyzed by microdilution assays. AMPs were determined by enzyme-linked immunosorbent assay (ELISA), polymerase chain reaction (PCR), and immunohistochemical analysis. The impact of proinflammatory triggers on AMP release from pleural mesothelial cells was evaluated.

Results. Antimicrobial activity assays revealed high bactericidal properties of postoperative pleural drainage fluids. They effectively killed gram-negative pathogens (*Escherichia coli*, *Pseudomonas aeruginosa*) as well as gram-positive pathogens (*Staphylococcus aureus*, *Streptococcus pneumoniae*, and *Streptococcus pyogenes*). A variety of AMPs was detected at constantly high concentrations in the pleural fluids. They mainly derived from leukocytes and pleural epithelium. Although proinflammatory cytokine levels were elevated in the postoperative pleural fluids, AMP expression could not be augmented by Toll-like receptor (TLR) triggering or by the proinflammatory cytokines interleukin (IL)-1β and tumor necrosis factor (TNF)α.

Conclusions. We provide the first evidence of a high abundance of AMPs in postoperative pleural fluids. Our findings might explain the broad protection against infectious complications of the pleural space after major lung operations.

(Ann Thorac Surg 2014;98:1042–50)

Lung resections account for the majority of surgical activity in the field of thoracic surgery. To access the lung, the pleural cavity, which is a highly sterile region of the body, has to be opened. Inevitably, the sterile state of the pleural cavity cannot be maintained because of contact with lung parenchyma during the operation. The lung itself is considered a nonsterile organ. Colonization by pathogens can be found in 20% to 50% of patients undergoing lung resections [1–3]. Although the sterile state of the pleural cavity is lost during lung operations, postoperative pleural empyema is rare and occurs in less than 5% of all patients [4]. It is of interest that most of the published studies showed only a marginal impact of prophylactic antibiotic regimens on the infectious outcome after major lung operations [5–7]. Thus an internal protective factor has to exist to protect the pleural space from infectious complications.

Antimicrobial peptides (AMPs) are primitive and evolutionarily conserved components of the innate immune system. They protect the organism against environmental pathogens and exhibit considerable antimicrobial activity against gram-negative and gram-positive bacteria, mycobacteria, enveloped viruses, and fungi [8]. In addition, AMPs link the innate and the adaptive immune system by acting as chemoattractants to dendritic cells and T cells [9]. Some AMPs are constitutively expressed, providing an ever-present defense, whereas others can be rapidly induced during infection. In humans, the most thoroughly characterized AMPs are the alpha- and beta-defensins, S100 proteins (S100A7, S100A8/A9), ribonucleases (RNAses), and cathelicidin. They can be found in skin and intestinal epithelium and in body fluids (saliva and lacrimal and vaginal fluids) and are produced either by phagocytic

Accepted for publication April 21, 2014.

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The Appendix can be viewed in the online version of this article [http://dx.doi.org/10.1016/j.athoracsur.2014.04.106] on http://www.annalsthoracicsurgery.org.

0003-4975/$36.00
http://dx.doi.org/10.1016/j.athoracsur.2014.04.106

cells or by the epithelial layer [10]. It is currently unknown if AMPs are present in the pleural fluid.

We hypothesized that the pleural space must have a high natural antimicrobial barrier and that AMPs might effectively protect the pleural cavity from infectious complications after lung operations.

Patients and Methods

Patient Selection

Patients who were operated on at the Department of Thoracic Surgery, Medical University of Vienna were prospectively recruited for this study. A positive ethics committee vote was obtained (EK No. 585/10), and all participants gave their written informed consent before inclusion. Patient characteristics are summarized in Figure 1 and Appendix Table 1. Preoperative evidence of pneumonia, emphysema, or necrotizing parts in the tumor as well as any signs of systemic infections at the time of operation were defined as exclusion criteria.

Collection of Pleural Fluids, Parietal Pleura, Blood, and Serum Samples

Pleural fluid samples (10 mL) were collected immediately after opening the chest and twice daily during the postoperative course (0, 2, 18, 26, and 42 hours postoperatively). For the postoperative collection, chest tubes were first cleared of old material, then clamped, and the new portion of fluid was obtained by sterile puncture of the chest tube line. For some experiments, pleural fluids were centrifuged to separate cellular components and passed through a 40-μm cell strainer. Specimens were aliquoted and immediately frozen at –80°C until used in further experiments. In a subgroup of patients (wedge resections, n = 5; lobectomy, n = 6; pneumonectomy, n = 1), whole blood and serum samples were collected. Serum was stored at –80°C, and whole blood was analyzed immediately after the blood draw by flow cytometry. Parietal pleura samples (n = 3) were obtained during pleurectomy procedures for recurrent pneumothoraces. Pleural specimens were either fixed with 4% formaldehyde or shock-frozen in liquid nitrogen for further processing.

Enzyme-Linked Immunosorbent Assay

Levels of human beta-defensin (HBD)1 (antibodies-online.com, Atlanta, GA), HBD2 (PeproTech, Rocky Hill, NJ), RNAse5 (R&D Systems, Minneapolis, MN), RNAse7 (antibodies-online.com, Atlanta, GA), S100A7 (MBL International Corporation, Woburn, MA), S100A8/A9, cathelicidin (both Hycult, Uden, the Netherlands), interleukin (IL)-1β, IL-2, IL-6, tumor necrosis factor (TNF)α, IL-8, IL-10, transforming growth factor-β, soluble ST-2 (all R&D Systems, Minneapolis, MN) in serum and pleural fluid samples were analyzed by enzyme-linked immunosorbent assay (ELISA) technique following the manufacturers' instructions.

Establishment of Mesothelial Cell Culture

Normal pleura (NP)-1 and NP-2 cell lines were established from parietal pleura specimens from surgically treated patients with pneumothorax following published protocols [11]. Cells demonstrated mesothelial

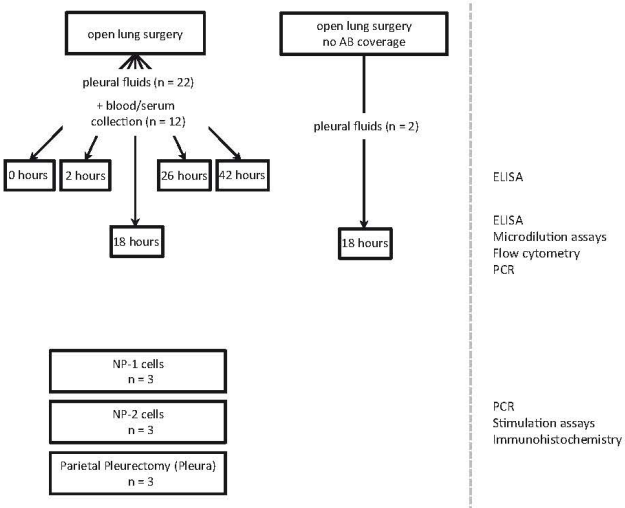


Fig 1. Study design and patient recruitment. (AB = antibiotic; ELISA = enzyme-linked immunosorbent assay; NP = normal pleura; PCR = polymerase chain reaction.)

morphologic features as described previously [12] and stained positive for vimentin and negative for CD31.

Polymerase Chain Reaction

Tissue specimens of parietal pleura were homogenized in RNA lysis buffer using the Precellys system (Peqlab, Erlangen, Germany), and cellular components of pleural fluids were isolated by a centrifugation step before lysis. NP-1 and NP-2 cells were lysed directly. RNA was purified using a standard protocol, and quantitative real-time polymerase chain reaction (PCR) was performed on a LightCycler using a SYBR Green I Master Kit (Roche Diagnostics, Basel, Switzerland) as described previously [13]. The primers used are presented in detail in Appendix Table 2.

Immunohistochemical Examination

Immunohistochemical staining was performed on paraffin-fixed samples of parietal pleura following the procedure described elsewhere [14]. The following antibodies were used: HBD1 (2 μg/mL; Abcam, Cambridge, UK), HBD2 (1 μg/mL; Abcam, Cambridge, UK), S100A7 (psoriasin) (5 μg/mL; Abcam, Cambridge, UK), anti-S100 A8/A9 (2 μg/mL; Acris, Hiddenhausen, Germany), RNAse5 (5 μg/mL, Abcam, Cambridge, UK), and RNAse7 (2 μg/mL, Abcam, Cambridge, UK).

Flow Cytometry

Single-cell suspensions from postoperative pleural effusion were stained with a fluorescein isothiocyanate–conjugated anti-CD45 antibody (BioLegend, San Diego, CA). Fluorescein isothiocyanate–IgG1 was used as appropriate isotype control (BioLegend, San Diego, CA). Dead cells were excluded with 7-amino-actinomycin-D (7-AAD; Calbiochem, Darmstadt, Germany). For subgroup analysis, anti-CD4, anti-CD8, anti-CD19, and anti-CD56 antibodies were used (all Beckman Coulter, Brea, CA). Flow cytometry analyses were performed on a FACSCalibur (Becton Dickinson, Franklin Lakes, NJ), and data were analyzed using FlowJo software (Tree Star, Ashland, OR).

In Vitro Induction of AMP Expression

For in vitro stimulation analysis, NP-1 cells were incubated for 6 hours and 24 hours with the following Toll-Like receptor (TLR) ligands (all InvivoGen, San Diego, CA): 1 μg/mL Pam3CSK4 (TLR1/2 ligand), 10 μg/mL HKLM (TLR2 ligand), 20 μg/mL Poly(I:C) (TLR3 ligand), 100 ng/mL lipopolysaccharide from *Escherichia coli* K12 (TLR4 ligand), 500 ng/mL Flagellin from *Salmonella typhimurium* (TLR5 ligand), 500 ng/mL FSL1 (TLR6/2 ligand), 10 μg/mL Imiquimod (TLR7 ligand), 10 μg/mL single-stranded RNA40 (TLR8 ligand), and 2.5 μM ODN2006 (TLR9 ligand). Expression pattern of AMPs was determined by PCR following the procedure described earlier.

Antimicrobial Activity of Pleural Fluids

The antimicrobial activity of pleural fluids was determined by a microdilution assay [14] using the following pathogens: *E coli* (ATCC 11303), *P aeruginosa* (ATCC 27853), *S aureus* (ATCC 33592), *S pneumoniae,* and *S pyogenes*. Pathogens were incubated for 3 hours with 20% diluted pleural fluids in physiologic NaCl solution, containing 1% (v/v) trypticase soy broth at 37°C. Thereafter, suspensions were applied to LB agar plates. The antibiotic activity was analyzed by plating serial dilutions of the incubation mixtures on LB agar plates and determining the number of colony-forming units the next day.

Statistical Methods

All data collected in this study were analyzed by PASW Statistics, version 19 (SPSS, Chicago, IL) or GraphPad Prism, version 6 (GraphPad Software, LaJolla, CA) software. Data sets are depicted as mean ± standard error of the mean (error bars) in the figures. Unpaired 2-sided *t* tests were used to compare 2 independent groups. For multiple-group comparison, 1-way analysis of variance with post hoc Bonferroni correction was applied. *p* values equal to or less than 0.05 were considered statistically significant.

Results

Postoperative Pleural Fluids Show Strong Antimicrobial Activity Against Different Bacteria

To test the hypothesis of a high endogenous antimicrobial barrier of postoperative pleural fluids, we obtained pleural effusions by sterile puncture of the chest tube lines 18 hours after the end of the operation. Postoperative pleural fluids showed a strong antimicrobial activity against gram-negative bacteria (*Escherichia coli, Pseudomonas aeruginosa*) as well as gram-positive bacteria (*Staphylococcus aureus, Streptococcus pneumoniae,* and *Streptococcus pyogenes*) (Fig 2A). All these patients received perioperative parenteral antibiotic coverage with cefuroxime 3 g twice daily for 2 days starting with the skin incision. To rule out the possibility that the observed antimicrobial effects were attributable to the antibiotics, we collected postoperative fluids from 2 patients who did not have antibiotic coverage. A significant impact of perioperative antibiotics was found for all gram-positive bacteria tested but not for gram-negative bacteria. However, except for group A streptococci, considerable antimicrobial activity remained even if antibiotic prophylaxis was omitted (Table 1).

Postoperative Pleural Fluids Contain High Amounts of AMPs

Pleural fluids obtained during and after the operation were analyzed by ELISA for levels of different AMPs. HBD1, HBD2, S100A7, S100A8/A9, RNAse5, RNAse7, and cathelicidin were highly abundant in the postoperative pleural effusion, with concentrations ranging from 100 pg/mL to 100 ng/mL (Fig 2B). A detailed analysis of fluids within the first 2 postoperative days showed that AMPs were already present in high concentration in pleural fluid specimens obtained immediately after opening the chest cavity. During the postoperative course, they

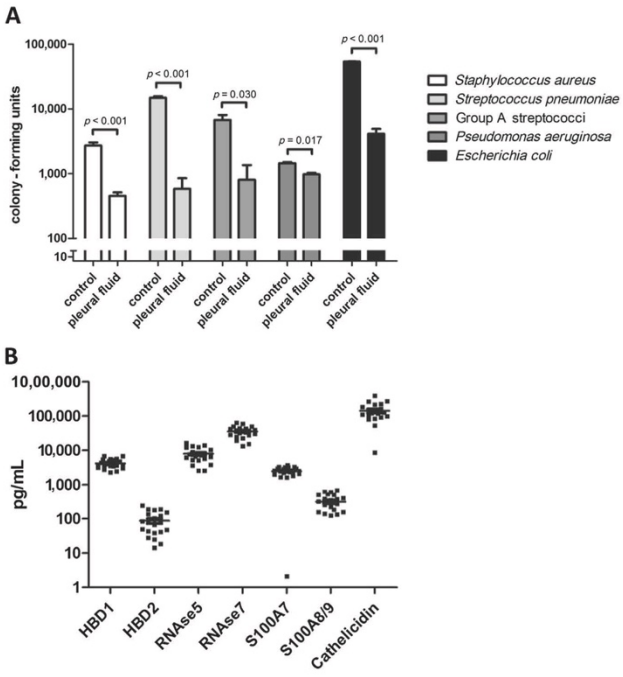


Fig 2. Postoperative pleural fluids showed evidence of profound antimicrobial activity against different pathogens. (A) High bactericidal activity against gram-positive bacteria (Staphylococcus aureus, Streptococcus pneumoniae, and Streptococcus pyogenes) and gram-negative bacteria (Escherichia coli, Pseudomonas aeruginosa) was found in microdilution assays. (B) Levels of different antimicrobial peptides (AMPs) in postoperative pleural fluids (18 hours) were determined by enzyme-linked immunosorbent assay (ELISA). Human beta-defensin (HBD) 1, HBD2, RNAse5, RNAse7, S100A7, S100A8/A9, and cathelicidin were highly abundant in the pleural effusion after major lung operations.

remained constantly high within the first 36 hours after the operation (Appendix Fig 1A). Interestingly, the extent of the operation (wedge resection, lobectomy, pneumonectomy) had no impact on AMP expression level (Table 2).

Table 1. Antimicrobial Activity of Pleural Fluids in Patients With and Without Perioperative Antibiotic Coverage[a]

Organism	Antibiotic Coverage	No Antibiotic Coverage	*p* Value
Staphylococcus aureus	86.6±1.3%	53.5±3.8%	<0.001
Streptococcus pneumonmiae	97.4±1.3%	67.0±3.8%	<0.001
Group A streptococci	95.5±4.3%	0.0±0.0%	<0.001
Pseudomonas aeruginosa	30.5±4.0%	23.4±7.2%	0.628
Escherichia coli	86.8±3.7%	62.4±2.2%	0.081

[a] In a subgroup of patients, no antibiotic coverage was applied during the perioperative period. Antibiotic coverage resulted in a more effective clearing of bacteria; however, strong bactericidal activity of pleural fluids remained even when perioperative cefuroxime prophylaxis was omitted. Values illustrate relative decrease of bacterial growth by addition of pleural fluids. (unpaired 2-sided *t* tests).

Cellular Composition of Postoperative Pleural Fluid Is Restricted to Inflammatory Cells

In the human body, the majority of AMPs is either produced by epithelial cells or leukocytes. Both cell types could be the source of the AMPs found in pleural fluids. We therefore determined the cellular content of pleural fluids. Flow cytometry analysis revealed no scaled-off mesothelial cells in the fluids (Fig 3A). The leukocyte infiltration initially contained exclusively granulocytes, which were partly substituted by monocytes within the first 36 hours (Fig 3B).

AMPs Are Produced by Inflammatory Cells and by Pleural Epithelium

In a further set of experiments, we performed PCR analyses with isolated cellular components of pleural fluids, specimens of parietal pleura (containing mesothelial cells and tissue-residing immune cells), and primary mesothelial cell cultures (NP-1, NP-2). We found that pleural epithelium/mesothelium was the major source for S100A7, because expression was high in samples of the parietal pleura and the NP-1 and NP-2 cells but not in the lavage samples containing high numbers of inflammatory cells. Accordingly, leukocytes were found to be the major source for S100A8/A9 and RNAse5 and RNAse7.

Table 2. Levels of AMPs in Postoperative Pleural Fluids Grouped Into Extent of Surgical Resection[a]

AMPs	Wedge Resection	Lobectomy	Pneumonectomy	*p* Value
HBD1 (pg/mL)	3,952±258	4,078±547	3,806±800	0.939
HBD2 (pg/mL)	107±26	91±21	57±22	0.399
RNAse5 (pg/mL)	9,550±1125	7,388±1,540	5,372±1,306	0.153
RNAse7 (pg/mL)	39,064±4443	3,1051±5,768	30,649±5,513	0.451
S100A7 (pg/mL)	2,534±180	2,084±353	2,270±414	0.559
S100A8/A9 (pg/mL)	175±57	322±42	394±97	0.064
Cathelicidin (pg/mL)	14,5366±23930	13,4381±4,3274	13,2648±3,0388	0.961

[a] The impact of resection techniques on AMP levels in the postoperative fluid (18 hours) is shown. No significant differences in AMP levels could be detected when comparing patients undergoing wedge resection, lobectomy, or pneumonectomy (1-way analysis of variance with post hoc Bonferroni correction).

AMPs = antimicrobial peptides.

Expression levels of the latter AMPs were significantly increased in pleural lavage samples compared with the mesothelial NP-1 and NP-2 cells (Fig 3C). These findings could be confirmed by immunohistochemical staining of pleural specimens (Fig 4). S100A7 was exclusively found on the epithelial lining; HBD1, HBD2, and RNAse5 staining were only weakly positive in the epithelium but were more prominent in scattered leukocytes, whereas S100A8/A9 was solely expressed in leukocytes.

Cytokine Milieu in Pleural Effusion

The local cytokine milieu of the postoperative pleural effusion was determined by ELISA. Different proinflammatory and antiinflammatory cytokines could be found in high amounts in pleural fluids within the first 3 postoperative days but were only marginally elevated in serum samples. Proinflammatory cytokines (IL-1β, IL-6, TNFα, and IL-8) were highest in the first portion (2 hours postoperatively) of drained pleural fluid and decreased thereafter, whereas antiinflammatory components (IL-10 and soluble ST2) increased on postoperative day 1 or remained at a high level throughout the whole observation period (Appendix Fig 1B).

AMP Production Is Independent of Inflammatory Stimuli

Previously published observations in epithelial cell lines showed that AMP production can be boosted by proinflammatory cytokines. Because we found a considerable proinflammatory milieu in the postoperative pleural effusion, we further aimed to determine the influence of TLR triggering and proinflammatory cytokines on AMP production in the mesothelial NP-1 cell line. Coincubating NP-1 cells with TLR ligands for TLR1, TLR2, TLR3, TLR4, TLR5, TLR6, TLR7, TLR8, TLR9, IL-1β, and TNFα, however, did not increase the production of HBD1, RNAse5, RNAse7, S100A8/A9, and cathelicidin (Fig 5). Interestingly, TNFα could induce S100A7 mRNA expression by 3.7-fold ($p = 0.029$).

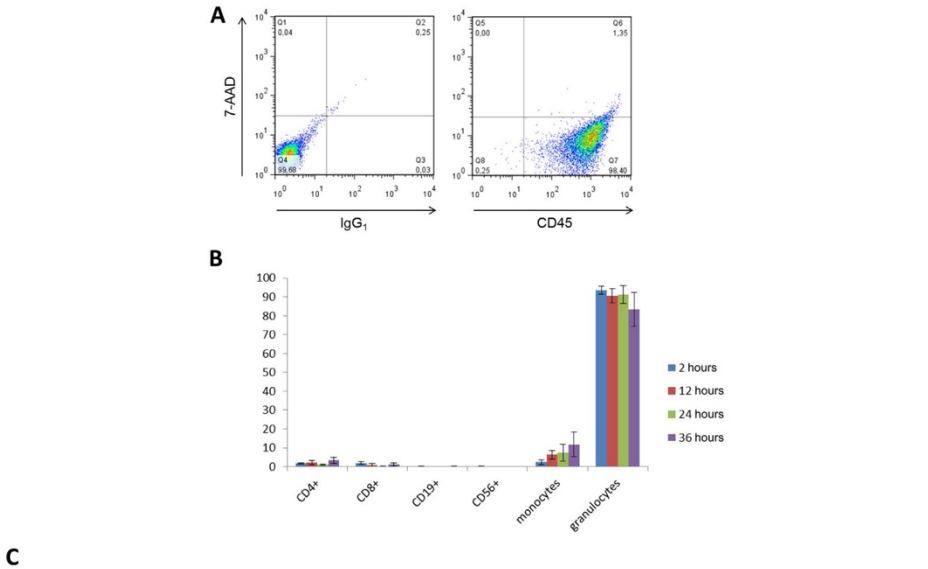
Comment

This work gives first evidence that the postoperative pleural fluid unfolds a remarkable antimicrobial activity against lung pathogens. The growth of different gram-positive and gram-negative bacteria was considerably reduced by incubating pathogens with cell-free pleural fluids. As for the source of this antimicrobial activity, a high abundance of different AMPs was found in the pleural fluids.

During the normal postoperative course, the production of pleural fluid is increased to 400 to 600 mL/day [15]. This fluid is drained by chest tubes placed in the thoracic cavity at the end of the operation to prevent compression of the remaining lung tissue perioperatively. Our data give a new insight that this increased postoperative pleural fluid production may also play a central role in the defense against pathogens. Surgical interventions in the thoracic cavity are by definition nonsterile because the lung is constantly colonized with different microorganisms. In a thorough study by Belda and colleagues [4], bronchoscopic brushings and lavage revealed perioperative bronchial colonization in 83% of all patients, including potentially pathogenic microorganisms. The pleural space, in contrast, is highly sterile. This sterile state is, however, lost during major lung operations through an inevitable contact with the potentially contaminated lung parenchyma. Nevertheless, infectious complications of the pleural space after major lung operations are rare [16]. According to a recently published retrospective analysis including more than 1,400 patients, postoperative pleural empyema after lung cancer operations developed in only 0.9% of cases [17]. The high abundance of AMPs in postoperative pleural fluids may serve as a form of protective shield, effectively preventing postoperative infections of the pleural cavity.

AMPs provide a rapid first line of defense against exposure to bacteria and fungi. They are either constitutively expressed or can be produced and released within a short time. Because of this they are considered a key factor in the first line of response to pathogens.

AMPs in the postoperative pleural fluid could either originate from the pleural epithelium or from leukocytes infiltrating the pleural cavity [18]. We performed flow cytometry analyses to determine the number and distribution of leukocytes in the postoperative pleural effusion. The prevailing inflammatory cell population was granulocytes, which were partly replaced by monocytes during the first 48 hours. Detached pleural epithelium could not be found in the pleural fluids



	HBD-1	HBD-2	HBD-3	RNAse5	RNAse7	S100A7	S100A8	S100A9	Cathelicidin
Pleura versus lavage	ns			$P = 0.081$	*	*	***	***	ns
NP-1 versus lavage	**			*	*	*	***	**	*
NP-1 versus pleura	$P = 0.085$			$P = 0.052$	$P = 0.061$	ns	***	**	*
NP-2 versus lavage	ns			*	*	**	***	**	$P = 0.15$
NP-2 versus pleura	ns			$P = 0.067$	ns	ns	***	**	ns
CP value (mean±SD)	33.3 ± 0.8	> 50	> 50	25.5 ± 0.9	27.7 ± 0.9	39.1 ± 1.0	21.6 ± 1.2	22.7 ± 1.5	31.2 ± 0.6

Fig 3. (A) Flow cytometry analyses revealed that the cellular content in postoperative fluids invariably consists of CD45+ cells. (B) The majority of these cells were granulocytes (> 90%), which were partly replaced by infiltrating monocytes within the first 36 hours after the operation. (C) Polymerase chain reaction (PCR) evaluations of pleural fluids (lavage), pleural specimens, and a pleural mesothelial cell culture (normal pleura (NP)-1 and NP-2) showed a predominantly pleural origin of S100A7 in contrast to a mainly leukocytic origin of human beta-defensin (HBD)1, RNAses, and S100A8/A9. (7-AAD = 7-amino-actinomycin-D.)

because all detectable cells were $CD45^+$. To determine whether AMPs were of leukocytic or pleural epithelial origin, we performed PCR analysis with leukocyte-rich pleural effusions and a pleural epithelium cell culture (NP-1, NP-2). HBD1, RNAses, S100A8/A9, and cathelicidin were highly expressed in the lavage samples, but

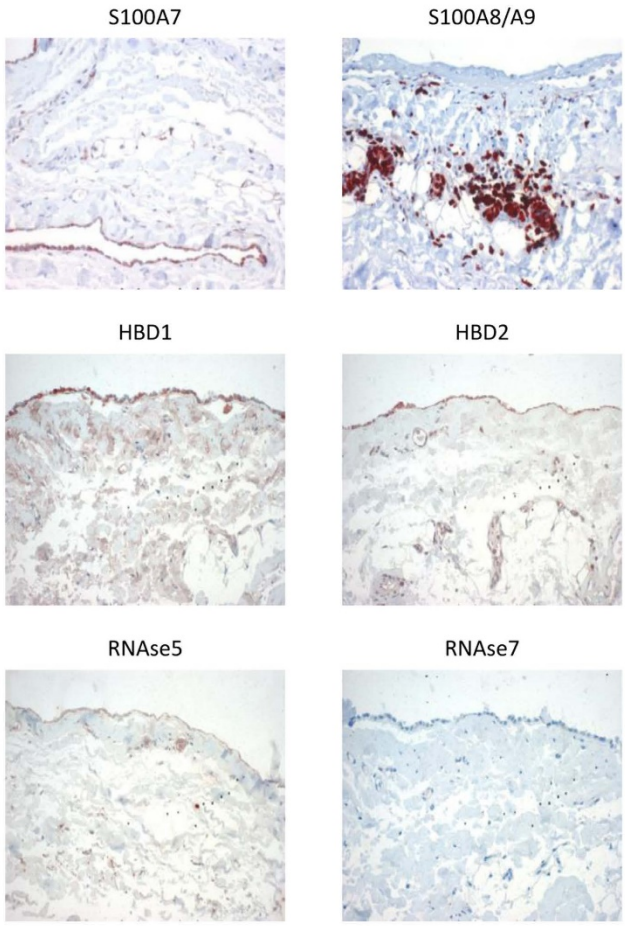


Fig 4. Immunohistochemical evaluation of pleural samples underlined the polymerase chain reaction (PCR) analyses. Pleural resident leukocytes showed a positive staining pattern for S100A8/A9, human beta-defensin (HBD)1, HBD2, and RNAses, whereas S100A7 was exclusively found in the epithelial lining of the pleural mesothelium (×200 magnification).

only a baseline expression of these AMPs was found in the primary cell line. In contrast, S100A7 was produced only in the pleural cell culture and was not detected in the lavage samples. These findings suggest that both the pleural epithelium and infiltrating leukocytes account for the high abundance of AMPs.

In human epithelial layers, AMPs are either constitutively expressed or can be induced during a proinflammatory response after contact with pathogens. Cytokines such as IL-1, IL-6, TNFα, and interferons trigger the upregulation of S100A8/A9 in keratinocytes. Secretion of S100A7 and S100A8/A9 can be directly induced by TLR stimulation [13, 19]. The pleural space is covered by mesothelium, a specialized epithelial cell type of mesodermal origin. It plays a central role in maintaining the immunologic equilibrium of the pleural space [20]. During inflammatory diseases, many mediators are secreted into the pleural space, including cytokines (IL-1α, IL-1β, IL-6), chemotactic factors (ENA-78, MCP-1, MIP-1α), and growth factors (transforming growth factor–β, fibroblastic growth factor, insulin-like growth factor) [21]. We were able to show that the surgical trauma elicits an immediate local immune response with increased levels of IL-1β, IL-6, TNFα, and IL-8. However, this proinflammatory status could not increase AMP production in vitro using primary

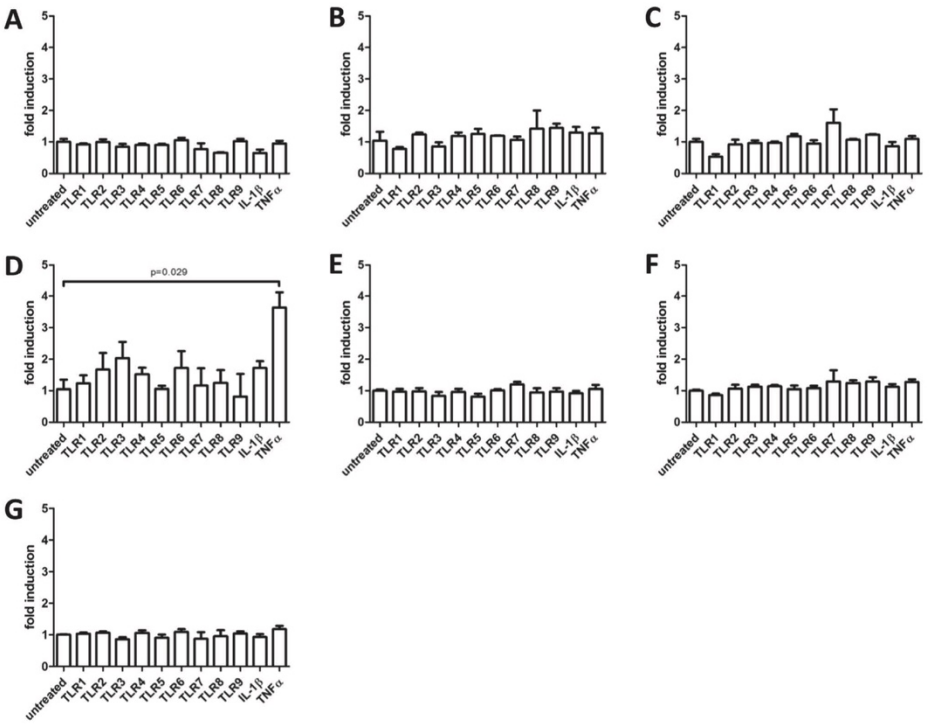


Fig 5. Expression of different antimicrobial peptides (AMPs) upon triggering with Toll-like receptor (TLR) ligands, interleukin (IL)-1β, and tumor necrosis factor (TNF)α was determined by polymerase chain reaction (PCR). AMP expression was largely independent of proinflammatory stimuli in the pleural epithelial normal pleura (NP)-1 cell culture. However, S100A7 production could be augmented by coincubation of NP-1 cells with TNFα ($p = 0.029$). (A) HBD1; (B) RNAse5; (C) RNAse7; (D) S100A7; (E) S100AB; (F) S100A9; and (G) cathelicidin.

mesothelial cell cultures (Fig 5). In addition to that, AMPs were already highly abundant in the first portion of pleural fluids obtained directly after opening the chest.

The patients included in this study received intravenous perioperative coverage with cefuroxime 3 g twice daily. Although antibiotic coverage is recommended by most guidelines, evidence is weak. Several prospective randomized controlled trials have been performed; however, no clear conclusion can be drawn on the basis of published data. A meta-analysis revealed that no differences between infectious complications after lung operations could be observed when comparing patients with and those without antibiotic coverage [2]. When reviewing our data, we observed a mild impact of antibiotic prophylaxis on the antimicrobial activity of postoperative pleural fluids. However, pleural fluids obtained from patients who did not receive an antibiotic regimen were also highly bactericidal. The only exception was complete inactivity against group A streptococci. Nonetheless, *S pyogenes* are usually not pathogenic to the lung, with only a few published case reports of pleural empyema in the literature [22, 23].

Recent studies have shown that the extent of the surgical procedure correlates with rates of postoperative infectious complications [24, 25]. In contrast, the exposure to potentially contaminated lung parenchyma is greater in wedge resection than in pneumonectomy. Both factors could have an impact on AMP levels in the postoperative fluids. We therefore performed a subgroup analysis, categorizing patients according to the amount of lung tissue resected. However, levels of AMPs were comparable in patients undergoing wedge resection, lobectomy, and pneumonectomy.

In conclusion, this study demonstrates for the first time that AMPs are highly abundant in the postoperative pleural fluid. This results in broad antimicrobial activity against gram-positive and gram-negative pathogens. Our findings, at least partly, explain the comprehensive

protection of the pleural cavity against infectious complications after lung operations.

The authors thank Walter Berger and Mir Alireza Hoda (Institute of Cancer Research, Medical University of Vienna) for the kind gift of the mesothelial cell cultures and Elzbieta Motuk for technical support.

This study was supported by the Christian Doppler Laboratory for Cardiac and Thoracic Diagnosis and Regeneration, Medical University of Vienna.

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Appendix Table 1 Patients' demographics

	Sex	Age	Indicaction	Procedure	Definitive histology	Samples	Antibiotics
#1	m	54	multiple pulmonary lesions	Wedge resection	fibrous tumor	PF, B, S	yes
#2	w	54	non verified lesion	Wedge resection	necrobiotic granuloma	PF, B, S	yes
#3	w	39	non verified lesion	Wedge resection	fibrotic scar	PF	yes
#4	m	75	non verified lesion	Wedge resection	SCC pT1a pN0	PF	yes
#5	m	70	multifocal ADC	Wedge resection	ADC pT1b pN0	PF	yes
#6	m	76	multiple pulmonary lesions	Wedge resection	ADC	PF	yes
#7	m	67	non verified lesion	Wedge resection	necrobiotic granuloma	PF, B, S	yes
#8	m	50	non verified lesion	Wedge resection	necrobiotic granuloma	PF, B, S	yes
#9	m	55	sup. interstitial lung disease	Wedge resection	NSIP	PF, B, S	yes
#10	m	52	FDG pos, non verified lesion	Lobectomy	inflammatory lesion	PF	yes
#11	m	52	SCC	Lobectomy	SCC pT1b pN1	PF, B, S	yes
#12	w	47	ADC, St.p. CHT	Lobectomy	ADC ypT1b pN2	PF	yes
#13	w	68	non verified lesion	Lobectomy	ADC pT1a pN0	PF, B, S	yes
#14	m	53	ADC	Lobectomy	ADC pT3 pN1	PF, B, S	yes
#15	w	78	ADC	Lobectomy	ADC pT2a pN2	PF, B, S	yes
#16	w	69	ADC	Lobectomy	ADC pT1a pN0	PF, B, S	yes
#17	m	61	non verified lesion	Lobectomy	ADC pT1a pN0	PF, B, S	yes
#18	m	53	SCC, St.p. CHT	Pneumonectomy	SCC pT2a pN1	PF	yes
#19	m	80	susp. NSCLC	Pneumonectomy	LCLC pT3 pN1	PF	yes
#20	m	46	SCC	Pneumonectomy	SCC pT3 pN2	PF	yes
#21	w	32	ADC, St.p. CHT	Pneumonectomy	ADC ypT2a pN1	PF	yes
#22	m	57	ADC	Pneumonectomy	ADC pT3 pN0	PF, B, S	yes
#23	m	53	non verified lesion	Wedge resection	CRC metastasis	PF	no
#24	m	72	ADC	Lobectomy	ADC pT1b pN0	PF	no
#25	m	18	Pneumothorax	Pleurectomy	-	PP	yes
#26	m	26	Pneumothorax	Pleurectomy	-	PP	yes
#27	w	45	Pneumothorax	Pleurectomy	-	PP	yes

Appendix Table 1: Chart summarizing patients' characteristics

Appendix Table 2 Primers used for PCR experiments

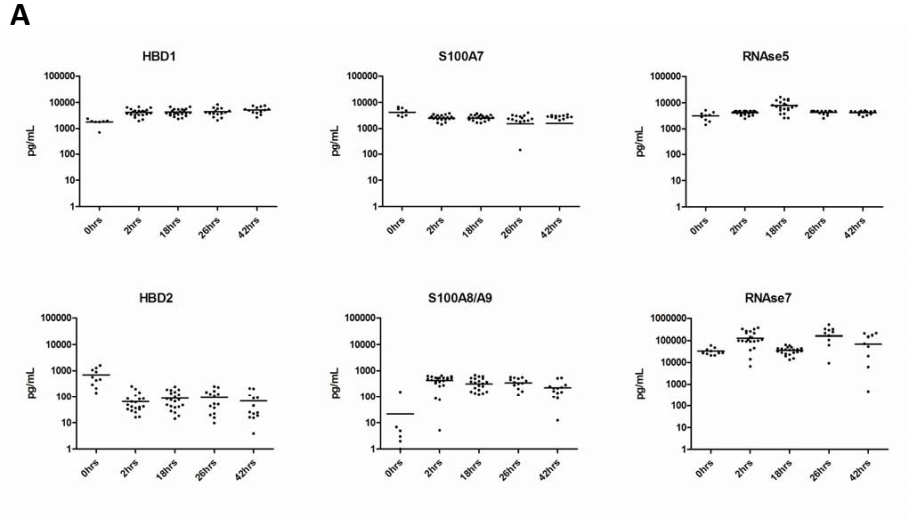
Gene	Forward primer	Reverse primer
β2M	5'-GATGAGTATGCCTGCCGTGTG-3'	5'-CAATCCAAATGCGGCATCT-3'
HBD-1	5'-CCCAGTTCCTGAAATCCTGA-3'	5'-CTTCTGGTCACTCCCAGCTC-3'
HBD-2	5'-ATCAGCCATGAGGGTCTTGT-3'	5'-GAGACCACAGGTGCCATTTT-3'
HBD-3	5'-AGCTGTGGCTGGAACCTTTA-3'	5'-CGATCTGTTCCTTCCTTTGGA-3'
S100A7	5'-GGAGAACTTCCCCAACTTCCTT-3'	5'-GGAGAAGACATTTTATTGTTCCT-3'
S100A8	5'-ATGCCGTCTACAGGGATGAC-3'	5'ACGCCCATCTTTATCACCAG-3'
S100A9	5'-CAGCTGGAACGCAACATAGA-3'	5'-TCAGCTGCTTGTCTGCATTT-3'
RNase5	5'-AGAAGCGGGTGAGAAACAAA-3'	5'-TGTGGCTCGGTACTGGCATG-3'
RNase7	5'-GAGTCACAGCACGAAGACCA-3'	5'-GGCTGCATGTGCTGAATTT-3'
Cathelicidin	5'-GCTAACCTCTACCGCCTCCT-3'	5'-GGTCACTGTCCCCATACACC-3'

Appendix Table 2: Primers used for AMP PCR analyses.

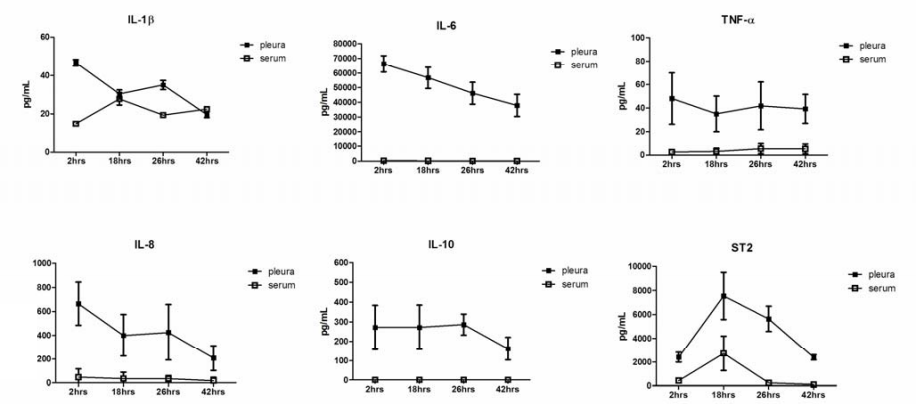
Figure Legend

Appendix Figure 1: AMP levels are highly abundant in the first portion of pleural fluid obtained after thoracotomy and remained at a high level throughout an observation period of 42hrs after the operation **(a)**. A detailed analysis of pro-inflammatory and anti-inflammatory cytokines is depicted in **(b)**. Pro-inflammatory cytokines were highest in samples obtained immediately after the operation and dropped thereafter, whereas anti-inflammatory cytokines (IL-10, soluble ST2) increased or remained at an unchanged high level within the first 24hrs postoperatively.

A


HBD1
S100A7
RNAse5
HBD2
S100A8/A9
RNAse7
pg/mL
0hrs
2hrs
18hrs
26hrs
42hrs

B


IL-1β
IL-6
TNF-α
IL-8
IL-10
ST2
pg/mL
pleura
serum
2hrs
18hrs
26hrs
42hrs

2.2 Interlude

The knowledge and experience of the human body's own production and release of antimicrobial peptides by leukocytes and the pleural epithelial layer in the pleural cavity induced us to analyse blood mononuclear cells for their antimicrobial content and activity. More specifically, after our research group had previously shown many different therapeutic effects of the MNC secretome, we were highly interested in whether the blood's MNC secretome contained antimicrobial properties.

The wound healing process is an interplay between various molecular and cellular events, depending on many factors to restore skin integrity. Of special note with respect to chronic wounds are patients with DFUs. Thus, because of the difficult treatment of DFUs, particularly due to polymicrobial infections, our interest was directed towards therapeutic use of the secretome of MNCs in the treatment of this disease. However, a rigorous analysis of antimicrobial potency of the MNC secretome and its antimicrobial content, and the effect of cell death inducing radiation on that antimicrobial property, was still missing. In this paper we thus focused on the antimicrobial properties of the supernatant of MNCs, and we investigated the effect of irradiation on this feature.

This study was published in the "European Journal of Clinical Investigation".

The author of this thesis contributed to the final version of the following manuscript by designing the study in consultation with Jan Ankersmit, preparing the secretome of human MNCs (non-GMP), performing the microdilution assay determining the antimicrobial potency of MNC secretome, RNA isolation from the irradiated and non-irradiated MNCs and performing quantitative real-time PCR analysis. The Author also carried out the animal experimental assay using Sprague Dawley rats, determined the levels of AMPs in the human experimental MNC secretome (non-GMP), human MNC secretome prepared according to GMP protocol and rat serum using commercially available ELISA systems. He furthermore wrote the paper with input from all authors, processed the generated experimental data and performed the computational analysis.

2.2.1 Second Paper

DOI: 10.1111/eci.12667

ORIGINAL ARTICLE

Dying blood mononuclear cell secretome exerts antimicrobial activity

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ABSTRACT

Background Several activities are attributed to antimicrobial peptides (AMPs), including bacterial killing, leucocyte recruitment and angiogenesis. Despite promises of advanced cellular therapies for treatment of diabetic foot ulcer, it is currently accepted that paracrine factors rather than cellular components are causative for the observed effects. Whether AMPs are present in the mononuclear cell (MNC) secretome (MNC-sec) of white blood cells that are beneficial in experimental wound healing is not known.

Materials and methods Antimicrobial activity of the secretomes of nonirradiated (MNC-sec) and γ-irradiated MNCs (MNC-sec rad) was analysed by microdilution assay. AMPs were determined by quantitative real-time PCR (RT-PCR) and enzyme-linked immunosorbent assay (ELISA). Whether human MNC-sec rad causes AMP secretion *in vivo* was examined in an experimental rat model. Image flow cytometry was used to determine the type of cell death induced in MNCs after exposure to γ-radiation.

Results The antimicrobial activity assay revealed a bactericidal activity of MNC-sec rad and to a lesser degree also of MNC-sec. Image flow cytometry showed that γ-irradiation of MNCs induced early apoptosis followed mainly by necroptosis. RT-PCR and ELISA revealed a high abundance of different AMPs in the secretome of MNCs. In addition, human MNC-sec elicited an increase in *de novo* endogenous AMP production in rats *in vivo*.

Conclusion We provide evidence that the secretome of MNCs has direct and indirect positive effects on the immune defence system, including augmentation of antibacterial properties. Our data further suggest that necroptosis could play a key role for the release of paracrine factors and the therapeutic action of MNC-sec rad.

Keywords Antimicrobial peptides, diabetic foot ulcer, MNC secretome, mononuclear cell, peripheral blood mononuclear cells.

Eur J Clin Invest 2016; 46 (10): 853–863

Introduction

Antimicrobial peptides (AMPs) are part of the innate immunity with broad-spectrum activity against bacteria and fungi. Among several activities attributed to them, chemotactic properties such as recruitment of other leucocytes along with direct bacterial killing have been investigated [1,2]. Several groups have proposed them as offering a promising new approach to common antibiotic therapies in the case of bacterial resistance [3] or nonhealing wounds like diabetic foot ulcer (DFU) [4]. According to recent reports, DFUs are infected in the majority of cases [5,6], and one in six patients requires amputation; however, successful treatment of DFU highly depends on complex events, including adequate angiogenesis or antibacterial actions [7,8].

Advanced cellular therapies hold promise as therapeutic agents for their known antimicrobial activity [9], angiogenesis [10] and wound healing [11]. Several studies have shown that mesenchymal stem cells (MSCs) represent an effective therapeutic agent in a variety of experimental models. For instance, Maharlooei *et al.* [12] showed that adipose tissue-derived MSCs enhance diabetic wound healing in a diabetic rat model. Krasnodembskaya *et al.* studied the effect of human bone marrow-derived MSCs on the bacterial growth of

Gram-negative and Gram-positive bacteria. They showed that MSCs and their conditioned medium both have inhibitory effects on bacterial growth [9]. The idea of therapeutically using the secretome of MNCs originates from stem cell research results and suggests that the observed beneficial effects of stem cell therapy are due to paracrine factors rather than direct cellular components [13]. In this study, we focused on secretomes derived from living (MNC-sec) or dying (MNC-sec rad) peripheral blood MNCs which Korf-Klingebiel *et al.* [14] showed is only moderately different from the stem cell secretome.

Previously, our group identified the following effects of MNC-sec rad in preclinical investigations: (i) regeneration of infarcted myocardium in an acute myocardial infarction (AMI) model [15]; (ii) reduction in microvascular obstruction after AMI [16]; (iii) protection against autoimmune myocarditis [17]; (iv) reduction in infarction area in a preclinical stroke model [18]; and (v) enhancement of wound healing in a murine model of wound healing [19].

Agerberth *et al.* [20] showed that some AMPs are expressed by specific lymphocyte and monocyte populations from MNCs and can be enhanced by proinflammatory cytokines *in vitro*. Mildner *et al.* [19] reported that MNC-sec can improve wound healing in a murine model *in vitro*. Moreover, and more relevant to the human wound setting, we showed that the topical application of irradiated MNC-sec improves the quality of the regenerating skin and increases angiogenesis in the wound area in a porcine burn injury and skin grafting model [21].

In 2014, we began a first secretome-based regenerative Phase I study to investigate the safety and tolerability of topically administered autologous good manufacturing practice (GMP) protocol MNC secretome (ApoSec; Marsyas I clinical trial; EudraCT Nr.: 2013-000756-17; NCT02284360). This trial is intended as a first step towards a clinical proof-of-concept study of DFU patients (Phase II). Because treatment of DFUs is complicated by its poly-factorial pathogenesis, including polymicrobial infection in the wound area, we became interested in whether MNC-sec also comprises antimicrobial activities. To address this question, we produced MNC-sec rad under GMP conditions and investigated AMP content and functional activity *in vitro* and *in vivo*. Our preclinical data support the notion that MNC-sec rad is able to directly kill bacterial pathogens, a much desired effect in the treatment of DFU.

Material and methods

Internal review board and ethics

This study and all experimental procedures were approved by the ethics committee of the Medical University of Vienna (vote number: 1236; 2013) and conducted according to the principles of the Helsinki Declaration and Good Clinical Practice. Written informed consent was obtained from all participants aged 18 40 years. Exclusion criteria were any treatment with immunomodulatory medication during the past 4 weeks and any signs of acute infection. All animal procedures were approved by the Animal Research Committee of the Medical University of Vienna (Prot. Nr.: 66·009/0220WF/II/3b/2014).

MNC-sec preparation

Human MNC secretome were prepared as described previously by Beer *et al* [22.], Irradiated and nonirradiated MNCs and their supernatants were collected separately after 0, 4 and 24 h of incubation and served as experimental groups.

Production of MNC secretome from humans and control medium according to good manufacturing practice protocol for experimental setting

Human GMP MNC-sec samples were prepared as described previously by Altmann *et al.* [18].

Investigation of cell death by ImageStream analysis. We adapted the method described by Pietkiewicz *et al.* for the FlowSight(R) cytometer to the ImageStream (Amnis(R), part of EMD Millipore, MilliporeSigma, The life science business of Merck KGaA, Darmstadt, Germany) cytometer. In brief, cells were stained with AnnexinV FLUOS and propidium iodide (Annexin V FLUOS staining kit; Roche Diagnostics, Basel, Switzerland) in incubation buffer according to the manufacturer's instructions and analysed immediately. In the flow cytometer, debris and doublets were gated out, and focused cells that were excited with the 488 nm laser were used for analysis. Per condition, at least 20 000 events (=pictures) were recorded in the bright field (430 480 nm), Annexin V- FLUOS (505 560 nm) and PI (430 480 nm) channels. Colour compensation from single-labelled samples was performed as described before [23]. For analysis, IDEAS 6.2 software (MilliporeSigma) was used, and focused, single cells were identified using the gradient root mean square (RMS) of the BF image, followed by bright field image area and aspect ratio intensity. The fluorescence intensity of Annexin V-FLUOS and PI allowed to distinguish between double negative (live and healthy) cells, Annexin V-positive (early apoptotic) cells and double-positive cells that can either be late apoptotic or necroptotic. By applying the image-based features 'intensity threshold [> 30%]' and 'contrast morphology' for the PI channel as described in Pietkiewicz *et al.* [23], we could distinguish between late apoptotic (small fragmented nuclei) and necroptotic cells (nonfragmented enlarged nuclei).

Total RNA isolation and quantitative real-time polymerase chain reaction

Total RNA isolation and quantitative real-time polymerase chain reaction (qPCR) were performed as described previously

by Beer *et al.* [22]. The following primer pairs were used: forward primer 5′-AGAAGCGGGTGAGAAACAAA-3′ and reverse primer 5′-TGTGGCTCGGTACTGGCATG-3′ for Angiogenin/RNase5; forward primer 5′-CAGCTGGAACGCA ACATAGA-3′ and reverse primer 5′-TCAGCTGCTTGTCTGCA TTT-3′ for S100A9; forward primer 5′-GCTAACCTCTAC CGCCTCCT-3′ and reverse primer 5′-GGTCACTGTCCCCATA CACC-3′ for cathelicidin; forward primer 5′- GAGTCACAGCA CGAAGACCA-3′ and reverse primer 5′- GGCTGCATGTGC TGAATTT-3′ for RNase7; and forward primer 5′-GATGAGT ATGCCTGCCGTGTG-3′ and reverse primer 5′-CAATCCA AATGCGGCATCT-3′ for B2M. The relative expression of target genes was compared to the housekeeping gene B2M using a formula described by Pfaff *et al* [24.]

AMP enzyme-linked immunosorbent assay analysis of human experimental/GMP MNC-sec and rat serum

AMP levels were measured using commercially available enzyme-linked immunosorbent assay (ELISA) kits, following the manufacturers' instructions (human beta-defensin 124: catalog# SEQ656Hu; human ribonuclease A3: catalog# SEB758Hu; all Uscn Life Science, Wuhan, China; human LL-37, cathelicidin: catalog# HK321; human calprotectin: catalog# HK325; all Hycult Biotech, Uden, the Netherlands; human angiogenin, RNase5: catalog# DY265 R&D Systems, Minneapolis, MN, USA; and rat calprotectin: catalog# HK321; Immundiagnostik AG, Bensheim, Germany). Optical density values were measured at 450 nm on an ELISA plate reader (Victor3 multilabel plate reader; PerkinElmer, Waltham, MA, USA).

Antimicrobial assay and MNC-sec

The antimicrobial potency of MNC-sec was determined by a microdilution assay [25] using the following pathogens: *Pseudomonas aeruginosa* (ATCC 27853), *Escherichia coli* (ATCC 11303), *Staphylococcus aureus* (ATCC 33592) and *Streptococcus pyogenes*. Pathogens were incubated for 3 h with the secretome of irradiated MNCs (250×10^6) at 37 °C. Thereafter, suspensions were diluted and applied to Columbia agar plates with 5% sheep blood. The antibiotic activity was analysed by determination of the number of colony-forming units the next day. The protocol of the bacterial assay is presented in detail in Appendix 1.

Furthermore, we investigated the antimicrobial activity of MNC-sec after blocking two of the major AMPs by neutralizing antibodies. The following antibodies were used for blocking experiments: Angiogenin/RNAse5 (LSBio, Seattle, WA, USA; clone 27E10, 25 μg/mL) and Calprotectin (Santa Cruz, Dallas, TX USA; clone D-5, 25 μg/mL).

Rat AMP secretion after exposure to the human GMP MNC secretome

Adult male Sprague Dawley rats weighing between 300 and 350 g ($n = 21$, Department of Biomedical Research, Medical University of Vienna, Austria) were used for our experiments and housed under standard conditions. In the experiments, the animals were anesthetized with 1·5% isoflurane to assure proper intraperitoneal (i.p.) application of medium or GMP MNC-sec. Animals were divided into one Medium and one MNC-sec rad group and received 1 mL of Medium or GMP MNC-sec rad (the equivalent of 5×10^6 MNCs). Afterwards, six animals were anesthetized deeply with xylazine (10 mg/kg) i.p. and ketamine (100 mg/kg) i.p. after 2, 12 and 24 h (three animals for the MNC-sec rad group and three for Medium), and the inferior vena cava of each animal was punctured to draw blood. This draw was followed by a deep heart incision. The obtained blood samples were centrifuged at 3500 *g* for 15 min to obtain serum.

Statistical analysis

Statistical analysis was performed using GRAPHPAD PRISM4 software (GraphPad Software, La Jolla, CA, USA). Comparisons between groups at given time points were tested by Student's *t*-test. Data are represented as mean ± standard deviation (SD). Differences were also assessed graphically using bars and symbol connection lines. The calculations were performed separately for each experimental setting. A two-sided corrected *P*-value < 0·05 was considered significant.

Results

Antimicrobial activity of MNC-sec against Gram-negative and Gram-positive bacteria

To investigate whether irradiation of MNCs augments antimicrobial activity, we performed an antimicrobial microdilution assay with MNC-sec from irradiated and non-irradiated white blood cells. We observed increased antimicrobial activity of the secretomes derived from MNCs 24 h after irradiation (Fig. 1).

As shown in Fig. 1a, growth of the Gram-negative bacterium *P. aeruginosa* was significantly inhibited by both, MNC-sec rad (71 ± 21) and MNC-sec (14 ± 14; Medium as negative control, 0 ± 0% inhibition; all mean ± SD: Medium vs. MNC-sec rad, $P = 0·0001$; Medium vs. MNC-sec, $P = 0·03$). In contrast, the Gram-negative bacterium *E. coli* (Fig 1b) was also significantly inhibited by MNC-sec rad (66 ± 32) but not by MNC-sec (9 ± 16; Medium as negative control, 0 ± 0% inhibition; all mean ± SD: Medium vs. MNC-sec rad, $P = 0·0005$; Medium vs. MNC-sec, $P = 0·24$). In addition, we also found a

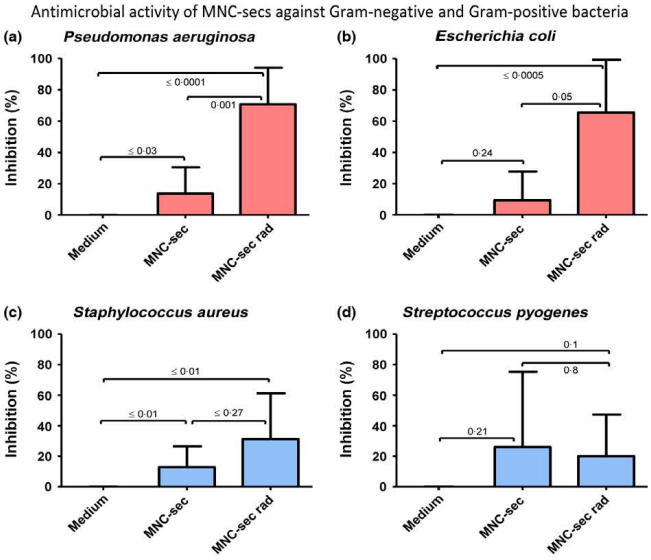

Figure 1 Antimicrobial activity of irradiated and nonirradiated MNC-sec against Gram-positive (blue bars) and Gram-negative (red bars) bacteria, presented data are mean ± SD of nine independent experiments shown as percentages of inhibition. Medium served as control. We show more than 70% inhibition of (a) *P. aeruginosa* growth and more than 60% significant inhibition of (b) *E. coli* growth compared to control (medium). Furthermore, we show up to 30% significant inhibition of (c) *S. aureus* growth and more than 20% insignificant inhibition of (d) *S. pyogenes* growth compared to medium. *P* values ≤ 0·05 were considered statistically significant. All MNC-sec are obtained 24 h after incubation of irradiated and nonirradiated MNCs.

significant growth inhibition of the Gram-positive bacterium *S. aureus* (Fig. 1c) treated with MNC-sec rad (31 ± 28) and MNC-sec (13 ± 12, mean ± SD; Medium as negative control, 0 ± 0% of inhibition, mean ± SD; Medium vs. MNC-sec, $P = 0{\cdot}01$, Medium vs. MNC-sec rad, $P = 0{\cdot}01$). Growth of *S. pyogenes* was only weakly inhibited by both secretomes (Fig. 1d) without reaching statistical significance (Medium vs. MNC-sec, $P = 0{\cdot}1$ and Medium vs. MNC-sec rad, $P = 0{\cdot}21$, respectively).

These results suggest that irradiation of MNC-sec significantly augments the antimicrobial activity against Gram-negative (*P. aeruginosa and E. coli)* and Gram-positive (*S. aureus)* bacteria, each of which important pathogens in DFU.

Gamma-irradiation of MNCs induces early apoptosis and necroptosis

To study how and to which extend MNCs are dying after exposure to γ-radiation, we next performed imaging flow cytometric analysis with ImageStream technology. In previous studies, we were able to show that 24 h after irradiation a huge substantial proportion of MNCs were Annexin V and PI double positive, leading to an increased secretory phenotype of the cells [22,26.] However, using conventional flow cytometry, we were not able to discriminate between late apoptosis and necroptosis. Using automated high-throughput morphological image analysis (ImageStream), we found that the dying cells displayed early apoptotic (26·1%) and necroptic (14·8%) phenotypes (Fig. 2). Early apoptotic cells expose phosphatidylserine on the cell surface, followed by membrane blebbing, nuclear fragmentation and decreased cell volume in late apoptotic cells [27]. In contrast, cells undergoing programmed necrosis show early plasma membrane rupture and fast cytoplasmic and nuclear swelling [28]. Compared to untreated cells (Fig. 2a), we found a strong increase in Annexin V and PI double-positive cells in γ-irradiated MNCs (Fig. 2b,c). Using the algorithm recently described by Pietkiewicz *et al* [23.], we could show that within the Annexin V and PI double-positive cell population 95% showed morphological features described for necroptotic cell death (Fig. 2d).

Irradiated and nonirradiated MNCs express and release antimicrobial peptides

To investigate whether MNCs express antimicrobial peptides, we performed RT-PCR at 0, 4 and 24 h after irradiation and cultivation. We showed a significant increase in angiogenin/RNase5 mRNA levels in irradiated MNCs compared to nonirradiated after 24 h (Fig. 3a; fold change, all mean ± SD, 15·7 ± 1 vs. 6·4 ± 2, $P = 0{\cdot}001$). Cathelicidin mRNA was also expressed in irradiated as well as nonirradiated MNCs and remained quite stable during the time course (Fig. 3b). Gene

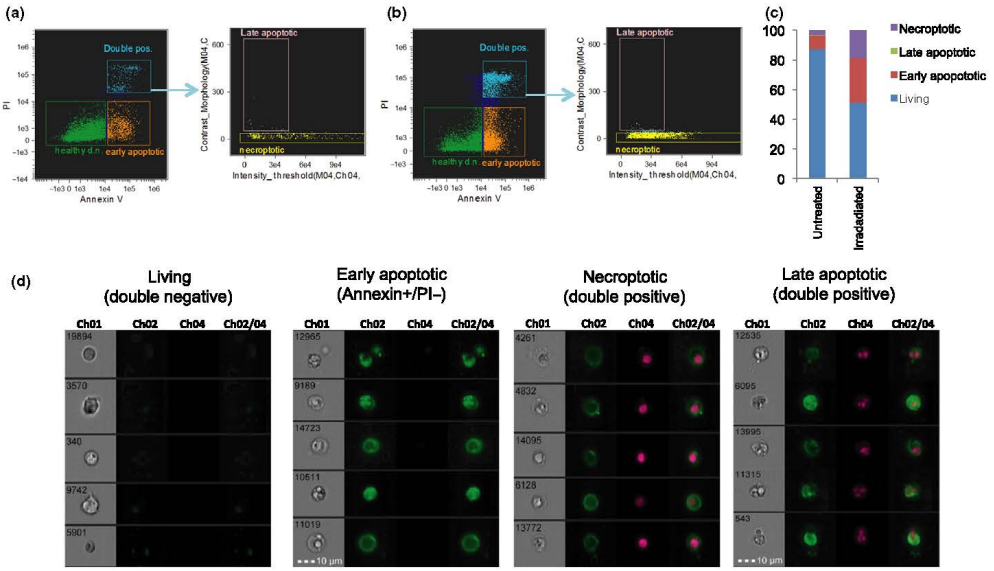

Figure 2 Analysis of the type of cell death. The type of cell death was investigated on MNCs 24 h after exposure to γ-radiation by ImageStream analysis. To detect the percentage of early and late stages of programmed cell death, the cells were stained with annexin V and propidium iodide. FACS analyses of nonirradiated (a) and irradiated (b) MNCs 24 h after γ-irradiation are shown. Viable cells are annexin V and propidium iodide-negative (lower left quadrant), whereas early apoptotic cells are annexin V-positive and propidium iodide-negative (lower right quadrant) and late apoptotic or necroptotic cells are double positive (upper right quadrant). (c) The percentages of the different cell states are show. (d) High-throughput morphological image analysis allows a clear discrimination between living, early apoptotic, late apoptotic and necroptotic cells. Channel 1 (Ch01) shows a light microscopical picture of the cells. Channel 2 (Ch02) shows the green fluorescence staining with Annexin V, channel 4 (Ch04) shows the red fluorescence staining with PI and channel 2/4 (Ch02/04) shows a merged picture of Ch2 and Ch3. One representative experiment of two is shown.

expression analysis of RNase7 decreased within the first 4 h and remained quite stable until 24 h (Fig. 3c). Interestingly, the mRNA expression level of S100A9 decreased in a time-dependent fashion (Fig. 3d). In addition, we analysed the gene expression of human β-defensins 1, 2, 3 and S100A7 and identified no expression in the irradiated or nonirradiated MNC gene pool (data not shown).

To further investigate AMP production on the protein level, we measured the concentration of released AMPs in the supernatant of both irradiated and nonirradiated MNCs after 4 and 24 h (Fig. 3e,f). Although S100A9 mRNA were decreases after 24 h, we found a significant released amount of calprotectin (a dimer of S100A8 and S100A9; Fig. 3e) and cathelicidin (Fig. 3f) after 24 h of culture. Furthermore, irradiation of MNCs further augmented calprotectin release (Fig. 3e; $P \leq 0{\cdot}0002$). Released Angiogenin/RNase 5 protein was not detected in either irradiated or nonirradiated MNC-sec. In addition, we detected high amounts of RNase3 and human beta-defensin 124 in irradiated MNC-sec, two proteins with putative, however so far unknown antimicrobial activity (Appendix 2). Blocking of the two major AMPs (calprotectin and Angiogenin/RNAse5) did not abrogate the antimicrobial activity (Appendix 3).

Presence of AMPs in irradiated MNC-sec produced according to the GMP protocol

The next aim was to investigate whether antimicrobial factors are present in our envisioned final drug product for the

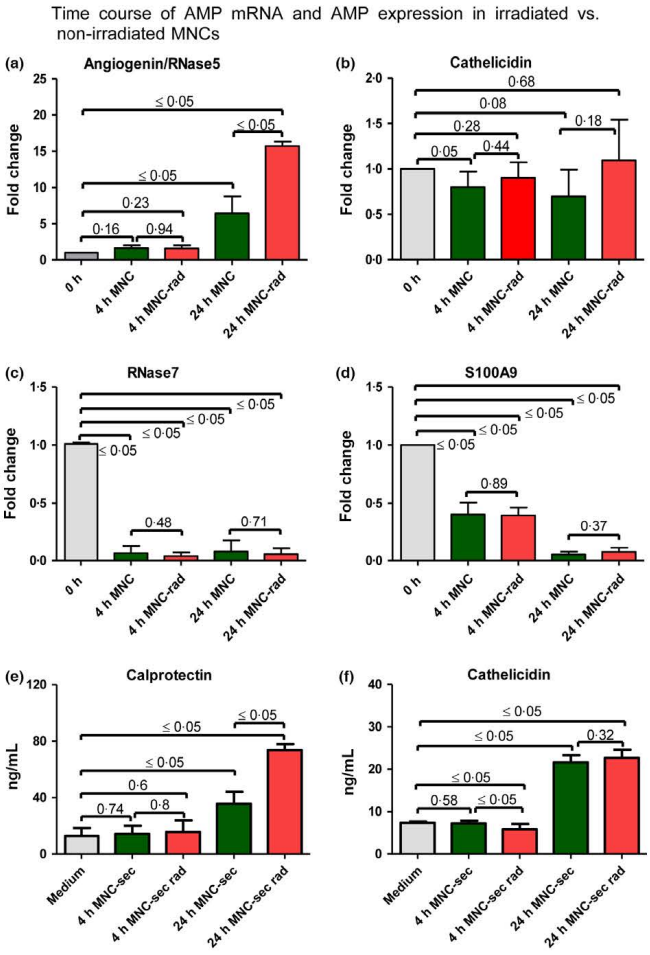

Figure 3 mRNA expression levels of selected AMPs and time course of AMP expression in MNC-sec. The AMPs were selected based on the coverage of different expression levels. The cohort for RT-PCR validation consisted of five patients. Cell culture supernatants derived from irradiated and nonirradiated MNCs ($n = 4$) were collected after 4 and 24 h. The levels of AMPs were evaluated by ELISA. Medium served as control. P values $< 0{\cdot}05$ were considered statistically significant. (a) Significantly higher angiogenin/RNase5 gene expression was detected in the supernatant of irradiated compared to nonirradiated MNCs after 4 h of increase in a time-dependent manner. (b) High levels of cathelicidin gene expression were detected in irradiated and nonirradiated MNCs with a tendency to an insignificant increase in irradiated MNCs. (c) Gene expression of RNase7 was highest at the beginning and decreased within the first 4 h and remained quite stable until 24 h. (d) Gene expression of S100A9 was highest at the beginning and decreased in a time-dependent manner within the first 4 h. (e) Irradiation of MNCs induced higher calprotectin release in the supernatant of irradiated MNCs after 24 h. (f) the concentration of cathelicidin increased significantly in the supernatant of both irradiated and nonirradiated MNCs in a time-dependent manner.

treatment of DFU. Cathelicidin, calprotectin, RNase3 and human beta-defensin 124 were determined to be present in concentrations similar to those of experimental non-GMP-manufactured MNC-sec. These data indicated that the twofold measure of viral clearance (methylene blue and high-dose gamma-irradiation at 25 000 Gy) did not alter AMP content in the final drug product (Table 1).

GMP MNC-sec stimulates endogenous AMP production in rats

To further explore whether GMP MNC-sec induces *de novo* AMPs *in vivo*, we injected human MNC-sec into rats and investigated plasma levels of rat calprotectin at 2, 12 and 24 h. These experiments were similar to those performed by Altmann *et al.* and

Table 1 Concentrations of AMPs in human experimental- and GMP-irradiated MNC-sec (pooled)

AMPs	GMP MNC-sec ng/ml	Experimental MNC-sec ng/ml
Cathelicidin	24·75 ± 5	22 ± 1
Calprotectin	82·25 ± 4	73 ± 4
RNase 3	19·75 ± 3	9 ± 5
DEFB 124	2·75 ± 1	6 ± 1

AMP levels were measured by ELISA. Data are mean ± SD of four independent experiments.

Haider *et al.* showing that human MNC-sec induced *de novo* rat brain-derived neurotrophic factor and CXCL1 chemokine [18,29]. Rat calprotectin concentration was measured by rat-specific ELISA, and we found a highly elevated level of rat calprotectin 24 h after i.p. administration of irradiated MNC-sec (1849 ± 495 compared to 0 h, 404 ± 32; $P = 0{\cdot}007$). This increase was highly significant compared to the Medium group after 24 h (362 ± 66; $P = 0{\cdot}007$), indicating that xenogeneic MNC-sec induces secretion of rat AMPs *in vivo* (Fig. 4).

Discussion

DFU is a major complication of diabetes mellitus with a huge economic burden. Because wound healing is a dynamic and complex process with multifactorial aetiology, the management of DFU requires different actions, including debridement when necessary, optimal control of blood glucose, antibiotic therapy [30]. However, despite all of the efforts made, 14–20% of patients with DFU end up with amputation [31], and more than 50% of those amputees have less than 5-year survival rates [32].

Our group previously demonstrated the regenerative properties of MNC-sec rad. The therapeutic effects of MNC-sec rad include inhibition of microvascular obstruction, vasodilation [16], angiogenesis, enhanced migration of human primary keratinocytes and fibroblasts, reduction in scar formation after burn injury [21] and improvement of wound healing [19], all of which play major roles in wound-healing mechanisms. Here, we investigated whether antimicrobial activity is also a property of MNC-sec because it is well known that infection complicates treatment of DFU and is responsible for at least half of the cases of lower limb amputations [33]. Until recently, only scarce evidence had been published indicating that MNCs secrete AMPs and thus demonstrate antimicrobial activity in functional assays [20,30]. The special focus of our study was on the investigation of the antimicrobial properties of MNC-sec rad which is produced under GMP conditions including a twofold measure of viral clearance (methylene blue and high-dose gamma-irradiation at 25 000 Gy).

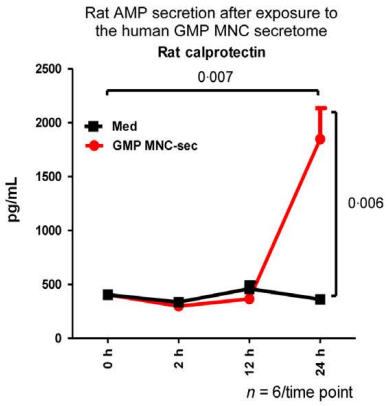

Figure 4 Time course of AMP expression in rat serum ($n = 6$/time point). Secretome MNCs derived from irradiated MNCs after 24 h were injected into healthy male rats. Rat serum samples were obtained at 2, 12 and 24 h after injection. AMP levels were measured by rat-specific ELISAs. A highly significant increase in rat calprotectin 24 h after injection of irradiated MNC-sec compared to medium was detected. All values are shown as mean ± SD. P values < 0·05 were considered statistically significant.

Our findings provide the first evidence that high-dose gamma-irradiation, inducing cell death, has a positive effect on AMP production and secretion of MNCs. Which pathway of cell death accounts for the observed effects of MNC-sec rad is an interesting but still open question. Approximately 50% of the irradiated cells enter a form of programmed cell death. Interestingly, this high dose of ionizing radiation induced early apoptosis, followed by necroptosis. This finding is of particular interest as we have shown previously that γ-irradiation indeed induces a secretory phenotype in MNCs [26] which might be due to the induction of necroptosis. Our data suggest that a very complex interplay between several different forms of cell death contributes to the observed positive activities of MNC-sec rad. The exact mechanisms, however, are still not known and our study builds a basis for future investigations.

In addition, we could demonstrate for the first time that MNC-sec rad displays a direct antimicrobial activity. In particular, the growth of Gram-negative *P. aeruginosa* and *E. coli.* and Gram-positive *S. aureus* was considerably reduced by incubation of these pathogens with the secretome of irradiated MNCs, and to a significantly lesser degree, with the secretome of nonirradiated MNCs. As for the source of this antimicrobial activity, a high abundance of different AMPs was found in the

conditioned medium. Interestingly, blocking two major AMPs present in our secretome did not abrogate its antimicrobial activity, suggesting multifactorial components accounting for the observed bactericidal action of the secretomes. This is in line with one of our previous studies, were we demonstrated that blocking the major survival factors (VEGF, IL-8, ENA-78) with blocking antibodies was not sufficient to abolish the cytoprotective activity of the secretome [34]. In our exploration, we showed a significant enhancement of antibacterial activity of irradiated MNC-sec. In respect to DFU and its potential favourable treatment with MNC-sec, *Pseudomonas* and *S. aureus* are most frequently found in this disease entity [35]. In addition, we also showed a significant increase in the antibacterial activity of irradiated MNC-sec against Gram-negative *E. coli* as compared to nonirradiated MNC-sec. The potential use of MNC-sec for systemic and topical treatment of *S. aureus* relates to the fact that a wide variety of human illnesses, ranging from relatively benign soft tissue infections to life-threatening toxic shock syndrome, necrotizing pneumonia and infective endocarditis, is still in need of alternative treatment options [36,37].

To further substantiate the presence of antimicrobial components in MNC-sec, we performed gene expression analyses that were validated by ELISA at the protein level. RT-PCR validation of angiogenin/RNase5 showed that irradiation led to a massive increase in mRNA production after 24 h ($P < 0{\cdot}0001$). These results supported our primary hypothesis that irradiation causes increased AMPs in MNC-sec. Despite high mRNA expression, we were not able to detect angiogenin/RNase5 at the protein level in our MNC-sec, suggesting that high amounts of the protein are stored intracellularly. For the release of the protein further triggers, such as pathogen recognition, might be necessary. However, to fully elucidate this mechanism, further experiments are needed.

Cathelicidin mRNA was also expressed in both irradiated and nonirradiated MNCs, findings that were corroborated using a commercially available ELISA system to verify protein release. This finding was of particular interest as cathelicidin has been previously identified as the responsible factor in the antimicrobial activity of MSC secretomes [9].

In addition, we found high levels of secreted calprotectin in irradiated MNC-sec. Interestingly, while secreted calprotectin protein was increasing, calprotectin mRNA decreased in a time-dependent manner. This might represent a mechanism by which rapid antimicrobial action is provided, but overproduction leading to strong inflammatory reactions is tempered by downregulation of mRNA production. A similar mechanism could also account for RNase7. However, due to technical limitations, we were only able to analyse RNase7 expression on the mRNA level. The exact underlying mechanisms remain to be elucidated.

It should be emphasized that the analysed substance contained the secretome of 25×10^6 MNCs. However, as previously described [20], we obtained the supernatant of 250×10^6 cells for the microdilution assay. Therefore, we argue that the effective concentration of soluble AMPs in MNC-sec is far beyond that measured.

In spite of the limited information about the role of AMPs in the pathogenesis of DFU, β-defensins are known to be overexpressed in DFUs, whereas the AMP cathelicidin shows low or no expression in comparison with healthy skin. These data suggest that possible MNC-sec treatment in DFU, containing cathelicidin and others (calprotectin, RNase3, DEFB124), would be an appropriate measure to promote wound healing by preventing secondary infections [5].

In the setting of topical and systemic infection, autochthonous production of AMPs would be a most desired effect in MNC-sec-treated patients because AMPs are well characterized and include recruitment of macrophages, granulocytes and lymphocytes like T cells by α-defensins [38 40] or chemotactic activity of cathelicidin with the highest affinity for CD4 T cells [20].

Our findings revealed that naive rats exposed to xenogeneic GMP MNC-sec showed a marked increase in rat calprotectin in a time-dependent fashion. Thus, we present here for the first time compelling evidence that MNC-sec rad can stimulate the secretion of endogenous AMPs *in vivo*. Whether or not irradiation of MNCs is indeed necessary to induce endogenous AMP expression remains to be elucidated.

Taken together, our results suggest that *in vitro* and *in vivo* effects on vascularization and wound healing can be associated with angiogenic and antimicrobial/chemotactic AMPs like cathelicidin [41]. Furthermore, we provide strong evidence that MNC-sec rad can have direct and indirect positive effects on the immune system, including exerting antibacterial properties.

Acknowledgements

The Christian Doppler Research Association provided funding for manuscript editing. We thank Ionela-Mariana Nagelreiter for technical help with the image stream analysis.

Disclosure

The Christian Doppler Research Association, APOSCIENCE AG and the Medical University of Vienna funded this study (Patent number: PCT/EP09/67534, filed 18 Dec 2008; Patent name: Pharmaceutical Preparation Comprising Supernatant of Blood Mononuclear Cell Culture). H.J. Ankersmit is a shareholder of APOSCIENCE AG, which owns the rights to commercialize MNC-sec for therapeutic use. All other authors declare that they have no competing financial interest.

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Received 14 September 2015; accepted 9 August 2016

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Appendix 1

Bacterial microdilution essay protocol

To store bacterial colonies over a period of years we use glycerol/broth slurries stored at −80 °C. So we transport bacterial colonies from a Plate or a stab by a sterile plastic or metal loop into glycerol/broth slurry. Glycerol is a cryoprotectant, which will help the culture to survive under frozen conditions. These frozen cultures are stored at −80 °C and are used for 'plating out' colonies.

Plating out bacterial colonies

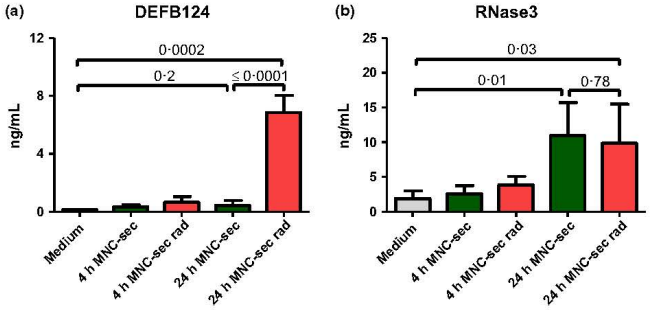
1 Place plates on bench top to let them come to room temperature.
2 Fetch the frozen glycerol/bacteria solution from freezer (never allow it to thaw).
3 Lightly scratch the surface of the frozen slurry by an inoculating loop.
4 Streak the loop all over the plate surface.
5 Incubate plates inverted at 37 °C overnight.

We can store streaked plates in a refrigerator for less than a week but for a bacterial assay it's important always to use freshly streaked colonies.

Bacterial assay procedure

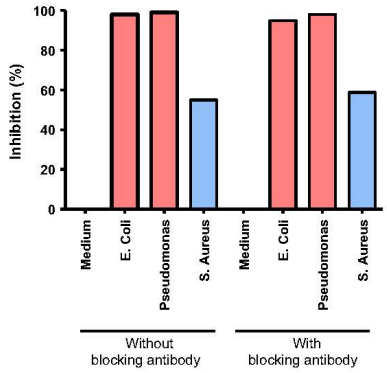
1 Gently scrape off 2 4 bacterial colonies from the overnight incubated plates by a cotton swab.
2 Put the cotton swab into a 10 mL tube, resuspend with 10 mL 1% Tryptic Soy Broth (TSB) and vortex for 5 s.
3 Mix 100 μL of bacterial solution with 100 μL medium, MNC-sec or MNC-sec rad, and incubate for 3 h at 37 °C with shaking.
4 Dilute bacteria mixture 1 : 10 000 with 1% TSB and plate 100 μL on a Columbia Agar palte.
5 Incubate the the agar plates for 24 h at 37 °C.
6 Count the colonies on the next day.

Appendix 2



Time course of antimicrobial peptide expression in irradiated vs. non-irradiated MNC-sec. ($n = 4$), P values < 0·05 were considered statistically significant. Cell culture supernatants derived from irradiated and non-irradiated MNCs were collected after 4, and 24 h. The levels of AMPs were evaluated by ELISA. Medium served as control. (a) irradiation of MNCs induced higher DEFB124 release in the supernatant of irradiated MNCs after 24 h ($P = 0·0002$), significantly so compared to non-irradiated ($P < 0·001$); (b) the concentration of RNase3 increased significantly in the supernatant of both irradiated and non-irradiated MNCs in a time-dependent manner ($P = 0·03$, $P = 0·01$ respectively).

Appendix 3



Blocking of antimicrobial peptides in irradiated MNC-sec. Antimicrobial activity of irradiated MNC-sec against Gram-negative and Gram-positive bacteria with and without blocking two of the major AMPs by neutralizing antibodies, presented data are one representative experiment of two ($n = 1$) shown as percentages of inhibition. Medium served as control. Blocking of the two major AMPs (Calprotectin and Angiogenin/RNAse5) did not abrogate the antimicrobial activity.

CHAPTER THREE: DISCUSSION

3.1 General Discussion

Years before the discovery of penicillin, scientist, among them Alexander Fleming, had limited knowledge about the innate antimicrobial activity of the human body, in particular human blood and human body secretions. Some antimicrobial components of human body fluids could be determined so far. For example, Fleming's investigation on lacrimal secretion revealed bactericidal activity due to presence of a potent antimicrobial agent called lysozyme. In the early Years of the 21st century research focused on antimicrobial components of the respiratory tract.(216) In addition to lysozyme a wide range of other antimicrobial peptides have been identified in the airway surface liquid expressed by mucosal cells, submucosal glands and immune cells.(217, 218) However some issues still remained to be elucidated. For example, the bactericidal activity of the pleura specially in a post-operative setting and the antimicrobial content along with the cellular source still remained unanswered.

Moreover, direct antimicrobial activity of human whole blood against different bacteria have also been observed by Fleming.(216) However the specific identification of white blood cell subpopulation responsible for that antimicrobial effect took place in the late 19th century where accumulated data evidenced the antimicrobial activity of some MNCs against gram negative and gram positive bacteria.(219-221) Separate from direct antimicrobial activity some researcher were also interested in existence of antimicrobial factors, such as AMPs in human blood. Thus O. Sørensen showed AMP in human plasma.(222) Followed by Agerberth(223) who finally reported of AMP expression in some MNCs isolated from the human whole blood. However the question was still unanswered, whether the specific features of the MNCs were comparable with that of the secretome of MNCs particularly in regard of antimicrobial activity. Moreover the impact of conditioning via irradiation was still to be investigated.

3.2 Antimicrobial Peptides in Postoperative Pleural Fluid

Open lung surgery is a frequent procedure performed in the majority of thoracic surgical operations. Indeed, opening the pleural cavity, which is considered sterile, is inevitable while performing thoracotomy. In contrast, the lung is basically considered non-sterile and is contaminated very often. Consequently, the operation field turns into a non-sterile area because of opened lungs. Belda *et al.*, demonstrated over 80% perioperative bronchial colonization, partly by pathogenic microorganisms, amongst patients who had undergone lung surgery. Nevertheless post-operative infectious complications have still been reported to be very rare, as demonstrated by Yamauchi *et al*.(224, 225) Furthermore, the production of pleural fluid increases up to 400-600 ml/day during the postoperative period compared to a daily production of 200-250 ml/day(91) in healthy non-operated subjects, and this may contribute to an augmented defence mechanism against postoperative infection.

We thus conducted the following study, analysing the pleura and its components for its own internal protective factors, with special emphasis on the antimicrobial pattern of pleural fluid. Our antimicrobial potency evaluation of postoperative pleural fluid, performed by bacterial microdilution assays, revealed high antimicrobial activity of pleural fluid against different gram negative bacteria, such as E. coli and P. aeruginosa, and gram positive bacteria, such as S. aureus, S. pneumoniae and S. pyogenes. Nevertheless, the patients included in the study received perioperative antibiotic coverage. Comparing our results with those from patients who had not received antibiotic coverage, revealed only marginal differences of antimicrobial activity, indicating a high abundance of antimicrobial agents in the pleural fluid, barely augmented by antibiotics. This result encouraged us to investigate the presence of AMPs in pleural effusions.

To show whether AMPs are present in postoperative pleural fluid we analysed the concentration of different known AMPs in that fluid using commercially available ELISA systems. Our results revealed high concentrations of diverse

AMPs, including hBD1, hBD2, S100A7, S100A8/A9, RNAse5, RNAse7, and cathelicidin.

It is already known, that AMP expression takes place mostly at body sites with high pathogen exposure such as pleura, which is under constant exposure to lung pathogens. However, the expression pattern differs depending on the cells and tissue type. After detecting high concentrations of AMPs in postoperative pleural fluid, and with the knowledge that AMPs in pleural fluid could either originate from infiltrating leukocytes or from pleural epithelium(96), we performed flow cytometry analysis to further investigate the cellular content of that fluid. Interestingly, no epithelial component could be detected, and the leukocyte population consisted mainly of CD45+ leukocytes with the majority being granulocytes (> 90%), which were partly replaced by monocytes in a time dependent manner. To further characterize the cellular source of the detected AMPs, investigations have been directed towards gene analysis, determining whether AMPs were of leukocytic or pleural epithelial origin. According to the literature, pleural mesothelial cells are the most common cell types in the pleural space, and are the first line of response against invading pathogens and other immune stimuli. They release diverse proteins upon activation, including AMPs that play a crucial part in the immunity of the pleura. For that purpose, to further substantiate the cellular source of AMPs, PCR analyses have been performed using cellular components of pleural fluid, parietal pleural specimens and pleural mesothelial cell cultures. Pleural mesothelium has been shown to be one significant source for S100A7. In contrast, high gene expression for S100A8/A9, RNAse5/angiogenin, cathelicidin and RNAse7 has been shown in pleural fluid containing high amounts of leukocytes, indicating that these two immune cell types are the major cellular source for these AMPs. We further confirmed our findings by immunohistochemical stainings of pleural specimens. S100A7 was exclusively detected on the epithelial linings confirming epithelial origin of that peptide, whereas HBD1, HBD2 and RNAse5/angiogenin were mainly expressed in the dispersed tissue leukocytes and showed weak epithelial expression. On the other hand, S100A8/A9 has been detected exclusively in leukocytes, with no epithelial expression.

Moreover, compelling evidence suggest that the induction and activation of AMPs is triggered upon inflammation and/or injury. For example by pro-inflammatory cytokines secreted into the pleural space in response to TLR activation following pathogen encounter. The important mediators include IL-1α, IL-1β, IL-6, ENA-78, MCP1, MIP-1α, transforming growth factor–β, fibroblast growth factor and insulin-like growth factor.(90) Elevated concentrations of several pro-inflammatory cytokines, including IL-1b, IL-6, IL-8 and TNFα have been shown in postoperative pleural effusions in terms of immediate immune response, which decreased during the postoperative days. In contrast, increasing concentrations of anti-inflammatory cytokines, including IL-10 and soluble ST2, have been observed on the first postoperative day and stayed stable during the observation period.

Accumulated data suggested that AMP concentrations can be upregulated in diverse human epithelial layers during a proinflammatory response after contact with pathogens. For example, upregulation of the human AMPs calprotectin and psoriasin has been shown in keratinocytes in response to release of pro-inflammatory cytokines, such as IL-1, IL-6, TNFα, and interferons upon triggering via TLRs.(39, 226) In addition, according to Hussain *et al*. staphylococcal peptidoglycan dependent activation of TLR in murine primary pleural mesothelial cells induces upregulation of the AMP mBD-2.(33)

We therefore investigated the influence of pro-inflammatory cytokines on postoperative AMPs in pleural mesothelial cell culture upon triggering with different TLR ligands: TLR1, TLR2, TLR3, TLR4, TLR5, TLR6, TLR7, TLR8, TLR9, IL-1b, and TNFα. However, despite an increase of diverse cytokines in terms of local immune responses immediately after surgical trauma, no changes on AMP gene expression were observed, except an increase of S100A7 gene expression induced by TNFα. This independence of AMP expression of pro-inflammatory stimuli in the pleural epithelial cell culture suggests a rather leukocytic origin of the detected AMPs in postoperative pleural fluid.

In addition, our detailed analysis revealed high AMP concentrations in pleural fluid samples obtained immediately after surgical chest opening, irrespective of

the extent of the operation (wedge resection, lobectomy, pneumonectomy). The measured concentration remained constant during the postoperative period.

In studying our data, we noted a high bactericidal activity of pleural fluid in both patient populations receiving perioperative antibiotic prophylaxis and patient populations without antibiotic coverage, with a slight trend towards higher antibacterial activity in the latter group. This data is of interest because the positive impact of perioperative antibiotic coverage is still controversial, despite several prospective randomized controlled trials having been performed. No difference in postoperative infectious complications after lung operations could be seen between patients with and without antibiotic prophylaxis.

3.3 Antimicrobial Property of MNC Secretome

Only limited data have so far been obtained with respect to AMPs secreted from MNCs, but in functional assays these provide evidence of antimicrobial activity. We thus undertook to investigate the antimicrobial activity of the MNC secretome in more detail, together with its response to irradiation. Our research results provide the first evidence for positive effects of irradiation on antimicrobial and immunomodulatory properties of the MNC secretome. Our analysis revealed that the secretome of irradiated MNC significantly inhibited the growth of Gram-negative Pseudomonas, as compared to non-irradiated MNCs. Pseudomonas is known as one of the most frequently detected bacteria in DFU.(227). and the second most prominent biofilm former amongst DFU colonizing pathogens.(173)

In contrast to its activity against Pseudomonas, we could only detect an insignificant trend towards higher antibacterial activity of the irradiated MNC secretome against E. coli compared to the non-irradiated MNC secretome. However, the non-irradiated MNC secretome, did show a significant antibacterial activity against S. Aureus which together with E. coli are the leading colonizing pathogens of DFUs. S. aureus accounts for a vast diversity of severe human diseases, with methicillin-susceptibility in only 64% of cases, and

is the predominant biofilm former amongst isolated biofilm forming pathogens.(173) E coli, in addition have been shown to lead the bacterial population producing extended spectrum of beta lactamase.

In light of these observations the MNC secretome is likely to be a promising alternative treatment option for infected DFUs, considering high rates of methicillin-resistence and the consequential limitation of antibiotic medication for both systemic and topical treatment.

To identify antimicrobial components in the secretome of MNCs we conducted analyses at the gene level by RT-PCR, followed by analyses at the protein level by ELISA. We showed expression of different well-known AMPs in irradiated MNCs, as well as in non-irradiated MNCs, in a time dependent manner. RT-PCR results showed an increased transcription of the angiogenin gene 24 hours after irradiation. This result was highly significant compared to angiogenin gene expression in non-irradiated MNCs, confirming our primary hypothesis that irradiation can upregulate AMP secretion in the MNC secretome. Unfortunately, despite high expression levels of this gene, no protein product could be detected in the subsequent ELISA analyses. This may be either due to the fact that translation of the gene information requires the passing of many regulatory stages that control transcription and translation, thus preventing information transfer into protein production, unless further triggering mechanisms are activated. Alternatively, large amounts of the peptide may already have accumulated intracellularly, but still require further triggering mechanisms to be released. Hence further experiments are necessary to entirely explain the underlying mechanism. As mentioned above, angiogenin (RNase5) is an AMP with potent angiogenic activity, and a key stimulator of angiogenesis, induced by other angiogenic factors, like VEGF, EGF or FGF. Analyses by Trujillo *et al.* on effects of calcitriol in the promotion of angiogenesis in DFU revealed compromised angiogenin expression in DFU compared to healthy controls at both the gene and protein level. He, furthermore, noted stimulation and augmentation of angiogenin production following incubation of keratinocytes with calcitriol *in vitro*, indicating the angiogenic role of angiogenin in the induction of angiogenesis.(228)

Further characterization of antimicrobial components in the MNC secretome revealed high gene expression of cathelicidin in irradiated as well as non-irradiated MNCs, with an insignificant trend towards increased expression in irradiated MNCs. Furthermore, high amounts of cathelicidin protein could be detected in the secretome of MNCs by commercially available protein detection ELISA systems, indicating that the gene was being translated.

Cathelicidin, as a potential antimicrobial therapeutic for chronic wounds has been reported to accelerate re-epithelialization in many different models of acute and chronic wounds. However, of the multiple beneficial activities attributed to cathelicidin the following are likely to be necessary for treating DFU: potent wound healing capacities, together with enhanced re-epithelialization and granulation tissue formation, angiogenesis, keratinocyte migration and, of course, the broad spectrum antimicrobial activity against several bacterial species including, E. coli, P. aeruginosa, Klebsiella pneumoniae, Acinetobacter baumanii, Salmonella typhimurium, Stenotrophomonas maltophilia, Proteus vulgaris, Neisseria gonorrhoeae, Staphylococcus spp., Streptococcus spp., Enterococcus spp., Bacillus spp., Lactobacillus acidophilus, Lysteria monocytogenes and Propionibacterium acnes.(25, 28-30) Furthermore, fungicidal activity against Candida albicans can also be advantageous in the therapy of DFU.(31) Interestingly, diabetic patients have been shown to suffer from compromised cathelicidin gene expression in peripheral blood cells. Concomitantly, lack of gene expression of that peptide in the skin of those patient populations, especially in diabetic wounds in comparison with healthy skin, indicates susceptibility of those patients to infectious diseases, suggesting a crucial role of that peptide in the healing capacity of DFUs.(25, 27) In line with existing literature, we therefore propose that cathelicidin is one responsible factor for the antimicrobial activity of MNC secretome. This data provide an interesting approach for treatment of DFU with the secretome of MNCs.(61)

Calprotectin levels increased significantly by irradiation in MNC-sec while calprotectin gene expression decreased in a time dependent fashion and was not affected by irradiation. This may be because of the activation of a negative

feedback effect on gene expression necessary after a quick upregulation of the gene product, preventing an inflammatory response by overproduction of the mentioned factors. Calprotectin, a member of S100 calcium-binding protein family, characterized by his heterodimeric shape composed of S100A8 and S100A9, implicates an anti-inflammatory and antimicrobial response of the immune system. It is a well characterised AMP which is involved in many different cellular processes like cell signalling, cell cycle regulation, cell growth and cell differentiation. Broad spectrum antimicrobial activity has been described for this peptide, ranging from bacteria, including S. aureus, S. epidermis, E. coli, Klebsiella, Borrelia burgdorferi, Capnocytophaga sputigena, to fungi, including Candida albicans. Increased amounts of Calprotectin have been identified in psoriatic lesions of patients suffering from psoriasis.(39)

We furthermore evidenced a significant attenuation of RNase7 gene expression in irradiated as well as non-irradiated MNCs in a time dependent manner, whilst the expression of this protein has been shown to be suppressed constitutively in chronic non-healing wounds.(229) Unfortunately, we could not measure the concentration of the protein product in our investigated secretome due to technical limitation. However, similar to calprotectin, we assume that decreasing gene expression prevents an overproduction of the protein product avoiding an unnecessary inflammatory response which, of course, needs further elucidation. As a recognized AMP of human skin, RNase7 shows comprehensive antimicrobial activity against different sorts of microbial pathogens, including gram-positive and gram-negative pathogens such as S. aureus, Propionibacterium acnes, P. aeruginosa, E. coli, and vancomycin-resistant Enterococcus faecium, one of the most challenging nosocomial pathogens to treat, and fungi such as Candida albicans.(48, 230)

In addition, utilizing commercially available ELISA systems, we detected two further AMPs in our investigated secretome of MNCs. Expression of Eosinophil cationic protein, also called RNase 3, was increased equally in irradiated as well as non-irradiated MNC-sec. In contrast, DEFB124 was increased significantly by irradiation. RNase3 is another AMP from the RNase A superfamily, with accumulating reports of its high antimicrobial activity against different sort of

pathogens namely bacteria, viruses, fungi and helminths. More interesting, a potent eradication activity against P. aeruginosa biofilm has been noted for this peptide in previous literature. For example, antibacterial activity of RNase3 against P. aeruginosa and its biofilm has been shown by Pulido *et al*.(176) Moreover, Domachowske *et al*. demonstrated direct antiviral activity against subtype B of human respiratory syncytial virus *in vitro*.(231)

In the treatment of local and systemic infection, especially in DFUs, enhancement of endogenous AMP secretion is one major desirable objective. However, some AMPs are well characterised for their capacity to recruit immune cells such as granulocytes, macrophages, and T lymphocytes, for example by alpha-defensins or chemotactic activity of cathelicidin.(223, 232-234) In an animal experiment with adult male Sprague–Dawley rats we demonstrated a time-dependent upregulation of rat calprotectin following intraperitoneal injection of human MNC secretome. We thus provide compelling evidence that the *in vivo* application of MNC secretome, produced under a GMP protocol, leads to augmentation of organism specific AMPs, indicating a positive immunomodulatory property of the MNC secretome in general, and independent of the species of origin. However, this is more likely due to the chemotactic properties of the soluble AMPs than to any immunogenicity of the MNC secretome itself. Haider *et al*. showed that the cytokines IL-10, TNF-α, and IL-1β, were not detectable up to 24 h after intraperitoneal administration of human MNC secretome in healthy rats compared to control animals.(71) In addition, Hoetzenecker *et al.* showed that the MNC secretome has immune suppressive properties *in vivo*.(67) As we know, the MNC secretome consists of many different soluble pro- and anti-inflammatory cytokines, including IL-6 and interferon-ɣ.(78) This could be one possible mechanism behind the increased presence of AMPs *in vivo* after intraperitoneal administration of human MNC-secretome, as described by Mørk et al.(235) and Agerberth et al.(223)

According to all the data provided above, the MNC secretome, comprising various AMPs such as cathelicidin, calprotectin, RNase3, angiogenin, or DEFB124, is a promising therapy option for the management of DFU. Furthermore, particular conjoined unique characteristics making it an

appropriate therapy for DFU include wound healing promotion and augmentation of angiogenesis, together with neuroprotection and, more importantly, elimination of the existing and prevention of a secondary infection, along with enhancement of endogenous AMP secretion.

To further identify factors which could contribute to the beneficial effect of the MNC secretome we inactivated two of the major AMPs present in it (angiogenin and calprotectin) by performing a neutralizing antibody mediated blocking assay. Interestingly, neutralizing these AMPs did not abrogate antimicrobial activity, suggesting multifactorial genesis of the observed effects of the MNC secretome. This confirms our previous results, where cytoprotective activity of the MNC secretome could not be altered after inhibiting the activity of survival factors, including VEGF, IL-8 and ENA-78 with specific blocking antibodies.(78)

Within this thesis for the first time we provide compelling evidence that high-dose gamma-irradiation of MNCs affects their secretory pattern, inducing antimicrobial peptide production *in vitro* and *in vivo*. Positive therapeutic outcomes as a result of the changes of this secretory pattern range from vasodilatation, amelioration of microvascular obstruction, neo-angiogenesis, neuroprotection and neuro-regeneration, to augmented anti-infective effects. Altogether these events relie on the ability of the cells to secrete a mixture of multiple different factors that mediate a various diverse reaction, contributing to an enhanced and improved wound healing capacity of the MNC secretome. As an explanation for the observed positive changes in the secretion of MNCs in response to irradiation, induction of cell death should be noted. Different kinds of cell death have been observed in MNCs subjected to irradiation, including apoptosis and necroptosis. As mentioned previously, necroptosis is a form of regulated and programmed necrosis, induced in replay to diverse stimuli, including radiation. It is characterized by specific morphological changes, such as early loss of membrane integrity followed by membrane rupture, fast cytoplasmic and nuclear swelling, and a modified secretory phenotype.(73) Considering the fact that about 50% of all irradiated MNCs undergo a kind of early apoptosis followed by necroptosis, we can argue that a complex interplay between diverse kinds of cell death account for the observed secretory changes

in irradiated MNCs.(236) The exact understanding of the underlying mechanism remains to be elucidated, however, our study may serve as a basis for future investigations.

3.4 Diabetic Foot Ulcer

DFU, as an example for chronic wounds, is defined as a diabetic foot with a chronic wound, characterized by impaired healing capacity and dysfunctional regeneration ability. It is an extremely common complication of poorly controlled diabetes, subjecting a large percentage of the affected patients to the most feared consequences, such as limb amputation. Aside from worsening patients' physical and mental health, leading to decreased patients' quality of life, the associated socioeconomic burden, including medical expenditures, are considerable. Therefore, in view of the negative impact on healthcare systems, a thorough evaluation of a diabetic foot is important for the prevention of an ulcer. The development of almost all chronic wounds, including DFU, usually begins with a neglected small cut or abrasion. An insufficient or even arrested repair process, due to various factors, leads to the chronification of the wound healing process. Recently, a great deal of research has been directed towards understanding the critical factors that influence poorly healing wounds such as DFU. The most significant risk factors for developing DFU include peripheral neuropathy with the subsequent anatomical deformities, together with vascular insufficiencies, followed by an array of dysfunctions, including coagulation, homeostasis, inflammation, proliferation, and remodelling deficiencies. Although infection is not primarily a part of the aetiology of DFU, such ulcers are extremely susceptible to infection once the wound is present, making the infection one of the most important characteristic factors of DFU. Moreover, due to the multifaceted nature of DFU, numerous different comorbidities must usually be taken into account prior to developing a treatment plan. Once the DFU has occurred an adequate description of ulcer characteristics is essential for the choice of the correct treatment method in order to achieve adequate healing. Thus, management of DFU and its sequela is a challenging obstacle,

needing a multidisciplinary approach due to the multifactorial aetiology. Along with diabetic diet, regular exercise, and optimal control of glycaemia, further treatment, including adequate debridement, offloading the wound area, together with the use of wound dressings and sufficient antibiotic treatment in the case of contaminated or infected wounds, must be considered when planning an individual-specific treatment. By definition, an ideal therapy should comprise early intervention to achieve sufficient healing and prevent recurrence of the lesion once it has healed. However, regardless of the efforts made in developing numerous different topical and systemic products to treat DFU, only a few have succeeded to be efficacious, while the rest are considered to be far from ideal. Moreover, the conventional therapies are still not satisfying enough, and a great percentage of this patient population ultimately face limb amputation, followed by low survival expectancy. Hence, there is an intense medical need for therapies that accelerate the sustainable closure of these cutaneous ulcers, control the disease and prevent progression, both for the well-being of the patients, and to reduce costly hospitalisations due to the sequelae of the ulcers.

Extensive evaluation, laboratory investigations and clinical studies have yielded a wealth of information on management of DFU with cellular therapy, characterized by cells from various sources with regenerative and multi-differentiational potential, with especial focus on the field of stem cell therapy. Stem cells, as biologically undifferentiated cells, possess the unique capacity to differentiate into diverse type of tissues. Some studies have demonstrated effective treatment outcomes by taking advantage of particular stem cell specific characteristics, including cell generation, cell differentiation and cell proliferation with respect to vascularization, neuroregeneration, regulation of inflammation, remodelling of ECM and so on. All of these are important aspects of sufficient wound healing. However, despite several benefits of stem cell therapy, including enhancement of angiogenesis, elevation of anti-inflammatory cytokines and growth factor levels and differentiation into numerous tissues types, particular comparative disadvantages confront this promising therapy modality. In particular, the use of embryonic stem cells involves destruction of

blastocysts from fertilized eggs, and this is ethically problematical. Furthermore, due to the novelty of these technologies, lack of data regarding long-time unwanted effects is still an issue. Moreover, associated surgical risks, particularly in the matter of bone marrow mesenchymal stem cells (MSC) biopsies, must be considered prior to sampling. Low stem cell concentrations and proliferation capacity, for example, of haematopoietic stem cells, used mainly to treat patients suffering from leukaemia, delineates another imperfection. Even though functional multi-lineage Haematopoietic stem cells (HSCs) (called induced-HSCs), have been developed, which have an improved capacity to regenerate and differentiate, thereby making them more suitable for transplantation, the *in vivo* application is still challenging.(237) Another matter is the lack of a standardized consensus of stem cell isolation protocols, since stem cells need to be isolated by various purification techniques and from various different sources in advance to their therapeutic usage. Finally, therapeutic capacities of stem cells often decline after administration, and the maintenance of multipotential capacities have been seen only in a limited numbers of cells during *in vitro* experiments.(237)

In addition, the concentrations of transplanted cells and their performance are considerably influenced by patients' comorbidities. For instance, stem cells from DFU patient's as well as other chronic wounds have been shown to be impaired and defective.(56) To overcome this obstacle, delivery of healthy stem cells is necessary which, on the other hand, confronts patients receiving this therapy with other feared consequences, including increased risk of malignancy, unwanted immune responses and the transmission of adventitious agents.(238) Based on the existing data, various approaches have been developed and numerous medical products have been promoted for DFU care, but with unfortunately limited therapeutic efficacy. For example, the tumorigenic potential of stem cell therapy, especially MSCs, has been demonstrated in different studies.(239) This increased rate of malignancy has been associated with several factors, including isolation, expansion, *in vitro* culturing, the source of the cells, cell dosage and the route, as well as timing, of administration.(239) On the whole, the safety of these different approaches still remains

questionable, especially following cell modification and manipulation, which is associated with higher tumorigenic potential. As mentioned above, another possible consequence of stem cell therapy is that it can affect the host's immune system. The induction of an immune response may occur directly by leukocyte infiltration, as shown by Nussbaum *et al.*, leading to graft rejection(240), or may happen indirectly by immunomodulatory effects on the host's immune system. Interestingly, the immunogenic potential of stem cells has been shown to amplify upon differentiation, making the *in vivo* application more unpredictable.(238) Stem cell therapy as a cell-based product does not undergo pathogen inactivation or a removal step, nor does it undergo a purification step, making it one of the serious risk factors for pathogen transmission between donor and host. This disadvantage applies to all cell based therapeutic entities.(241) Because of these limitations, and while advantages and disadvantages of cellular therapy are still being considered for *in vivo* therapeutic utilization, a good deal of research has focused on paracrine factors from the same cells. In contrast to earlier assumptions, where the benefits of cellular therapy were accredited to the cells and certain cellular capacities, a number of researchers focused on the paracrine components from cellular sources. They pursued the idea of therapeutically using conditioned media and tried to prove that the achieved therapeutic successes are, on the contrary, due to paracrine constituents in the supernatant of the respective cells. For example, Gnecchi *et al.,* provided evidence for the promotion of tissue regeneration by soluble paracrine factors released from MSCs.(60) The emergence of this novel kind of therapeutic strategy has been supported by others who were able to demonstrate antimicrobial, angiogenic and wound healing capacities in the secretome of MSC.(61-63) Recently, this therapy modality has been taken advantage of in the management of DFU. Increasing evidence shows high levels of various growth factors, including insulin-like growth factor (IGF-1), transforming growth factor-β (TGF- β), vascular endothelial growth factor (VEGF), interleukin-8 (IL-8), Hepatocyte growth factor (HGF), Fibroblast growth factor (FGF), and cytokines in the secretome of MSC. Many diverse therapeutic effects could be attributed to the secretome when

applied to DFU, such as angiogenesis, epidermal regeneration, amelioration of inflammation and the recruiting of other stem cells to the wound site.(242)

Similar effects have been described for the secretome of blood MNC by Korf-Klingebiel *et al.*(64) and our group, who demonstrated tissue protection and regenerative properties along with other therapeutic effects for the same secretome in several preclinical and clinical investigations. The most notable findings are (i) recovery of infarcted area after acute myocardial infarction in an animal model(65); (ii) attenuation of microvascular obstruction after heart attack(66); (iii) protection against autoimmune myocarditis(67); (iv) reduction in infarction area after an acute ischemic injury in a rat stroke model(68); (v) enhancement of wound healing together with accelerated re-epithelialization and neo-angiogenesis and up-regulation of pro-survival signaling pathways in a murine model of wound healing(69); (vi) augmentation of wound healing along with neo-angiogenesis and decreased numbers of mast cells in a porcine burn wound model(70); (vii) improved neurological function following spinal cord trauma(71) along with many further effects, including pro-angiogenic, vasodilatatory and inflammation-modulating capacities, as well as enhancing migration of human primary keratinocytes and fibroblasts.

Interestingly, Lichtenauer *et al.* have shown therapeutic benefits of MNC secretome after detecting high amounts of different growth factors and cytokines in the secretome of conditioned MNC including IGF-1, transforming growth factor-β (TGF-β), VEGF, IL-8, HGF , FGF, and many other cytokines.(78). Since compromised growth factor levels have been shown in patients suffering from DFU, they have received great attention in terms chronic wound management. Low expression levels for the following growth factors have been detected: FGF, TGF-β, PDGF, and VEGF, indicating that the MNC secretome could be a promising alternative therapy for DFU.(243)

Moreover, Altmann *et al.* provided evidence for the neuroprotective and neuroregenerative effects of the MNC secretome.(68) He demonstrated an enhancement of neuronal sprouting *in vitro* along with upregulation of cytoprotective gene products. In addition, Haider *et al.* found an improved functional recovery, together with reduced neuronal injury, following spinal cord

trauma. His histological evaluation demonstrated increased vascular density in *ex vivo* spinal cords of treated animals, indicating that the MNC secretome can be utilized as an advantageous novel therapy for diabetic neuropathy, which accompanies peripheral nerve injury. This could be taken advantage of in the therapy of DFU with sensory disturbance.(71)

The MNC secretome is also a promising strategy treating microvascular deficiencies associated with DFU, prior to surgical considerations. For example, deterioration of nitric oxide-mediated vasodilation due to decreased availability of endothelial nitric oxides (NOS), together with increased leukocyte-vascular wall interactions are well known to be accompanied by DFU. Furthermore, the resultant inflammation and oxidative stress aggravate NO attenuation, platelet activation and platelet aggregation, leading to progression of microvascular obstruction.(151, 244) This is in line with existing evidence provided by Höetzenecker *et al.* who investigated the effect of the MNC secretome on vasodilation and platelet aggregation. He reported upregulation of vasodilatory mediators such as phospho-endothelial constitutive nitric oxide synthase (p-eNOS), inducible nitric oxide synthase (iNOS), Prostaglandin I2 (PGI2) and Nitric Oxide (NO) following MNC secretome application, suggestive of an indirect vasodilatory property. He further presented compelling evidence of a direct vasodilatory capacity for the MNC secretome via NO, detected in significant concentrations, along with an anti-platelet-aggregatory capacity due to vasodilator-stimulated phosphoprotein (VASP)-mediated inhibition of platelets.(66) At this juncture, however, it is worth emphasizing that the treatment of the underlying ischemia is essential in order to achieve a successful outcome. Assessing a vascular status, especially of the affected limb, is highly recommended prior to any treatment of DFU, as ischemia accounts for poor healing prognosis of the chronic wound. Currently surgical consultation for vascular intervention, in particular angioplasty, is accepted as first line therapy in the treatment of chronic wounds for selected patients suffering from distal ischemic events.

Currently, several active treatments of ulcers have received attention, but unfortunately they have failed to successfully enter the market due to their

limited efficacy. Some approaches are based on paracrine factors isolated from blood, others use cell-based skin substitutes for wound healing. There is, therefore, an intense medical need for a curative therapy of DFU. No active wound care product has yet become the standard of care, and this provides an enormous opportunity for treatment with MNC, a novel and affordable therapeutic approach for the restoration of the internal regenerative capacity. The MNC secretome promises to restore damaged tissues by coordinating the intrinsic regenerative processes upon injury, and thus provides a unique and highly innovative approach to regenerative medicine. Currently the standard of care for DFU is moist wound dressings, which are insufficient to control the disease, and prevent progression, frequently resulting in eventual amputation. Based on data provided above, the MNC secretome represents a preferable primary candidate for the treatment of microvascular obstruction in diabetic foot patients prior to any surgical intervention. In addition, combination of this therapy modality with surgical treatments is also worth considering.

3.5 Conclusion & Future Prospects

In this research work, we provide the first evidence for innate antimicrobial defence of the human body fluids including postoperative pleura fluid and the secretome of MNCs.

For the first time we identified antimicrobial peptides in postoperative pleural fluid and antimicrobial activity against various gram negative and gram positive bacteria. We showed that high abundance of diverse AMPs in the pleural fluid act as a protective shield against pathogens causing infections of the pleural cavity. Further, we described for the first time that neutrophil granulocytes, mesothelial cells, monocytes and other MNCs serve as cellular sources for the AMPs. Inflammatory events lead to migration of inflammatory cells into the pleural space building up an inflammatory cell rich pleural effusion with augmented protein content. This indicates the role of the pleura in inflammatory processes and partly explains the comprehensive protection of the pleural

cavity against postoperative infections and the low incidence of postoperative pulmonary infectious complications following major lung operations.

Moreover, we demonstrated for the first time that the secretome of irradiated MNCs, comprises high amounts of soluble AMPs and exerts antimicrobial activity against various gram negative and gram positive bacteria. In addition, our results provide evidence for direct and indirect positive effects of this secretome on the immune system, along with antibacterial properties. More interestingly, we showed for the first time that diverse specific types of cell death might be responsible for the cell morphological changes in response to irradiation and the augmented secretory phenotype. Thus we argued that the observed beneficial effects, are probably a consequential outcome of apoptotic and necroptotic cell death. Thus the secretome of irradiated MNCs might be a therapeutic adjunct in the management of chronic wounds, including DFU, and can be utilized as a novel therapy strategy due to the several clinical advantages and favourable unique features including enhancement of angiogenesis and wound healing.

However, considering the high rates of multidrug resistance and the consequential limitation of antibiotic medication for both systemic and topical treatment of DFU, secretome of irradiated MNCs represents a promising alternative treatment, or at least an additional option to supplement the common antibiotics.

Our pioneering work – shedding light on features and clinical applicability of APMs and on MNC secretome - might serve as an important reference and base to students, researchers, and clinicians for further research and trials towards a better understanding of the human defence mechanisms, especially in the field of chronic wounds.

CHAPTER FOUR: MATERIALS & METHODS

4.1 Materials and Methods First Paper

4.1.1 Patient Selection

Patients undergoing operations in the Department of Thoracic Surgery, Medical University of Vienna, were prospectively recruited for this study. A positive ethics committee vote was obtained (EK No. 585/10), and all participants gave their written informed consent before inclusion. Pneumonia, emphysema, or necrotizing tumorous parts in the lung preoperatively, as well as any systemic signs of infections at the time of surgery, were defined as exclusion criteria.

4.1.2 Collection of Pleural Fluids, Parietal Pleura, Blood, and Serum Samples

The collection of 10 ml pleural fluid took place immediately after opening the thorax. Further pleural fluid samples were collected at 2, 18, 26, and 42 hours after the operation. These samples were obtained by sterile puncture of the chest tube line after clamping the tube, following clearance of the chest tube of old material. Preparing pleural fluids for some experiments was performed by centrifugation and separation of the cellular content followed by filtering the fluid through a 40-mm cell strainer. Specimens were then aliquoted and stored immediately at –80°C until further experiments. In a subgroup of patients (wedge resections, n = 5; lobectomy, n = 6; pneumonectomy, n = 1), whole blood and serum samples were collected. Serum samples were placed immediately at -80°C. Whole blood samples were analysed by flow cytometry immediately after collection. Parietal pleural samples (n = 3) were obtained during pleurectomy procedures for recurrent pneumothoraces. Pleural specimens were either fixed with 4% formaldehyde or shock-frozen in liquid nitrogen for further processing.

4.1.3 Enzyme-Linked Immunosorbent Assay

Pleural fluid and serum was evaluated for the following proteins using commercially available enzyme-linked immunosorbent assay (ELISA) kits, according to the manufacturer's instructions: human beta-defensin (human beta-defensin1: antibodiesonline.com, Atlanta, GA; human beta-defensin2: PeproTech, Rocky Hill, NJ; Human angiogenin RNase5: catalog# DY265 R&D Systems, Minneapolis, MN, USA; RNAse7: Antibodies-online.com, Atlanta, GA; S100A7: MBL International Corporation, Woburn, MA; human LL-37(cathelicidin): catalog# HK321; human S100A8/A9 (calprotectin): catalog# HK325; all Hycult Biotech, Uden, the Netherlands; interleukin (IL)-1b, IL-2, IL-6, tumor necrosis factor (TNF)a, IL-8, IL-10, transforming growth factor–b, soluble ST-2 (all R&D Systems, Minneapolis, MN). Optical density values were measured at 450 nm on an ELISA plate reader (Victor3 multilabel plate reader; PerkinElmer, Waltham, MA, USA). Tobsy

4.1.4 Establishment of Mesothelial Cell Culture

Mesothelial cell lines (NP-1 and NP-2) were established following published protocols from parietal pleural specimens of patients with pneumothorax who had undergone surgical treatment. Cells demonstrated mesothelial morphologic features as described previously, and stained positive for vimentin and negative for CD31.

4.1.5 Polymerase Chain Reaction

Following centrifugation and isolation of cellular components of pleural fluids, the pleural tissue specimens were homogenized in RNA lysis buffer using the

Precellys system (Peqlab, Erlangen, Germany), NP-1 and NP-2 cells were lysed directly. RNA was purified using a standard protocol, and quantitative real-time polymerase chain reaction (PCR) was performed on a LightCycler using a SYBR Green I Master Kit (Roche Diagnostics, Basel, Switzerland). The following primer pairs were utilized for the analysis: forward primer 5′-AGAAGCGGGTGAGAAACAAA-3′ and reverse primer 5′-TGTGGCTCGGTACTGGCATG-3′ for Angiogenin/RNase5; forward primer 5′-GAGTCACAGCACGAAGACCA-3′ and reverse primer 5′-GGCTGCATGTGCTGAATTT-3′ for RNase7; forward primer 5'-GGAGAACTTCCCCAACTTCCTT-3' and reverse primer 5'-GGAGAAGACATTTTATTGTTCCT-3' for s100A7; forward primer 5'-ATGCCGTCTACAGGGATGAC-3' and reverse primer 5'ACGCCCATCTTTATCACCAG-3' for S100A8; forward primer 5′-CAGCTGGAACGCAACATAGA-3′ and reverse primer 5′-TCAGCTGCTTGTCTGCATTT-3′ for S100A9; forward primer 5'-CCCAGTTCCTGAAATCCTGA-3' and reverse primer 5'-CTTCTGGTCACTCCCAGCTC-3' for hBD1; forward primer 5'-ATCAGCCATGAGGGTCTTGT-3' and reverse primer 5'-GAGACCACAGGTGCCATTTT-3' for hBD2; forward primer 5'-AGCTGTGGCTGGAACCTTTA-3' and reverse primer 5'-CGATCTGTTCCTTCCTTTGGA-3' for hBD3; forward primer 5′-GCTAACCTCTACCGCCTCCT-3′ and reverse primer 5′-GGTCACTGTCCCCATACACC-3′ for cathelicidin; and forward primer 5′-GATGAGTATGCCTGCCGTGTG-3′ and reverse primer 5′-CAATCCAAATGCGGCATCT-3′ for B2M.

4.1.6 Immunohistochemical Examination

Paraffin-embedded samples of healthy parietal pleura obtained from patients suffering from recurrent pneumothoraces were analysed. Immunohistochemical staining was performed on 5-µm thick sections of formalin-fixed, paraffin-

embedded tissues. After deparaffinization and hydration, sections were pre-treated with microwave in citrate buffer (Dako, Vienna, Austria) as described by Abtin(226) and stained with following antibodies: The following antibodies were from Abcam, Cambridge, UK: HBD1, HBD2, S100A7, RNAse5, RNAse7 and the following antibodies were from Acris, Hiddenhausen, Germany: anti-S100 A8/A9.

4.1.7 Flow Cytometry

To investigate the cellular content of postoperative pleura fluid following chest operations, pleural effusions were obtained by sterile punctuation of chest tube lines, and cell suspensions from postoperative pleural effusion were stained by fluorescein isothiocyanate conjugated anti-CD45 antibody (BioLegend, San Diego, CA). Fluorescein isothiocyanate IgG1 served as isotype control (BioLegend, San Diego, CA). 7-amino-actinomycin-D (7-AAD; Calbiochem, Darmstadt, Germany) was used to exclude dead cells. Anti-CD4, anti-CD8, anti-CD19, and anti- CD56 antibodies were used for subgroup analysis (all from Beckman Coulter, Brea, CA). Flow cytometry analyses were performed on a FACSCalibur (Becton Dickinson, Franklin Lakes, NJ), and data were analyzed using FlowJo software (Tree Star, Ashland, OR).

4.1.8 In Vitro Induction of AMP Expression

To evaluate and analyse AMP production upon stimulation, NP-1 cells were incubated for 6 hours and 24 hours together with the following Toll- Like receptor (TLR) ligands (all InvivoGen, San Diego, CA): 1 mg/mL Pam3CSK4 (TLR1/2 ligand), 10 mg/mL HKLM (TLR2 ligand), 20 mg/mL Poly(I:C) (TLR3 ligand), 100 ng/mL lipopolysaccharide from Escherichia coli K12 (TLR4 ligand), 500 ng/mL Flagellin from Salmonella typhimurium (TLR5 ligand), 500 ng/mL FSL1 (TLR6/2 ligand), 10 mg/mL Imiquimod (TLR7 ligand), 10 mg/mL single-

stranded RNA40 (TLR8 ligand), and 2.5 mM ODN2006 (TLR9 ligand). Further determination of expression pattern of AMPs was done by PCR.

4.1.9 Antimicrobial Activity of Pleural Fluids

To evaluate the antimicrobial activity of pleural fluids a microdilution assay was performed according to the protocol mentioned by Mildner et al (245), using the following pathogens: Pseudomonas aeruginosa (ATCC 27853), Escherichia coli (ATCC 11303), Staphylococcus aureus (ATCC 33592) and Streptococcus pyogenes and Streptococcus pneumoniae. Briefly, pathogens were co-incubated with 20% diluted pleural fluids in physiologic NaCl solution, containing 1% (v/v) trypticase soy broth at 37 °C for 3 hours. Afterwards, 25µl of the suspensions were plated onto LB agar plates and spread with a sterile Trigalsky spatula. This was repeated and serial dilutions of the incubation mixtures were performed. The antibiotic activity was analysed by determining the number of colony-forming units the next day.

4.1.10 Statistical Methods

All data collected in this study were analyzed by PASW Statistics, version 19 (SPSS, Chicago, IL) or GraphPad Prism, version 6 (GraphPad Software, LaJolla, CA) software. Data sets are depicted as mean ± standard error of the mean in the figures. Unpaired 2-sided t tests were used to compare 2 independent groups. For multiple-group comparison, 1-way analysis of variance with post hoc Bonferroni correction was applied. p values ≤0.05 were considered statistically significant.

4.2 Materials and Methods Second Paper

4.2.1 Ethics Statement

The local ethics committee at the Medical University of Vienna approved this study, including all experimental practices (vote number: 1236; 2013). This study has been conducted according to the principles of the Helsinki Declaration and Good Clinical Practice. All participants, aged 18–40 years, provided written informed consent. Exclusion criteria were any immunomodulatory therapy in the past 4 weeks and any signs of acute infection.

All animal experiments were approved by the Animal Research Committee of the Medical University of Vienna (Prot. Nr.: 66·009/0220WF/II/3b/2014).

4.2.2 MNC-sec Preparation

Whole blood samples were obtained by peripheral venepuncture from four healthy male volunteers. After the blood withdrawal, cell separation was done by Ficoll-Paque (GE Healthcare Bio-Sciences AB, Sweden) density gradient centrifugation. Immediately after blood withdrawal, anticoagulated blood specimens in heparin tubes were processed and diluted 1:2 in Hanks balanced salt solution (HBBS, Lonza, Basel, Switzerland). Diluted blood specimens were transferred carefully to 50 ml tubes containing Ficoll–Paque solution (GE Healthcare Bio–Sciences AB, Sweden) and were centrifuged for 15 minutes at 800 g at room temperature without braking. After the centrifugation, the buffy coats with MNCs were carefully obtained using a pipette. MNCs were resuspended in CellGro® serum-free medium (CellGenix, Freiburg, Germany; 25 × 106 cells/ml) after washing in HBSS. Cell concentrations were determined using a Sysmex automated cell counter (Sysmex Inc., USA). To induce apoptosis MNCs from the four donors were γ-irradiated using a Caesium-137 source with a total dosage of 60Gray (Gy). Irradiated cells were then cultivated

in a phenol-red free and serum-free CellGro® cell culture medium, at a concentration of 25 × 10^6 cells per ml for 24 hours. To investigate time dependant irradiation induced changes in MNCs, irradiated and non-irradiated cells from the same donor were incubated for 0 hours, 4 hours, and 24 hours. After these time points the cells and their supernatants were collected separately and served as the experimental entity.

4.2.3 Production of MNC Secretome from Humans and Control Medium According to the Good Manufacturing Practice Protocol for Experimental Settings

To authorize a blood derived product two pathogen reduction methods are mandatory for the regulatory authorities. These two methods are: photodynamic treatment with methylene blue (MB) plus visible light, and gamma irradiation, and have been developed to inactivate viruses and other pathogens. Regulatory authorities demand that these products be produced in a good manufacturing practice (GMP) facility according to good manufacturing practice protocols.

The preparation of human irradiated MNC secretomes according to GMP protocols was conducted as follows: whole blood samples were obtained by peripheral venepuncture from four healthy male volunteers. After the blood withdrawal cell separation has been done by Ficoll-Paque (GE Healthcare Bio-Sciences AB, Sweden) density gradient centrifugation. Immediately after the blood obtainment anticoagulated blood specimens in heparin tubes were processed and diluted 1:2 in Hanks balanced salt solution (HBBS, Lonza, Basel, Switzerland). Diluted blood Specimens were transferred carefully to 50 ml tubes containing Ficoll–Paque solution (GE Healthcare Bio–Sciences AB, Sweden) and were centrifuged for 15 minutes at 800 g at room temperature without braking. After the centrifugation generated buffy coats with MNCs were carefully obtained by a pipette. MNCs were resuspended in CellGro® phenol-red free and serum-free medium (CellGenix, Freiburg, Germany; 25 × 10^6 cells/ml) after washing in HBSS. Cell concentrations were determined using a Sysmex automated cell counter (Sysmex Inc., USA). To induce apoptosis

MNCs from the four donors were γ-irradiated using a Caesium-137 source with a total dosage of 60Gray (Gy). Then, irradiated cells were cultivated in a phenol-red free and serum-free CellGro® cell culture medium at a concentration of 25 × 10^6 cells per ml for 24 hours. After the collection of supernatants, methylene blue (MB) plus light treatment was performed in the Theraflex MB-Plasma system (MacoPharma, Mouvaux, France), using the Theraflex MB-Plasma bag system (REF SDV 0001XQ) and an LED-based illumination device (MacoTronic B2, MacoPharma). Light energy was monitored and reached 120 J/cm^2. Each bag system contained 85mg MB, reaching a concentration range of 0.8 to 1.2 mM MB per unit. MB and photoproducts were removed by Blueflex filtration immediately after treatment. Prior to the second step of pathogen removal, supernatants were lyophilized. Then, in order to achieve a homogeneous distribution of irradiation dose to the whole product, the virally inactivated lyophilized supernatant was placed in metal sterilization containers, which moved in a meandering path in five layers through the irradiation vault around the emitting centre of the irradiation and were irradiated by a Mediscan gamma irradiation unit (Gammatron 1500, Mediscan, Seibersdorf, Austria). The source of the gamma rays was Cobalt 60. Polymethyl methacrylate (PMMA) was utilized as a dosimeter to validate a predetermined dose rate of 25000 Gy after 23 hours of irradiation. The treated pathogen-free and lyophilized supernatant of MNCs was stored at -80°C until experimental use. For the control group, cell culture medium of phenol-red free and serum-free CellGro® was treated as above, *ie* the medium was subjected to cultivation and the two step pathogen reduction processes, including irradiation and lyophilization. The treated lyophilizates of the medium were also stored at -80°C until experimental use.

4.2.4 Investigation of Cell Death by ImageStream Analysis

As mentioned in the second paper, the method described by Pietkiewicz et al.(246) for the FlowSight® cytometer was adapted to the ImageStream (MilliporeSigma, Merck KGaA, Darmstadt, Germany) cytometer. Briefly, cells

were stained with Annexin V FLUOS and propidium iodide (Roche Diagnostics, Basel, Switzerland) as follows: After the induction of cell death by stimulation, cells, including the medium, were harvested and transferred into 1.5 ml vials. Cells were collected after centrifugation at 500 ×g for 5 min at 4 °C, were washed with 1 ml phosphate buffered saline (PBS, Life Technologies Corporation, UK) and resuspended in 100 µl staining buffer (Annexin V FLUOS staining Kit, Roche) by mixing 2 µl of Annexin-V-FLUOS and 2 µl propidium iodide (PI) in incubation buffer according to manufacturer's protocol. After incubation for 15 min in the dark at room temperature, cells were centrifuged at 500 ×g for 5 min at 4 °C, and were resuspended in 100 µl PBS and analysed immediately by FlowSight® flow cytometer (Amnis®, EMD Millipore) according to the manufacturer's protocol. A laser emitting at 488 nm was used to excite the stained cells, and debris and doublets were gated out. At least 20000 pictures of every cell, were recorded in the bright field (430–480nm), Annexin V FLUOS (505–560nm) and PI (430–480nm) channels. Because of overlapping absorption spectra of Annexin V FLUOS and PI, a colour compensation was conducted to eliminate false positive results. In addition, single-labeled dead samples, stained with Annexin V FLUOS or PI, served as positive controls. IDEAS 6.2 software (MilliporeSigma) was applied for analysis, and focused single cells were determined using the gradient root mean square of the bright field image, and thereafter bright field area and aspect ratio intensity. Fluorescence intensity of Annexin V FLUOS and PI was utilized for discrimination between double negative (representing live and healthy) cells, Annexin V positive (representing early apoptotic cells) and double positive (representing late apoptotic or necroptotic) cells. Image based characteristics of the PI channel, including intensity threshold and contrast morphology, allows a clear differentiation between late apoptotic cells with small fragmented nuclei and necroptotic cells with nonfragmented enlarged nuclei.

4.2.5 Total RNA Isolation and Quantitative Real-Time Polymerase Chain Reaction

After the final cell cultivation step, as extensively described above, irradiated and non-irradiated MNCs were collected at 0 hour, 4 hours and 24 hours after irradiationor sham treatment. Immediately thereafter, total RNA isolation from approximately 25×10^6 cells was performed, using Trizol® Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions, and as described previously by Beer *et al.*(79) The quantification of RNA was performed using a NanoDrop-1000 spectrophotometer (Peglab, Erlangen, Germany), and RNA quality control was performed by an Agilent 2100 Bioanalyzer (Agilent, Böblingen, Germany). All RNA samples used in further steps had an RNA integrity score between 5.7 and 10. In total 28 samples were generated from four different donors. Subsequently, quantitative real-time polymerase chain reactions were performed, using the following primer pairs: forward primer 5′-AGAAGCGGGTGAGAAACAAA-3′ and reverse primer 5′-TGTGGCTCGGTACTGGCATG-3′ for Angiogenin/RNase5; forward primer 5′-CAGCTGGAACGCAACATAGA-3′ and reverse primer 5′-TCAGCTGCTTGTCTGCATTT-3′ for S100A9; forward primer 5′-GCTAACCTCTACCGCCTCCT-3′ and reverse primer 5′-GGTCACTGTCCCCATACACC-3′ for cathelicidin; forward primer 5′-GAGTCACAGCACGAAGACCA-3′ and reverse primer 5′-GGCTGCATGTGCTGAATTT-3′ for RNase7; and forward primer 5′-GATGAGTATGCCTGCCGTGTG-3′ and reverse primer 5′-CAATCCAAATGCGGCATCT-3′ for B2M. The relative expression of target genes was compared to the housekeeping gene B2M using a formula described by Pfaffl et al.(247)

4.2.6 AMP Enzyme-Linked Immunosorbent Assay Analysis of the Human MNC-sec and Rat Serum

AMPs in the secretome of MNCs and rat serum were evaluated at the protein level, using commercially available enzyme-linked immunosorbent assay (ELISA) kits, according to the manufacturers' instructions (human beta-defensin

124: catalog# SEQ656Hu; human ribonuclease A3: catalog# SEB758Hu; all Uscn Life Science, Wuhan, China; human LL-37, cathelicidin: catalog# HK321; human calprotectin: catalog# HK325; all Hycult Biotech, Uden, the Netherlands; human angiogenin, RNase5: catalog# DY265 R&D Systems, Minneapolis, MN, USA; and rat calprotectin: catalog# HK321; Immundiagnostik AG, Bensheim, Germany). Optical density values were measured at 450 nm on an ELISA plate reader (Victor3 multilabel plate reader; PerkinElmer, Waltham, MA, USA).

4.2.7 Antimicrobial Activity of the Human MNC Secretome

The antimicrobial activity of the secretome of MNCs was evaluated by an adapted microdilution assay from Mildner et al (245), using the following pathogens: Pseudomonas aeruginosa (ATCC 27853), Escherichia coli (ATCC 11303), Staphylococcus aureus (ATCC 33592) and Streptococcus pyogenes. In brief, bacterial colonies were transferred from a plate by a sterile metal loop into a glycerol/broth slurry and were stored at -80°C. Glycerol, is a cryoproctectant, and prevents the bacterial culture from freezing under sub-zero conditions. These frozen cultures served as sources for bacterial experiments. For the experiments, the frozen glycerol/bacteria solutions were lightly scratched on the surface by a sterile inoculating loop, and the loop was then streaked over a sterile Columbia agar plate with 5% sheep blood. After an overnight incubation at 37 °C, the plates with freshly grown bacteria were used as a source for bacterial experiments. 2-4 bacterial colonies were transferred from the plates into 10ml greiner and resuspended with 10ml 0,9% sodium chloride using a sterile cotton swab. The same mixture was prepared for all bacterial strains and served as pathogen source for further steps of the assay. To determine antimicrobial potency of the MNC secretome, supernatants from 250 × 10^6 irradiated cells or 250 × 10^6 non-irradiated cells were mixed with the suspension containing the bacteria. A mixture of bacterial solution and CellGro® medium served as control. After a short incubation time at room temperature for 30 min, 25µl of each suspension was applied to Columbia agar plates and spread with a

sterile Trigalsky spatula. The number of colony-forming units analysed the next day served as determinants for the antimicrobial activity of MNC secretome.

A blocking assay was also performed, using neutralizing antibodies to investigate antibiotic activity of antimicrobial factors in the MNC secretome in particular. The antibodies used are as follows: Angiogenin/RNAse5 (LSBio, Seattle, WA, USA; clone 27E10, 25 µg/mL) and Calprotectin (Santa Cruz, Dallas, TX USA; clone D-5, 25 µg/mL).

4.2.8 Rat AMP Changes Following Exposure to the Human MNC Secretome

For this experiment adult male Sprague–Dawley rats with weights from 300 to 350 g (n = 24, Department of Biomedical Research, Medical University of Vienna, Austria) were used and housed under standard conditions. Animals were anesthetized with 1.5% isoflurane prior to intraperitoneal injection to ensure the correct intraperitoneal application of medium or MNC secretome. Animals were divided into two groups of CellGro® medium and MNC secretome, receiving 1 ml of either medium or 1 ml of the equivalent of 5×10^6 MNC cells of the MNC secretome. Subsequently, six animals were anesthetized with Xylazine (10 mg/kg, i.p.) and ketamine (100 mg/kg i.p.) at 2 hours, 12 hours and 24 hours after the injection of medium or MNC secretome, and the inferior vena cava of each animal was punctured to obtain blood. Each time point contained three animals for the MNC secretome group and three for the medium group. This blood withdrawal was followed by a deep heart incision. The blood samples were centrifuged at 3500g for 15 min to obtain serum, then immediately prepared for investigation.

4.2.9 Statistical Methods

The software Graphpad prism 4 (GraphPad Software, La Jolla, CA, USA) was used for statistical analysis. Student's t-test was used to compare between groups at given time points. Data are shown as mean ± standard deviation (SD). The graphical presentation of differences was also assessed using bars and symbol connection lines. The calculations were performed separately for each experimental setting. A two-sided corrected P-value < 0.05 was considered significant.

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Curriculum Vitae

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■ Education

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■ CLINICAL TRAINING

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08.2016-10.2016 SMZ Ost – Donauspital, residency at the pediatric and adolescent surgery
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■ publications

Mohammad Mahdi Kasiri, Lucian Beer, Shahrokh Taghavi, Thomas Haider, Elisabeth Simader, Christian Gabriel, Denise Traxler, Michael Mildner and Hendrik Jan Ankersmit: Secretome of Apoptotic Peripheral Blood Mononuclear Cells possess antimicrobial activity and induce de novo AMPs in vivo (european journal of clinical investigation 2016)

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