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Construction and use of medical devices for the selective removal of bacteria and endotoxins from biological fluids

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COORDINATOR
Prof. Lorenza Trabalzini

TUTOR:
Prof. Alessandro Pini

DOCTORAL THESIS OF:
Giovanni Cappello

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"I am among those who think that science has great beauty"

Marie Curie

ABSTRACT

The infections by multidrug-resistant (MDR) pathogens are steadily increasing and very often result in numerous cases of sepsis. For this reason, it is necessary to find not only new drugs active against these multidrug-resistant bacteria but also new strategies to counteract the evolution of sepsis. SET-M33 peptide is an antimicrobial peptide with a great ability to kill bacteria and bind bacterial wall components such as lipopolysaccharide (LPS) from Gram-negative bacteria and lipoteichoic acid (LTA) from Gram-positive bacteria. It is a cationic non-natural peptide synthetized in a tetra-branched form that makes it more resistant to degradation in biological fluids.

SET-M33 peptide is characterized by high antimicrobial activity both *in vitro* and *in vivo*, anti-inflammatory capacity through neutralization of endotoxins such as LPS, low hemolytic activity, lack of immunogenicity, and is active against biofilm-forming bacteria.

This paper reports the construction of a new device based on the antimicrobial peptide SET-M33 for the multiple and selective removal of both bacterial toxins (LPS and LTA) and live bacteria (*Escherichia coli O111:B4* and *Staphylococcus aureus USA 300*) from biological fluids of patients in sepsis.

The device was initially constructed by immobilizing the peptide on a resin constituted by agarose beads via a thiol residue at the c terminal of the peptide.

Subsequently, the peptide was bound to a polyether sulfone (PES) resin by high-energy beta irradiation. In both cases, the devices demonstrated excellent retention of bacterial toxins and bacteria.

For the preparation of the devices, the peptide was synthesized with amino acids in L configuration.

The peptide to be bound to agarose beads was synthesized with a thiol residue at the c terminal end to allow thioether bonding with agarose resin, the peptide for PES matrix was prepared without any modification to the original structure

as it was immobilized by exploiting radical formation due to high-energy beta irradiation.

Stability studies and endotoxin removal (LPS) experiments in *ex vivo* animal models were also performed in this work.

The safety of the devices was tested by electrophoretic analysis and hematochemical tests performed on pools of healthy and sepsis patients.

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INTRODUCTION

HISTORICAL BACKGROUND

Throughout history, epidemics of infectious diseases in humans have been observed and well documented. The discovery of microbes, including bacteria, fungi, and viruses, as the causative agents of infections, occurred only during the 19th century. Antimicrobials are probably one of the most successful forms of chemotherapy in the history of medicine (Aminov, 2010).

In 1928, Fleming discovered penicillin. He detected that the substance produced by a common mold, the so called *Penicillum notatum*, was able to block the growth of *Staphylococcus aureus* in a plate. The antibiotic was named Penicillin, and it came into clinical use in the 1940s (Abraham and Chain, 1988) (Saga and Yamaguchi, 2009). The discovery of penicillin changed the course of history. The following development of antimicrobial treatments has had a revolutionary impact on human health over the past century. It has prevented people from dying of various infectious diseases, such as bacterial pneumonia, sepsis, or gonorrhea, and has allowed actuation of various medical approaches, like organ transplantation, cancer chemotherapy and surgery (Lobanovska and Pilla, 2017).

During the subsequent two decades, new classes of antimicrobial drugs were developed one after another, leading to a golden age of antimicrobial chemotherapy. After the discovery of β-Lactams (penicillin) numerous other classes of drugs were discovered (Aminov, 2017). Gerhard Domagk was the first to identify in 1935 a molecule of the Sulphonamides class that became the first class of antimicrobials that went into the truly large-scale production (Jeśman et al., 2011). Albert Schatz purified streptomycin (Aminoglycosides class) from two strains of *Streptomyces* active against the tubercle *bacillus (*Wainwright*, 1991)*. The first antibiotic of Tetracyclines class, was discovered in 1945 from *Streptomyces aureofaciens*, and the antibiotic purified from it was marketed under the trade name Aureomycin (Unemo et al., 2014). Chloramphenicol (Amphenicols class), which was discovered and isolated from *Streptomyces venezuelae* by David Gottlieb in 1947 (Ehrlich et al., 1948). Nalidixic acid (Quinolones class) was discovered by mistake during the synthesis of chloroquine (Naeem et al., 2016). Finally, at the turn of the 1930s and 1940s, it was discovered that various strains of the soil bacterium

Brevibacillus brevis produced linear and cyclic peptides (Gramicidin and Tyrothricin) which inhibited a number of pathogenic bacteria and even fungi (Van Epps, 2006). These latest molecules, subsequently identified also in numerous plant and animal species, contributed to the birth of the class of antimicrobial peptides (AMPs). Improvements in each class continued to achieve a broader antimicrobial spectrum and higher antimicrobial activity (Saga and Yamaguchi, 2009).

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SEPSIS: INCIDENCE AND PATHOGENESIS

Sepsis is a syndrome that is still not fully understood either biologically or pathologically, and for these reasons its definition has undergone changes over time and is still not perfect.

In 1991, the first definition of sepsis was formulated (Bone et al., 1992), in which the view of sepsis as a process resulting from SIRS (Systemic Inflammatory Response Syndrome) prevailed. "Severe sepsis" is a later stage of sepsis and involves organ dysfunction; this can evolve into "septic shock," which is manifested by persistent sepsis-induced hypotension despite rehydration treatments. Currently, sepsis is identified as a "life-threatening organ dysfunction caused by a dysregulated host response to infection" (Singer et al., 2016), i.e., a potentially fatal organ dysfunction due to a response toward an infection, effectively complementing even the old definition of "severe sepsis." Septic shock, on the other hand, is identified as a subset of sepsis in which circulatory, cellular, and metabolic alterations are present that go on to significantly increase the mortality rate.

In 2017, the World Health Organization (WHO) recognizes sepsis as a global health priority (Reinhart et al., 2017), despite this, sepsis continues to be a major health problem with high mortality rates that are 22% for sepsis and 46% for septic shock (in England) (Shankar-Hari et al., 2017), in addition, morbidity rates and post-discharge issues are not to be underestimated.

Unfortunately, regarding epidemiological data concerning sepsis, despite their importance in enabling prevention policies and resource allocation, there is no truly correct estimation as only data from hospitalized patients and from upper-middle-income nations are exploited (Rudd et al., 2020).

Recently, data from other sources have also been exploited to consider non-hospitalized patients and a wider area of territory. Hence the result of the "Global Burden Disease" studies, which monitoring the period 1990-2017 estimated 48.9 million cases in 2017 with 11 million deaths (19.7 percent of the total), while still emphasizing an overall decrease in incidence (37 percent

decrease) and mortality (52.8 percent decrease) compared to 1990 (Rudd et al., 2020).

Studies on the geographic distribution of sepsis cases (Fig. 1) indicate both higher incidence and higher mortality in inverse relation to the SDI index (index considering per capita income, average educational attainment, and fertility rate) (GBD 2017 Causes of Death Collaborators, 2018), thus denoting higher incidence in regions such as sub-Saharan Africa, Southeast Asia. (Rudd et al., 2020).

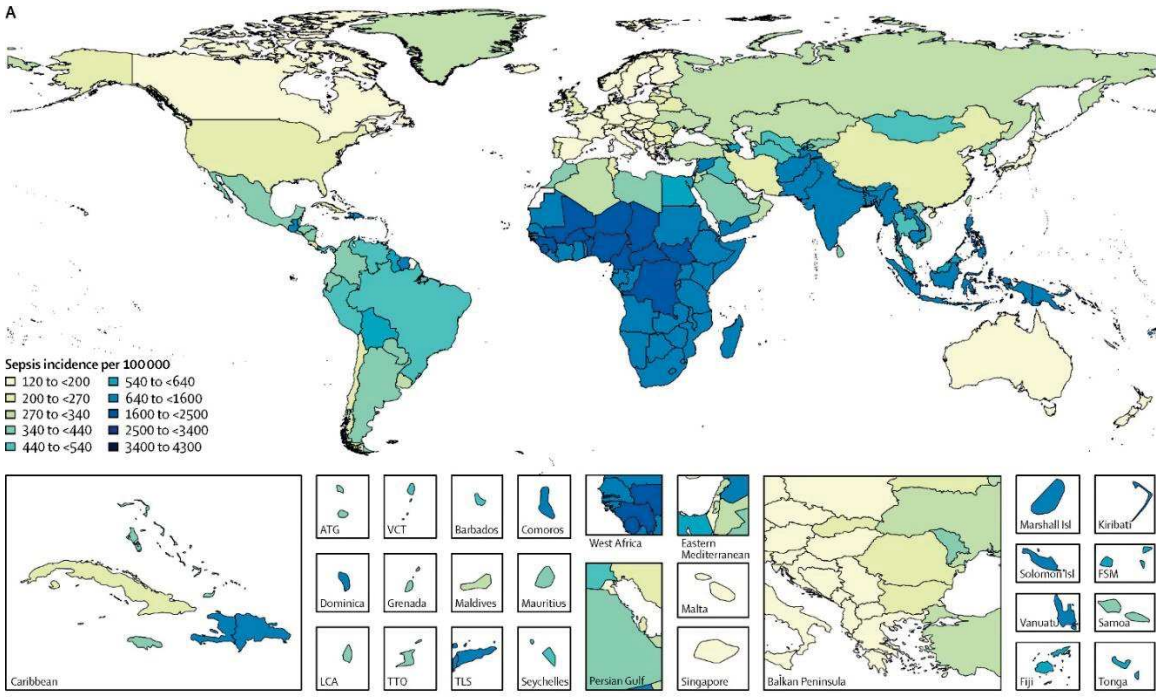

Figure 1. Incidence of sepsis per 100˙000 population. (Rudd et al., 2020).

The most affected age groups are the "extreme" age groups, namely childhood (especially the < 1 year old age group) and the elderly. There is also higher mortality in the male sex than in the female (Rudd et al., 2020; Rubin et al., 2014).

The main sites of infection onset are 3 (80% of cases): the respiratory tract, with particular focus on pneumonia; abdominal infections, following ruptured

appendicitis and penetrating wounds; and the urogenital tract. (Esper et al., 2006; Annane et al., 2003; Alberti et al., 2002).

The main comorbidities recorded, especially given the older age group, are diabetes mellitus, chronic obstructive pulmonary disease, cancer, end-stage renal disease, HIV, and cirrhosis (Esper et al., 2006).

Pathogens found in sepsis cases appear to be predominantly bacteria, but fungi (especially in the nosocomial environment), viruses, and parasites may also be involved. Gram-positive bacteria are currently predominant over Gram-negative bacteria, but these data are variable and there are still multiple differences in community- and nosocomial-acquired infections (Rubin et al., 2014). In addition, it is very important to emphasize the concern arising from the increase in cases of infections with Multi Drug Resistant (MDR) bacteria (Annane et al., 2003).

In patients with sepsis, there is an inflammatory response that loses its localized meaning in favor of a systemic action with further errors in regulation, which in its progression is characterized by fever, increased respiratory rate, and other metabolic, hormonal, and coagulative alterations, eventually leading to hypotension (Rubin et al., 2014; Singer et al., 2016; Abbas et al., 2017).

Early diagnosis of a clinical picture of sepsis allows a better and faster clinical outcome. Lactic acid (Lac), procalcitonin (PCT) and sequential organ failure assessment (SOFA) score are useful biomarkers to assess the sepsis severity, predict the prognosis in septic shock and make an earlier diagnosis. In numerous studies it has been observed that in the group of dead patients there were numerous elderly people and with increased SOFA score and increased Lac concentration. (Yu et al., 2019).

A correlation was observed between altered Procalcitonin (PCT) levels and sepsis-related mortality. Procalcitonin has shown promising results in diagnosing and predicting outcomes in sepsis patients. Nowadays microbiological cultures are the gold standard for sepsis-diagnosis, but they are time-consuming, and the result might not be positive in patients receiving

antimicrobials. Other markers, such as C-reactive protein (CRP) and erythrocyte sedimentation rate (ESR), report poor sensitivity and specificity (Malik et al., 2021).

Sepsis is characterized by several overt symptoms:

- Sepsis-Associated Neuropath (SAE): Manifestations of confusion, delirium and comatose state. Other neurological signs are very frequent (50% patients in intensive care unit) in individuals with sepsis (Eidelman et al., 1996) (Zhang et al., 2022). In fact, the phenomenon is referred to as SAE (Sepsis-Associated Neuropathy)/SIBD (Sepsis-Induced Brain Dysfunction), the pathophysiology of which is still unknown but with an undoubted role of alteration of the blood-brain barrier, with subsequent damage then contributed by bacterial toxins, neutrophils and other pro-inflammatory components, and possible further promotion of damage by drugs and hypoxia (Sonneville et al., 2017).

- Sepsis-Induced Cardiomyopathy (SICM): is a form of transient cardiac dysfunction associated with sepsis. Factors inducing such depression at the myocardial level may be cytokines, PAMPs, complement components, oxidative stress and mitochondrial dysfunction (L'Heureux et al., 2020) (Lin et al., 2020).

- Disseminated Intravascular Coagulation (DIC): The currently used definition is "an acquired syndrome characterized by intravascular activation of coagulation with localized loss of character, which can cause small-caliber vessel damage and subsequent organ dysfunction," coined by the International Society of Thrombosis and Haemostasias (ISTH) (Taylor et al., 2001). DIC occurs in 30% to 50% of patients with sepsis (Papageorgiou et al., 2018) (Iskander et al., 2013).

- Acute respiratory distress syndrome (ARDS) is noncardiogenic pulmonary edema that manifests as rapidly progressive dyspnea and hypoxemia (Meyer et al., 2021).
The ARDS is a complication that can lead to death of the individual in sepsis as it is associated with multi-organ failure. Multiple factors are involved such as: components of the coagulation cascade, components involved in

inflammation, neutrophil NETs, and DAMPs (Singer et al., 2016). Instead, there is a tendency to refer to SCI (Sepsis-Induced Coagulopathy) as an early stage, in which it is easier to act with any anticoagulant therapies, and this is instead defined as "organic dysfunction and infection-induced coagulopathy" (Iba et al., 2019).

Survival to sepsis itself poses significant health difficulties; in fact, more than 40 percent of patients who survive the early phase later develop quite severe chronic conditions, which can lead to more than 14 days in intensive care units (Stortz et al, 2018).

There is also an increased health risk, resulting in re-hospitalizations (Cavaillon et. al. 2020), particularly regarding bacterial infections with subsequent significant reduction in the patient's quality of life; finally, neuromuscular weakness, chronic pain and post-discharge depression are also reported.

After bacterial infection there is a triggering of cellular mechanisms. Bacterial membrane components, mainly lipopolysaccharide (LPS), are the main triggers of the innate immunity response. This process triggers one of the first responses of innate immunity and leads to the production of cytokines. These are produced by multiple cell types, including macrophages, dendritic cells, mast cells, endothelial cells, and epithelial cells. Cytokines possess numerous functions at both paracrine and endocrine levels: they induce inflammation, prevent viral replication, promote T lymphocyte activation, and possess different roles in immune responses (Abbas et al., 2017). The main cytokines involved in septic shock include TNF-α, which plays a very important role in endotoxin shock, IL-1, IL-6, IL-17 that together with bacterial-derived factors lead in increased levels of G-CFS, which is responsible for upregulation in the production of mature and non-mature neutrophils (Shen et al., 2017).

Host processes are the ones that in their dysregulation bring the multiple complications. Among them we have the activation of the inflammatory response which involves the release of killing factors (e.g., reactive oxygen species) that are harmful not only to pathogens but also to the organism itself

(Annane et al., 2005; Singer et al., 2016). Activation of the complement system, which is fundamental in defense against infection, is implicated in damage to a variety of organs, especially by the action of anaphylatoxins (Fattahi et al., 2018). In addition, there are alterations on a variety of cell types, which appear to be powered by cellular communication mechanisms based on the extracellular release of vesicles containing miRNAs with proinflammatory action (Danesh et al., 2018; Monte Real et al., 2018).

However, inflammation is not the only factor that takes a severe clinical picture in sepsis patients; immunomodulatory factors play a very important role since their stimulation with the wrong timing can promote the inflammatory process resulting from any secondary infections (Rubin et al., 2014). To emphasize this role, the term "immunosuppression" is often used, related to the problem concerning the "reprogramming" of circulating leukocytes (Cavaillon et al., 2019). In conjunction with this phenomenon, increased spontaneous apoptosis of lymphocytes and a reduction of this in neutrophils has also been demonstrated.

Indeed, errors concerning tissue homeostasis, and thus, proliferative and apoptotic processes, are important factors in this pathology and are found to be altered variably among different cell populations, leading to increased susceptibility to secondary and recurrent infections. A large proportion of the cells of the innate immune response tend to undergo apoptosis, these include in particular dendritic cells, immature macrophages, and NK lymphocytes; in contrast, neutrophils have less tendency to undergo apoptosis. Adaptive response cells are not excluded from such alterations: both CD4+, CD8+ and B lymphocytes go against cell death, in contrast T-regulatory cells show extensive resistance resulting in loss of defensive action against secondary and opportunistic infections and immune tolerance phenomena (Shen et al., 2017; Rubin et al., 2014; Luan et al., 2015).

Also particularly relevant is the process leading to increased neutrophil survival, which is due to several factors (Fig. 2): increased levels of the antiapoptotic protein Mcl-1, hyperactivation of ERK-1/2 (Regulated Protein Kinase) and PI-3K (Fostatidyl-Inositol 3 Kinase) mediated by LPS and C5a

resulting in increased expression of Bcl-XL (antiapoptotic protein), decreased levels of Bim (pro-apoptotic), and phosphorylation of Akt that prevents formation of apoptosome (Shen et al, 2017; Perianayagam et al., 2004; Guo et al., 2006); finally, decreased LPS-induced function of caspase 8 is observed (Jia et al., 2008).

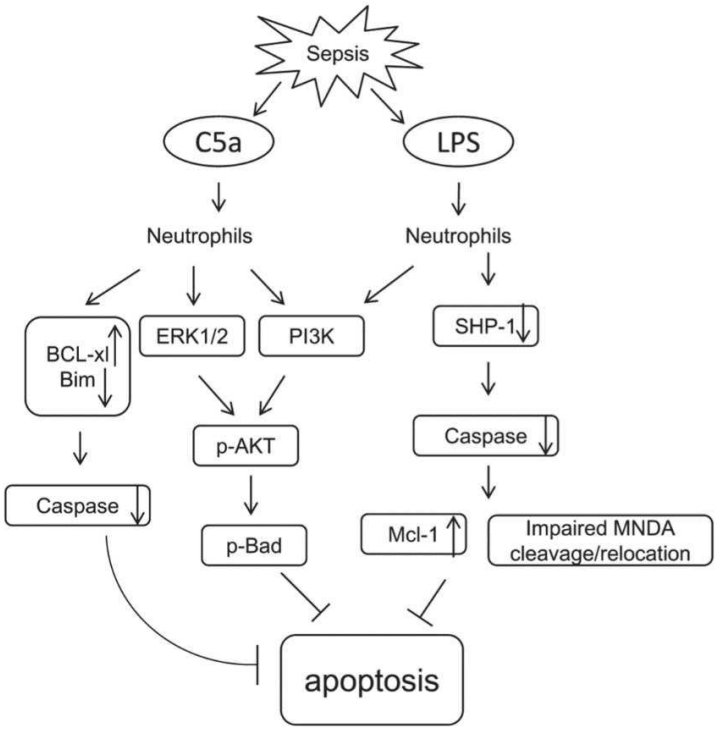

Figure 2. Schematic diagram of the mechanisms leading to loss of regulation of neutrophil proliferation (Shen et al., 2017).

The process underlying DIC is also attributable to errors in the host response and mainly exploits two mechanisms of action: on the one hand, there is the role of neutrophil NETs, which stimulate fibrin and act as a platform for components of the coagulation cascade, allowing activation of the factor XII pathway (intrinsic pathway), platelets (Delabranche et al, 2017; Fuchs et al., 2012) and TF (Tissue Factor, extrinsic pathway) (Iba et al., 2020). On the other hand, there is an absent activation of the fibrinolytic pathway, mediated by enzymes derived from neutrophil granulocytes trapped in NETs, which can inhibit the formation of TFPI (TF Pathway Inhibitor) and other anticoagulants such as thrombin and protein C (Shen et al., 2017).

The remaining tissue and circulatory damages are mainly attributable to metabolic dysfunction. These are characterized by a reduction in oxygen supply, caused by several factors such as erythrocyte deformation; increased vessel permeability with loss of plasma material, enabled by alterations in endothelial cells, which overexpress iNOS (Nitric Oxide Synthase in its inducible form) (Rubin et al, 2014); and by mitochondrial dysfunction, found in multiple tissues (Singer 2014), which bring about a switch to glycoside metabolism, with a reduction in ATP production to which organic dysfunction then follows (Singer, 2014).

LIPOLYSACCHARIDES (LPS), LIPOTEICHOIC ACIDS (LTA) AND THEIR ROLE IN SEPSIS.

Gram-negative bacteria are characterized by the presence of a double membrane, each with classical double-layer structure, and periplasmic space between them, in which a thin layer of peptidoglycan and hydrolytic enzymes are located. Of particular interest is the outer membrane, which is stiffer than the inner membrane. Its structure sees a full asymmetry regarding the inner and outer layers, the former formed by phospholipids, while the latter rich in lipopolysaccharide (LPS) presented OMPs (Outer Membrane Proteins) (Bertani and Ruiz, 2018) (Fig. 3).

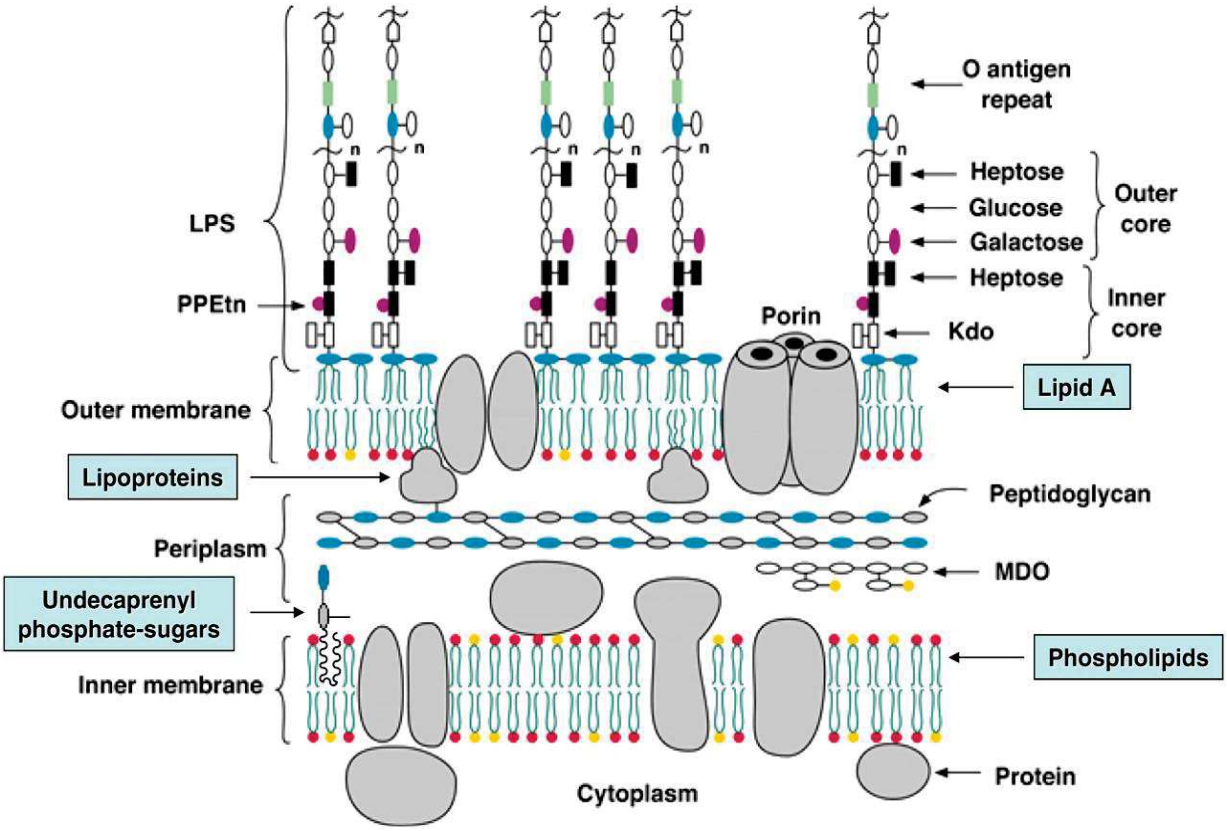

Figure 3. Membrane structure of Gram-negative bacteria (Raetz et al., 2002).

LPS is a molecule composed of 3 parts: lipid A, a nonrepeating oligosaccharide core, and antigen-O. The antigen-O and the non-Kdo (3-deoxy-D-manno-oct-2-ulosonic acid) residues of the oligosaccharide core are predominantly found in Wild-Type bacteria, enhancing their resistance to antibiotics and

environmental stresses, but are not required for growth in the laboratory (Raetz et al., 2002). Such molecules are found only in Gram-negative bacteria, but despite the wide conservation there are differences in the LPS structure of different bacterial species.

Lipid A is a glucosamine-based domain, also known as "endotoxin". It acts as a hydrophobic anchor for the LPS molecule and, given its fairly conserved structure, is an interesting pharmacological target (Raetz et al., 2002). The synthesis and use of numerous antibiotics targeting lipid A has led to the emergence of resistance mechanisms, such as against Polymyxin B due to the arn operon (which regulates an *E. coli* and S. typhimurium gene cluster) that allows the formation of the lipid L-Ara4N by adding 4-Amino-Arabinose (Moffatt et al., 2019).

The central oligosaccharide core is composed of the inner and outer cores, but to talk about it properly, it is necessary to make a distinction between the Smooth and Rough bacterial phenotypes.

In the Smooth phenotypes the inner portion contains the Kdo residues and the heptose sugar portion (Fig. 3, Fig. 4) (Raetz et al., 2002); the outer portion contributes to the heterogeneity of the LPS as it is more variable.

In Rough phenotypes, on the other hand, the inner core lacks heptose sugar components leading to greater membrane instability with hypersensitivity to substances such as dyes or antibiotics (Raetz et al., 2002).

The antigen-O (or O-polysaccharide) relies on a very strong heterogeneity of structure with even intraspecies differences leading to the formation of different serovars (Antonelli et al., 2017).

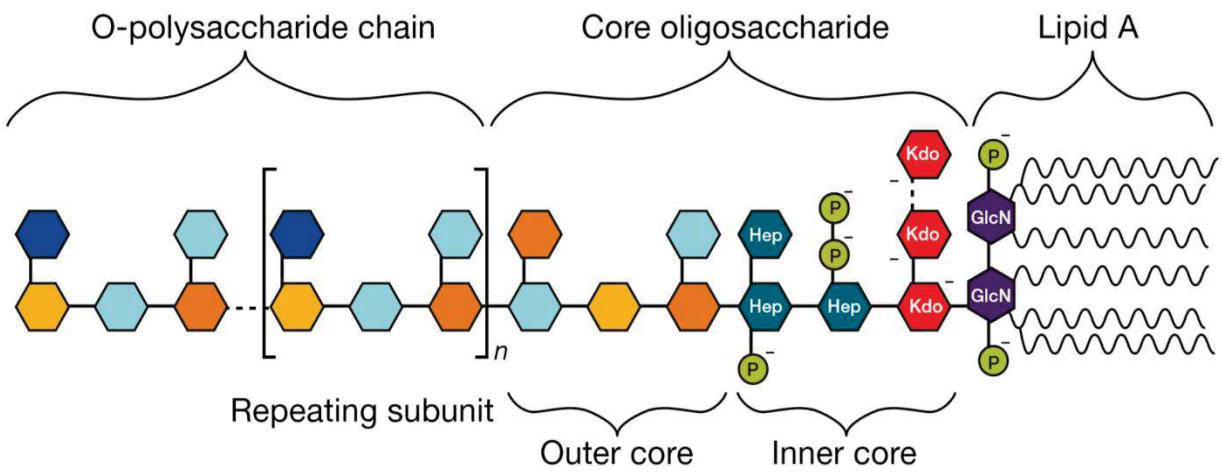

Figure 4. Schematic structure of the LPS.

The mechanism that leads to the initiation of sepsis is the same physiologically involved in the inflammatory response to a pathogen. There are several factors (proteins, lipopolysaccharides, nucleic acids) that trigger this signaling pathway and are encapsulated under the general term PAMPS (Pathogen-Associated Molecular Patterns) (Pontieri et al., 2010).

These factors are recognized and bound by a receptor group called PRRs (Pattern Response Receptors), especially by the family from TLRs (Toll-Like Receptors). Toll-Like Receptors are type I transmembrane receptor proteins present in all cell types involved in the body's defense (including endothelial cells and fibroblasts). Ten of them are identified in humans and possess roles not only in mechanisms typical of innate immunity, but also in adaptive immunity (Roach et al., 2005).

The TLR4 receptor plays an important role in Gram-negative bacteria infection. The TLR4 receptor recognizes and is activated by circulating LPS; this binding results in dimerization of TLR4. However, this process involves some intermediate steps: LBP (LPS-Binding Protein) binds a circulating LPS molecule, thereby interacting with the CD14 protein (generally associated with the membrane of macrophages and monocytes but also present in the circulation (Pugin et al., 1993), allowing it to act as a chaperone to give the correct conformation to the TLR4/MD-2 complex. MD-2 is a coreceptor of

TLR4 capable of binding LPS Lipid A, subsequently allowing its proper exposure to TLR4 and subsequent dimerization of the latter (TLR4/TLR4' complex) (Park et al., 2009). Such dimerization also involves the cytosolic portions of TLR4, which allow the activation of two mutually exclusive pathways (Guven-Maiorov et al., 2015): the TRIF-dependent pathway, which involves internalization into endosome and promotes IFN-β production, and MyD88-dependent pathway.

Following dimerization of TLR4, the dimer of the intracellular Toll/Interleukin 1 Receptor (TIR) domains recruiting TIRAP (TIR domain-containing adaptor protein) is formed, this goes on to form a homodimer that anchors to the membrane. This complex allows the arrival of MyD88, which forms the binding complex for IRAK2 and IRAK4 (Interleukin-1 Receptor-Associated Kinases), forming the next complex called the "myddosome," the latter promoting autophosphorylation of IRAK4. TRAF-6 (TNF-Receptor Associated Factor 6) goes on to bind to the complex forming a trimer and thus E3-ubiquitin-ligase binding occurs, promoting polyubiquitination on TRAF6, which is then recognized by TAB2 and TAB3. The latter are the adaptors of the IKK complex responsible for activation of TAK1 leading to phosphorylation of the NF-kB inhibitor (I-kB). Finally, NF-kB that enables transcription of TNF-α, il-1β AND il-6 (Fig.5) (Kuzmich et al., 2017).

It is worth to mention that DAMPs (factors derived from endogenous damage) can act as additional stimulators of the TLR-4 receptor and can be produced as a result of damage by bacteria during sepsis (Iba et al., 2020).

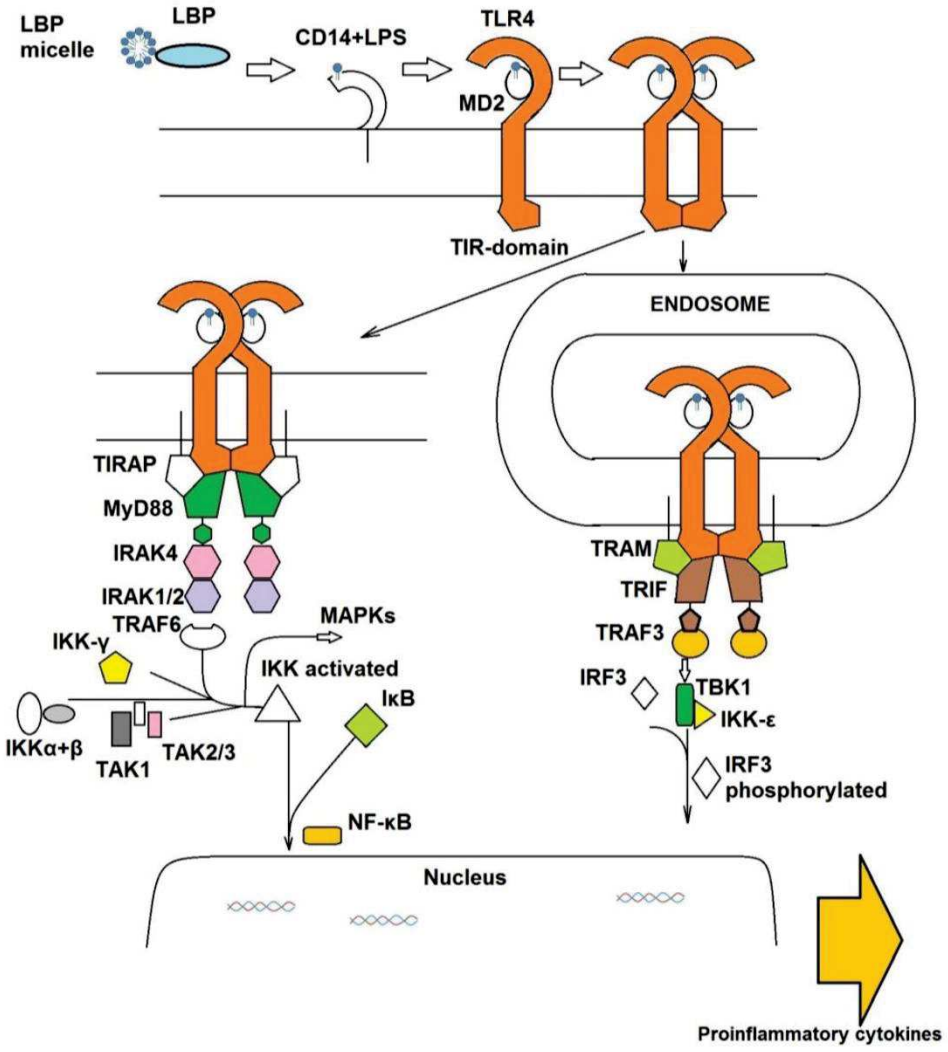

Figure 5. Illustration of both LPS-induced stimulation pathways (Kuzmich et al., 2017).

Lipoteichoic acid (**LTA**) is a major constituent of the cell wall of Gram-positive bacteria. These organisms have a cytoplasmic membrane and, externally, a peptidoglycan layer. The structure of LTA varies between the different species of Gram-positive bacteria, but it is an amphiphile molecule, commonly composed of a hydrophilic backbone with repetitive glycerophosphate units and D-alanine or hexose substituents as well as a lipophilic glycolipid (Morath et al., 2005) (Fig.6).

LTA is anchored to the cell membrane through diacylglycerol. It acts as regulator of autolytic wall enzymes (muramidases). It has antigenic properties being able to stimulate specific immune response.

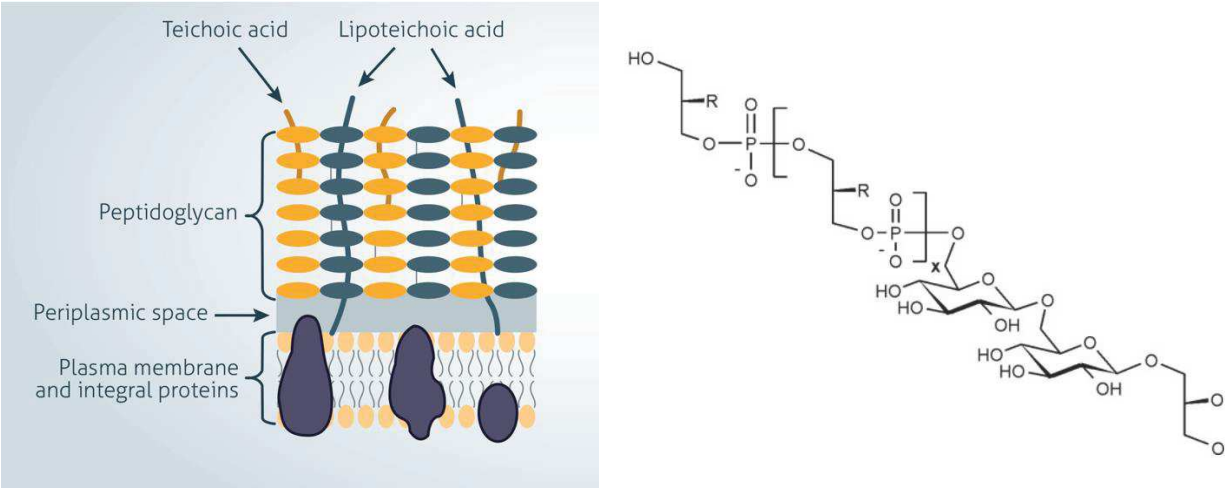

Figure 6. Gram-positive bacterial membrane and chemical structure of LTA from *S.aureus*.

The analogy of the structure/function relationship between LPS and LTA motivated many studies in the 1980s and 1990s, which aimed at investigating the role of LTA in Gram-positive septic shock. Many of these investigations concluded that LTA represents an important immunostimulatory principle. However, the often-conflicting publications left the role of LTA controversial. Especially, LTA of *Staphylococcus aureus*, the most prevalent Gram-positive pathogen, obtained from a commercial source and purified by a standardized procedure, proved to be biologically inactive (Morath et al., 2005).

LTA is released from the bacterial cells mainly after bacteriolysis induced by lysozyme, cationic peptides from leucocytes, or beta-lactam antibiotics. It binds to target cells either non-specifically, to membrane phospholipids, or specifically, to CD14 and to Toll-like receptors. LTA bound to targets can interact with circulating antibodies and activate the complement cascade to induce a passive immune kill phenomenon (Ginsburg, 2002).

Overview of the innate immune response to LTA. LTA from *S. aureus* activates macrophages via TLR-2, CD14 and CD36; the role of LBP is still unclear. This activation induces a strong recruitment signal for granulocytes via IL-8 and G-CSF release. Due to a lack of IL-12 production by macrophages, lymphocytes and NK cells are not induced to release IFN-γ. LTA activates the complement system by direct binding to L-ficolin (Ginsburg, 2002).

ANTIBIOTICS AND ANTIMICROBIAL PEPTIDES (AMPs)

We may use many different antibiotics depending on different pathogens, but we have to avoid resistances mechanisms of pathogens. Globally, an estimated 214,000 deaths due to neonatal sepsis are thought to be caused by resistant pathogens (Pant et al., 2021).

The history of antibiotics begins in 1928 with the discovery of penicillin by Alexander Fleming, with subsequent industrial-scale production and the first prescription in 1940. Since that time, antibiotics have reduced infant mortality rates and increased life expectancy, not only because of their ability to resolve bacterial infections but also to prevent them from occurring following surgery and chemotherapy, going on to play a prominent role socially as well (Blair et al., 2015; Aslam et al., 2018).

In the same way that bacteria naturally develop resistance to antimicrobials naturally produced by a variety of organisms, resistance to commercially available antibiotics, whether naturally derived or synthetic, has also been recorded (Blair et al., 2015; Aslam et al., 2018). The emergence of antibiotic resistance has resulted in what is a real global threat, both economically, according to the "World Economic Forum Global Risks," and health-wise, as reported by multiple bodies such as the "Centers for Disease Control and Prevention (CDC)" and the "World Health Organization (WHO)" (Aslam et al., 2018). Unfortunately, the excessive and not correct use of antibiotics led to development of many resistances and consequently of multi-drug resistant (MDR) bacteria, pathogens not sensible to multiple classes of antibiotics (McLeod et al., 2021). With the emergence of MDR (Multi-Drug Resistant) bacteria, particularly Gram-negative XDR (extremely drug-resistant) and TDR (totally drug-resistant), which fear the risk of a return to a "pre-antibiotic" era (Rossolini et al., 2014). The existence of such bacteria is recorded not only in nosocomial settings, but also in the community and in animals. In fact, the following are cited as major factors that have contributed to the accelerated development and spread of such strains: overuse of antibiotics in the human

clinic, use in animal husbandry and clinics as well as use in settings with poor hygiene standards (Aslam et al., 2018).

Before briefly mentioning the mechanisms of drug resistance, it is necessary to make a mention of MGEs (Mobile Genetics Elements), moreover transmissible between bacteria characteristic of humans and animals (Mølbak, 2004), and the three means of horizontal gene transfer. In fact, bacteria can acquire resistance-coding genes through plasmids, prophages, and conjugative and integrative transferable genetic elements (ICEs). Gene transfer is enabled by transformation mechanisms, an acquisition of heterologous genetic material from the post-capacitation environment that can also lead to the formation of "mosaic genes" (Sibold et al., 1994); conjugation mechanisms, a contact-dependent horizontal exchange; and transduction mechanisms, a horizontal exchange promoted by bacteriophages (Antonelli et al., 2017).

There are two types of antibiotic resistance, the first is intrinsic resistance, while the other is due to gene acquisition or mutation followed by environmental selection. Intrinsic resistance results from the presence of pre-existing enzymes or structures characteristic of the bacterium, for example: the outer membrane of Gram-negatives, which confers resistance to multiple compounds, such as vancomycin, unable to cross it (Rice, 2012); the capsule in many Gram-positives; biofilms, or bacterial communities layered on top of each other; and the capacity for intracellular replication (Minasyan 2019).

Instead, developed resistance can make use of multiple mechanisms: the reduction of permeability especially in Gram-negatives, with downregulation of non-specific channels and exposure of specific channels; increased efflux, which consists of overexpression of MDR efflux pumps; mutation in antibiotic target genes, with conformational or polarity change in the target site without altering enzyme function; protection of drug targets by modifications not directly affecting the target gene, but often its regulatory genes or those with a role in synthesis; direct action on antibiotics, either by hydrolysis, by enzymes such as lactamases, or by inactivation by addition of acyl, acetyl, phosphate and nucleotide groups, to which aminoglycosides are particularly

susceptible (Blair et al. , 2015). Finally, there are L bacterial forms, i.e., which are induced in a wall-free state post-stimulation with antibiotics for which it is targeted, effectively making them resistant to a broad spectrum of drugs (Allan et al., 2009) (Fig. 7).

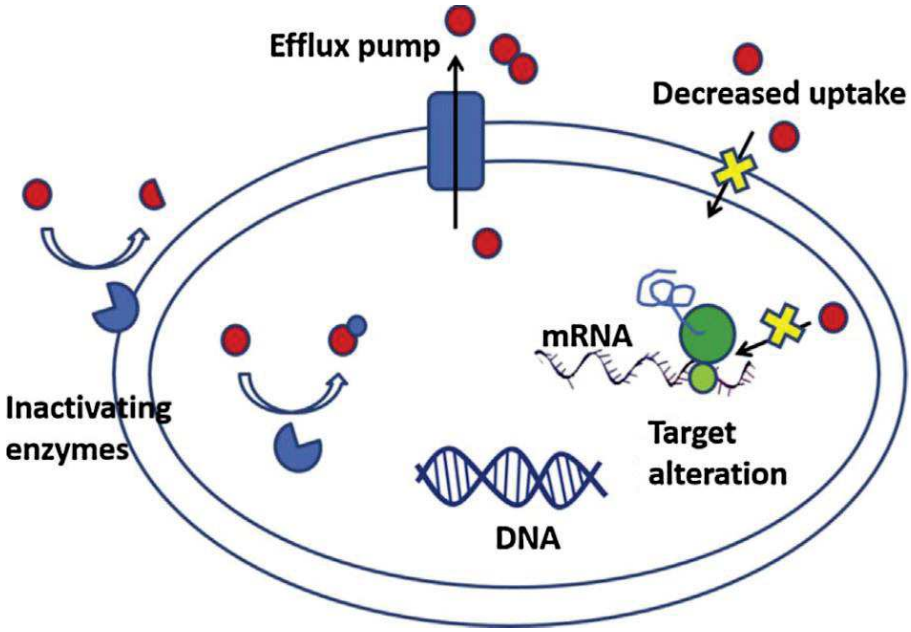

Figure 7. Examples of antibiotic resistance mechanisms in bacteria.

AMPs (Antimicrobial Peptides), also known as HDPs (Host Defensive Peptides), are ubiquitously occurring polycationic peptides. These peptides generally vary in length, with the most active ones often at a length between 20-50 (Patel et al., 2017; Ageitos et al., 2017). AMPs, in higher organisms, are involved in innate defense; instead, they play a role in the creation of biological niches in bacteria (Cogen et al., 2010). They have been shown to be used in multiple fields (oncological, antiviral, fungal, antibiotic), as they are thought to have an important advantage over traditional antibiotics, as described below. In the antimicrobial field, they possess a rather general mechanism of action, rather than one with a single, specific target, and therefore also a broad spectrum of efficacy (Ageitos et al., 2017; Hancock et al., 2006). Their mechanism of action involves multiple targets: the bacterial membrane, cell wall biosynthesis, cell division, nucleic acid, and protein di synthesis, as well as the folding action of

chaperone proteins and other cytoplasmic enzymes. Indeed, high conservation of these targets results in a lower likelihood of resistance development (Mansour et al., 2014).

Structurally, AMPs possess an excess of positive charges and a portion of hydrophobic residues between 30 and 50 percent; this amphipathicity is associated with their action and affinity for negatively charged membranes, such as bacterial membranes, which are rich in LPS (Gram-negative) and LTA (Gram-positive) (Hancock et al., 2006, Kumar et al., 2018).

Three structural classes are currently characterized (Fig. 8): α-helix, β-sheet, and extended-helix peptides.

α-helix AMPs often lack a defined structure in aqueous solution and tend to acquire one only once in contact with membrane, micelles or liposomes. The β-sheet structure is more structured in solution due to the stabilization promoted by disulfide bridges between cysteine residues. The last class has an extended structure with 2 or more proline residues, which acquires structure only post contact (Kumar et al., 2018). The three main characteristics of AMPs, with strong correlation to their action, are charge, hydrophobicity, and amphipathicity. Changes in any one of these 3 characteristics can dramatically alter the function of the peptide. There are values for charge and hydrophobicity that improve the antimicrobial capacity and pharmacokinetics of the peptide, but there are also "threshold" values beyond which there is a deterioration that can result in decreased selectivity, increased hemolytic activity, and increased involvement of mammalian cell membranes (Dathe et al., 2001; Chen et al., 2007). Alterations in amphipathicity due to mis-localization of residues in the secondary structure, also lead to such defects (Lee et al., 2016).

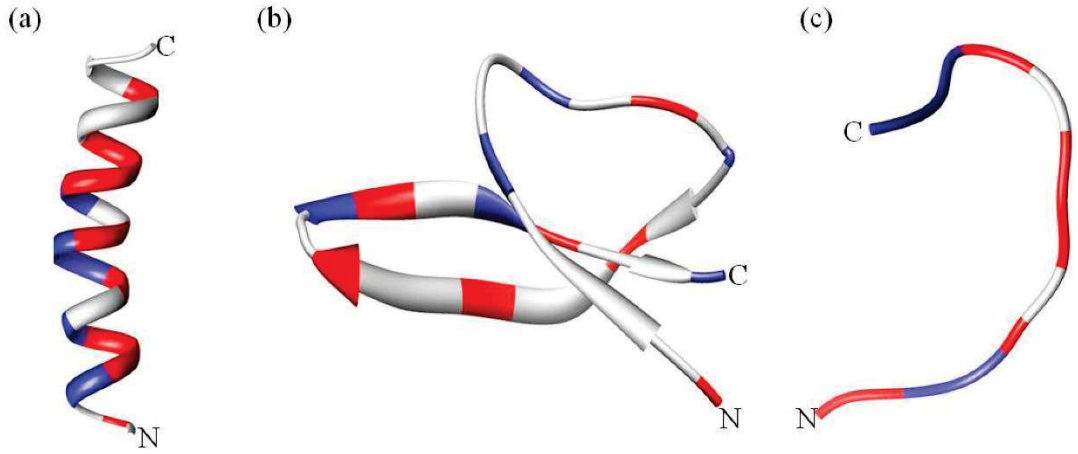

Figure 8. Secondary structure representation of the 3 structural classes of AMPs, made with CHIMERA software. The peptides represented are: Magainin (a), human Defensin 5 (b) and indolicin (c). Hydrophobic residues are in red and positively charged are in blue (Kumar et al., 2018).

The selectivity of AMPs toward prokaryote membranes compared with those of mammalian cells relies on a variety of factors: bacterial membranes possess a strong negative charge not only because of the presence of LPS or LTA, but also because of the richness in phosphatidylglycerol and cardiolipin in the outer leaflet. In contrast, mammalian membranes may rely on a richness in neutral zwitterionic phospholipids, such as phosphatidylcholine and sphingomyelin in the outer layer and with the most negatively charged residues located in the inner membrane leaflet (Matsuzaki et al., 1995). Also indicative of this statement is the presence of antitumor activity of some AMPs, as there is often dysregulation in the expression (overexpression) of highly negatively charged elements (Bechinger et al., 2017). Other factors in binding specificity, such as membrane curvature, lipid ratios, ionic conditions, temperature, and other environmental conditions, are also considered important (Bechinger et al., 2017).

The mechanisms of action of AMPs fall into 2 categories, direct killing mechanisms on pathogens and immunomodulatory mechanisms reflecting their role in organisms:

- *Killing mechanisms:* These can be further subdivided into membrane targeting and non-membrane targeting mechanisms, although it is extremely likely that each AMP may use more than one mechanism. However, the initial phase of interaction is common to both mechanisms and involves electrostatic AMPs-membrane interactions and an association at the surface, leading to peptide accumulation, until a threshold is reached, and bactericidal activity is given (Huang, 2006). Regarding membrane permeabilization there are three models, the "barrel-stave" and "toroidal" for pore formation and the "carpet" (Fig. 9). In the first, the hydrophilic and hydrophobic structure of the membrane is maintained, with the peptides inserting with the hydrophobic portions towards the outside of the aqueous pore and the hydrophilic portions towards the inside; whereas in the second, there is an alteration of this balance as the lipid heads remain bound to the peptide, thus resulting in transient pores due to membrane curvature, it is therefore likely that these peptides exploit this mechanism to enter and hit intracellular targets. The third model, on the other hand, involves the membrane being covered with peptide to such an extent that changes in energy and fluidity are created, leading to its disintegration into micelles, with detergent-like action. Mechanisms not involving the membrane, on the other hand, are those that involve targeting intracellular elements, including wall biosynthesis, enzyme, or chaperone expression, and blocking duplication, transcription, or translation (Lee et al., 2016; Kumar et al., 2018).

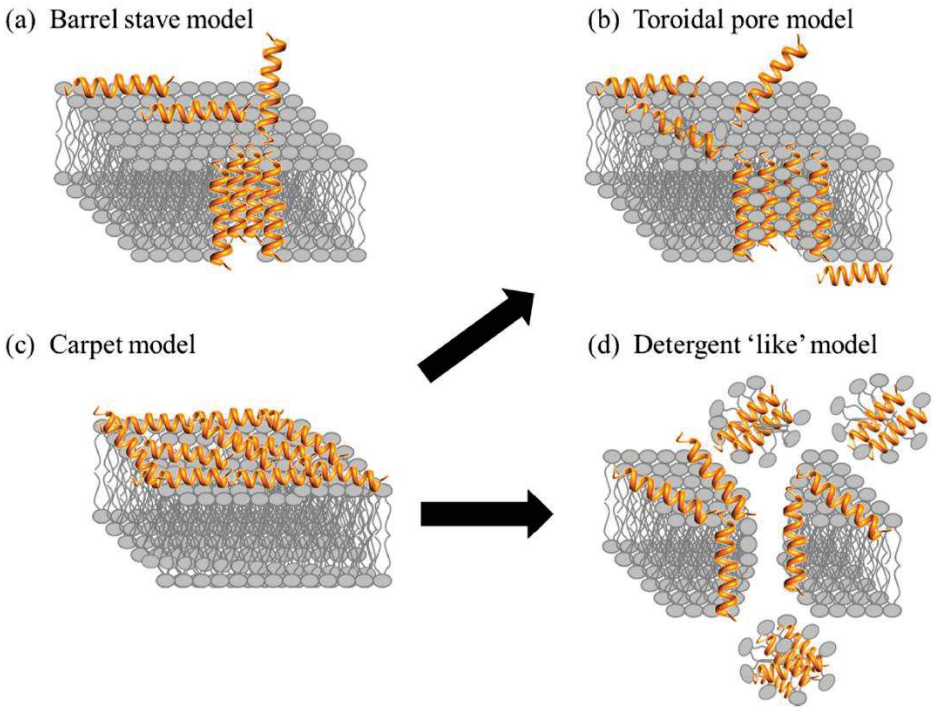

Figure 9. Models of action of AMPs on bacterial membranes.

- *Immunomodulation mechanisms:* They involve direct or non-direct recruitment of effector cells, with promotion of maturation and differentiation, "wound-healing" action and regulation of apoptotic mechanisms (Nijnik et al., 2012), also strong in structural similarity with chemokines that might indicate some evolutionary relationship (Yount et al., 2006).

However, AMPs can have disadvantages, the three main ones being *in vivo* toxicity, poor half-life, and cost, reasons why there are few AMPs currently used in the clinic (Roscia et al., 2013).

Toxicity is related to nonspecific actions on eukaryotic membranes due to the tendency to aggregate or undesired actions at the immunomodulatory level. (Mansour et al., 2014)

The poor half-life is due to the nature of the peptide, which appears to be sensitive to multiple host proteases, such as digestive enzymes, making oral administration impossible, and serum proteases. Toxicity and poor half-life are also the reason why many AMPs are approved for topical use only. Finally,

alterations in the function of AMPs can be recorded *in vivo* as physiological conditions change the levels of, for example, inorganic cations, polyanions (such as heparin), and mucins (Mansour et al., 2014; Kumar et al., 2018).

Furthermore, it is worth to consider the high costs of production, as a solid-phase synthesis with Fmoc (Fluorenylmotoxylcarbonyl) chemistry can lead to variable costs ranging from $50-$600 per gram, to which must be added the difficulty of production by recombinant technology in bacteria, due to toxicity to the bacterium itself. Large-scale recombination-fermentation-purification systems are currently widespread for bacteriocins, for example (Mansour et al., 2014; Hancock et al., 2006).

Despite the increased difficulty of the emergence of resistance to AMPs by bacteria, some examples are still present, the main ones being surface charge changes (as is the case with Ara4N), bacterial protease activity, active efflux (as with traditional antibiotics), and the formation of arrays of proteins and polysaccharides in biofilms, which are able to create electrostatic repulsion and capture the peptide. Also of concern are possible mechanisms of cross-reaction with natural AMPs, such as α-defensin HNP1, post-treatment with therapeutic AMPs (Bechinger et al., 2017; Andersson et al., 2016).

Multiple strategies have subsequently been developed to improve the characteristics of AMPs, which can be divided into chemical modifications and delivery systems.

These systems include:

- Chemical modifications:

- Use of D-amino acids. The creation of peptides, containing amino acids with different conformation that can confer improved efficacy with regard to resistance to proteases, both serum and bacterial, as well as improved pharmacokinetics and in some cases greater specificity and therefore less toxicity (Falciani et al., 2012; Di Grazia et al., 2015; Li et al., 2016).

- Incorporation of non-natural amino acids. The term "non-natural amino acids" is used to define amino acids that are not exploited in ribosomal

synthesis; therefore, one can exploit the replacement of cationic natural amino acids with non-natural ones having similar physicochemical characteristics to improve stability to proteolysis. However, the results are variable and need further investigation as there are cases where such modifications can be problematic (Gentilucci et al., 2010; Haney et. al 2019).

- Dendrimeric forms. Synthesis in the form of MAP (Multiple Antigen Peptide) results in increased serum stability of the peptides as well as improved clearance. Such branching is enabled by radially arranged lysine cores (Fig. 10) on whose amino terminal group the peptides are synthesized (Bracci et al., 2003; Pini et al., 2008). The mechanisms underlying this phenomenon appear to be the steric clutter of the MAP form, which prevents access to the catalytic site by proteases, and the possible formation of hydrophobic clusters and aggregations (Falciani et al., 2007).

- Cyclization. Cyclization generally involves increased stability and specificity, generally enabled by the formation of disulfide bridges between N- and C-terminal cysteine residues, but other alternatives are also possible (Nguyen et al., 2010; Gentilucci et al., 2010).

- Acetylation. This modification results in partial neutralization of the positive charges, increasing hydrophobicity and thus peptidase resistance, but generally at the expense of the peptide's antimicrobial activity (Nguyen et al., 2010; Papanastasiou et al., 2009).

- Peptidomimetics. The term "peptidomimetics" generally denotes all sequences that mimic a peptide structure without being based solely on α-amino acid backbone; this is possible because it is the maintenance of secondary structure that is critical for proper interaction with the target. For example, α-peptoids (side chains bound on Nα instead of C-α) and β-peptoids can be highlighted as being able to maintain the activity of the original peptide while possessing greater stability in serum and lower toxicity (Molchanova et al., 2017).

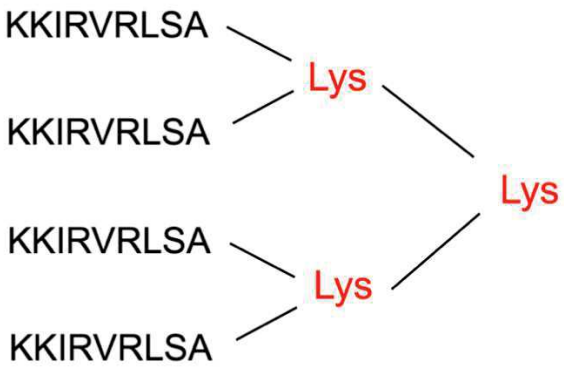

Figure 10. Lysine core structure of the antimicrobial peptide SET-M33. For the peptide sequence is indicated one letter amino acid code, for the lysine core was used three letter amino acid core.

- Delivery systems:

- Polymer conjugation. Polymer conjugation has long been used to improve the characteristics of biomolecules. The main technique is PEGylation, or conjugation to Polyethylene Glycol, a nontoxic and nonimmunogenic polymer that can reduce the biomolecule's clearance rate, improving its solubility, decreasing its immunogenicity, and improving its stability (Veronese et al., 2008).

- Encapsulation. Encapsulation can occur in both organic (lipids, surfactant, dextran) and inorganic (graphene, carbon nanotubes, metals) materials. Such carriers can bring benefits, such as reduced toxicity of AMP (Ritter et al., 2020) or improved persistence and lower clearance in the target organ (Cresti et al., 2022b) (Falciani et al., 2020).

THE ANTIMICROBIAL PEPTIDE SET-M33

SET-M33 (Fig. 11) is an antimicrobial peptide of 9 amino acids (KKIRVRLSA), synthesized in the form tetra branched (MAP). It is a synthetic AMP, selected by phage display from a synthetic library. The phage display is a technique that uses a peptide library or Antibody exposed to bacteriophages (M13 in most cases) with genotype phenotype matching (Falciani et al., 2005; Pines, 2011). Initially the lead peptide L1 was obtained (QEKIRVRLSA, M1 when in tetrameric form) which was then followed by modification processes rational, which led to the birth of the peptide M6 (QKKIRVRLSA) (Pini et al., 2005), to which finally N-term glutamine was removed with the following birth of SET-M33 (Pini et al., 2010).

This peptide showed high efficacy against a broad spectrum of Gram-negative bacteria, including also, MDR clinical isolates, with MIC comparable to polymyxin B (Pini et al., 2010; van der Weide et al., 2017; van der Weide et al., 2019) and an even lower resistance appearance frequency ($5*10^9$ CFU) (Brunetti et al., 2016a). It also provoked a strong immunomodulatory activity with reduced expression of NFkB and other proinflammatory cytokines, and showed low genotoxicity (Brunetti et al., 2016b) and low hemolytic activity (van der Weide et al., 2017). Moreover, it had a strong antibacterial activity *in vivo* in models of sepsis and lung and skin infections (Brunetti et al., 2016a).

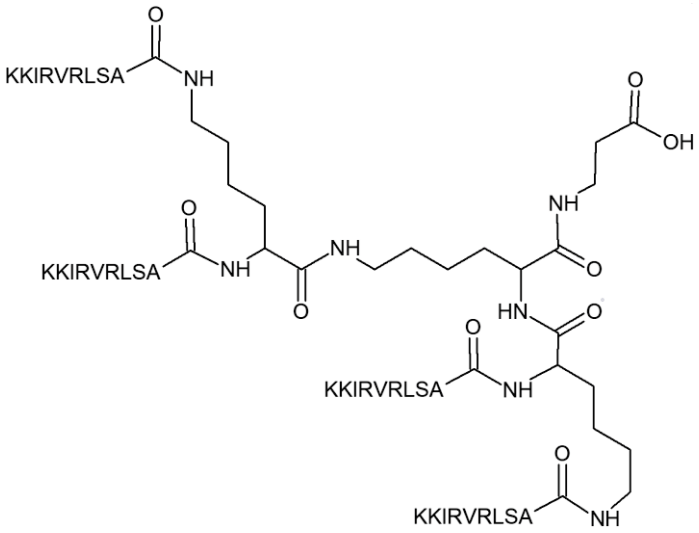

Figure 11. Representation of the structure of SET-M33.

From the molecular point of view, the killing mechanism of SET-M33 consists of 2 phases: the first phase consists in the binding of LPS thanks to the peptide positive charges, the second phase leads to the destruction of the bacterial membrane (Brunetti et al., 2016; Pini et al., 2007), the swelling and damage of the membrane are evident in electron microscopy (Fig. 12 and 13). However, the amphipathic α-helix structure responsible for the destruction of the bacterial membrane is acquired only post-contact with the target membrane (van der Weide et al., 2017), (Castiglia et al., 2019).

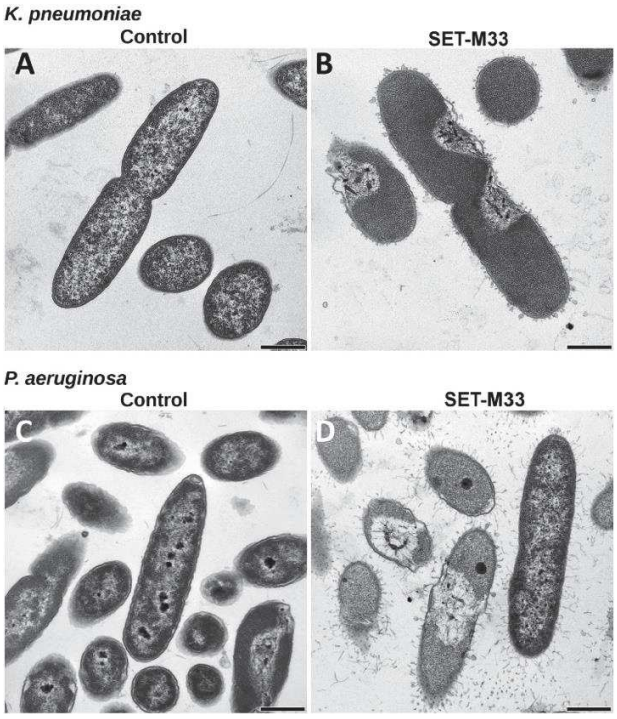

Figura 12. TEM (transmission electron microscopy) images depicting normal and post-incubation K. pneumoniae and P. aeruginosa (control) for 30 minutes with SET-M33 (van der Weide et al., 2017).

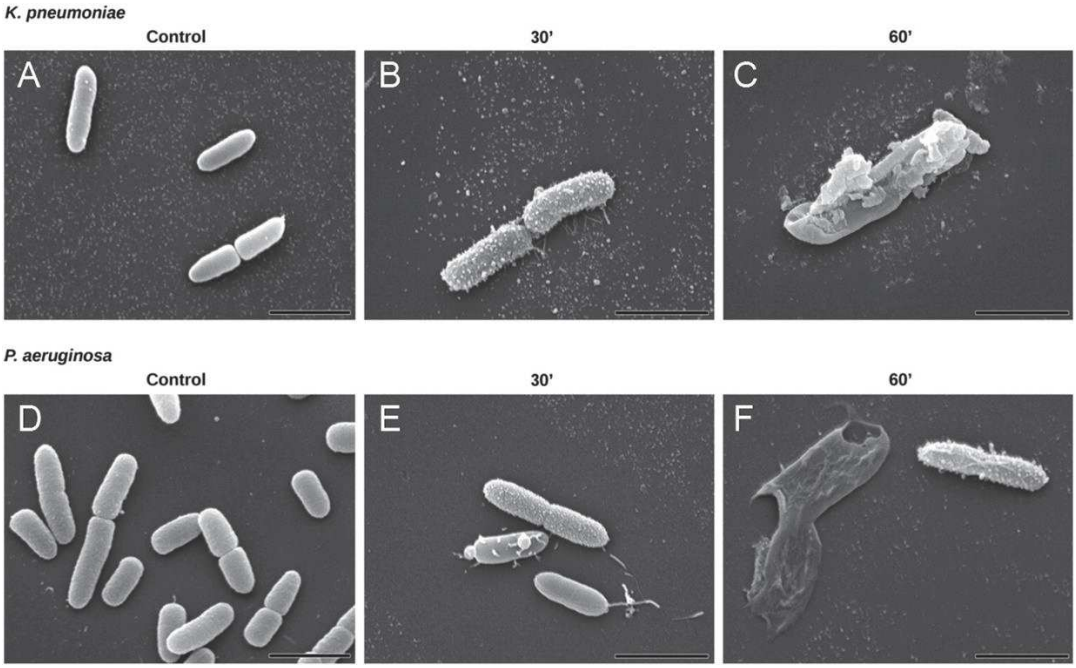

Figure 13. SEM (scanning electron microscopy) images depicting non treated K. pneumoniae and P. aeruginosa (A, D), 30 min post-incubation with SET-M33 (B, E) and 60 min post-incubation with SET-M33 (C, F) (van der Weide et al., 2017).

To allow greater efficacy *in vivo*, the peptide has undergone further modifications: synthesis takes place in acetate form and no longer as a trifluoroacetate salt (TFacetate), through the use of a resin for ion exchange, with a strong decrease in toxicity *in vivo* (Pini et al., 2012); PEGylation, i.e. the addition in C-term of a PEG4 molecule, which allowed a further improvement to the already improved half-life of the MAP form, through a reduction in renal clearance speed and a better resistance to P. aeruginosa elastase (Falciani et al., 2014).

EXTRACORPOREAL HEMOPERFUSION: EXISTING DEVICES

Extracorporeal blood purification (Fig.14) is a valuable therapy in sepsis to be used alongside antibiotic drug administration to kill bacteria and remove endotoxin from the bloodstream (Li et al., 2022).

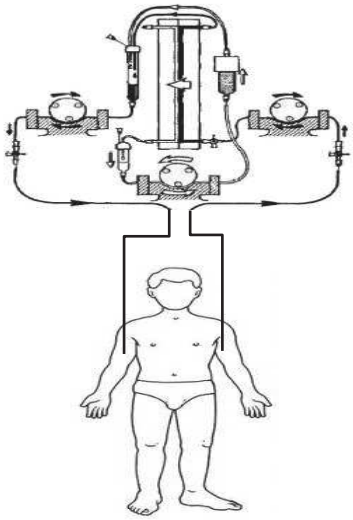

Figure 14. Extracorporeal blood purification mechanism.

Several devices, using different peptides, resins and retention methods are available today that attempt to remove endotoxins, bacteria, and cytokines from the blood:

- OXiris (Baxter, Meyzieu, France): it is a filter which combine removal of endotoxin (LPS) and cytokines (Zhou et al., 2022). The cytokine storm induced by LPS may lead to septic shock and acute kidney injury (AKI). OXiris may be used for continuous renal replacement therapy (CRRT) (Pickkers et al., 2019). This filter is composed of the AN69 membrane, which contains positively charged amine groups that interact with negatively charged cytokines and endotoxins and allow their removal from the bloodstream.

  In addition, the membrane has a heparin graft that confers antithrombotic activity (Guan et al., 2022). The adsorption mechanism

of OXiris is mainly due to ionic interactions, and this device is the only one able to remove both endotoxins and cytokines (Malard et al., 2018). OXiris has received FDA approval for the treatment of COVID-19 patients in intensive care units (ICUs), for the removal of inflammation mediators present in the patients' bloodstream.

- Toraymyxin (Toray industries, Japan): it is a hemoperfusion column which use the polycationic antibiotic polymyxin B linked to polystyrene fibers to remove endotoxin (LPS) and pyrogenic factors from blood (Tani et al., 2019) (Fig.15). Treatment with this extracorporeal hemoperfusion device lasts about 2 hours (Tani et al., 2019). Polymyxin B is one of the few antimicrobial polypeptides, currently used in the clinic, that can bind and neutralize LPS. In addition, this AMP is also able to reduce the level of pro-inflammatory cytokines, such as TNF-α, usually induced by endotoxins (Sharp et al., 2010). Limitations to the use of Polymyxin B, like many other antimicrobial peptides, relate to the development of resistance and nephrotoxicity and neurotoxicity in human subjects (Velkov et al., 2018). For these reasons, alternative uses of these peptides have been found. As early as the 1990s, for the first time, polymyxin B was immobilized on a polystyrene-derived fiber to remove endotoxins from blood. Immobilization was accomplished through a covalent bond between polymyxin and polystyrene. This device was used to filter blood externally using an extracorporeal circuit (Ronco et al., 2014). In 1994 in Japan the first application of hemoperfusion with Toraymyxin column to treat patient with multiple organ failure was performed, reporting decreased level of LPS. In 2005, it has been demonstrated safety of Toraymyxin and its ability in improve renal function (Vincent et al., 2005). Over the years, studies have been conducted on this device that have given conflicting opinions: the EUPHAS (Early Use of Polymyxin Hemoperfusion in Abdominal Septic Shock) study demonstrate that Toraymyxin treatment highly reduce mortality in sepsis patient; the ABDO-MIX trial conducted in France failed to show benefits of treatment compared with conventional ones (Shimizu et al., 2017). Nowadays there are 3 different Toraymyxin

columns available in Japan: PMX-20R ®, PMX-05R ® and PMX-01R ® respectively used for the treatment of septic shock in adults, pediatric patients and premature born patients (Nakamura et al., 2007) (Nishizaki et al., 2016).

- Cytosorb ® (CytoSorbents Corporation, USA): This device is mainly devoted to the removal of inflammatory cytokines. Hem-adsorption using this new technology allow to manage the cytokine storm and to control inflammatory conditions in sepsis and septic shock patients (Fig.15). This device allows to remove bacterial exotoxins, myoglobin, free hemoglobin, and inflammatory cytokines (Borthwick et al., 2017). Studies have shown that the device has better results when used in combination with other therapies and when applied within 24 hours of sepsis diagnosis (Paul et al., 2021). CytoSorb® can be used in other inflammation-related conditions, such as severe pancreatitis or cardio-pulmonary bypass (Huber et al., 2019). Cytosorb® is made up of polystyrene and divinylbenzene beads with a larger surface area, which allow removal of cytokyne from blood by size selection and adsorption. In a study to evaluate clinical outcomes of this device, it has been observed reduction in all the biomarker levels, such as procalcitonin, C-reactive protein and serum lactate, and inflammatory markers, like IL-6, IL-10 and TNF) (Mehta et al., 2020). Therapy with Cytosorb report increased survival rate and safety without adverse events, but new studies are underway to assess whether the device can be considered a viable new treatment for sepsis (Kogelmann et al., 2017).
- High Volume HemoFiltration (HVHF): it's a continuous hemofiltration with higher volumes compared with other devices that allow to remove high number mediators from plasma. No mortality benefits were observed, and an important negative aspect was seen during studies: due to the large volume exchange, this device also removes antibiotics, vitamins, and essential nutrients from plasma (Govil et al., 2020).
- High cut-off membrane (HCO): it was designed to remove more middle-weight molecule (50 to 60 kDa). Its validity remains controversial because some studies demonstrated that HCO membrane therapy can

result in lower ICU mortality and reduced circulating levels of inflammatory mediators, but recently other studies revealed no benefits from this type of therapy (Zhang et al., 2021).

- Coupled Plasma Filtration Adsorption (CPFA Bellco, Italy): it is a blood purification technology useful to remove pro and anti-inflammatory cytokines and LPS from plasma using a resin sorbent cartridge. The free cytokyne plasma is then reinserted in circulation thanks to a dialyzer (Ankawi et al., 2018). This device is that there is no direct contact between blood cells and sorbent filter, resulting in higher biocompatibility. Previous studies, such as COMPACT and ROMPA, do not demonstrate mortality benefit (Zhang et al., 2021).
- Alteco LPS adsorber (Alteco medical AB, Sweden): it is a cartridge with a polyethylene plate inside containing a peptide able to bind lipid A of LPS with high affinity (Fig.15). This device has no ability to remove bacteria or inflammatory cytokines. Some studies demonstrate the efficacy of this device only if the therapy starts few hours after sepsis diagnosis (Adamik et al., 2015).
- JAFRON HA330 (Jafron Biomedical, Guangdong, China): it is an hemoperfusion cartridge with electrical and porous resin for the removal of cytokines and endotoxins. Sazonov et al, report that this device eliminates IL-6 better than Cytosorb and it is also efficient for the treatment of septic shock patients (Sazonov et al., 2021).
- Seraph ® 100 Microbind® Affinity Blood Filter (ExThera, Martinez, CA): this device is composed by polyethylene beads with linked heparin. It can remove many resistant bacteria and also viruses and it can be used both alone and in combination with kidney replacement procedures (Fig.15). This filter was recently approved for the treatment of severe COVID19 infections, due to ability in binding nucleocapsid protein of SARS-CoV-2 (Schmidt et al., 2022). An important advantage of Seraph 100 is that it does not induce development of resistances due to its biomimetic features (Rifkin et al., 2022).
- Selective cytopheretic inhibitory device (SCD): this uses a novel strategy to treat sepsis patients, consisting of a synthetic biomimetic membrane

able to bind activated leukocytes in blood. This extracorporeal device uses a circuit consisting of a cartridge containing biocompatible polysulfone (Tumlin et al., 2015).

- Matisse-Fresenius system (Fresenius SE, Germany): it is an endotoxin adsorption system containing human serum albumin immobilized on polymethacrylate beads. Numerous studies have given conflicting opinions on the effectiveness of this device (Esteban et al., 2013).

Figure 15. Medical devices in preclinical phase and in clinical use: (a) Toraymixin, (b) Alteco, (c) CytoSorb, (c) Seraph 100.

NEW SET-M33-BASED MEDICAL DEVICE FOR THE SELECTIVE REMOVAL OF ENDOTOXINS AND BACTERIA

Patients suffering from sepsis are usually treated with intravenous antibiotics, fluids, and oxygen, to maintain a stable blood pressure level. Sometimes also dialysis is used in particular cases.

Early diagnosis of the clinical picture of sepsis reduced hospital mortality, but the immunomodulatory drugs used to date have not shown particularly positive results (Hotchkiss et al., 2016).

In addition, antibiotics play a decisive role in the pathophysiological process of septic shock, mainly through their ability to liberate immunologically active components of the bacterial cell wall (LPS and LTA) during destruction of the microorganism. As reported above LPS is a potent inducer of the pro-inflammatory cytokine response in individuals suffering from Gram-negative sepsis or septic shock. In the case of Gram-positive infection, LTA may exert similar biological effects (Cohen, 2002).

The antimicrobial peptide SET-M33 demonstrates ability to bind LPS, LTA and bacteria , along with low propensity to develop resistances and activity against both Gram-positive and Gram-negative multi drug resistant (MDR) bacteria (Pini et al., 2010). These features allow to design devices containing SET-M33 to remove LPS, LTA and bacteria from sepsis and septic shock patients.

For many years SET-M33 peptide was studied for the parenteral administration but, despite its effectiveness, efficacy dosage resulted close to the toxic one. At high dosages it demonstrated renal toxicity in dogs and rats and irreversible variations on epithelium of vagina, uterine cervix, and bone marrow (Cresti et al., 2022a). For these reasons, the peptide has been assessed for its possible use in extracorporeal device avoiding direct administration.

AIM OF THE PROJECT

My doctoral project focuses on the construction of a new effective medical device for the treatment of septic patients, in addition to standard therapy.

This project, aimed at developing a device for clinical use in sepsis patients, originated a few years ago at Setlance srl, a small company which financed my PhD fellow.

This device was analyzed for the removal of living Gram-negative, Gram-positive and MDR bacteria from the serum of septic patients. The ability to remove endotoxinsand LTA, which are responsible for the worsening clinical picture of these patients, was also evaluated.

My PhD project initially focused on a device containing a matrix of agarose beads conjugated with the antimicrobial peptide SET-M33. Later, in collaboration with the company Medica spa, the focus turned to a device using the same antimicrobial peptide linked to a new biocompatible matrix.

METHODS

SET-M33-CYS SYNTHESIS, PURIFICATION AND CHARACTERIZATION

SET-M33-cys peptide (Fig.16), in a branched form (MAP), was synthesized on a Syro automated machine (MultiSynTech, Witten, Germany) using 9-fluorenylmethoxycarbonyl (Fmoc) chemistry and activation of the carboxylic group with O-(benzotriazol-1-yl)-N,N,N, N-tetramethyluronium hexafluorophosphate (HBTU)/N, N-disopropylethylamine (DIPEA). A TentaGel® S RAM resin (Rapp Polymere GmbH) and a polymer insoluble solid support (poly(oxyethylene)-2,4-dimethoxybenzhydrylamine) was used for the synthesis. Side-chain-protecting groups were tert-butoxycarbonyl (Boc) for Lys, 2,2,4,6,7- pentamethyldihydrobenzofuran-5-sulfonyl (Pbf) for Arg, and tert-butyl ether (tBu) for Ser. The first amino acid immobilized on the membrane was Fmoc-NH-Cys(Trt)-COOH, Trt (titryl group), followed by the addition of Fmoc-NH-PEG(4)-CH2-CH2-COOH with the second coupling and then the addition of Fmoc-Lys(Fmoc)-OH twice for the establishment of the lysine core on which the four KKIRVRLSAs are assembled. After the synthesis was finished, the product was detached from the resin and laterally deprotected in a solution of TFA (trifluoroacetic acid), TIS (triisopropylsilane), water (95:2.5:2.5), EDT (1,2-ethanediol) and precipitated with diethylether. The crude peptide was purified by high-performance liquid chromatography (HPLC) using a reversed-phase column for HPLC (XBridge peptide BEH C18 Waters) that has C18 (octadesilane) chemistry, 300Å pores, pH range 1-12 and P.M. range 1-15KDa.

A linear gradient of 83%A-70%A was used for elution for 40 min (A is a 0.1%TFA solution in water and B is acetonitrile).

The identity and purity of the peptide were finally confirmed both by reversed-phase chromatography, exploiting Phenomenex C18 analytical column (based on C18 chemistry with 300Å pores), and by Matrix assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectrometry. Characterization in mass spectrometers was done with ULTRAFLEX TOF UTX-00699 (Bruker Daltonix), using a positive voltage linear acquisition with a 5-

20Kda method. Cyano-4-hydroxycinnamic acid matrix dissolved in a 50 percent solution of acetonitrile in water was used. 2µL of matrix and 2µL of sample were mixed and plate on MTP 384 target plate for analysis.

Finally, the peptide underwent lyophilization by freezing at -20°C overnight in 5% aqueous solution of acetic acid and was subjected to a freeze-drying process for 48 inside a ScanVac freeze dryer.

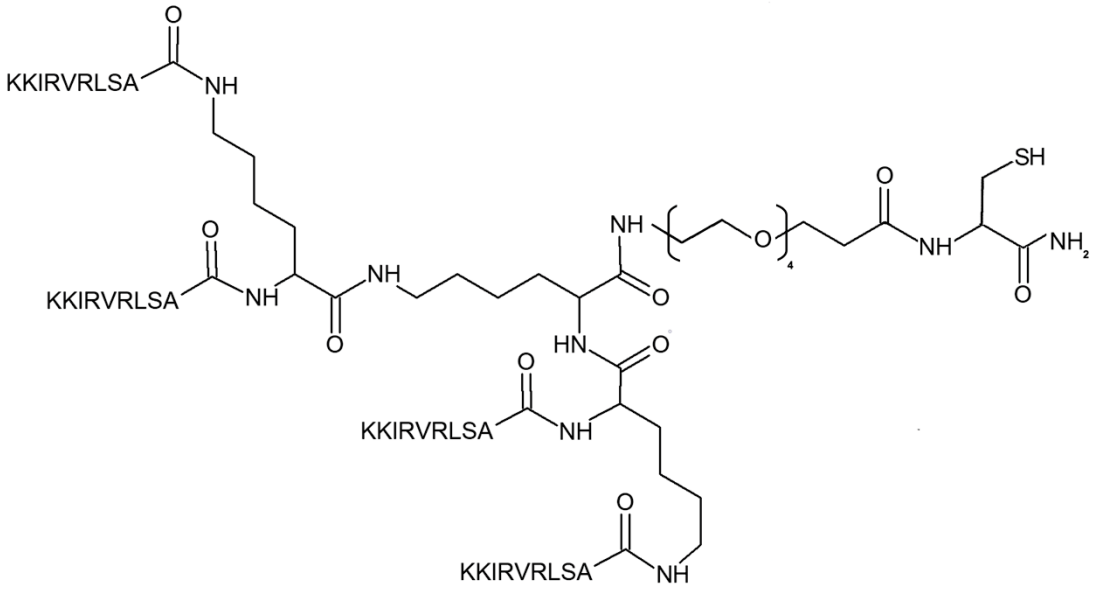

Figure 16. SET-M33-Cys peptide.

SET-M33-CYS IMMOBILIZATION IN DEVICE ON BIOCOMPATIBLE MATRIX

Tetra branched SET-M33-Cys peptide was conjugated to the agarose beads (SulfoLink™ Coupling Resin) with two rounds of conjugation.

The SulfoLink Coupling Resin is derivatized to contain iodoacetic groups that react specifically with free sulfhydryl at pH 7.5-9.0 (Fig. 17), present in the peptide.

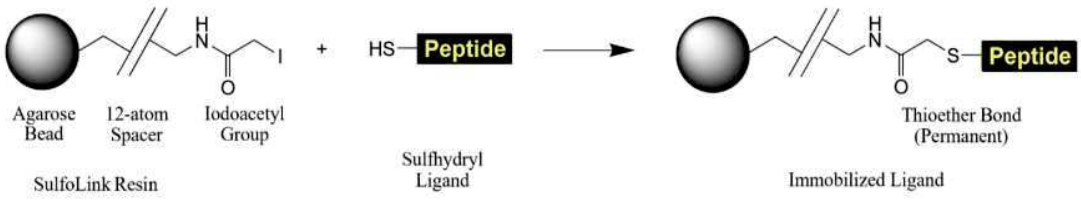

Figure 17. Mechanism of interaction and conjugation of the peptide with agarose beads.

The 12-atom spacer arm minimizes steric hindrance, ensuring efficient endotoxin-peptide and bacteria-peptide binding interactions. Depending on the stability of the immobilized peptide, the column may be used multiple times without significant loss in binding capacity.

After removing the storage solution from SulfoLink resin, the peptide was dissolved 1mg/ml in Coupling Buffer (50 mM Tris, 5 mM EDTA-Na; pH 8.5). The solution was incubated with the agarose beads to allow conjugation. Two rounds of conjugation of the peptide to the agarose beads were performed. The peptide was incubated with the beads for 45minutes. The binding rate of the peptide to the agarose beads was assessed by measuring the peptide present in the flow-through of the 2 immobilization cycles. Measurement was made by High Performance Liquid Chromatography, by comparing the area of the peptide peak before and after immobilization cycles. The resin was washed

with Wash Solution (1.0 M NaCl, 0.05% NaN3) 3 times and equilibrated with Coupling Buffer. Then the nonspecific sites of the agarose beads were saturated with 50mM L-Cysteine-HCl solution in Coupling Buffer. After a 45-min incubation, the agarose beads with the immobilized peptide were equilibrated with PBS (0.05% sodium azide). The bond between the peptide and agarose beads (Fig. 19) is very stable and long-lasting.

Peptide-conjugated agarose beads were dispensed into devices of different sizes (1ml, 4ml), stored at 4°C. Columns containing peptide conjugated with beads were washed with PBS before being used.

A at the end of peptide conjugation with agarose beads, the cartridge (Fig. 20) can be used or stored at 4C°.

QUANTIFICATION OF LPS BY LIMULUS AMEBOCYTE LYSATE TEST

The Pierce Chromogenic Endotoxin Quant Kit (ThermoFisher Scientific) is a quantitative and highly sensitive assay using amebocyte lysate derived from Limulus blood for quantification of bacterial endotoxin within samples. Limulus red blood cells (amebocytes) clotted in the presence of low levels of endotoxin. The working principle of the test is based on the ability of bacterial endotoxin to catalyze the activation of a proenzyme contained in Limulus amoebocyte lysate (LAL), which results in its conversion into an active enzyme. After the addition of the colorless substrate Ac-Ile- Glu-Ala-Arg-pNA, the enzyme cleaves the pNA (p-Nitroanilina). The colorimetric intensity is proportional to the amount of endotoxin present in the sample and can be quantified by absorbance reading on the spectrophotometer at a wavelength of 405 nm.

Sera at the different concentrations were analyzed at various dilutions using the LAL test to identify which of the concentrations tested yields values that fall within the range of the standard curve.

To perform the test, a standard curve was prepared by diluting the endotoxin stock of *E. coli* O111:B4 (0.1-1.0 EU/mL). 50 µL of each sample was added to the wells of the plate followed by 50 µL/well of LAL. After 12 minutes of incubation at 37°C, 100 µL/well of chromogen substrate was added, and after 6 minutes of incubation at 37°C, the reaction was stopped with 50 µL/well of 25% acetic acid. Absorbance values were obtained using the plate reader (Ascent software) at a wavelength of 405 nm. The optical density values were converted to EU/mL concentration based on the standard curve using the software CurveExpert.

QUANTIFICATION OF LTA BY ELISA

The LTA content of a sample has been quantified by the use of commercial LTA ELISA kit (MyBioSource). This assay has high sensitivity and excellent specificity for detection of LTA. This kit was based on sandwich enzyme-linked immune-sorbent assay technology. A standard curve (7.8-500 pg/mL) was prepared using a lipoteichoic acid from *S. aureus* (control standard) in Sample/Standard dilution buffer, and serially diluted.

100 µl of sample is added to micro-plate wells pre-coated with LTA antibody. After incubation (90 minutes at 37°C) and washing, 100 µl of LTA detection antibody labeled with biotin is added. After appropriate incubation (60 minutes at 37°C) and washing, 100µl streptavidin-horse radish peroxidase conjugate with Streptavidin (HRP-Streptavidin) is added followed by incubation (30minutes at 37°C) and washing. After addition of 90µl chromogenic 3,3',5,5'-tetramethylbenzidine (TMB) solution the sample is incubated for 50 minutes at 37°C. The HRP enzyme reaction is stopped by addition of a acid stop solution(50µl). The intensity of color developed is proportional to the concentration of the lipoteichoic acids present in the sample and read at 450nM using a plate reader.

All sample was processed in duplicate.

BACTERIAL COLONY COUNTING

Gram-negative (*E. coli* serovar ATCC 33780™) and Gram-positive (*S. aureus* USA300) bacteria were tested. The *S. aureus* strain was chosen to test the efficacy of the devices against a multi drug-resistant bacteria. Living bacteria were removed from saline in preliminary tests, and subsequently by human serum.

For the production of the serum containing bacteria, a pre-culture of bacteria was prepared in medium (Luria Broth for *E. coli* and Tryptic Soy Broth for *S. aureus*). The pre-culture was incubated overnight in agitation at 37°C 220RPM. A 1:100 dilution of the overnight pre-culture in liquid medium was made, then incubated until an absorbance value of O.D. 1 (600nm) was reached on the spectrophotometer.

After making two dilutions (1:25 and 1:20) in decomplemented serum (2h at 56°C), the sample was incubated in column at room temperature in slow rotation (2h). Serum inactivation of serum complement (56°C, 2h) was necessary because the active complement system killed bacteria. Serum collection was performed by withdrawing the supernatant from the column.
To quantify the bacterial retention capacity after device circulation, samples were plated in Petri dishes with agar medium. Samples were collected and serial dilutions (undiluted serum to 106) were made of serum not incubated on column (INPUT), serum incubated on column with peptide (OUTPUT-M33), and serum incubated on column without peptide (OUTPUT-CTR).Gram-negative bacteria (*E. coli* 0111:B4) were plated in Luria broth agar to be counted, and Gram-positive bacteria (*S.aureus USA300*) were plated in Mueller-Hinton agar to be counted. 100µl of sample was plated in plates with agarized medium (Luria Broth Agar for *E. coli* and Tryptic Soy Broth for *S.aureus*). After overnight incubation at 37°C, bacterial colony counts were performed. Data were processed using GraphPad Prism 6 software.

MEASUREMENT OF HEMATOCHEMICAL PARAMETERS

Device safety was assessed by evaluating alterations in the composition of various serum components. Sera were processed for verification of various clinical parameters. Pools of sera from healthy and sepsis patients were tested. The sera, before being processed for evaluation of clinical parameters, were respectively fed into SET-M33-containing (M33) and peptide-free (CTR) device circuits. The pools of sera were in recirculation at room temperature for 2 hours and then samples were taken and analyzed. In addition, sera were analyzed before being put into the circuit (INPUT), as a control.

These analyses were performed in collaboration with Clinical Pathology Laboratory "Azienda Ospedaliera Universitaria Senese, Policlinico le Scotte" Siena, Italy.
Percentage of serum proteins present in the samples in g/L was analyzed by capillary electrophoresis.
In addition, other parameters were measured such as: procalcitonin, total proteins, albumin, IGG, IGA, IGM, C3, C4, transferrin, ferritin, creatinine, uric acid, urea, glucose, total bilirubin, and direct bilirubin.

LPS REMOVAL FROM MURINE SERUM (*EX VIVO* TEST)

CD1 female mice (Envigo) were used for the experiment. Preliminary experiments were executed to determine the best amount to simulate a real sepsis condition. Among 15 mg/Kg, 5 mg/kg, 1 mg/kg, 0.1 mg/kg, 0.05 mg/kg, and 0.01 mg/kg the best concentration for the purpose resulted 0.05mg/kg.

CD1 mice were injected (intraperitoneal injection) with LPS from *E. coli* (O111:B4 serotype) (Sigma-Aldrich). Blood was withdrawn after 4 hours, when animals began to show the first manifest signs of sepsis, and processed in the medical device for LPS removal.

The serotype of LPS used for the experiments was the same of the quantification kit.

Health conditions were monitored hourly. Detailed observation regarding clinical signs were made during dosing. Blood samples were drawn from the retro-orbital plexus of all animals under light isoflurane anesthesia. At the end of the experiment, the animals were sacrificed via CO2.

The samples were collected early in the working day and processed in the same day. Blood was collected into tubes without EDTA-K3 or other anticoagulant to obtain serum.

The serum was extracted by centrifugation at 3000 RPM for 10 minutes from the whole blood. The circuit was prepared in which the serum was filtered with a device containing 2ml of agarose beads functionalized with 2 mg of peptide SET-M33. The serum was recirculated for 2 hours at a flow of 1 ml/min. After the passage in the circuit, the serum was analyzed by the LAL test assay (Chromogenic Endotoxin Quant Kit) following the instructions of the kit, for the quantification of endotoxin.

All procedures involving the use of animals have been approved by the Italian Ministry of Health, January 14, 2016, Protocol 34/2016-PR.

NEW DEVICE WITH A COMMERCIAL BIOCOMPATIBLE MATRIX

The collaboration of Setlance srl and Medica Spa produced a new medical device using Poliethersulfone (PES), a commercial biocompatible matrix, conjugated with SET-M33. New device is currently under development exploiting the same antimicrobial peptide, but without the cysteine residue.

- **SET-M33 IMMOBILIZATION IN DEVICE COLOUMN ON BIOCOMPATIBLE MATRIX**

Conjugation of the peptide to PES was carried out by beta irradiation. The irradiation doses applied are in the range of 30 to 300 kGy. Irradiation was performed in a nitrogen (N2) atmosphere with O2 amount < to 10 ppm, using an electron accelerator. The voltage and current were set to 160kV and 10 mA, respectively. The modified membrane was then washed with water for 1.5 h and then dried for 30 min (Schulze et al., 2012).

Irradiation, in addition to conjugation of the peptide with the resin, allows removal of all possible contaminants and spores.

- **SET-M33 RELEASE TEST**

After irradiation of the device, a release test was performed to verify the successful immobilization. Two cartridges were used for the experiment, one with SET-M33 and one without as control (CTR). The solution containing the peptide used for immobilization was removed from the cartridge, 3 washes were performed with 2-4 mL of saline solution and finally a recirculation was performed with 8 mL of saline solution for two hours using a peristaltic pump.

The five samples collected have been used to evaluate the peptide immobilization on the matrix trough high performance liquid chromatography (HPLC) and Matrix Assisted Laser Desorption/Ionization (MALDI-TOF).

- **REMOVAL OF BACTERIA FROM SALINE SOLUTION**

To test the ability to remove bacteria from a solution, *E. coli 0111:B4* was used for the experiment.

Bacteria were incubated overnight at 37 degrees at 220 rotation per minute (RPM); then, they were diluted 1:100 in LB medium and incubated to reach optical density (OD) equal to 1 at 600 nm wavelength. After that, bacteria were diluted 1:20 and 1:25 in saline solution and inserted in recirculation system with a peristaltic pump for two hours. Saline solutions were collected to quantify bacteria by plating in LB agar plates.

- **BACTERIAL QUANTIFICATION**

Sample containing bacteria not incubated in cartridge (INPUT), sample containing bacteria after incubation in recirculation system with cartridge not containing SET-M33 (CTR) and sample containing bacteria after incubation in recirculation system with cartridge containing SET-M33 (M-33) were spread in LB agar plates. These solutions were serially diluted from not diluted to 10^6 and then 100 µL were plated on LB-agar plate and incubated overnight at 37°C. Bacterial colonies were counted after 14 hours. Data were processed using GraphPad Prism 6.

- **QUANTIFICATION OF LPS FROM SALINE SOLUTION**

To test the ability to remove LPS from a solution have been used LPS of *E. coli 0111: B4.*

A solution containing 6000 EU/ml of LPS was prepared. Two separate parallel circuits were set up using two different extracorporeal circulation machines, one with CTRL cartridge (0.8g PES without peptide) and one with M33 cartridge (0.8g PES functionalized with peptide 1mg/ml).

Saline solution was in the circuit for 120 minutes, peptide amounts were measured by LAL assay (Chromogenic Endotoxin Quant Kit) following the instructions of the kit, at different times (0, 15, 30, 60, 90, 120 minutes).

RESULTS

SET-M33-CYS SYNTHESIS, PURIFICATION AND CHARACTERIZATION

For the peptide conjugation with the biocompatible resin (agarose beads), it was necessary to modify SET-M33 by adding a free sulfhydryl group. This residue allows the formation of a disulfide bridge, building a very strong bond between the peptide and the agarose beads.

After synthesis the peptide was purified by reversed-phase HPLC (Jupiter C18 Phenomenex) and characterized by mass spectrometer Q-Exactive Plus (ThermoFisher).

The final purification produced an HPLC purity higher than 95%. the retention time of peptide was 21,32 minutes (Fig. 18A).

The MS profiles revealed a peak at molecular mass of 4960,2 Da, accordingly to the expected molecular mass ($C_{220}H_{421}N_{77}O_{49}S$ MW 4960.24 Da) (Fig. 18B).

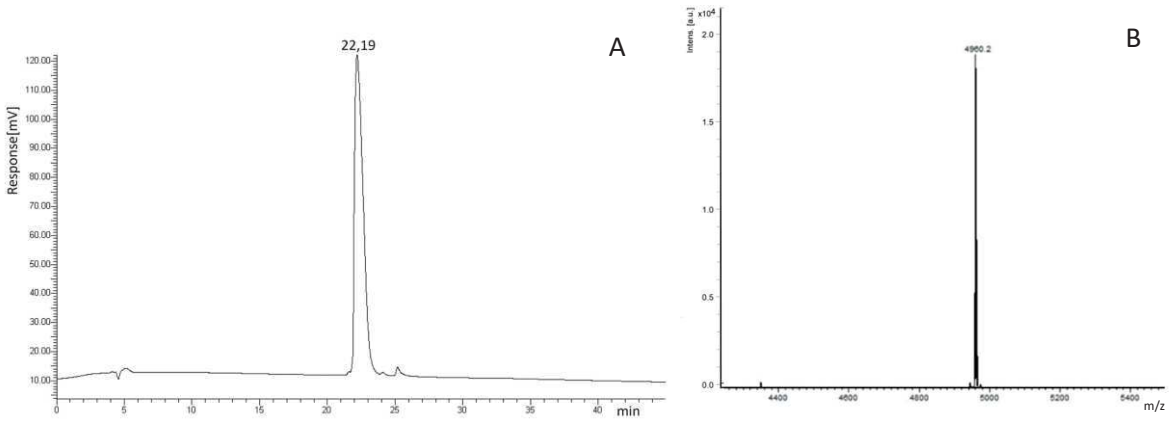

Figure 18. A. HPLC profile of SET-M33-Cys. The method was: Phenomenex analytical Column Jupiter C18 300Å 5µm 4.6 x 250mm, eluent A: 0.1%TFA / H2O, eluent B: Acetonitrile, gradient from 83:17 A/B to 70:30 A/B in 40min. B. MALDI-TOF mass spectrometry profiles of SET-M33Cys.

SET-M33-CYS CONIUGATION WITH AGAROSE BEADS

The SulfoLink® Coupling resin allows conjugation of the SET-M33-Cys peptide with covalent bond (Fig. 19). Peptide-conjugated agarose beads are contained within a cartridge (Fig. 20). Agarose beads have an iodoacetyl group with a 12-atom spacer (Fig. 17). The reaction of the iodoacetyl group incomes by nucleophilic substitution of iodine with a sulfur atom from a sulfhydryl group (-SH) of sidechain of cysteine in the peptide C-terminus, at 7.5-9.0 pH, resulting in a stable thioether linkage.

The binding efficiency was evaluated by HPLC test, analyzing the flow-through collected during the conjugation steps of the peptide with the agarose beads, the results (not reported) confirm the successful binding of the peptide to the beads.

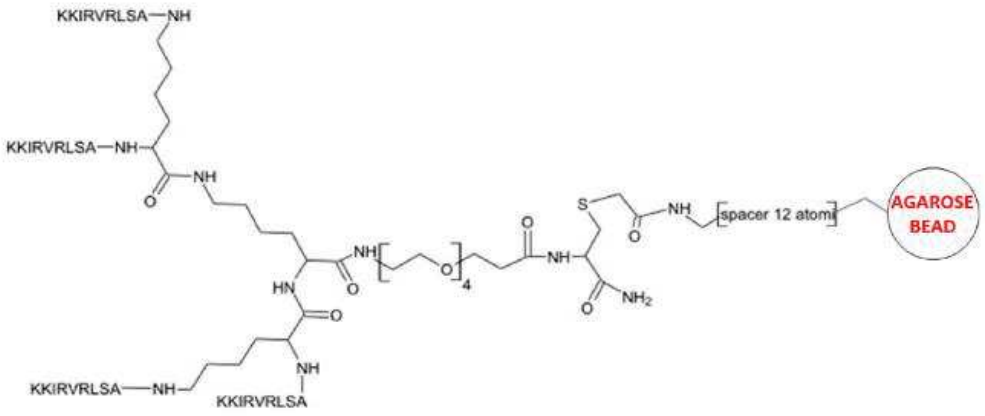

Figure 19. SET-M33-Cys peptide conjugated to agarose beads.

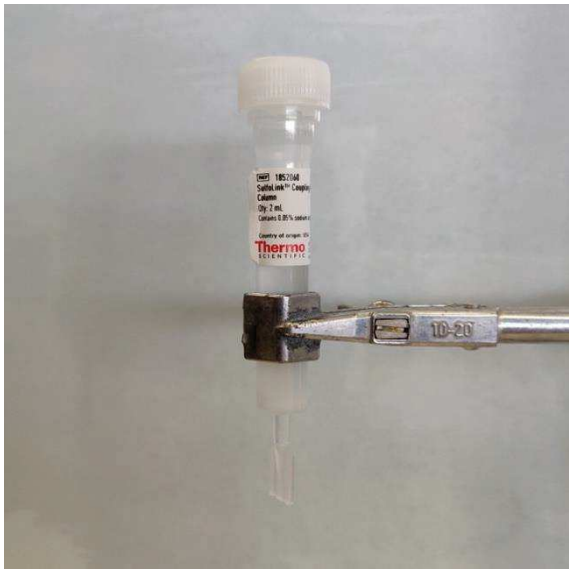

Figure 20. SulfoLink™ cartridge.

DEVICE WITH SET-M33-CYS AND STABILITY TEST

The device has shown an excellent ability to remove endotoxins and bacteria from biological fluids, but with a view to future use in the clinic it must be able to guarantee good efficacy even after long periods after production.

For this reason, the efficacy of the device was evaluated after 1 month from production, under different storage conditions. The efficacy of the devices was evaluated by testing the ability to remove bacteria from serum at time zero and after 30 days stored at 4° C.

The bacterial removal experiment was performed as described in Methods.

After two hours of incubation, serum containing *E. coli 0111:B4* bacteria was taken from the columns, diluted and plated (100 µl/plate) into LB agar plates. The plates were incubated at 37°C overnight.

This procedure has been carried out 2 times:

- At time zero (T0), using one column without peptide (CTR) and one with peptide SET-M33-Cys immobilized to resin (M33)
- After 30 days, using one column without peptide (CTR) and one with peptide SET-M33-Cys immobilized to resin (M33), in which the resin was stored at 4°C in the dark.

  The data show that the column stored at 4°C maintains the bacterial removal characteristics almost unchanged (Fig.21).

The reported percentages correspond to the removal of living bacteria from the serum.

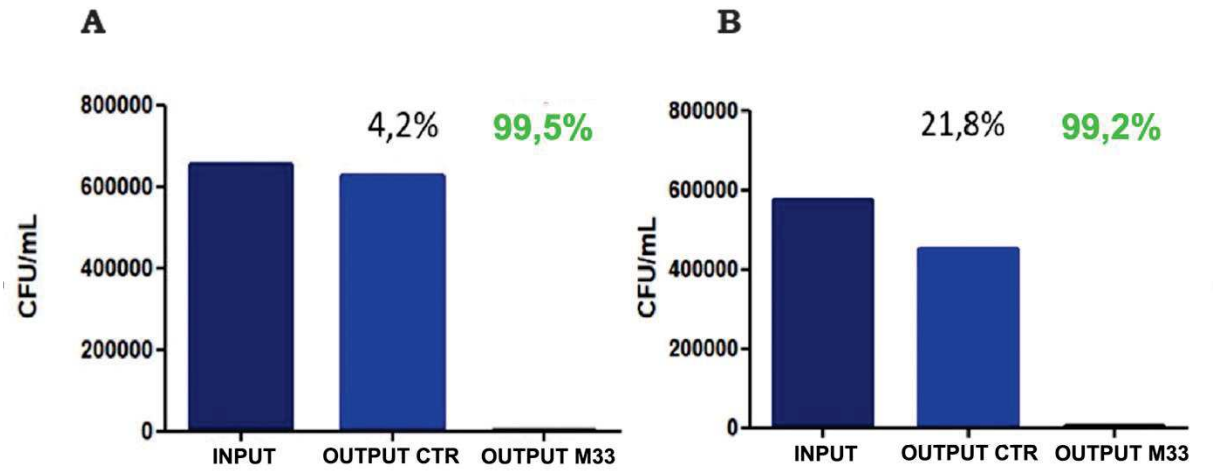

Figure 21. A. Graphical representation of bacterial removal in columns stored at time zero. B. Graphical representation of bacterial removal in columns stored 30 days at 4°C. The INPUT histogram corresponds to the serum containing the bacteria before passing in the column, the OUTPUT CTR represents the serum in the column without peptide and OUTPUT M33 represents the serum in column with peptide. The standard deviation was not reported because the experiment was performed only once.

LPS REMOVAL FROM HUMAN SERUM

5mL column, containing 2 mL of resin (agarose beads) conjugated with 2 mg of SET-M33-Cys peptide, was used. 2 mL of human serum containing LPS from *E. coli* O111:B4 (Sigma, St. Louis, MO) (75 Eu/mL) were incubated with the resin into the cartridge for 2 hours at room temperature under constant rocking. Then the sample was collected and measured for the LPS amount by the LAL test.

As a negative control, the same amount of resin not loaded SET-M33-Cys was incubated with the same amount of serum and its LPS content determined as above. In another control, the LPS content of an untreated serum aliquot was also measured (Fig. 22).

The serum used came from healthy patients without any treatment.

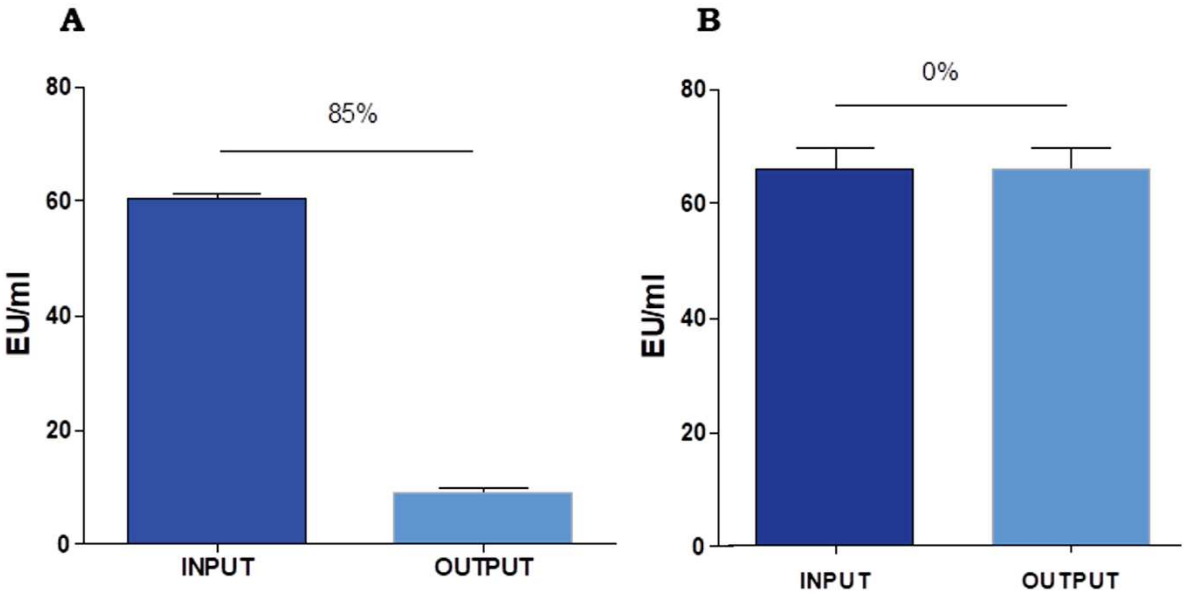

Figure 22. E. *coli* LPS removal from human serum. A The sample was flown through the cartridge containing the resin conjugated with SET-M33-Cys. B The sample was flown through the cartridge containing the resin without SET-M33-Cys. Y axis indicates the amount of endotoxin per ml. INPUT histograms are related to the serum containing LPS before the passage through the cartridge. OUTPUT histograms are related to sera after the passage through the cartridge.

LTA REMOVAL FROM HUMAN SERUM

In this experiment 2ml of SET-M33-Cys-loaded resin were used in its 5mL column, 2 mL of PBS containing LTA from *S. aureus* (MyBiosourc) (500 pg/mL) were incubated with the resin into the cartridge for 2 hours at room temperature under constant rocking. Then the sample was collected and measured for the LTA amount by the Human LTA ELISA Kit as described.
As a negative control, the same amount of resin not loaded with SET-M33-Cys was incubated with the same amount of PBS and its LTA content determined as above. As further control, the LTA content of an untreated PBS aliquot was also measured (Fig. 23). The values were calculated using Graph Pad Prism software.
Thee loaded resin can remove 83% of LTA from *S. aureus* in human serum diluted 1:10 in physiologic salt solution, whereas the unloaded resin removes just 8% of LTA.

The serum used came from healthy patients and has not had any treatment.

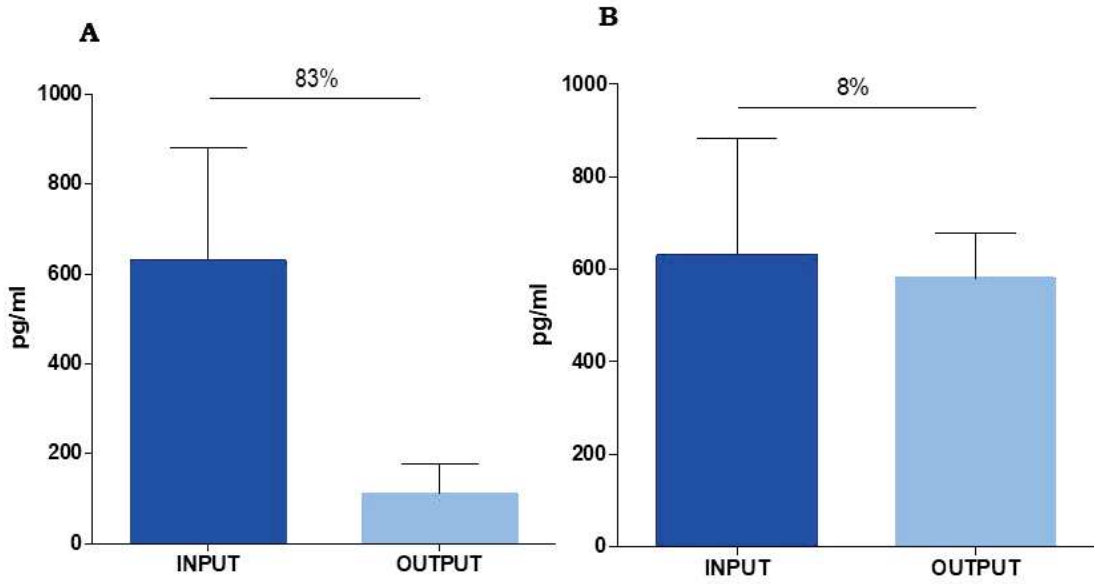

Figure 23. S. *aureus* LTA removal from human serum diluted 1:10 in physiologic salt solution. A The sample was flown through the cartridge containing the resin conjugated with SET-M33-Cys. B The sample was flown through the cartridge containing the resin without SET-M33-CYS. Y axis indicates the amount of endotoxin per ml. INPUT histograms are related to the serum containing LPS before the passage through the cartridge. OUTPUT histograms are related to sera after the passage through the cartridge.

LPS REMOVAL FROM *EX VIVO* MURINE SERUM

In order to evaluate the removal capacity even in biological matrices deriving from living beings, we set up an *ex vivo* experiment where serum of mice challenged with LPS, was processed in the SET-M33 device.

Preliminary experiments were carried out for the identification of the right amount of LPS to be used. Mice inoculated with LPS at different concentrations were monitored for one day and LAL test was performed on the collected sera.

Mice inoculated with 15 mg/kg LPS showed after about 2 hours severe signs of malaise such as reduced activities, difficulty in movements, reduced locomotion, bristly fur, and abnormal posture with arched trunk and lowered head. The condition of the animals deteriorated rapidly, and about 20 hours after inoculation they died. Serum at the concentration of 15 mg/kg analyzed by the LAL test gave a high optical density value, above the highest point of the standard curve (1500 OD, 1 EU/mL), making quantification in EU/mL impossible.

Mice inoculated with LPS at the concentrations of 5 mg/kg and 1 mg/kg about 2 h after inoculation showed moderate reduction in movement, bristly fur and abnormal posture. After 24 hours after inoculation, the animals were still alive, and at the end of the experiment they were sacrificed. Again, it was not possible to quantify the LPS content within the serum at either the 5 mg/kg or 1 mg/kg concentration because the optical density values obtained exceeded the highest point of the standard curve.

In mice inoculated with LPS at the concentrations of 0.1 mg/kg, 0.05 mg/kg and 0.01 mg/kg, a slight slowing in movements and shaggy fur were evident. After 24 hours after inoculation, the animals were still alive, and at the end of the experiment they were sacrificed. The optical density values obtained from the LAL test performed on the sera at all three concentrations were within the range of the standard curve. This allowed us to quantify the serum LPS content of approximately 50 EU/mL

Serum obtained from mice inoculated with LPS at a concentration of 0.05 mg/kg was incubated in SulfoLink column containing SET-M33-Cys for 2 h in rotation at room temperature, 200 μL of unfiltered serum was kept aside. To measure the amount of LPS retained by the device, an LAL assay was performed on the serum before and after filtration. Once the EU/mL concentration was calculated, the graph showing an 81% reduction in LPS was constructed (Fig. 24).

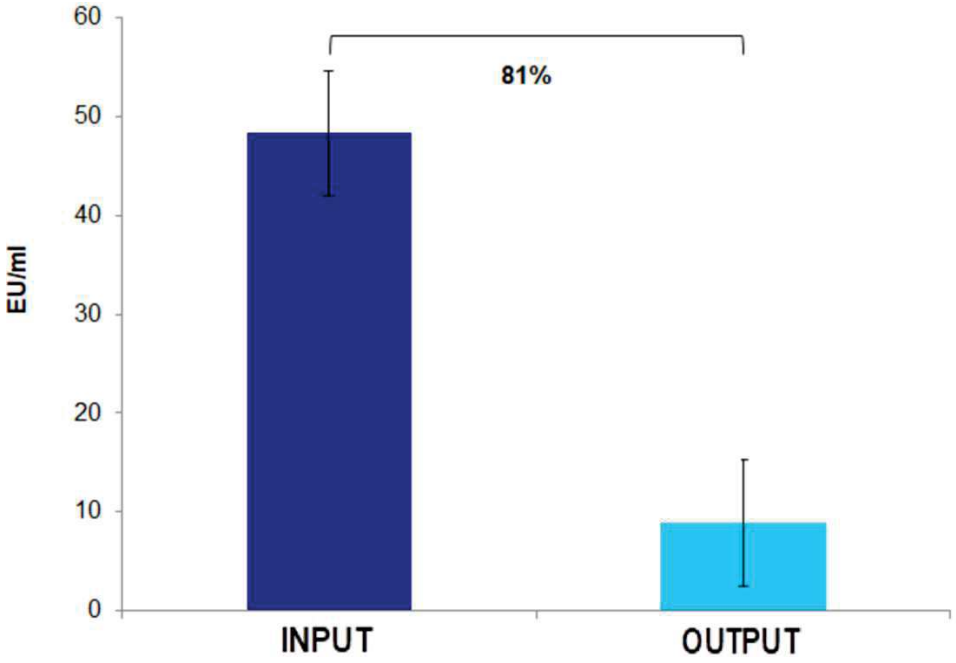

Figure 24. Reduction of LPS content within serum at the concentration 0.05 mg/kg. An 81% reduction is shown between unfiltered serum (INPUT) containing 48.3 EU/mL of LPS and serum collected after filtration (OUTPUT) containing 8.9 EU/mL of LPS.

REMOVAL OF LIVE GRAM-NEGATIVE AND GRAM-POSITIVE BACTERIA FROM HUMAN SERUM

Bacterial removal tests were performed with devices containing peptide-functionalized agarose beads (M33), non-functionalized agarose beads (CTR) and samples before being loaded into the device (INPUT). The strains used in the experiments were *E. coli ATCC 37780* (0111: B4) and *S. aureus* USA300.

Serum samples with *E. coli ATCC 37780* and *S. aureus USA 300* bacteria were diluted, then plated (100µl/plate) in LB agar and MHA plates respectively, and the plates were incubated overnight at 37°C.

The data obtained showed that the column with the immobilized peptide can significantly decrease the bacterial amount in serum of both Gram-negative and Gram-positive (Fig. 25 and 26).
In the serum incubated in the column with SET-M33-Cys peptide (M33), significantly lower numbers of bacteria were found compared with both the control serum (CTR) and the serum not incubated in the column (INPUT). 99% reduction in the number of *E. coli* and *S. aureus* was observed. Retention of bacteria in the serum incubated in the peptide-free column was also observed; this may be attributable to the fact that during the resin sedimentation step, some of the bacteria become entangled in the resin. In the case of *S. aureus* an increase of colonies in the CTR device is evident, this can be due to the growth of bacteria in absence of the antimicrobial peptide (Fig 26).

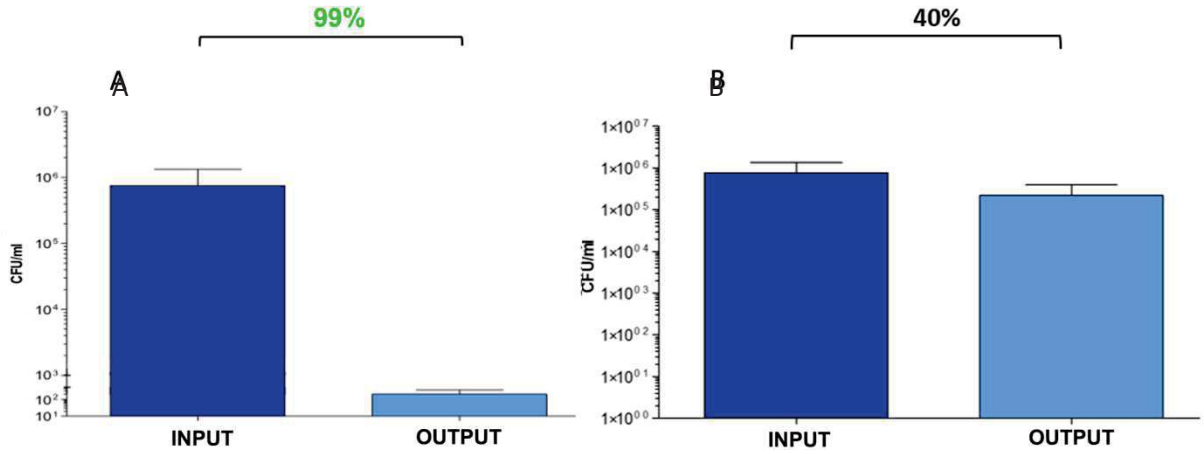

Figure 25. *E. coli* (O111:B4 serotype) removal from Human serum. The sample was flown through the cartridge containing the resin conjugated with SET-M33. Image A refers to the devices with M33, image B refers to the device without M33. The INPUT histograms correspond to the serum containing bacteria before the passage through the cartridge. The OUTPUT histograms correspond to sera after the passage through the cartridge.

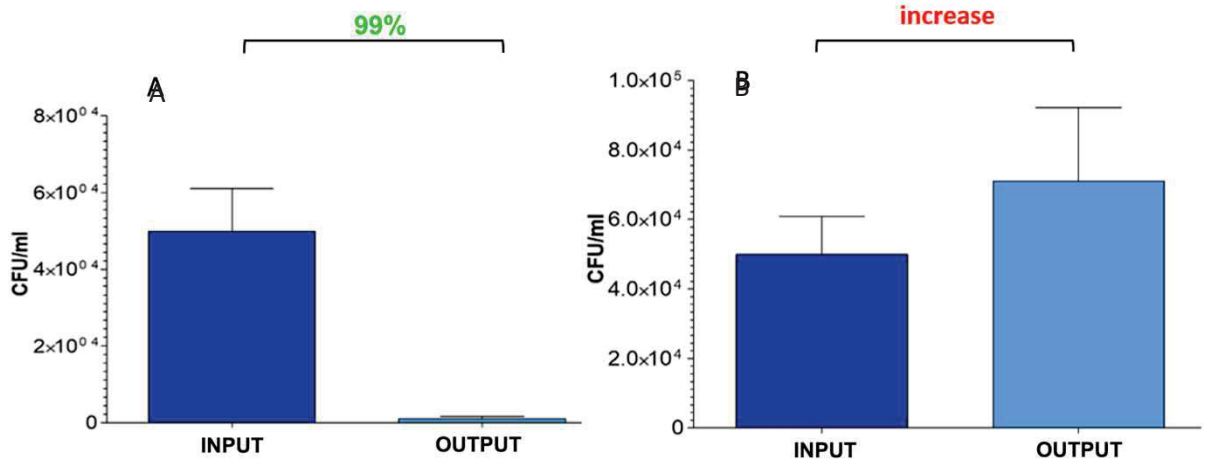

Figure 26. *S. aureus* (USA300) removal from human serum. The sample was flown through the cartridge containing the resin conjugated with SET-M33. Image A refers to the devices with M33, image B refers to the device without M33. The INPUT histograms correspond to the serum containing bacteria before the passage through the cartridge. The OUTPUT histograms correspond to sera after the passage through the cartridge.

BIOCOMPATIBILITY TEST (CAPILLARY ELECTROPHORESIS AND MEASUREMENT OF HEMATOCHEMICAL PARAMETERS)

In collaboration with the Clinical Pathology Laboratory, Azienda Ospedaliera Universitaria Senese, Policlinico le Scotte, Siena, Italy, studies on the alteration of clinical parameters and capillary electrophoresis analysis were performed.

Human serum, from pools of healthy and sepsis patients, before and after column passage with peptide (M33) and without peptide (CTR) was analysed with clinical device Capillarys. Fig. 27 describes the electrophoresis profiles of human serum before and after passage onto the SET-M33-Cys loaded resin. Serum protein content is not modified (Tab.1 and 2).

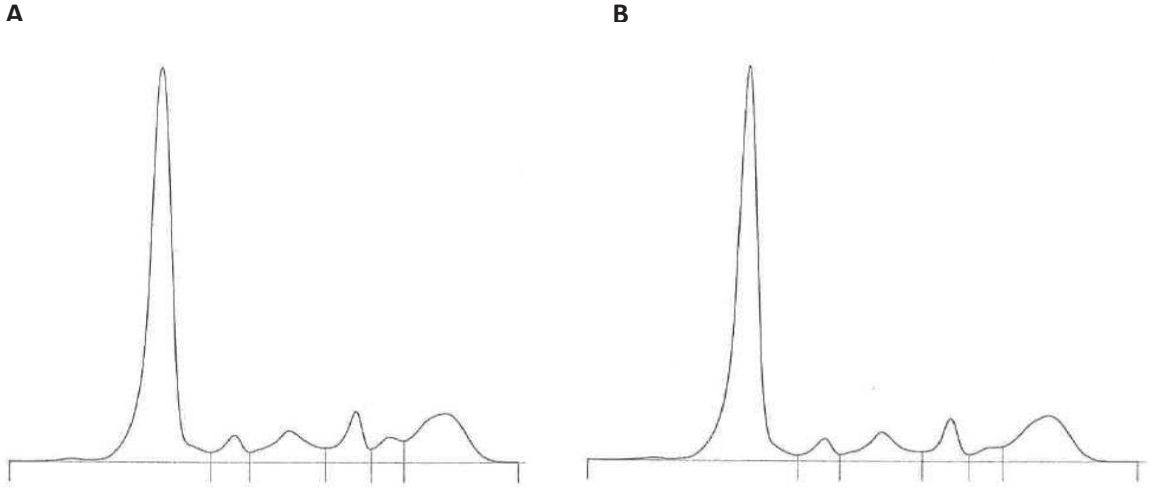

Figure 27. Protein electrophoresis profiles. A, serum sample pooled by healthy patients before its passage through the resin conjugated with SET-M33-Cys. B, Same serum sample after its passage through the resin conjugated with SET-M33-Cys.

Table 1. Percentage comparison of blood serum proteins electrophoresis of sera pools from sepsis patients before and after passage through CTR column and M33 column.

BLOOD SERUM PROTEINS ELECTROPHORESIS	REFERENCE VALUES	SERUM OF PATIENTS IN SEPSIS	SERUM OF PATIENTS IN SEPSIS CTR CARTRIDGE	SERUM OF PATIENTS IN SEPSIS M33 CARTRIDGE
ALBUMIN	53-66%	51,7	52,6	54,8
ALFA-1	1.9-4.5%	8,2	7,4	7,9
ALFA-2	6.5-3%	13,4	13,9	11,5
BETA-1	4-6%	4,9	4,9	5,7
BETA-2	1-3%	6,2	5,3	3,7
GAMMA	10.5-21%	15,6	15,9	16,4

Table 2. Percentage comparison of blood serum proteins electrophoresis of sera pools from healthy patients before and after passage through CTR column and M33 column.

BLOOD SERUM PROTEINS ELECTROPHORESIS	REFERENCE VALUES	SERUM OF HEALTHY PATIENTS	SERUM OF HEALTHY PATIENTS CTR CARTRIDGE	SERUM OF HEALTHY PATIENTS M33 CARTRIDGE
ALBUMIN	53-66%	61,7	60,2	63,5
ALFA-1	1.9-4.5%	4	3,8	3,6
ALFA-2	6.5-3%	9,8	10,6	9,3
BETA-1	4-6%	6,3	6,3	5,9
BETA-2	1-3%	4,4	3,8	3
GAMMA	10.5-21%	13,8	15,3	14,7

The parameters that were examined are both generic and specific for sepsis.

Important clinical markers of inflammation are C-reactive protein (CRP) and procalcitonin (PCT). Besides being markers of inflammation, they are molecules also involved in the inflammatory pathways that determine the pathogenesis of sepsis. CRP, under normal conditions binds to the membranes of bacteria opsonizing them, attracting complement and thus leading to cell lysis, in this case, an over expression due to the inflammatory state results in damage not only to bacterial cells but also to tissues. An increase in PCT helps distinguish bacterial from nonspecific infections; high PCT levels often coincide with the sepsis condition.

PCT and CPR resulted decreased in serum form sepsis patients passed through the SET-M33 cartridge, while the reduction was not observed in the same serum passed in the control column (Tab. 3). This is very promising even if further studies must be carried out to explain the reason of this reduction. It cannot be excluded that the stickiness of the charged peptide contributes to

the direct removal of these biologic factors. Serum by a pool of healthy individuals did not show any significant variation (Tab. 4).

Table 3. Comparison of blood serum proteins electrophoresis of sera pools from sepsis patients before and after passage through CTR column and M33 column.

CLINICAL DATA	REFERENCE VALUES	SERUM OF PATIENTS IN SEPSIS	SERUM OF PATIENTS IN SEPSIS CTR CARTRIDGE	SERUM OF PATIENTS IN SEPSIS M33 CARTRIDGE
PCR	< 0,50 mg/dl	10,18	8,53	0,41
Procalcitonin	< 0,05 ng/mL	8,21	5,67	4,47
Total Protein	6,4 -8,3 g/dL	5,2	4,3	3,4
Albumin	3.5 e 5.5 g/dl	3	2,4	1,9
IGG	800-1800 mg/dl	833	698	597
IGA	90-400 mg/dl	304	278	199
IGM	60-280 mg/dl	73	81	45
C3	M: 80-150 mg/dl F: 80-190 mg/dl	87	82	14
C4	M: 15 - 53 mg/dl F: 15 - 57 mg/dl	19	17	1
Transferrin	240 a 360 mg/dL	129	109	97
Ferritin	M: 20-200 ng/mL F: 20-120 ng/mL	624	565	469
Creatinine	0,6 a 1,3 mg/dl	2,47	1,79	1,53
Uric Acid	4 a 8 mg/dl	5,8	3,9	3,4
Urea	F: 15-42 mg/dl M: 18-45 mg/dl	75	56	50
Glucose	70 e 99 mg/dl	119	91	80
Total Bilirubin	0,2-1,1 mg/dL	1,2	0,9	0,6
Direct Bilirubin	0,3 mg/dL	inf 0,15	Inf 0,15	Inf 0,15

Table 4. Comparison of blood serum proteins electrophoresis of sera pools from healthy patients before and after passage through CTR column and M33 column.

CLINICAL DATA	REFERENCE VALUES	SERUM OF PATIENTS IN SEPSIS	SERUM OF HEALTHY PATIENTS CTR CARTRIDGE	SERUM OF HEALTHY PATIENTS M33 CARTRIDGE
PCR	< 0,50 mg/dl	0,09	0,06	<0,03
Procalcitonin	< 0,05 ng/mL	0,02	0,02	0,02
Total Protein	6,4 -8,3 g/dL	7	6,9	5,8
Albumin	3.5 e 5.5 g/dl	4,7	4,6	3,9
IGG	800-1800 mg/dl	1028	1113	951
IGA	90-400 mg/dl	180	202	147
IGM	60-280 mg/dl	101	121	72
C3	M: 80-150 mg/dl F: 80-190 mg/dl	110	122	15
C4	M: 15 - 53 mg/dl F: 15 - 57 mg/dl	23	24	1
Transferrin	240 a 360 mg/dL	277	292	261
Ferritin	M: 20-200 ng/mL F: 20-120 ng/mL	51	55	43
Creatinine	0,6 a 1,3 mg/dl	0,9	0,78	0,63
Uric Acid	4 a 8 mg/dl	4,5	3,6	3,1

Urea	F: 15-42 mg/dl M: 18-45 mg/dl	29	27	24
Glucose	70 e 99 mg/dl	72	66	60
Total Bilirubin	0,2-1,1 mg/dL	0,4	0,4	0,3
Direct Bilirubin	0,3 mg/dL	inf 0,15	inf 0,15	inf 0,15

LPS AND BACTERIA REMOVAL USING A DIFFERENT MATRIX

Following the results obtained with the device containing agarose beads functionalized with the antimicrobial peptide SET-M33-cys, a new device is currently being studied that exploits the same antimicrobial peptide, but without the cysteine residue.

This new project is the result of Setlance S.R.L.'s collaboration with Medica S.P.A.

The new device consists of a cartridge containing a new biocompatible material that would allow excellent conjugation with the antimicrobial peptide SET-M33 and have a 'large contact surface area for conjugation (Fig. 28). Polyethersulfone (PES) hollow capillary fiber polymer membrane was chosen for these reasons.

The short PES fibers are inert and do not alter the blood profile during passage in extracorporeal hemoperfusion. PES is a very stable material with little interaction, so it is very complex to conjugate it with other molecules.

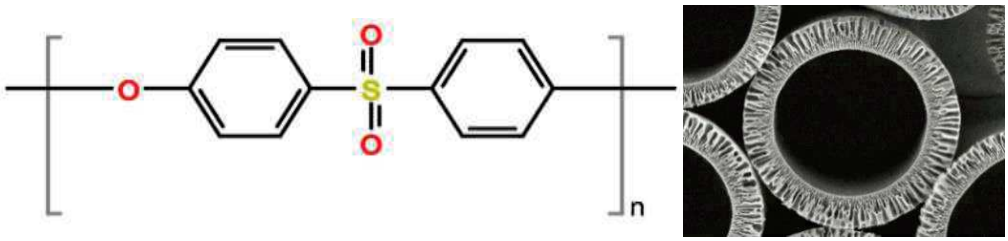

Figure 28. Chemical structure of polyetersulfone and image of the biocompatible matrix by scanning electron microscope.

The peptide SET-M33 in this case was used without any specific linker for conjugation with the biocompatible resin.

Conjugation of peptide to PES does not allow precise orientation of the peptide on the biocompatible matrix (Fig. 29), as the binding method is not directional. both peptide and PES are stable molecules. High-energy electron beams

destabilize both the PES and the peptide, leading to the formation of free radicals that interact and allow the conjugation of the peptide with the biocompatible matrix.

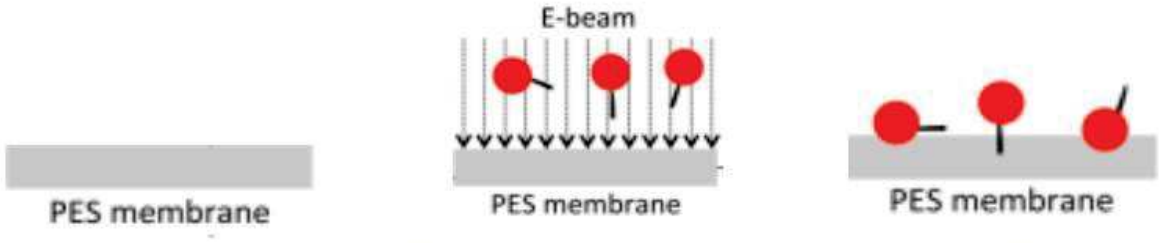

Figure 29. Conjugation of peptide with PES by electron beam ionization.

Previously, several studies have demonstrated the possibility of binding on PES flat membranes of antimicrobial peptides (Reinhardt et al., 2014) (Oliveira et al., 2008) by a nonspecific immobilization mechanism known as beta-irradiation. It consists of irradiation with an electron beam, without the use of catalysts, photo initiators or other toxic reagents (Schulze et al., 2010).

- **ANTIMICROBIAL PEPTIDE SET-M33 SYNTHESIS AND PURIFICATION**

  SET-M33 used in new devices has been synthetized and using a multiple automatic synthesizer (Fig. 30).

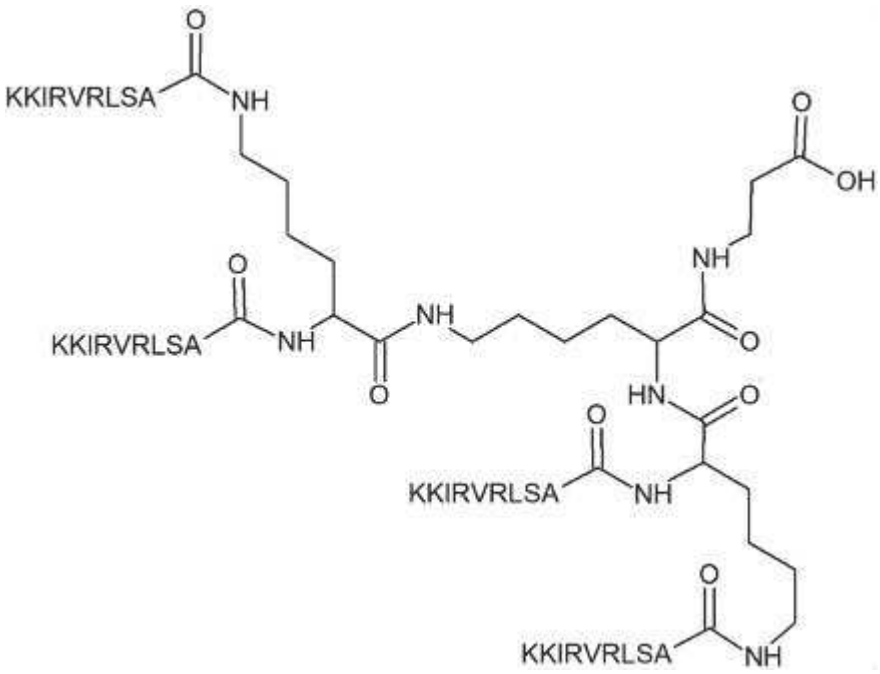

Figure *30*. SET-M33 structure representation.

The peptide after synthesis was purified by reversed phase HPLC (Fig. 31A). obtaining a purity of 95%.

Moreover, the peptide has been characterized by mass spectrometry (Fig. 31B). The peak on the graph correspond to molecular weight of the peptide.

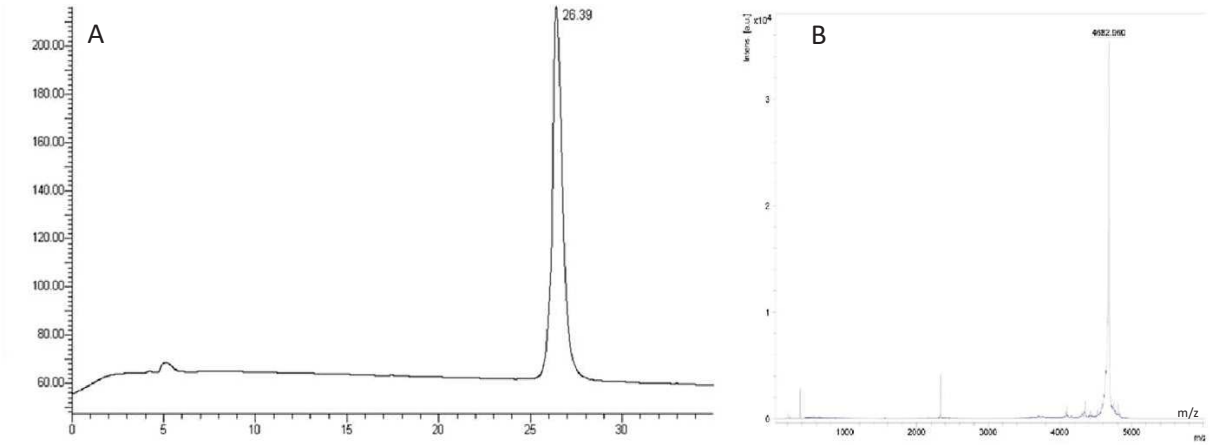

Figure 31. A. Graphical representation of HPLC purification of SET-M33 peptide. The peak corresponds to retention time. B. Graphical representation of the results obtained by mass spectrometry. The peak indicates the mass of the peptide.

- **CONJUGATION OF ANTIMICROBIAL PEPTIDE SET-M33 ON BIOCAMPATIBLE MATRIX AND RELEASE TEST**

In preliminary tests, different concentrations of peptide inside the cartridge have been used, (0.5 mg/mL, 1 mg/mL and 2 mg/mL). Moreover, cartridges containing different quantity of matrix have been tested (0.8 g, 0.6 g, 0.4 g). Combinations of amounts of resin and peptide were used to identify which was the best one for ideal removal. 1 mg/mL peptide and 0.8 g PES resulted the best one and was used for the experiment.

Cartridges with 2 mg/mL were not beneficial in terms of removal, and cartridges with 0.5 mg/mL showed a very low bacterial retention. Graphs of these tests were not reported in this thesis. The cartridge with 0.8 g PES showed the best results in terms of bacterial removal and flow.

After these preliminary tests, the first characterization step on the 1 mg/ml peptide 0.8 g PES cartridge was the evaluation of the amount of peptide not immobilized during beta irradiation and remained free in the solution inside the cartridge.

HPLC tests were performed for the following evaluations:

- presence and rough quantification of free peptide not immobilized on PES matrix (Fig. 32A)
- profile of control solution without peptide (1C) (Fig. 32B)
- presence and rough quantification of free peptide not immobilized on the saline solution of the three subsequent washes in the cartridge with SET-M33 (not reported)
- presence of free peptide not immobilized on the saline solution after recirculation for two hours in cartridges with SET-M33 (5P) (Fig. 32C)
- profile of the saline solution after recirculation for two hours in cartridges without the peptide (5C) (Fig. 32D)

The results showed peptide release in the solution containing the peptide used for immobilization and some residue in the three washes, but not in the sample that circulated for 2 hours.

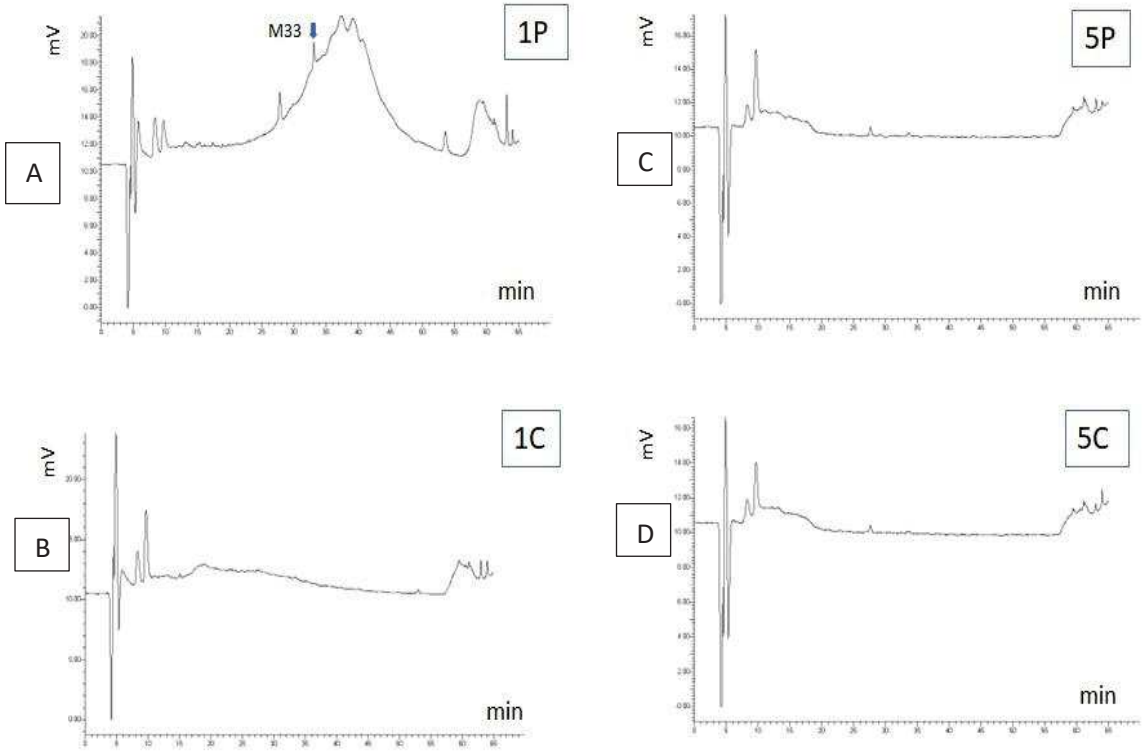

Figure *32.* HPLC chromatograms of saline solution in the cartridge containing SET-M33 (A) and in the control cartridge (B) and in the; saline solution after 2 hours recirculation in the cartridge containing SET-M33 (C) and in the control cartridge (D).

For a more accurate analysis the same samples were tested in mass spectrometry. The following samples were tested:

- the saline solution from the SET-M33 cartridge (1P) (Fig. 33A)
- the saline solution of the control cartridge without the peptide (1C) (Fig. 33B)
- the saline solutions used for the washing steps (graphs not shown)
- the saline solutions after 2 hours of recirculation in the SET-M33 cartridge (5P) (Fig.33C)
- the saline solutions after 2 hours of recirculation in the control cartridge without the peptide (5C) (Fig. 33D)

The results showed the presence of the peptide in the saline solution containing the peptide used for immobilization and in the saline wash

solutions (not reported). No traces of SET-M33 were found in the saline solution after 2 hours of recirculation.

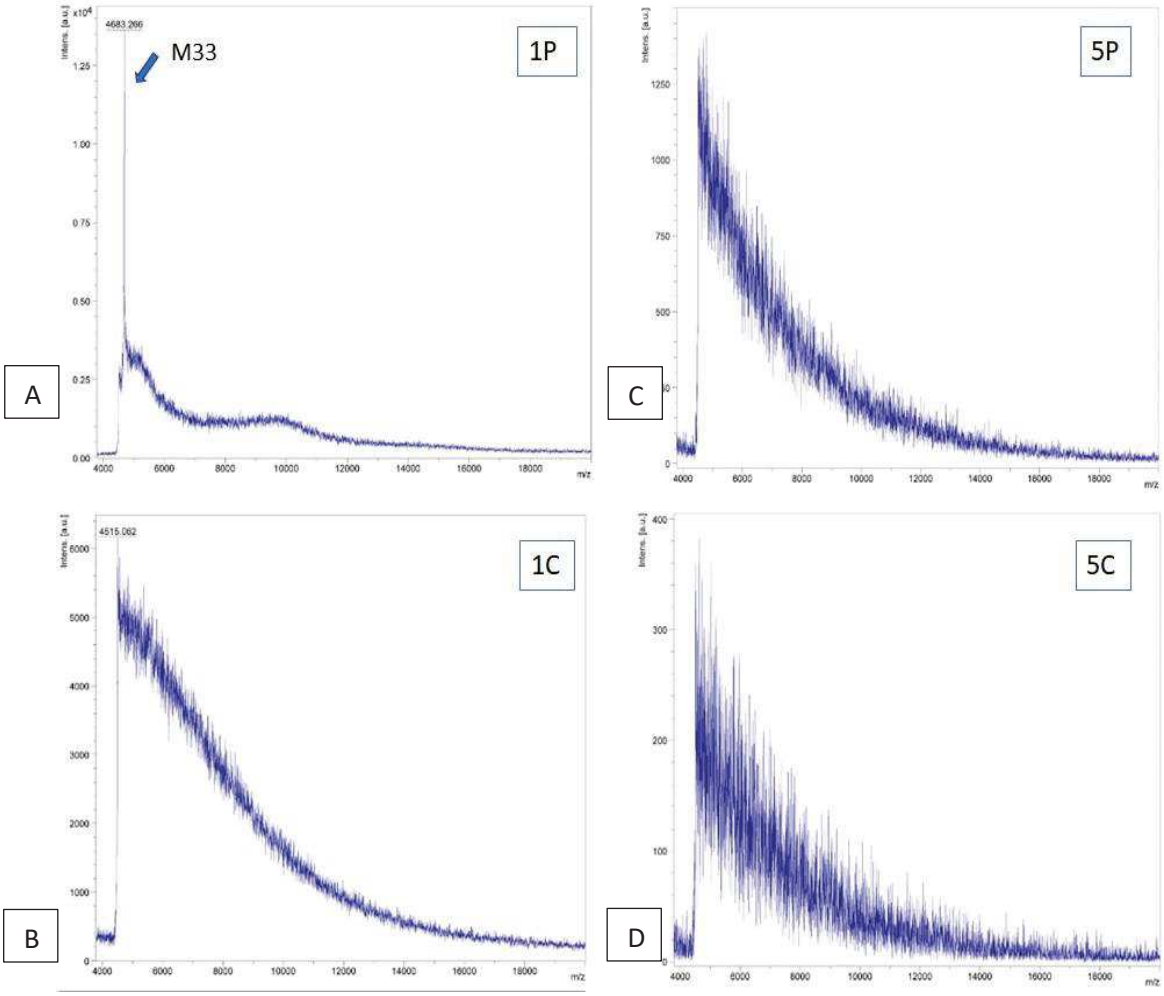

Figure 33. Mass spectrometry results of saline solution in cartridge containing SET-M33 (A) and in control cartridge (B); saline solution after 2 hours recirculation in cartridge containing SET-M33 (C) and in control cartridge (D).

The quantity of peptide not covalently bound to matrix and eliminated with washing steps has been quantified using the area of the peaks obtained by HPLC (Fig. 34).

Unbound peptide to the biocompatible matrix after conjugation corresponds to about 2 µG/ml, so the conjugation rate is greater than 99%.

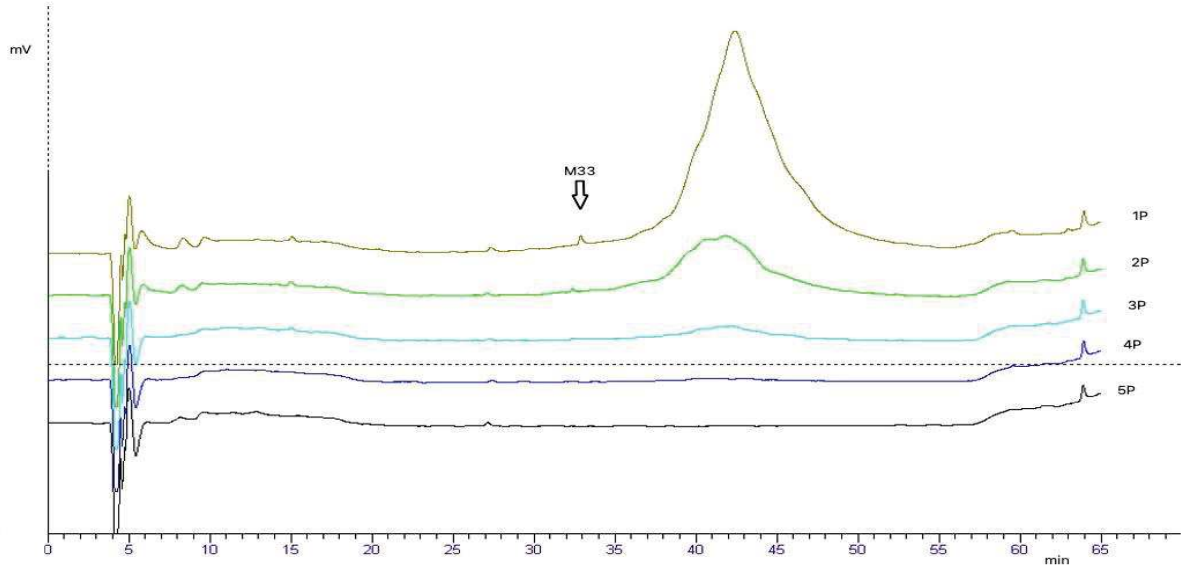

Figure *34.* Comparison of HPLC SET-M33 peaks obtained from saline solution (1P), saline solutions after first, second and third washing step (2P, 3P, 4P) and saline solution after 2 hours recirculation (5P).

- **QUANTIFICATION OF BACTERIAL REDUCTION USING PES MATRIX**

After preparing the saline solution containing the bacteria as described in methods, the circuit connected to the device without peptide (CTR) and the circuit connected to the device with peptide (M33) were set up.

After 2 hours of recirculation, saline solution containing bacteria is taken from the device and samples are diluted, plated in LB agar plates and incubated overnight at 37 degrees.

The result show 99,1% bacterial removal from saline solution after 2 hours recirculation in cartridge containing SET-M33 (OUTPUT M33). A strong nonspecific retention of bacteria was observed in the devices without peptide (OUTPUT CTR), evidently due to an interaction with the biocompatible matrix (Fig. 35). The standard deviation was not reported because the experiment was performed only once. The reasons for such marked nonspecific retention are not yet known. Studies on the nature of the matrix are currently underway to confirm a mechanical retention due to the dense network formed by the

polymer fragments. Regarding the removal rate, further experiments are planned to confirm the data.

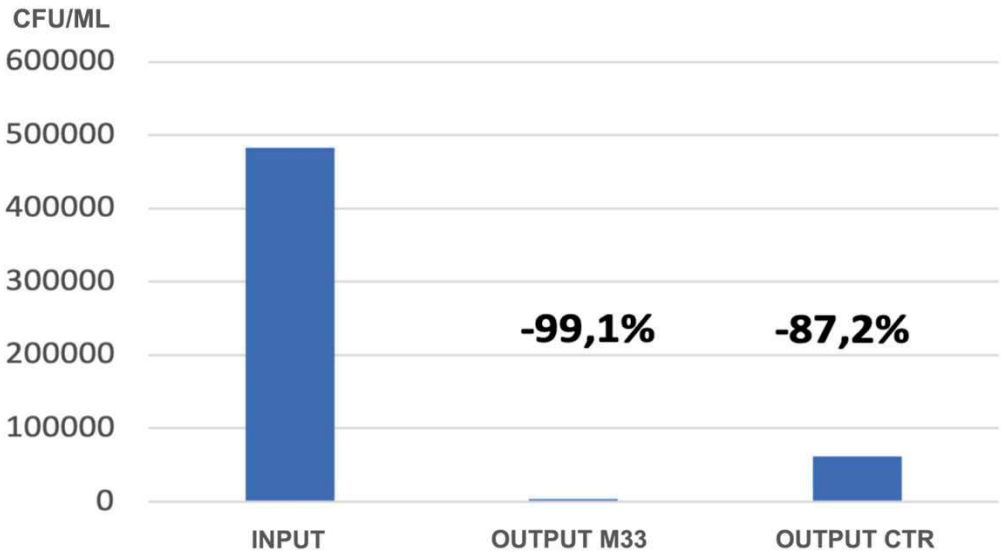

Figure *35.* Bacterial removal of SET-M33 linked to matrix inside cartridge. Saline solution containing bacteria before input into cartridge (INPUT), after 2 hours recirculation within cartridge containing SET-M33 (OUTPUT M33) and within cartridge without SET-M33 (OUTPUT CTR). The standard deviation was not reported because the experiment was performed only once.

- **QUANTIFICATION OF LPS REMOVAL**

The columns, containing 0.8g of PES conjugated with 1mg/ml of SET-M33 peptide, was used. Saline solution with LPS from *E. coli* O111:B4 (Sigma, St. Louis, MO) (6000 Eu/mL) were recirculated in 2 separate circuits for 120 minutes. A circuit (Fig.36) has been connected to the CTR device (0.8g of PES without peptide) and a circuit was connected to the device M33 (0.8g of PES conjugated with the peptide). The LPS concentration of the two circuits was monitored at different times. After 120 minutes, 99.9% LPS removal from the M33 device was obtained (Fig. 37).

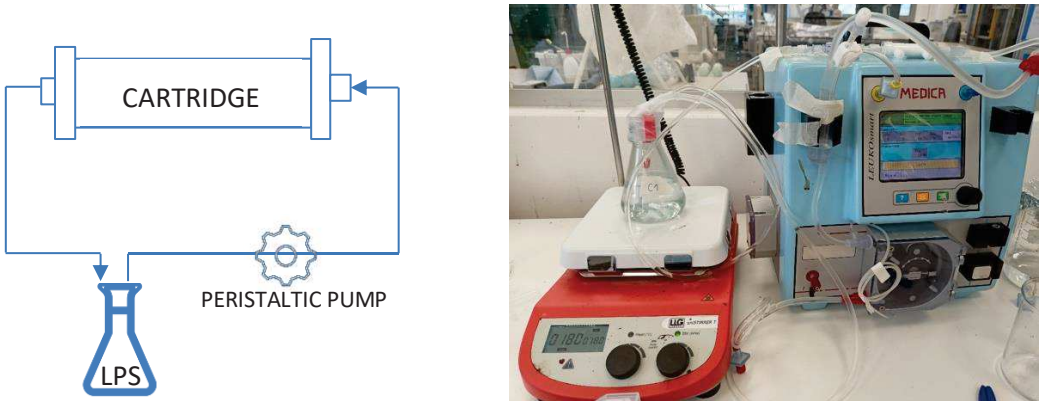

Figure 36. Schematic of the circuit used in the experiment.

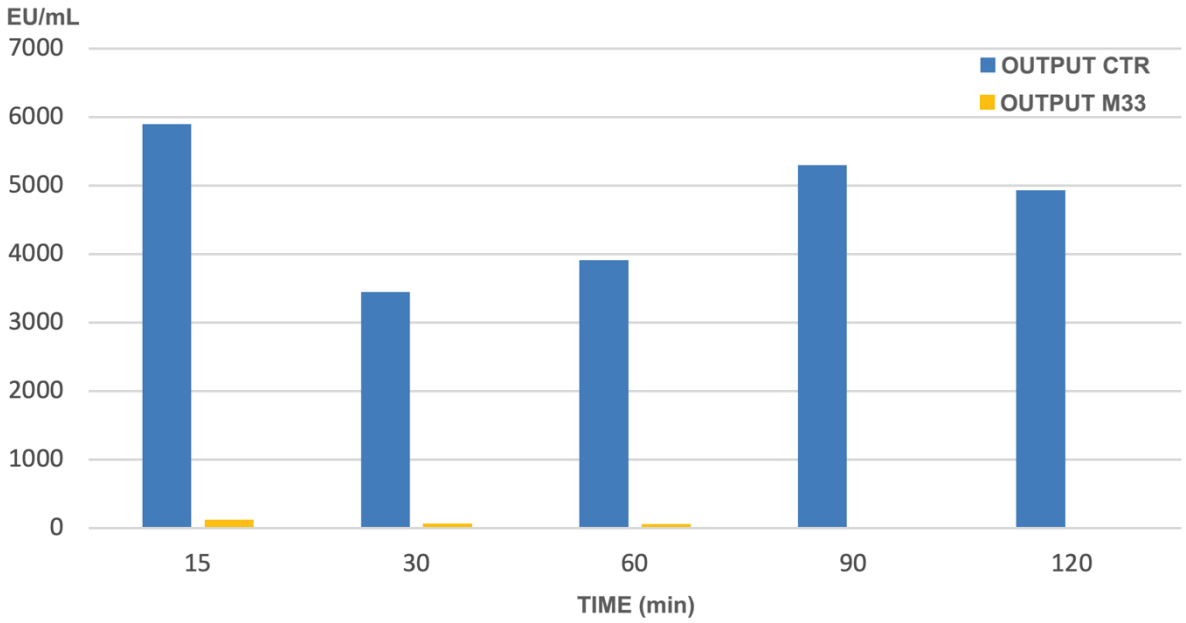

Figure 37. LPS removal from saline solution after 120 minutes. The histograms show the removal trend over time. The control cartridge histograms (OUTPUT CTR) show a fluctuating trend and minimal LPS reduction. The histograms of the cartridge with the peptide (OUTPUT M33) show an immediate and marked reduction of LPS (99.9%).

DISCUSSION

Bacterial infections have always been the cause of high mortality rates and morbidity. Sepsis is a systemic syndrome that can result in hypotension, organ failure and death. Sepsis has always been a subject of study because of its complexity and consequences for patients and the health care system. The discovery of antibiotic drugs has led to a great reduction in cases of sepsis and bacterial infections in general.

In recent years, an increase in infections caused by multidrug-resistant bacteria has been observed, these types of infections place humanity in a state of emergency.

Therefore, now with the presence of multi drug resistant bacteria, it is more necessary than ever to find alternative solutions to counter the problem.

In addition to the search for new antimicrobial drugs, research in recent years has turned its interest to the construction of medical devices with a wide and varied spectrum of action. These medical devices are "filters" with adsorptive capabilities used in extracorporeal hemoperfusion.

These devices are generally composed of a biocompatible synthetic matrix that forms a network capable of retaining various components (endotoxins, bacteria, cytokines) present in biological fluids.

However, these devices do not have the ability to retain different pathological components at the same time.

For retention of components present in serum, these medical devices use different strategies of action: different mesh size, charges of the biocompatible matrix, or presence of AMPs.

Many medical devices are currently still being studied for efficacy, while others are already being used in the clinic.

Devices currently used for the removal of bacteria, endotoxins and cytokines are: Oxiris, Toraymyxin, Cytosorb, Alteco and Seraph 100.

AMPs are molecules already partially used in clinical settings to support traditional antibiotic drugs, but antimicrobial peptides, like classical

antibiotic drugs, also have some problems (side toxicity, low biocompatibility, short half-life, etc.).

For these reasons, the use of AMPs in medical devices for extracorporeal hemoperfusion is very promising and some already experimented in the clinical practice, Polimixin B in the Toraymyxin device is an example.

AMPs have a positive net charge that allows them to selectively interact with anionic bacterial membranes and other negatively charged structures, such as LPS in Gram-negative bacteria or LTA in Gram-positive bacteria.

This binding to bacterial toxins allows bacterial lysis, but it also provides immunomodulatory activity as it sequesters endotoxins not allowing them to stimulate the immune system.

SET-M33 is an antimicrobial peptide obtained by phage display technology. This peptide has excellent antimicrobial characteristics, anti-inflammatory activity through selective neutralization of LPS, low hemolytic activity, lack of immunogenicity, and the ability to eradicate biofilms (Pini et al., 2008; Brunetti at al., 2016a; Brunetti at al., 2016b; Van der Weide et al., 2017).

For all these reasons, SET-M33 was selected as a good candidate for the construction of a medical device for the treatment of sepsis patients.

The use of the antimicrobial peptide SET-M33 conjugated to a resin (agarose beads) as a medical device is in the preliminary stage of development, with very promising results. Immobilization of SET-M33 to the resin demonstrated:

- the peptide's effectiveness in removing LPS from *E. coli* and LTA from *S. aureus*, showing a significant decrease in both bacterial toxins. SET-M33 was able to retain LPS and LTA in human serum, with a binding percentage of 85% and 83%, respectively.

- the reduction of LPS by 96% in *ex vivo* experiments.

- the retention of living bacteria (*E. coli* and *S. aureus*) from serum.

In addition, clinical analyses have been performed to evaluate the safety of the device. Protein electrophoresis of a serum sample has been performed before and after its passage through the resin. The serum profile was not altered by passage through the SET-M33-based device. In addition, the alteration of some clinical values was evaluated. The devices containing the peptide showed no alteration in the electrophoretic profile nor in the clinical parameters in the sera of healthy patients. The same experiment has been done with sera from sepsis patients. A decrease in clinical values stratigraphically related to stages of sepsis was observed.

Currently no statistical methods have been used for data analysis, but Student's t test (t test) and analysis of variance (ANOVA) will be the statistical methods used. Student's t test will be used to compare averages between two groups, while ANOVA will be used to compare averages between three or more groups.

The next step was to build a medical device that had the same retention capacity with a more sustainable industrial development. The new medical device, resulting from the collaboration between Setlance srl (the company which financed my PhD salary) and Medica Spa, uses the PES (biocompatible matrix) linked to the SET-M33 peptide by beta irradiation. The binding of the peptide to the matrix by beta irradiation made it possible to optimize production costs with a view to industrial development of the device.
The results obtained so far with the new device showed results that are completely comparable to the preliminary tests.
The last experiments carried out on devices with PES, are currently underway to verify the ability to remove LTA and Gram-positive bacteria.
The promising removal results allow to program a development of the device in terms of animal experimentation to be programmed in the pig to test the effectiveness of the device in extracorporeal circulation. If all goes according to plan, clinical trial and health ministry approval for commercialization are planned in a few years.

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