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**NOVEL GREEN STRATEGIES FOR PREPAREDNESS TO FUTURE
PANDEMIC DISEASES:**

**DEVELOPMENT OF A BIODEVICE FOR WATER REMEDIATION FROM
PATHOGENIC BACTERIA AND EVALUATION OF THE EFFECTIVENESS OF A NEW
SARS-COV-2 PLANT-BASED VIRUS-LIKE PARTICLE VACCINE**

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ABSTRACT

The global impact of the COVID-19 pandemic has underscored the urgent need for innovative and rapid strategies to enhance preparedness for future pandemics. The risk of the emergence of new infectious disease is extremely concrete and it is essential to invest in the development of vaccines and biotechnological drugs as well as to speedup of clinical trials.

Currently, one of the main focus is shifting to the global public health threat caused by the rise of antimicrobial-resistant (AMR) bacterial strains. Multidrug-resistant (MDR) pathogen infections are on increasing incidence and frequently lead to multiple events of sepsis. Therefore, it is necessary to discover both novel approaches and novel drugs active against these multidrug-resistant bacteria to prevent the spreading of sepsis. On the other hand, emerging viruses represent a real menace and it is essential to set-up and validate methods to monitor the spread of the diseases and speed up the release of vaccines and antiviral drugs.

This dissertation is divided in two main project:

The first project is focused on the development of a new SET-M33-based device 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 wastewater. The antimicrobial peptide SET-M33 has a remarkable capacity to both destroy and bind components of bacterial walls, including lipopolysaccharide (LPS) from Gram-negative bacteria and lipoteichoic acid (LTA) from Gram-positive bacteria. It is a cationic non-natural peptide synthesized in a tetra-branched form that makes it more resistant to degradation in biological fluids and water. Moreover, it shows a 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.

The device was realized by immobilization of the peptide on a biocompatible resin composed of agarose beads via a thiol residue at the C-terminal of the peptide. The peptide was synthesized with amino acids in L-configuration to facilitate the conjugation with the resin via the thiol residue at the C-terminal. Preliminary results showed that the peptide maintained its strong antimicrobial activity against both Gram positive and Gram Negative strains as well as bacterial molecules, released upon cell lysis.

A second part of the PhD was conducted inside Vismederi srl and was aimed to evaluate the effectiveness and immunogenicity of a SARS-CoV-2 plant-based vaccine manufactured through a *Nicotiana benthamiana* platform.

SARS-CoV-2 was directly responsible for the COVID-19 (Coronavirus Disease 2019) pandemic, an acute respiratory syndrome which led to significant health, social and economic consequences worldwide. Vaccination is the principal and single most cost-effective strategy of preventing COVID-19 and reduce illness and the severity of infection, especially in specific highrisk groups for complications.

In this context, microneutralization cytopathic effect-based (MN-CPE) assays were used to analyze the potency of this candidate vaccine to elicit a specific humoral immune response against five propagated variants of concern (VOCs) Alfa, Beta, Gamma, Delta and Omicron (BA.1) up to 21 days post-second immunization and up to 201 days after second immunization, in the context of a phase I clinical trial. This study was conducted in order to evaluate whether this novel COVID-19 vaccine was able to elicit a specific humoral immune response. Particularly, 540 human serum samples were analyzed revealing a strong humoral response against wt, Alpha and Delta variants, a moderate immunogenicity against Beta and Gamma and an important decrease against BA.1. Subsequently, serum neutralizing activity against the overmentioned five VOCs in hospitalized COVID-19 patients from the first pandemic wave was analyzed. Results were comparable to data obtained for the plant-based vaccine highlighting the occurrence of an antigenic drift event which required an periodic update of vaccine formulation. Moreover, this study highlights the importance of evaluating SARS-CoV-2 pre-existing immunity against emerging variants as a tool to foresee the immune escape and the extent of vaccine efficacy.

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Following the outbreak of the COVID-19 pandemic, focus is now shifting to another worldwide public health concern brought about by the increase in antimicrobial-resistant (AMR) bacterial strains. During the early 1900s, bacterial illnesses were the primary reason of mortality and morbidity. Later, in the 1940–1980 period, there was consistent development of new antibiotics, also called "miracle drugs," that increased lifespan and were celebrated by the public as major breakthroughs in microbiology. Starting from the mid-1980s, the pharmaceutical sector shifted its attention towards developing different types of drugs, leading to a significant decrease in the discovery of new antibiotics. As new anti-microbial drugs are no longer being discovered to counter the emergence of antibiotic resistance, bacteria have once again become a worldwide threat to modern healthcare, prompting worries among national and international public health organizations like the World Health Organization (WHO) (Baquero, 2021).

For this reason, during the last few years, other strategies such as the development of biotechnological drugs such as antimicrobial peptides or the use of vaccines have become a valuable alternative to prevent pandemics caused by AMR bacteria, for which currently available treatments are no longer or less effective. Antimicrobial peptides act by inhibiting bacterial growth and spread, avoiding any kind of resistance for their nonspecific activity, while vaccines can mitigate directly and indirectly the disease consequences. More specifically, vaccines prevent diseases by the elicitation of the immune response, reducing antimicrobial drug consumption, and the development of resistance. This is particularly important considering community and healthcare-associated infections with multidrug-resistant pathogens.

On the other hand, pandemics caused by emerging viruses represent another serious concern for global health. The appearance of new viruses that are pathogenic to humans, previously circulating only in other species is known as spillover. Most of these zoonoses occurred due to increased contact between humans and animals favored by the increasing ecosystem destruction, trade and market of live animals. Then, the emergence of infection is influenced by the interconnecting domains of determinants such as physical and environmental factors, ecological factors, genetic and biological factors, and social, political, and economic factors or more generally human behaviors (Quammen, 2013) (Quammen, 2022).

Both for AMR bacterial and emerging virus infections, it is crucial to encourage the use of new technologies and platforms for the development of novel vaccines or biological drugs. From this side, it is of great importance the need of novel drugs and technologies but also the set-up of appropriate robust and reliable methods to assess the immunogenicity, the effectiveness and the efficacy of this kind of novel drugs.

This P.hD work has been focused on two main tasks:

The first task was based on the set up of a filtration method based on a biocompatible filter derivatized with small peptides able to remove bacteria and toxins from water extracted from wells or previously contaminated with wastewater. The methods used are HPLC analysis, peptide attachment on biocompatible resins and microbiological techniques to assess the ability of the devices to remove different clinically relevant bacterial strains from wastewater. Specifically, this device was analyzed for the removal of living Gram-negative, Gram-positive and MDR bacteria from wastewater. The ability to remove endotoxins and LTA, was also evaluated. This part of the PhD work has been completely carried out inside the Setlance srl Laboratories and the laboratories of the department of biotechnologies of University of Siena.

The second part of the P.hD work has been conducted to evaluate the potential and the immunogenicity of a candidate SARS-CoV-2 vaccine, manufactured through a plant-based production platform of virus-like particles in tobacco plants, through the analysis of a phase I clinical study. In this context the stabilized prefusion form of the SARS-CoV-2 Spike protein is produced by transient expression in *Nicotiana benthamiana* and displayed at the surface of self-assembling virus-like particles that mimic the shape and the size of the virus. In order to evaluate if this novel COVID-19 vaccine was able to elicit a specific humoral immune response, a total of 540 human serum samples, including pre-vaccination and post-vaccination visits (up to 201 days post-second immunization), obtained from 180 healthy adults (18-64 years) subjects, were tested. These serum samples were tested through Micro-Neutralization-CPE assay. Upon a first screening with the original Wuhan strain, 57 sera were selected to subsequently evaluate the neutralizing titers against the five propagated variants of concern (VOCs) Alfa, Beta, Gamma, Delta and Omicron (BA.1). This part of the PhD work was completely carried out inside VisMederi srl.

TASK 1

INTRODUCTION

Antibiotic resistance and wastewater: overview

Antimicrobial resistance is considered one of the most global public health challenges by the world health organization (WHO), particularly due to the huge spreading of multidrug drug resistance (MDR) bacterial pathogens which have the ability to escape ordinary antibiotic treatments and to cause an increasing range of infections and hospital mortality (Murray *et al.*, 2019). This phenomenon occurs when a microorganism in the presence of an antimicrobial drugs, even at high concentration, evolve the ability to survive and to proliferate. The main mechanisms to evade antibiotic effects are a reduction in membrane permeability, an increasing expression of efflux pumps that limit the access to antibiotic targets and the enzymatic degradation or modification. Antimicrobial resistance can be native to the organism or acquired, under selective pressure (inherited mutations) or natural genetic mutations.

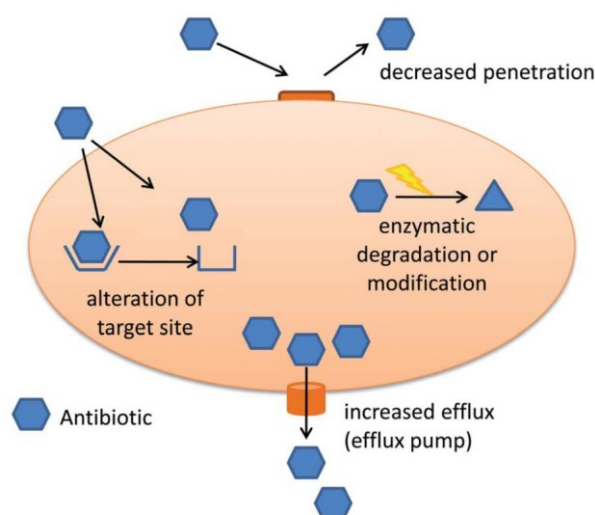


Figure 1 Examples of antibiotic resistance mechanisms in bacteria

Infectious Diseases Society of America (IDSA) enlists pathogens such as *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter spp.*, as the most concerning MDR bacteria (Rice *et al.*, 2008). Moreover, it is of great attention the study of many opportunistic pathogens, like *Escherichia coli*, *Enterococcus faecalis*, and *Burkholderia cepacia* that can develop MDR and cause severe infections and sepsis. The discovery of antibiotics at the beginning of the 20th century made aware the medical community of having won the fight against infectious disease. However, nowadays it is crucial and urgent to develop and release new antimicrobial molecules that can replace classical antibiotics, partially able to treat and solve infectious diseases. In 2023 WHO asserted that “we have arrived in the post antibiotic era” for the shortage of new antimicrobial drugs. Indeed, according to FDA (Food and Drug

Administration), since decades the released of completely new drugs has declined, considering that new drugs are preexisting molecules derivatized to improve their efficacy and to stem antimicrobial resistance which however rapidly recurs, either by alterations of the same resistance mechanisms observed with the original compounds or horizontal acquisition of new resistance genes.

A great contribution to AMR and to the dissemination of antibiotic resistance genes comes from the accumulation of antibiotics in the environment and wastewater. Conventional water treatment is not conducive to complete removal of antibiotics and bacteria, mainly due to insufficient removal of antibiotic-resistant bacteria. In this context, the horizontal transmission of antibiotic-resistant genes (ARGs) to other microorganisms in the aquatic environment contributes to the spread of antibiotic resistance. Therefore, water treatment processes have a pivotal role in disseminating or eliminating antibiotic resistance into the drinking water supplies.

Worldwide, water scarcity is a major problem even in areas where there is plenty of rainfall. A lack of clean water increases the risk of bacterial infections. Current water treatment purification plants assure safety physiochemical parameters, but it is avoided bacterial and antibiotic resistance monitoring. Therefore, the need for novel sustainable and effective technologies to obtain drinking water in disadvantaged and poor areas is also essential.

Sepsis

One of the main effects of antimicrobial resistant bacterial infections is sepsis, a phenomenon defined as a "life-threatening organ dysfunction caused by a dysregulated host response to infection" (Singer M *et al.*, 2016). It can cause septic shock upon systemic alteration of the organism, hence significantly contributing to increase the mortality rate. According to WHO, in 2020 following the result of a Global Burden Disease study, which analyzed the period of 1990-2017, 48.9 million of sepsis related cases have been estimated with 11 million sepsis-related deaths, hence representing 20% of all global deaths. The same study highlights that approximately 20 million of the estimated sepsis cases globally arise in children younger than five years old or old people, with the highest rates in lower-middle-income countries (LMICs). Indeed, the rate of incidence and mortality is inversely related to Sustainable Development Index (SDI) (Rudd *et al.*, 2020).

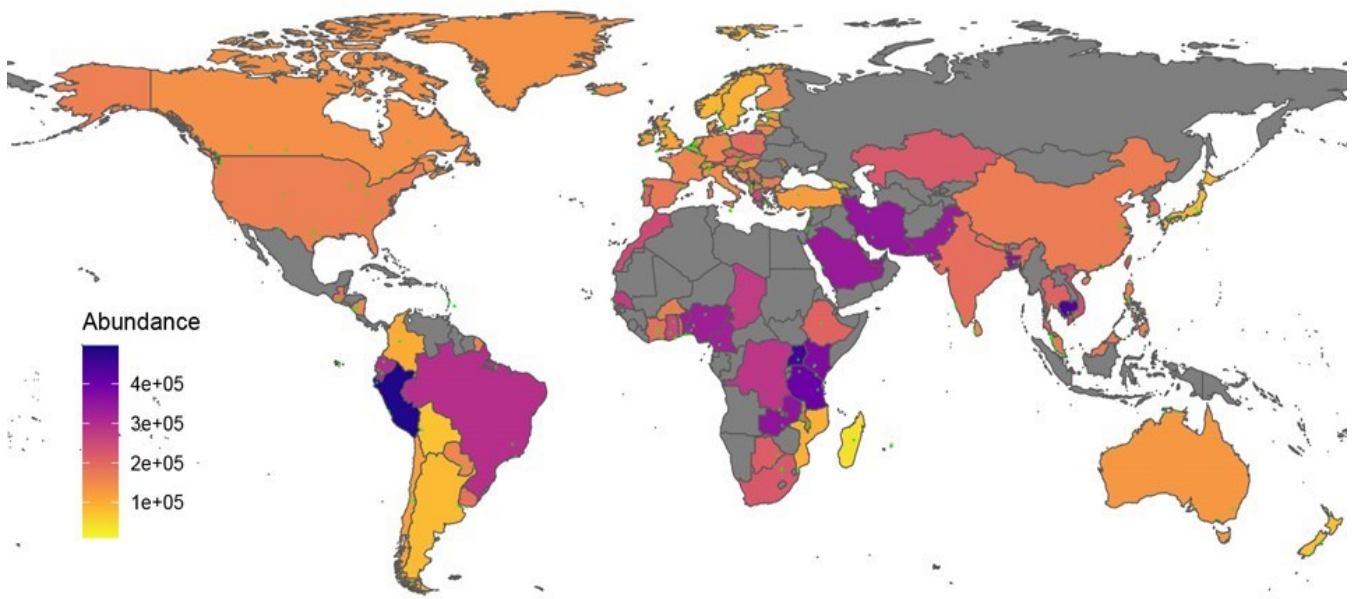


Figure 2 Global resistome based on sewage-based monitoring. Choropleth of the world is coloured by the country-wise average total AMR load. Small green dots show unique sampling sites contributing to the average. (Munk P *et al.*, 2022)

Sepsis mainly manifests by enhancing a systemic inflammatory response. All these events are triggered upon bacterial adhesion and entry into hosts tissues. Briefly, upon bacterial infection, pathogen-associated molecular patterns (PAMPs) such as lipopolysaccharide (LPS) are recognized and activate the first lines of innate immunity via the interaction with specific receptor on the innate immune cells (i.e. toll-like receptors). Another interaction can occur damage-associated molecular patterns (DAMPs) that may be intracellular material or molecules released from dead or damaged host cells. This interaction results in the activation of intracellular transduction pathways that guide to the release of proinflammatory cytokine for the recruitment of innate immune cells including macrophages, dendritic cells, mast cells and natural killer cells (NK cells) but also the activation of complement system. Particularly, in this context $\text{TNF-}\alpha$, IL-1, IL-6, IL-17 play a key role in the releasing of Granulocyte colony-stimulating factor (G-CFS), crucial for the maturation of neutrophils. Other cytokines such as IL-1 β or caspases are released upon aggregation of inflammasome complex (Gyawali *et al.*, 2019).

The large number of inflammatory molecules and reactive oxygen species released at this level is the main cause of organ and tissue failure. As a consequence, metabolic, hormonal and coagulative homeostasis is altered. Furthermore, a range of cell types exhibit changes that seem to be driven by

cellular communication pathways, as evidenced by the extracellular release of vesicles carrying proinflammatory miRNAs (Danesh *et al.*, 2018).

Clinical symptoms include Sepsis-Associated Encephalopathy (SAE), Sepsis-Induced Cardiomyopathy (SICM), acute respiratory distress syndrome (ARDS) and Disseminated Intravascular Coagulation (DIC). The latter specifically has a multifactorial origin. Indeed, it is thought to be triggered by the release of tissue damage signals from endothelial cells, monocytes or polymorphonucleates cells (Remick, 2007) which promotes the systemic activation of coagulation pathways, hence the downstream release of prothrombotic elements such as thrombin or platelets, resulting in hypoxia and organ failure due to microangiopathic thrombosis. Specifically, at the beginning of sepsis event, coagulation is counterbalanced with anticoagulant factors. With sepsis advancement, the level of these factors tends to decrease, resulting in a hypercoagulable state. Indeed, antithrombin level is reduced for high proteases and elastases activity. In parallel, also the expression of the anticoagulant protein C which normally degrades the factors Va and VIIIa and downregulate endotoxin-induced production of TNF- α , IL-1 β , IL-6, and IL-8, is inhibited. Finally, the tissue factor pathway inhibitor (TFPI) expression is dysregulated (Tsantes *et al.*, 2023). The hypercoagulable state is also caused by the suppression of fibrinolysis which depends on the balance between tissue plasminogen activator (t-PA) and tissue plasminogen activator inhibitor PAI-1. By the progression of the infection the level of PAI-1 exceeds the one of t-PA resulting in formation of clots (Bergmann *et al.*, 2007).

Bacterial pathogens are contained in a fibrin network at the infection site as a defense mechanism caused by coagulation, which prevents the infections from spreading to nearby tissues and the systemic circulation. Hypoxia results from the prevention of pathogens from spreading to other tissues at the expense of unrestricted oxygen delivery to these tissues; in this particular setting, there are benefits associated with fibrinolysis impairment, but there are also drawbacks (Tsantes *et al.*, 2023).

SICM is also a common symptom. It is related to the level of circulating cytokine which cause cardiac myocytes depression and disfunction, resulting in reduced venous return, hypotension and hypoperfusion of tissues and organs as well as the increased level of lactic acid and ROS which can cause the alteration of endothelial cells. The latter phenomenon is the main reason ARDS manifestation which consists of the accumulation of edema fluid in body cavities or interstitial spaces such as in lungs (Hu *et al.*, 2020).

Inflammatory events can also drive to the suppression and reprogramming of circulated immune cells by the increase of their spontaneous apoptosis. Dendritic cells, immature macrophages, and NK

lymphocytes but also CD4⁺, CD8⁺ and B cells are mainly induced to apoptosis, while T-regulatory cells and neutrophils revealed an extensive resistance (Shen *et al.*, 2017).

Antimicrobial peptides

Antimicrobial peptides (AMPs) are small proteins that are naturally produced by various organisms, including humans, animals, and plants. They can kill or inhibit the growth of microorganisms such as bacteria, fungi, and viruses. AMPs play an important role in the innate immune system and serve as a first line of defense against infectious pathogens.

The antimicrobial activity of AMPs is primarily due to their ability to disrupt the structure and function of microbial membranes. They can penetrate the membrane of microorganisms, causing structural damage and leakage of cellular contents, leading to cell death. Additionally, some AMPs can also modulate the immune response and promote wound healing, possess anti-inflammatory properties, anti-cancer activity and prevent biofilm formation.

One of the advantages of AMPs is their broad-spectrum activity, meaning they can target a wide range of pathogens rather than specific strains. This makes them attractive candidates for developing new antimicrobial therapies, especially in the face of increasing antibiotic resistance.

Scientists are conducting research to better understand the mechanisms of action of AMPs and their potential applications in medicine, food preservation, and agriculture. However, challenges remain in terms of optimizing their efficacy, stability, and production. Despite that, antimicrobial peptides hold promise as a potential solution in the fight against infectious diseases (Cresti *et al.*, 2024).

Properties of antimicrobial peptides and classification

AMPs can be classified into four main families based on the kind of secondary structure: i). linear α -helical peptides, ii) beta-sheet peptides with two or more disulfide bonds, iii) peptides with a mixed secondary structure of α -helica and beta-sheets, iv) peptides without secondary structure or with an extended structure (Xuan *et al.*, 2023) (Mojsoska *et al.*, 2015). In Figure 3 the structure of different families is reported. AMPs can be also cationic when sharing a net positive charge which confer an amphipathic or amphiphilic conformation upon the interaction with the bacterial membranes, negatively charged (Bechinger *et al.*, 2017).

AMPs can also be classified based on sources. Indeed, peptides are released for a defensive response. On this base, several antibiotic peptides can be identified in mammals, amphibia, microorganisms and insects and also plants (Huan *et al.*, 2020).

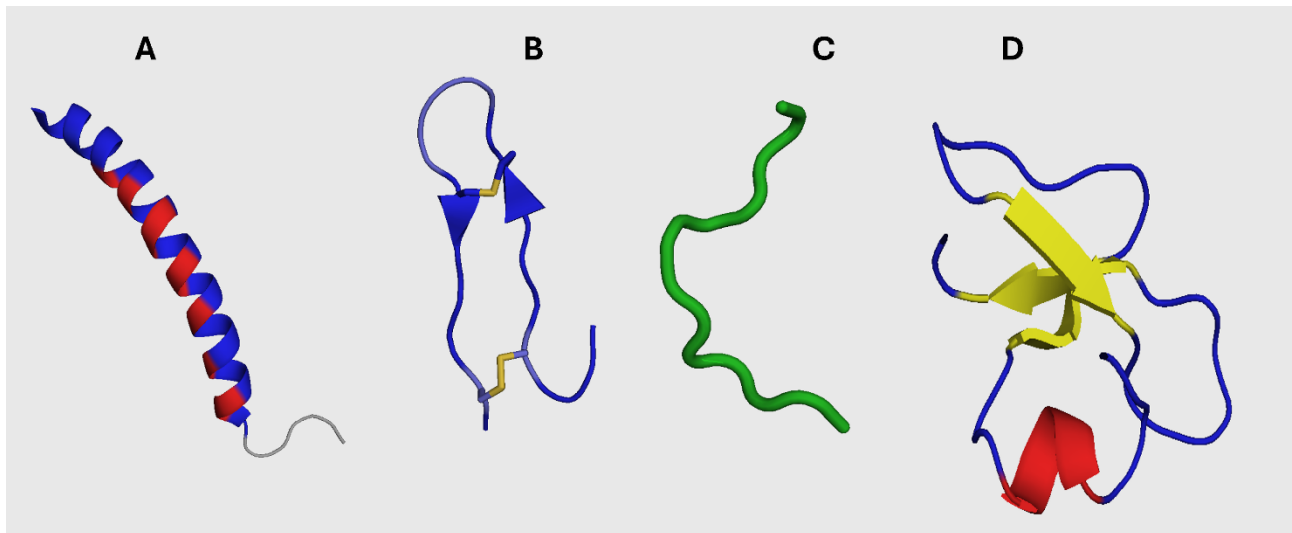


Figure 3 Four structural classes of antimicrobial peptides. (A) α -helical structure of human cathelicidin LL-37 (PDB code 2K6O); (B) β -sheeted polyphemusin (PDB code 1RKK); (C) extended indolicidin (PDB code 1G89); (D) and mixed structures like human β -defensin-2 (PDB code 1FQQ)

α -helical peptides are the widest and most studied class of AMPs either in natural sources or in biochemical field for synthetic analogs. They usually include 12-40 amino acids with helix stabilizing motifs like Ala, Leu or Lys (Takahashi *et al.*, 2010) and they do not contain cysteine residues (Luong *et al.*, 2020); thus, they are unable to form disulfide bridges. In nature they are predominantly found in insects and amphibia, e.g. melittin in bees or citropin or magainins in frogs (Steiner *et al.*, 1981). In particular the latter possess a strong antimicrobial activity against both Gram-positive and Gram-negative bacteria, virus and fungi (Zasloff *et al.*, 1987). In Mammalia a prominent example is the cathelicidin LL-37 (Figure 3A) that plays a key role in immunomodulatory and inflammation responses (Choi *et al.*, 2012). Helical peptides are usually unstructured in solution, but they can enhance their α helix on contact with membranes or above critical concentration of micelles and liposomes (Sato *et al.*, 2006). The combination of positive charged and hydrophobic residues leads to a wide variety of structures that allows a broad range of interaction including an efficient solubility into microbial membranes (Porcelli *et al.*, 2013). Nadezhdin *et al.* (2017) reported how in some cases the α -helical motif can be combined with other structural domains, as recently seen in an α -helical AMP identified as part of a modular spider toxin, OtTx1a, which is reported to combine an inhibitor cystine-knot domain and an AMP domain in a single molecule.

The second group of AMPs encompasses peptides with β -sheets topologies. This kind of peptide is structurally stabilized by the presence of conserved disulfide bonds. Thus, they are very stable in solution and do not go through conformational changes when passing from aqueous to membrane environment. Based on the cysteine and structural characteristics, they can be divided into two

groups: (i) β -hairpin peptides; and (ii) α -defensin peptides (Huan *et al.*, 2020). Some representative examples of β -hairpin peptides are thanatin from insects (Mandard *et al.*, 1998), protegrin-1 from porcine leukocytes (Kokryakov *et al.*, 1993), tachyplesin and polyphemusin (Figure 3B) from horseshoe crabs (Edwards *et al.*, 2017) (Tincu *et al.*, 2004). Particularly, the latter shows an amphipathic antiparallel β -hairpin structure connected by a type I- β -turn stabilized by two disulfide bonds (Figure 3); due to its characteristics this peptide displays antimicrobial activity against Gram-negative and Gram-positive species and fungi (Powers *et al.*, 2006).

On the other side, the α -defensins includes disulfide bonds in a 'trans' conformation that link three antiparallel β -strands. In this context a conserved salt bridge plays a pivotal role for a proper folding and proteolytic stability (Andersson *et al.*, 2012). α -defensin peptides are included into the multiple structural subgroup of defensins.

Mixed peptides are molecules with a structure containing either α -helix and β -Sheet elements. A very interesting example of this class is represented by β -defensin (Figure 3D), typically found in humans and mammals and also described in various invertebrate species and plants. These kinds of peptides are subclassified based on the different arrangements of the disulfide bonds, from three to five (Shafee *et al.*, 2016). In human and mammals, similarly to α -defensins, this subclass displays a trans conformation of disulfide bonds with the presence of a α -helix. Conversely, a Cis conformation, with two disulfide bonds coupling α -helices with the terminal portion of β -strands in a parallel arrangement, is typical of plants and invertebrates (Koehbach *et al.*, 2019). In human, β -defensin 2 is largely studied: this kind of peptide is rich in cysteine and arginine residues which stabilize its structure with three disulfide bonds (Cieřlik *et al.*, 2021). Their main function is to protect from bacterial infections and to modulate immune reactions. In the intestinal environment they also play a key role in preventing and healing epithelial wounds (Cobo *et al.*, 2013). Recent reports also support the role of HBD-2 in suppressing inflammation and promoting proliferation and angiogenesis (Yele *et al.*, 2023).

The fourth class of AMPs do not adopt any secondary structure in solution or on contact membranes, thereby they are included in the subclass of extended peptides. They are mainly rich in proline, glycine, tryptophan or histidine and they organize in their final structure by weak bonds with the membranes. A notable example is the family of cathelicidins (Figure 3C), which includes subfamilies rich in tryptophan and/or histidine like abaecin from honeybees (Casteels *et al.*, 1990), bovine indolicidin (Rokitskaya *et al.*, 2011), isolated from bovine neutrophils, human histatins (Oppenheim *et al.*, 1988) and hymenoptaecins from various insects (Casteels *et al.*, 1993). Another interesting subfamily

includes proline and arginine-rich molecules like the mammalian PR-39 which refolds in a typical PP-II helical conformation (Cabiaux *et al.*, 1994).

Recently a fifth group of AMPs with cyclic or complex topologies has been discovered. This class of peptides does not adopt the linear backbone belonging to the classes described above but reveals a typical “head to tail” and “head to side chain” cyclic topologies. Examples of head to tail backbone-cyclized peptides are bacteriocins, θ -defensins and plant cyclotides (Gabrielsen *et al.*, 2011) (Slough *et al.*, 2018). The first group does not share high homologies in primary sequences but display similar arrangements in 3D structures. On the other side, θ -defensins and plant cyclotides contain additional bonds such as disulfide bonds or thioether bridges that promote a steadier structure.

The head to side chain class exhibits unusual structural features that result in the presence of thioether bridges or a link between amino acids of the side chain and the peptide backbone. In the latter case especially, it is common to find an unusual threaded topology known as a lariat knot or lasso (Hegemann *et al.*, 2015). Briefly, in such structures a macrolactam cycle forms between the N terminus and the carboxylic acid side chain of a glutamate or aspartate residue, which is threaded by the C-terminal tail sequence. These bulky residues sterically lock the threaded tail in place and the structures are in many cases further stabilized by one or more disulfide bonds (Rosengren *et al.*, 2003).

Mechanism of antimicrobial peptide activity

Originally, AMPs mechanism of action was associated with their ability to bind membrane targets, therefore inhibiting bacterial activity. This mechanism is extremely different from the classical antibiotic activity. Later, other studies confirmed also a non-membrane targeting mechanism and an immune modulation mechanism. On this basis, AMPs activity is now divided into two groups: direct killing and immune modulation (Cresti *et al.*, 2024).

Direct killing is mainly characterized by specific interactions of cationic peptides with model membrane system. In this sense, the design of the membrane and the different composition of the lipid bilayer in eukaryotes and prokaryotes play a key role in the activity of antibacterial peptides and directly affect the sensitivity of procaryotic and eucaryotic cells. The outer monolayer (leaflet) of the lipid bilayer in bacteria membranes is primarily composed of lipids with negatively charged head groups, such as phospholipids sphosphatidylglycerol and cardiolipin whereas in mammalian membranes, most of these anionic lipids are located in the inner layer facing the cytoplasm and their outer layer is made up of zwitterionics phospholipids such as phosphatidylcholine, sphingomyelin and other neutral components such as cholesterol (Lohner *et al.*, 2009). Therefore, positively charged

AMP has strong electrostatic interactions with negatively charged phospholipids on the outer leaflet of the bacterial membrane (Hollmann *et al.*, 2018) (Mazurkiewicz-Pisarek *et al.*, 2023).

Furthermore, some AMPs are even sensitive to other lipid properties, not only in terms of charge but also of shape intended as the relative sizes of the hydrophobic head group and tail (Matsuzaki *et al.*, 1999) (Jouhet *et al.*, 2013); differences in lipid shape can exhibit several membrane curvature properties. Hence, for an initial interaction, in addition to the electrical properties of the membrane, the membrane curvature is crucial. (Nepal *et al.*, 2018) (Chen *et al.*, 2011) (Schmidt *et al.*, 2013).

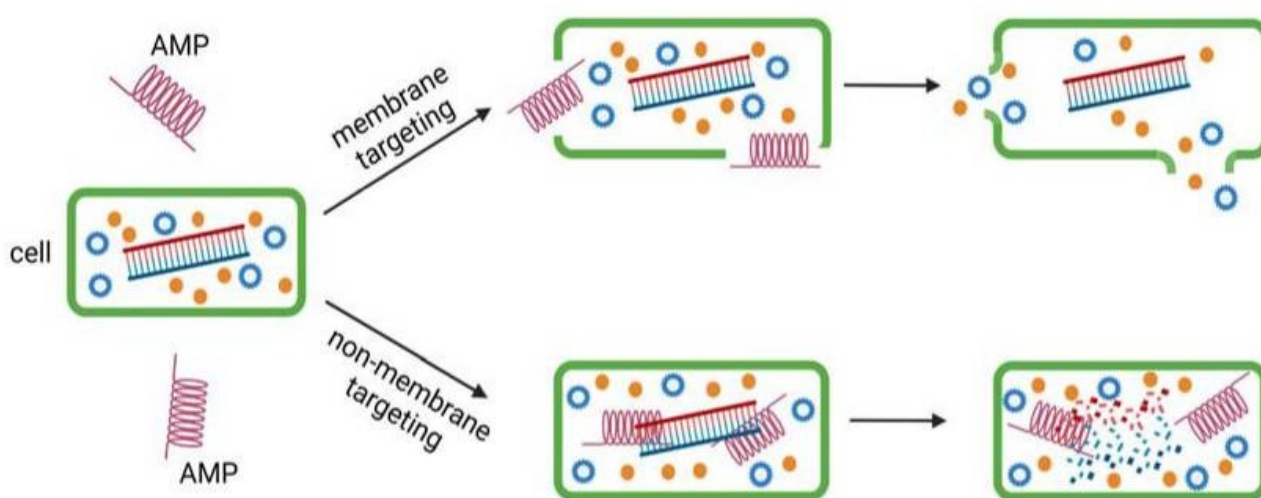


Figure 4 The direct killing mechanism of AMPs includes membrane targeting and non-membrane targeting. Green-bacterial membrane; Orange and Blue-cell organelles (Mazurkiewicz-Pisarek A *et al.*, 2023).

AMPs, mainly unfolded, aggregate onto the surface of bacterial cell membrane avoiding the assembly of outer or inner membrane and thereby leading to cell death. The first interaction is obtained by opposite electrostatic forces (Benfield *et al.*, 2020) (Brogden *et al.*, 2005). Indeed, the bacterial cell surface of Gram-positive and negative bacteria contains teichoic acids (LTA) and lipopolysaccharide (LPS), respectively, that confer a net negative charge and promote cationic peptides attraction (Shai *et al.*, 2002). In particular, in the case of Gram-negative, the higher affinity of peptides with LPS than those by the native divalent cations like Mg^{2+} and Ca^{2+} leads to a membrane perturbation and to the destabilization of normal barrier properties of the outer membrane (Clifton *et al.*, 2015). Peptides exploit these perturbed zones to translocate in the outer membrane in a process called self-promoted uptake. After the initial interaction and at a certain concentration, AMPs can self-assemble onto the cytoplasmic membrane. At this phase the action of AMPs has been described by several models classified under two vast groups: transmembrane pores and non-pore models (Epand *et al.*, 2015)

(Anderson *et al.*, 2016). Particularly, the transmembrane pore model comprises the barrel-stave pore and toroidal pore models.

In the first, AMPs originally place oriented parallel to the surface and then insert perpendicularly into the lipid bilayer. This event contributes to a lateral peptide-peptide interaction that eventually forms a pore similar to protein ion channel formation (Wimley *et al.*, 2010). Moreover, in this context the peptide structure is essential: indeed, only peptides with amphipathic structure (α -helix or β -sheets) have the ability to open a pore in the membrane, using the hydrophobic residues to interact with the lipids and the hydrophilic residues to form the lumen of the channels (Avci *et al.*, 2018).

The toroidal pore model consists of a direct perpendicular insertion of peptides in the lipid membrane without a peptide-peptide specific interaction. In this way peptides can induce the formation of a dynamic curvature of the bilayer and a transient toroidal pore (Sengupta *et al.*, 2008). This formation provides alternate surfaces for the lipid tail and the lipid head group to interact with. As the pores are temporary, some peptides can move in the inner part of the cell or in the cytoplasm where can bind and target intracellular components (Uematsu *et al.*, 2000). In the end both transmembrane pore models induce cell death upon membrane depolarization.

The transmembrane non-pore models are instead divided in carpet and micellar aggregate models; in these mechanisms antimicrobial peptides do not insert in the lipid bilayer to form transmembrane channels/pore or specific peptide structures.

In the carpet model, peptides gather parallel to the cell membrane with their hydrophilic portion facing extracellular space and their hydrophobic end adhered to the phospholipid bilayer. It was first described by Shai (Shai *et al.*, 2002) and it is the widely studied mode of action of AMPs for the disruption of microbial membranes. Similarly, to the previous described models, peptides approach and attach to the membrane due to their opposite charge with strong electrostatic interactions (Bedard *et al.*, 2018). Peptides accumulate themselves until they reach a critical threshold concentration to obtain a sort of carpet on the membrane. This event leads to the rupture of the membrane and creates an effect of detergent, which promotes the formation and the release of micelles. The latter step is referred to as a detergent-like model (Enany *et al.*, 2002). Some studies also propose the carpet model as a necessary step for the toroidal pore model (Lee *et al.*, 2016). The carpet mechanism does not need a peptide-peptide interaction of the peptide individuals bound to the membrane nor does it need the peptide to embed itself into the hydrophobic region to create transmembrane channels or specific peptide structures (Perez *et al.*, 2014). Moreover, this kind of models reveal how antimicrobial activity can be independent of the amino acid composition and length of the sequence; indeed, the carpet model is effective due to the amphiphilic composition of peptides and only at high

concentrations (Shai *et al.*, 2002). Some examples of these molecules are cecropin (Sitaram *et al.*, 1999) and aurein 1.2 (Fernandez *et al.*, 2012).

The previous models use an ATP-independent cellular uptake mechanism. However, ATP-dependent cellular mechanisms have been studied. These involve micropinocytosis events where the target plasma membrane folds inward along with the peptide to generate macropinosomes. Following the releasing of the vesicles, AMPs diffuse inside the cytoplasm and exhibit their antimicrobial effects. There are several other models by which peptides perform their antimicrobial activity. These include sinking raft models and electroporation, which create transient and unstable holes in the membrane that alter the charge on both sides of the membrane (Liu *et al.*, 2017) (Moravej *et al.*, 2018).

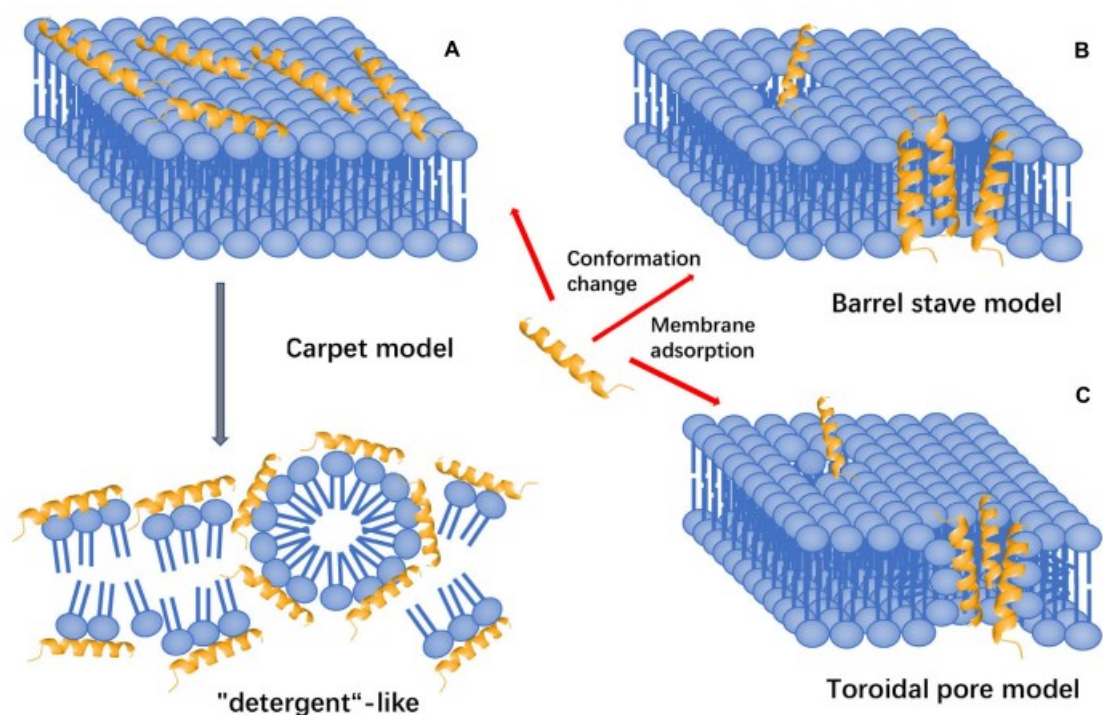


Figure 5 Models of action for extracellular AMP activity. **(A)** Carpet model: accumulation of AMPs on the surface and then destroy the cell membrane in the manner of “detergent”. **(B)** Barrel stave model: AMPs aggregate with each other and are inserted into the bilayer of the cell membrane in the form of multimers and arrange parallel to the phospholipids, then form a channel. **(C)** Toroidal pore model: accumulation of AMPs vertically embed in the cell membrane, and then, bend to form a ring hole (Huan *et al.*, 2020).

Besides direct killing mechanisms, more recent studies have suggested that AMPs have the capability to link innate and adaptive immune systems and modulate the activation of the immune response to prevent and protect against infection, control inflammation, and influence immune homeostasis. AMPs repertoire to modulate immune responses includes leucocyte recruitment, chemotaxis and angiogenesis stimulation, pro-inflammatory cytokine induction and release of chemokines and

reactive oxygen/nitrogen species, endotoxin neutralization, as well as activation and differentiation of immune cell lines (Hilchie *et al.*, 2013) (Lai *et al.*, 2009)

Some examples of AMPs that regulate innate immunity are α -defensins and cathelicidins. These molecules are found in granules of neutrophils and NK cells and play a key role in the intracellular killing of ingested pathogens; on the other hand, in the extracellular milieu, these peptides, like chemokines, are also involved in recruiting different cell types like neutrophils, eosinophils, mast cells, monocytes, and lymphocytes to the infection site (Auvynet *et al.*, 2009) (Hiemstra *et al.*, 2006). Additionally, this kind of peptides can increase the number of active macrophages and facilitate the phagolysosome formation (Tan *et al.*, 2006) (Soehnlein *et al.*, 2008).

Other AMPs are involved in affecting other cell types. This is the case of cathelicidin LL-37, which has been studied for the increasing of TLR4 expression in mast cells that improve the ability to detect pathogens (Yoshioka *et al.*, 2008). This same peptide has been also studied for the ability to increase the differentiation of monocytes to macrophages promoting a proinflammatory phenotype. Moreover, it can decrease the secretion of Interleukin-10 (IL-10) and increase that of Interleukin-12 (IL-12) in macrophages exerting an anti-inflammatory phenotype (van der Does *et al.*, 2010).

HNP1-3 is another notable example of pro- anti-inflammatory molecule. It has been reported how this peptide can regulate both pro- and anti-inflammatory response at the same time, by increasing the production of TNF- α , IFN- γ , and IL-1 whilst decreasing the production of IL-10 by monocytes (Duarte-Mata *et al.*, 2023).

Lastly, AMPs are also involved in various innate immune response processes like opsonization, activation or suppression of the complement system, and activation of the NLRP3 inflammasome (Prohaszka *et al.*, 1997) (Kahlenberg *et al.*, 2013).

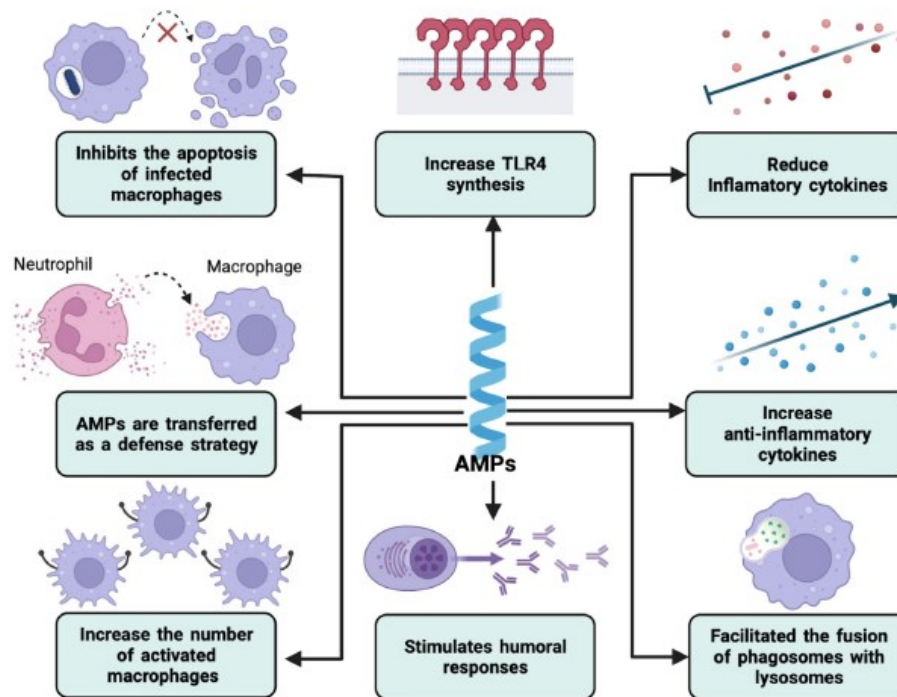


Figure 6 Immunomodulation by AMPs in intracellular bacterial infections. AMPs can modulate the host immune response in different ways. These include the regulation of the secretion of both anti- and proinflammatory cytokines and the expression of certain innate immune receptors. They can also increase the number of active macrophages and facilitate the phagolysosome formation. On the other hand, humoral immunity can also be modified (Duarte-Mata DI *et al.*, 2023).

Challenges of antimicrobial peptides application

Antimicrobial peptides remain promising candidates as new antibiotic agents to fight infections and antimicrobial resistance. Their combined antimicrobial, immunomodulatory and anti-inflammatory actions make them unique molecules, since none of the antibiotics used in clinical trials has these characteristics. Many examples of eukaryotic AMPs have been reported and characterized. However, only a few have been approved by US Food and Drug Administration for clinical use. Indeed, though most AMPs exhibit potent antimicrobial activities *in vitro*, many show reduced activity *in vivo*. This issue is due to cytotoxicity, low stability, high sensibility to serum proteins and also difficulties in large scale production. Natural antimicrobial peptides show a remarkable toxicity for eukaryotic cells due to their low selectivity that leads to a high degree of hemolysis. Moreover, they show a low stability for their susceptibility to human proteases of whole the digestive tract. (Jenssen *et al.*, 2006) (Andersson *et al.*, 2016) (Vaara *et al.*, 2009).

The latter issue as a consequence a systemic administration, e.g., by intravenous or topical way, resulting in short half lives *in vivo* due to the rapid renal or hepatic clearances (Haney *et al.*, 2013).

Therefore, in last years, new promising applications have been explored to exploit the strong antimicrobial activity of these molecules.

There are many examples of AMPs application in cosmeceutical field (Venkatesan *et al.*, 2017), biomedical and diagnostic field (Chen *et al.*, 2020) (Zhang *et al.*, 2020) and finally in environmental remediation field (Shi-Ying Ding *et al.*, 2020). However, in this context, scientists have to face other issues such as the ability to coat surfaces and their stability onto different surfaces, toxicity for eucaryotic cells and loss of antimicrobial activity.

To overcome these variables and to maintain the efficacy of AMPs at different stages, different strategies have been faced; for instance, these refer chemical modification of AMPs (Gentilucci *et al.*, 2010) and the study of different material formulations.

Strategies to improve antimicrobial peptides: Chemical modification

One of the main issues in the development of AMPs is their inability to maintain a broad-spectrum activity at low concentration (minimal inhibitory concentrations (MICs) of 1-10 μ M) from in vitro to in vivo models. In recent years advanced synthetic chemistry techniques have allowed to exploit structural diversity of naturally-occurring AMPs that can add another dimension to peptide structures agents and delivering to improve antimicrobial activity. There are several chemical modifications aimed at optimization of AMPs stability and effectiveness: these include (Cresti *et al.*, 2024)

- Peptide cyclization
- Terminal side chain modification
- Incorporation of D and non-natural amino acids
- Peptidomimetics
- Branched Peptides

Cyclization usually occurs in nature through the formation of amide bonds or disulfide bonds as showed for human β -defensins (hBDs) family (Han *et al.*, 2021). These reactions enhance antimicrobial activity, specificity and increase peptide stability. Disulfide bonds form in heterodetic cyclization which oxidate a side chain thiol of cysteines (Cys) creating single or multiple disulfide bridges. This reaction is considered the most frequently used method in peptide optimization due to their effectiveness. Indeed, the formation of multiple disulfide bridges increases the probability to form secondary structure that decreases protease susceptibility and the rate of degradation while

enhancing in vivo stability (Falanga *et al.*, 2017). Another strategy promotes the formation of homodetic cyclic peptides by amide bonds. This reaction can be obtained with different modes, e.g., head-to-tail, side chain-to-head/tail, and side chain-to-side chain and can also produce intramolecular peptide bonds (Han *et al.*, 2021).

A second approach includes the modification of N-/C termina by acetylation (N-terminal), methylation (N-terminal) or amidation (C-terminal). This kind of modification leads to the block of the peptidase and protease activity promoting an increasing in the proteolytic stability of peptides. However, on the other hand, this reaction results in the partial inhibition of antimicrobial activity for the removal of positive charge (Henninot *et al.*, 2018) (Papanastasiou *et al.*, 2009). In nature, amidation reactions of C-terminus are reported for peptides belonging to the temporin family (Mangoni, 2006). A second strategy is the modification of side chain groups by PEGylation and glycosylation. These increase biocompatibility and bioavailability by improving water solubility and blood half-life. Moreover, they can reduce nonspecific uptake in tissue, renal clearance, and immunogenicity (Veronese *et al.*, 2008). Finally, another strategy to improve antimicrobial activity, avoiding structural modifications, is the introduction of a lipid group to a lysine side chain or to the N-terminal residues. This modification aims to enhance hydrophobicity and membrane interaction as well as permeability and prevention from proteolytic cleavage. Since these modifications result in a more important selectivity for mammalian cells, it is crucial to maintain the hydrophilicity/hydrophobicity balancing, to stabilize peptide toxicity (Bellavita *et al.*, 2023).

Another strategy to improve peptide stability is the incorporation of non-natural or unusual amino acids. These molecules are those amino acids typically not belonged to protein sequences but are chemically synthesized or can be found in living organisms. In drug discovery, they are usually used to increase peptide activity and stability due to the different optical properties. Indeed, in nature amino acids can be found in two different forms, L- and D-, optically active (enantiomers) that can determine different biological activities (Grishin *et al.*, 2020). L-amino acids are the one usually found in proteins and are quite susceptible to proteolysis. On the other side, D-amino acids improve the resistance to proteolysis and allow to disguise AMPs from proteolytic enzymes by the alteration of stereochemistry (Kim *et al.*, 2010). Stereochemical modification usually does not affect antimicrobial activity of AMPs. However, the position of unnatural amino acids in the sequence can determine structure modifications and consequent loss of function (Hong *et al.*, 1999). Moreover, a partial substitution of D-amino acids can result in a different tertiary peptide structure that reduce biochemical properties, such as hydrophobicity (Li *et al.*, 2006). Therefore, the study of D-synthetic amino acids

with a wide variety of side chain groups is of great attention to improve peptide stability, activity and effectiveness in vivo.

A fourth strategy to stabilize AMPs is the synthesis of peptidomimetics. Briefly, it consists of the introduction of peptide-like compounds whose pharmacophore mimics a natural peptide or protein in 3D space with the ability to interact with the biological target and produce the same biological effect (Vagner *et al.*, 2008). The idea is that a short sequence of peptides or functional groups that are close together can be reproduced in smaller, conformationally similar to the epitopes of proteins. Peptidomimetics can be obtained by modification of both main- or side-chain of the original peptide and can be designed from protein epitopes with global or local conformational restrictions. Examples of global conformational constraints include cyclization of the peptide using nonpeptide moieties, lactam bridges or inclusion of penicillamine (dimethyl cysteine) to form disulfide bonds. Local conformational restrictions can be applied using backbone modifications at amino acid residues or between two amino acid residues in the peptide. (Gokhale *et al.*, 2014)

AMPs can be also synthesized in a branched form to bypass half-life and in vivo stability. This kind of molecules, called multiple antigens peptides (MAPs), form by covalently binding more copies of the sequence of the peptide of interest to a peptidyl scaffold of radially branched lysine residues (Cresti *et al.*, 2024). Since they have a higher steric hindrance, their access to the cleavage site of peptidase or protease is limited, thus their in vivo stability is enhanced. Additionally, their multimeric nature can increase the peptide efficacy for the larger number of interactions compared to the monomeric form.

SET-M33 peptide

SET-M33 is a nonnatural cationic antimicrobial peptide isolated upon phage display random screening against *Escherichia coli* cells. The peptide has been designed and synthesized in tetrabranch form (multiple antigen peptide, MAP) with four copies of SET-M33 sequence (KKIRVRLSA) linked to a three-lysine core to increase its resistance to degradation in biological fluids, thus improving the half-life and the suitability for in vivo applications.

It is obtained by 9-fluorenylmethyloxycarbonyl (Fmoc) chemistry, carried out on a multiple peptide synthesizer. Amino acids are gradually added on a solid support resin by a continuous cycles of Fmoc deprotection and carboxylic group activation. Then, the final product is cleaved from the solid support and deprotected by treatment with trifluoroacetic acid (TFA) containing triisopropylsilane and water and precipitated with diethyl ether.

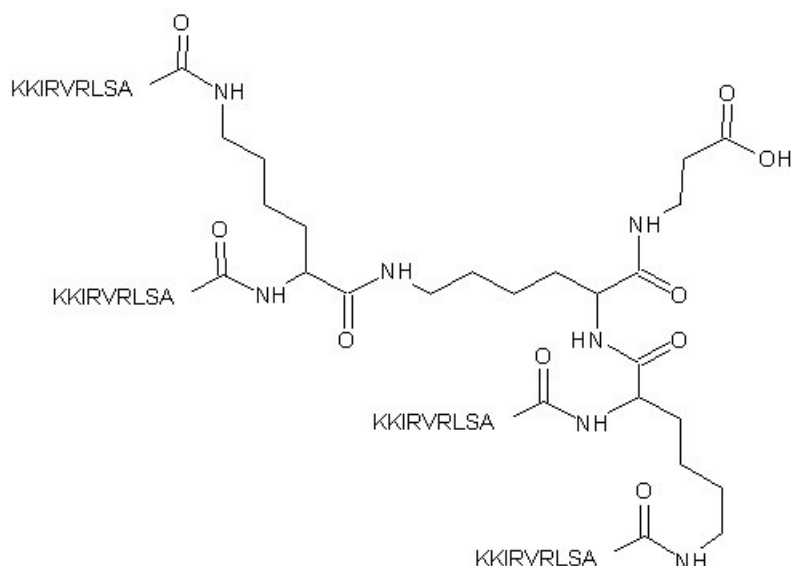


Figure 7 Structure of SET-M33 tetra-branched peptide

SET-M33 showed a strong antimicrobial activity against about 100 multidrug resistant pathogenic strains such as *Escherichia coli*, *Klebsiella pneumoniae*, and *Acinetobacter baumannii* and bacterial components including Lipopolysaccharide (LPS) and lipoteichoic acid (LTA), as well as eradication of biofilm from cystic fibrosis clinical isolates such as *Pseudomonas aeruginosa* (Pini *et al.*, 2010) (Falciani *et al.*, 2012). Particularly, the peptide showed a minimal inhibitory concentration (MIC) below 1.5 μM and 3 μM for clinical isolates strains of *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and MDR strains such as *Klebsiella Pneumoniae* (Pini *et al.*, 2012). In addition, this peptide revealed a consistent anti-inflammatory activity by the reduction of LPS-induced cytokine released, preventing septic shock (Brunetti *et al.*, 2016).

Bacterial strain and species		MIC (μM)	
		M33	Polymyxin B
<i>P. aeruginosa</i> ATCC 27583	Reference strain wild type	1.5	1.5
<i>P. aeruginosa</i> PAO-1	Reference strain wild type	1.5	1.5
<i>P. aeruginosa</i> VR-143/97	FQ ^r AG ^r ESC ^r NEM ^r (MBL/VIM-1)	1.5	1.5
<i>P. aeruginosa</i> AV65	FQ ^r AG ^r ESC ^r NEM ^r (MBL/VIM-1)	1.5	1.5
<i>P. aeruginosa</i> SC-MDr03-06	FQ ^r AG ^r ESC ^r NEM ^r	3	1.5
<i>P. aeruginosa</i> SC-VMr04-05	FQ ^r AG ^r ESC ^r NEM ^r	3	1.5
<i>P. aeruginosa</i> SC-DMr05-04	FQ ^r AG ^r ESC ^r NEM ^r	1.5	1.5
<i>P. aeruginosa</i> SC-BGr12-02	FQ ^r AG ^r ESC ^r NEM ^r	1.5	1.5
<i>P. aeruginosa</i> SC-MDm03-02	FQ ^r AG ^r ESC ^r NEM ^r	3	1.5
<i>P. aeruginosa</i> SC-GMm03-05	FQ ^r AG ^r ESC ^r NEM ^r	1.5	1.5
<i>P. aeruginosa</i> SC-CNm03-07	FQ ^r AG ^r ESC ^r NEM ^r	0.3	0.7
<i>P. aeruginosa</i> EF-OBG6-1	FQ ^r AG ^r ESC ^r NEM ^r (MBL/IMP-13)	1.5	0.7
<i>K. Pneumoniae</i> ATCC 13833	Reference strain wild type	1.5	0.7
<i>K. Pneumoniae</i> 7086042	FQ ^r AG ^r ESC ^r NEM ^r (MBL/VIM-1)	3	1.5
<i>K. Pneumoniae</i> C8-27	FQ ^r AG ^r ESC ^r NEM ^r (ESBL/CTX-M-15)	1.5	0.7
<i>K. Pneumoniae</i> FIPP-1	FQ ^r AG ^r ESC ^r NEM ^r (MBL/KPC-3)	3	1.5
<i>E. coli</i> ATCC 25922	Reference strain wild type	1.5	0.7
<i>E. coli</i> W03BG0025	FQ ^r AG ^r ESC ^r NEM ^r (ESBL/CTX-M-15)	0.7	0.7
<i>P. mirabilis</i> W03VA1017	FQ ^r ESC ^r (AmpC/CMY-16)	>24	>96
<i>E. aerogenes</i> W03BG0067	AG ^r ESC ^r (ESBL/SHV-5)	1.5	0.7
<i>E. cloacae</i> W03AN0041	ESC ^r (ESBL/SHV-5)	1.5	0.7
<i>A. baumannii</i> RUH 134	Reference strain European clone II	1.5	1.5
<i>A. baumannii</i> RUH 875	Reference strain European clone I	3	1.5
<i>A. baumannii</i> MR157	FQ ^r AG ^r ESC ^r NEM ^r (OXA/OXA-58)	3	1.5
<i>B. cepacia</i> ORB-99	Wild type	>24	>96
<i>S. aureus</i> ATCC 29213	Reference strain PEN ^r	6	96
<i>S. aureus</i> 3851	MR VAN	6	96

Table 1 Comparison of MIC values between SET-M33 and colistin against several clinical isolates; legend: FQ^r, resistant to fluoroquinolones; AG^r, resistant to aminoglycosides (gentamicin, amikacin, and/or tobramycin); ESC^r, resistant to expanded-spectrum cephalosporins; NEM^r, resistance to carbapenems, ETP^r resistance to ertapenem; ESBL, extended spectrum β-lactamase; MBL, metallo-β-lactamase; OXA, oxacillinase; MR methicillin-resistant; PEN^r resistance to penicillin; VANi, vancomycin-intermediate (Pini *et al* 2010).

The mechanism of action and the structure of SET-M33 peptide was evaluated using several biophysics and biochemical techniques such as NMR, circular dichroism, electron microscopy imaging (Van der Weide *et al.*, 2017). Scanning electron microscopy (SEM) and transmission electron microscopy (TEM) showed the ability to form bacterial superficial blisters and subsequently large holes at the cell poles, causing cell damage and death. NMR analysis revealed how SET-M33 in a free state explores a wide conformational space, assuming a random coil structure. In the same study, SDS micelles were used to mimic interactions of the peptide, either in a tetrabranch and

linear form (M-33 capped with Q), with membrane environment. The peptide transit from a non-structured conformation in water to a stable α -helix formation in the presence of micelles and it is attracted to the latter by electrostatic interactions between cationic amino acids (K and R) and negative net charges of sulfate groups or phosphate groups of LPS and LTA in bacterial outer membrane. Following this interaction, the amphipathic α -helix formation is also able to irreversibly damage Gram-negative and positive membranes by altering its homeostasis and functionality, thus causing bacterial death (Figure 8).

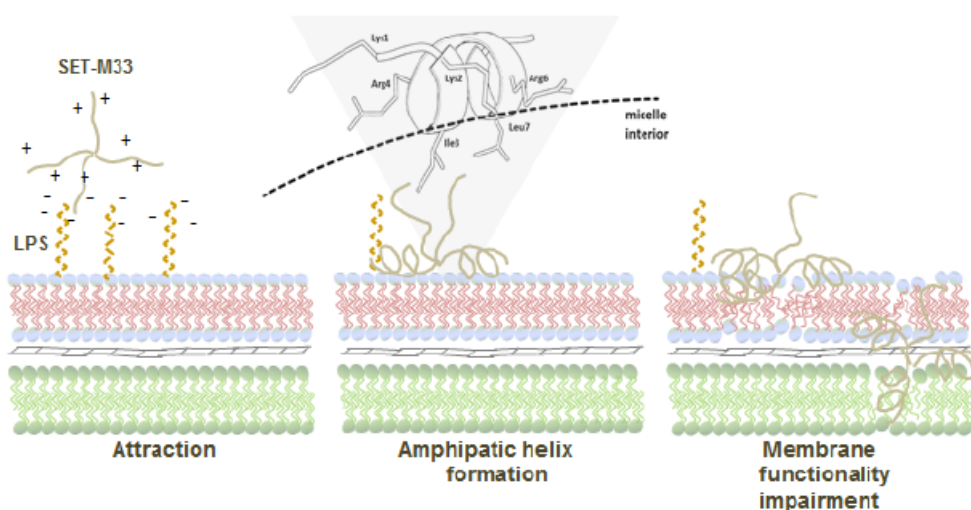


Figure 8 Mechanism of antimicrobial action of SET-M33 peptide against Gram-negative bacteria, based on NMR and CD results (Van der Weide *et al.*, 2017).

Acute toxicity in comparison with colistin and in vivo models of sepsis, pneumonia and skin infections caused by *Pseudomonas aeruginosa* were studied using optimized SET-M33 peptides (SET-M33L and SET-M33L-PEG) (Brunetti *et al.*, 2016). Moreover, in the same study, the bio-distribution of the radioiodinated peptide was evaluated. Neutropenic BALB/c mice were chosen for the experiments. Upon infection with a lethal dose of *P. aeruginosa* PAO1, the peptide was injected twice intravenously. For sepsis the rate of survivals reached 60-80%, for lung infections showed a survival rate of 80-90 %. Skin infections was evaluated using a modified strain of *P. aeruginosa* (P1242), expressing a luciferase gene. Analogously, to the previous models it was reported a consistent reduction of bacterial infection. Animals were then treated with 5-40 mg/Kg of the SET-M33L and colistin to evaluate acute toxicity. SET-M33 produced toxicity signs and mortality only at the maximum concentration, whilst colistin is already lethal at the minimum dose. All animals treated

with 40 and 20 mg/Kg showed less than 5% weight loss after 24 h. 48 h after the challenge they regained initial weight.

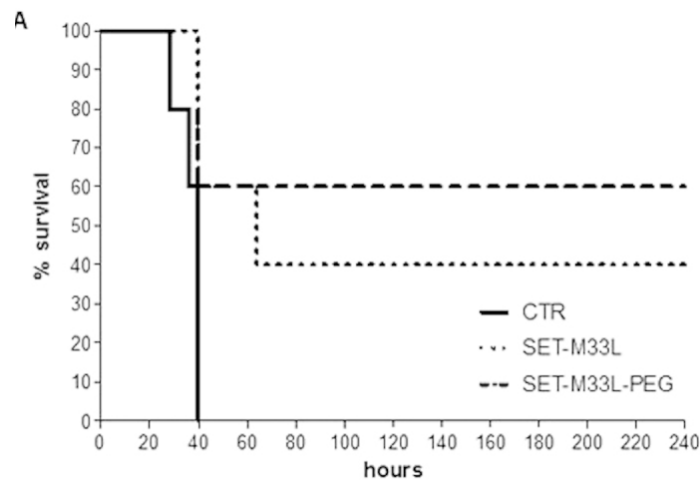


Figure 9 *In vivo* antibacterial activity of SET-M33 peptide in **pneumoniae** model (Brunetti *et al.*, 2016). BALB/c neutropenic mice were injected i.t. with a lethal amount of *P. aeruginosa* PAO-1 and then treated twice i.v. with SET-M33 (5 mg/Kg) 1 and 16 hours post-infection. Percentage survival (y-axis) is plotted as a function of time (x-axis); $p < 0.05$.

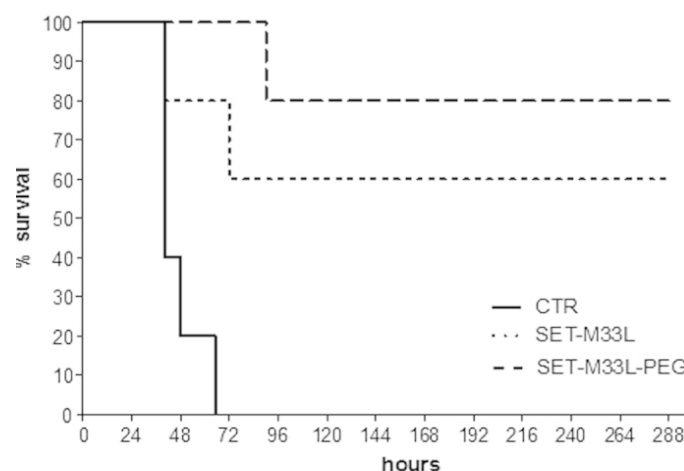


Figure 10 *In vivo* antibacterial activity of SET-M33 peptide in sepsis animal model (Brunetti *et al.*, 2016). 10 BALB/c neutropenic mice/group were injected i.p. with a lethal amount of *P. aeruginosa* PAOI and then treated twice i.v. with SET-M33 (5 mg/Kg), 24 and 72 hours post-infection. Percentage survival (y-axis) is plotted as a function of time (x-axis); $p < 0.02$.

SET-M33 was also evaluated for its *in vivo* efficacy, toxicity, bio-distribution and immunomodulatory effect at a pulmonary level upon inhalation administration in CD-1 mice. After a preliminary atmosphere analysis of the concentration of the peptide, clinical condition, body weight, food consumption, organ weight, macropathology, histopathology and toxicokinetic were observed.

No mortality was reported, and clinical signs were noted only at 23.1 mg/kg/day concentration, as showed in table 2. In addition, the therapeutic effect was also evaluated for the ability of the peptide in terms of inhibition of the cells and cytokines involved in inflammation. Preliminary studies had demonstrated the ability of the synthetic peptide to decrease TNF- α production and Nf-KB signal transduction factor in vitro in cell stimulated by LPS from *P. aeruginosa* and *K. Pneumoniae* (Pini *et al.*, 2010) (Brunetti *et al.*, 2016). In BALB/c mice the count of total cells recorded a strong activity for neutrophils and mononuclear cells and for the reduction of levels of IP-10, MCP-1, MIP1 and TNF- α cytokines. On the contrary, IL-6 was released in a dose-dependent manner (Cresti *et al.*, 2023). SET-M33 activity was also compared to the one of Budesonide, a corticosteroid drug used in clinical practice to reduce lung inflammation, proving its more efficient capability to control inflammatory effect provoked by LPS.

		Male			Female		
		0	4.34	23.1	0	4.34	23.1
		mg/kg/Day	mg/kg/Day	mg/kg/Day	mg/kg/Day	mg/kg/Day	mg/kg/Day
<i>SET-M33-related findings in the lungs</i>							
Inflammation, interstitial	Minimal	0	0	1	1	2	4
	Slight	0	0	4	0	0	2
	Total	0	0	5	1	2	6
Inflammation, granulomatous	Minimal	0	0	2	0	0	3
	Slight	0	0	3	0	0	0
	Total	0	0	5	0	0	3
Infiltrate, inflammatory cell, perivascular	Minimal	0	0	2	0	1	1
	Slight	0	0	0	0	0	1
	Total	0	0	2	0	1	2
Inflammation, alveolar ducts	Minimal	0	0	2	1	0	2
	Total	0	0	2	1	0	2
Fibrosis, alveolar ducts	Minimal	0	0	0	0	0	1
	Total	0	0	0	0	0	1
<i>SET-M33-related findings in the nose/turbinates</i>							
Atrophy/degeneration, olfactory epithelium	Minimal	0	2	1	1	4	0
	Slight	0	4	5	0	2	3
	Moderate	0	0	0	0	0	3

Figure 11 SET-M33-related findings in the lungs, nose/turbinates, larynx and tracheal bifurcation in CD-1 mice after treatment with inhaled peptide at 0, 4.34 and 23.1 mg/kg/day for 1 week. The findings were assigned a severity grade: minimal, slight, moderate, marked or severe (Cresti L *et al.*, 2023).

SET-M33 was also reported in a nanoparticle formulation such as dextran (Falciani *et al.*, 2020). More recently, a poly(lactide-co-glycolide) (PLGA) commercial nanoparticle was also evaluated to improve pulmonary delivery of the peptide. The peptide was loaded and encapsulated upon conjugation with rhodamine B and following a modified emulsion/solvent diffusion technique. PLGA

nanoparticles were conjugated with PEG 2000 Da or 5000 Da. TEM and FALT analysis confirmed nanoparticle shape and surface. In vitro release of the peptide was evaluated with kinetics and aerodynamics experiments. The evaluation of the interactions between the particles and major barrier components such as mucin, bacterial alginates or biofilm was also studied by a Transwell® multiplate assay. The ability to diffuse across these barriers were demonstrated. Antibacterial and antibiofilm activity was also measured and compared with free SET-M33 peptide at different incubation time, using *P. aeruginosa* (ATCC 27853™) as a bacterial model. Results confirmed the antimicrobial and antibiofilm activity of the peptide and revealed a persistent effect over the time, related to the sustained release provide by the nanoparticle. Cytotoxicity and in vivo acute toxicity were finally evaluated showing a reduction in manifest signs and toxic effects compared to the free SET-M33, confirming that the combination of polymeric NPs with this peptide can be considered an ideal antibacterial nanosystem for the development of a novel antibacterial agent (Cresti *et al.*, 2023).

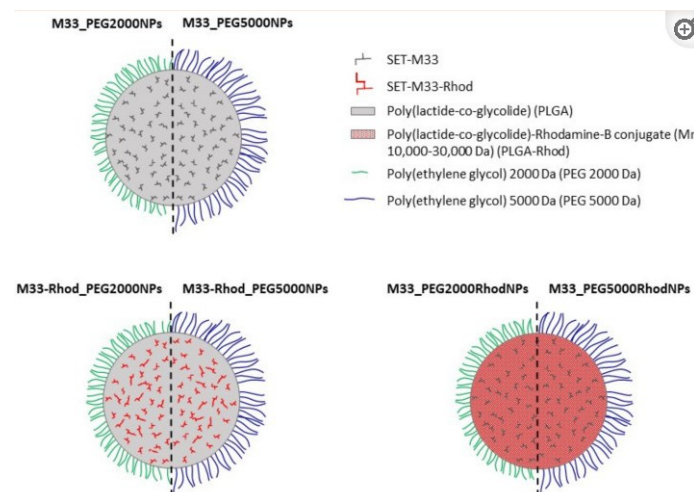


Figure 12 Diagram of the core–shell structure of SET-M33-loaded PLGA NPs, PLGA NPs containing SETM33 conjugated with rhodamine (SET-M33_Rhod), and NPs prepared by employing PLGA conjugated with rhodamine (PLGA-Rhod) (Cresti L *et al.*, 2023).

Peptide-based devices

Conjugation of AMPs on nanomaterials such as resins or membranes gives a potential answer for increment the strength yet decline the toxicity for AMPs. Nano-scale materials have an enormous explicit surface region, adaptable surface functionalization, and remarkable physicochemical properties. Moreover, NPs have a high loading potential for coated molecules due to their large surface area to volume ratio (Jiang *et al.*, 2015), and some nanomaterials have antimicrobial properties of their own (such as silver, zinc oxide, and titanium dioxide) that can adjuvant AMPs activity. Generally, this kind of systems can be divided in inorganic and polymer materials. Inorganic

materials include mesoporous silica particles, titanium, gold, silver graphene and carbon nanotubes, while polymeric materials are represented by fibers, polymer gels and polymer conjugates. NPs are considered the ideal candidate to load and use AMPs. AMPs have multiple functional groups, including amino and thiol which can immobilize them onto nanosurfaces by electrostatic interaction (Yang *et al.*, 2015).

Other strategies include self-assembly immobilization or physical methods to conjugate peptides to membranes or surfaces. For instance, Mohanraj *et al.*, describe a sulfone resin as a smart and effective material for the derivatization with peptides. Peptides and resins are conjugated upon by irradiation with UV-rays, generally used for sterilization. This mechanism allows the formation of interactive free radicals with respect to single polymers which are inert (Mohanraj *et al.*, 2018).

Antimicrobial peptides for water treatment

Antibiotic resistance is one of the major public health problems. By 2050, it is expected to become the widest death cause worldwide, if no preventive measures are universally adopted. Indeed, the environmental distribution of antibiotic resistance genes (ARG) and antibiotic-resistant bacteria (ARB) is growing, and no drugs are easily available for population. The dissemination of these two factors in the environment results from the inappropriate use of antibiotics in human and veterinary sectors, the increase in discharges of pharmaceutical industrial wastewaters, and also the reduced efficiency of wastewater and drinking water treatment plants (Duarte *et al.*, 2022). Additionally, the scarcity of water even in countries with adequate water resources represents a limit access to safe water for drinking and for practicing basic hygiene at home, in schools and in health-care facilities. Therefore, study and development of new efficient water treatment processes and devices is one of the critical strategies to stem antibiotic resistance.

Nowadays, the water purification processes are only based on physiochemical characteristics of water, such as coagulation/flocculation, sedimentation, filtration, disinfection, but the removal of ARB and ARG still remain a challenge (Sanganyado *et al.*, 2019). Some potentially great technology includes nanotechnology-based materials engineered with metallic particles or green biogenic nanomaterials, synthesized or derived from different microorganisms. These latter represent the most possible sustainable solution, but remain to implement for their instability, issues of synthesis and stability as well as manufacturing costs. Examples include nanotubes, nanomembranes, nanoparticles, cellulose, chitins, antimicrobial peptides-based devices. The latter kind of devices has been widely discussed and studied for extracorporeal blood purification in therapy for sepsis as alternative to antibiotic drug administration to kill bacteria, remove endotoxin or cytokine from the bloodstream.

Indeed, antimicrobial peptides can be easily engineered into various nanostructures, and their size, shape, morphology, functional groups, and other features may be tuned according to the antimicrobial applications. However, only few of this have been commercially approved for environmental treatment and drinking water purification.

Some examples include OXiris (Baxter, Meyzieu, France), Cytosorb® (CytoSorbents Corporation, USA), Coupled Plasma Filtration Adsorption (CPFA Bellco, Italy).



Figure 13 Examples of cartridge for water and fluid remediation

In literature several examples are reported for water treatment; Ding *et al.*, functionalized a Fe₃O₄ magnetic nanoparticles with antimicrobial peptides and demonstrated a massive rate of capture and remotion for pathogenic bacteria in water environments such as *E. coli* O157:H7, and a broad spectrum of capture ability towards various bacterial strains. The bactericidal effect is obtained upon interactions with bacteria's outer membrane (Ding *et al.*, 2020). Palmieri *et al.*, immobilize a de novo designed antimicrobial peptide (AMP) RiLK1 on a PLA-surface and demonstrated a relevant antimicrobial activity against waterborne pathogens such as *S. Typhimurium*, and *L. monocytogenes*. The direct conjugation of AMP to the surface is favored by the formation of amide bonds between the carboxyl groups generated on PLA by plasma treatment and the amine groups of the peptide (Palmieri *et al.*, 2020). Another study conducted by Nellore *et al.*, reported the conjugation of the antimicrobial peptide PGLa to carbon nanotubes. In this study, very interestingly, the obtained devices are able to disinfect aqueous solutions from Gram-negative bacteria and to remove toxic heavy metal (Nellore *et al.*, 2015).

Other examples comprise graphene-based membranes (Kanchanapally *et al.*, 2015). These kinds of materials maintain a strong antimicrobial activity against most of the multidrug resistant strains and biofilm formation and exhibited a prolonged efficacy in the biofilm and biofouling eradication. Moreover, the rate of toxicity is strongly decreased compared to the free peptide. Therefore, the use

of NPs derivatized with AMPs can be applied to several fields, from therapeutic to water treatment and environmental remediation. Recently, hollow fiber nanomembranes conjugated with AMPs have been released for water purification. These kits guarantee complete retention of bacteria, viruses, endotoxins and microplastics with a molecular weight greater than 1.5 kDa and a dimension of 0.005 μm .

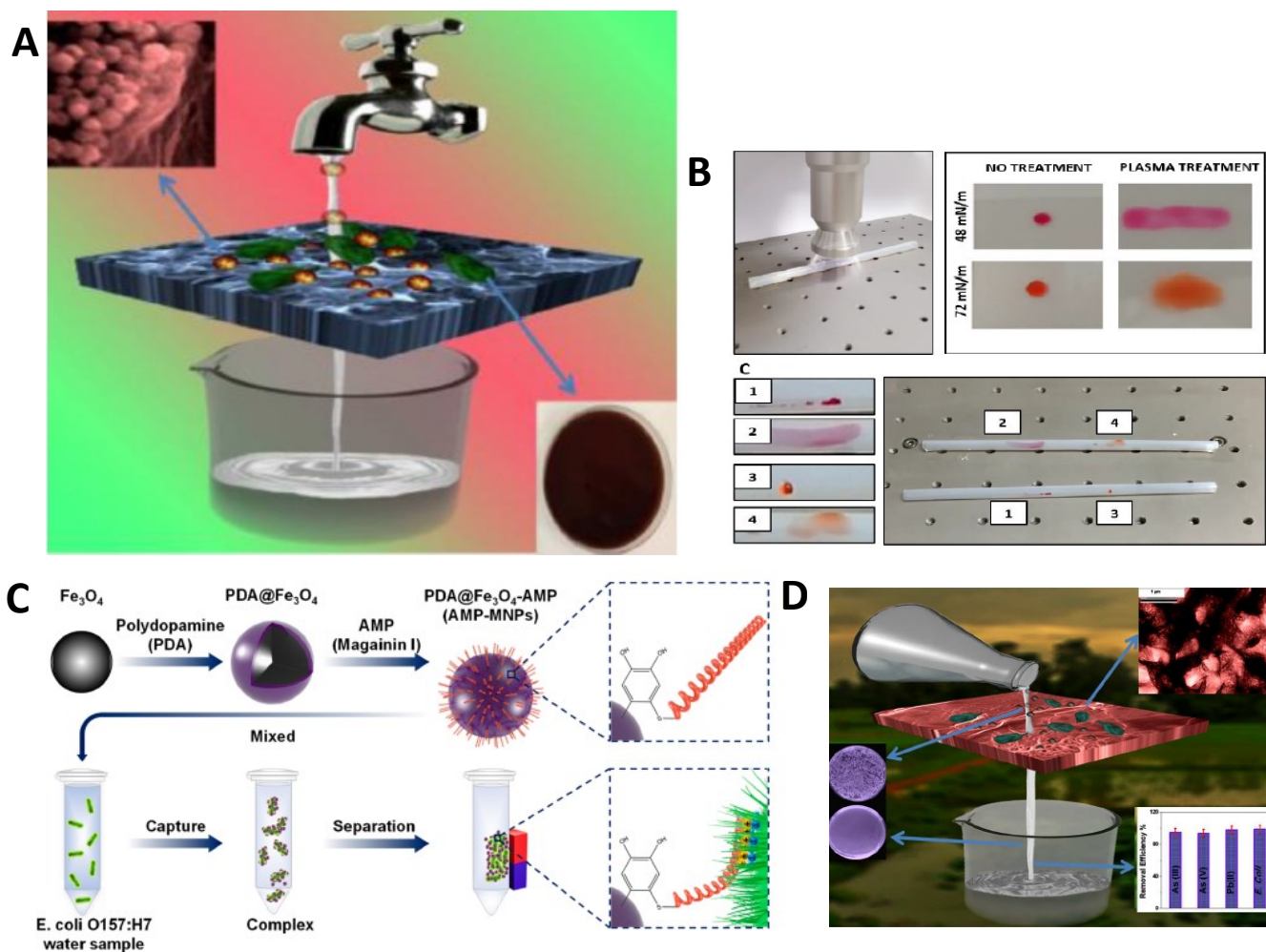


Figure 14 **A** Antimicrobial peptides conjugated to graphene-based membranes (Kanchanapally *et al.*, 2015) **B** Antimicrobial peptide (AMP) RiLK1 CONJUGATED on a PLA-surface (Palmieri *et al.*, 2020) **C** Magainin-functionalized magnetic nanoparticles (AMP-MNPs) consist of magnetic core (Fe_3O_4), polydopamine (PDA)(Ding *et al.*, 2020) **D** PGLa and glutathione-conjugated CNT bridged porous graphene oxide membrane (Nellore *et al.*, 2015).

MATERIALS AND METHODS

SET-M33-CYS synthesis, purification and characterization

SETM33 branched peptide (MAP), was produced by solid phase synthesis using standard 9-fluorenylmethoxycarbonyl (Fmoc) chemistry on Fmoc4-Lys2-Lys- β -Ala Wang resin with activation of the carboxylic group with O-(benzotriazole-1-yl)-N,N,N, N-tetramethyluronium hexafluorophosphate (HBTU)/N, N-disopropylethylamine (DIPEA) on a Syro automated machine (MultiSynTech, Witten, Germany). 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl (Pbf) for Arg, tert-butoxycarbonyl (Boc) for Lys, and tert-butyl ether (tBu)for Ser were used as protecting group for side chains. Fmoc-NH-Cys(Trt)-COOH, Trt (titryl group) was the first amino acid to be added on the resin. Subsequently, Fmoc-NH-PEG(4)-CH₂-CH₂-COOH and Fmoc-Lys(Fmoc)-OH twice were coupled to obtain the lysine core where four KKIRVRLSAs are assembled. The final peptide was cleaved from the resin and treated with TFA (trifluoroacetic acid) containing triisopropylsilane and water (95/2.5/2.5) for its deprotection. Diethylether was used for the precipitation. Reverse phase liquid chromatography (HPLC) was used to purify the crude peptide on a column for medium-scale preparation. A linear gradient form was used for 30 min using 0.1% TFA/water as eluent A and methanol as eluent B. The final product was obtained as trifluoroacetate salts (TFAcetate). Ion exchange was carried out using a quaternary ammonium resin in acetate form (AG1-X8, 100-200 mesh, 1.2 meq/mL capacity, BioRad, Hercules, CA, USA), with a resin: peptide ratio of 2000:1. Resin and peptide were stirred for 1 h, the resin was then filtered off and washed thoroughly. The purity of the peptide was verified reversed phase chromatography on a Jupiter C18 analytical column (Phenomenex, Torrance, CA, USA, 300 Å, 5 μ m, 4.6 $\times$ 250 mm), under the same conditions as for the HPLC purification and by MALDI ToF/ToF mass spectrometry (Ultraflex III, Brucker Daltonics, Bremen, Germany) (Cresti *et al.*, 2022).

Mass spectrometry characterization was carried out using a positive voltage linear acquisition with a 5-20 KDa method. The matrix used was composed of Cyano -4-hydroxycinnamic acid dissolved in a 50 % solution of acetonitrile/water. 2 μ L of matrix and 2 μ L of sample were mixed and plate on MTP 384 target plate for analysis. Ultimately, the peptide was lyophilized by freezing overnight at -20°C in a 5% aqueous solution of acetic acid, and then it was freeze-dried for 48 hours inside a ScanVac freeze drier.

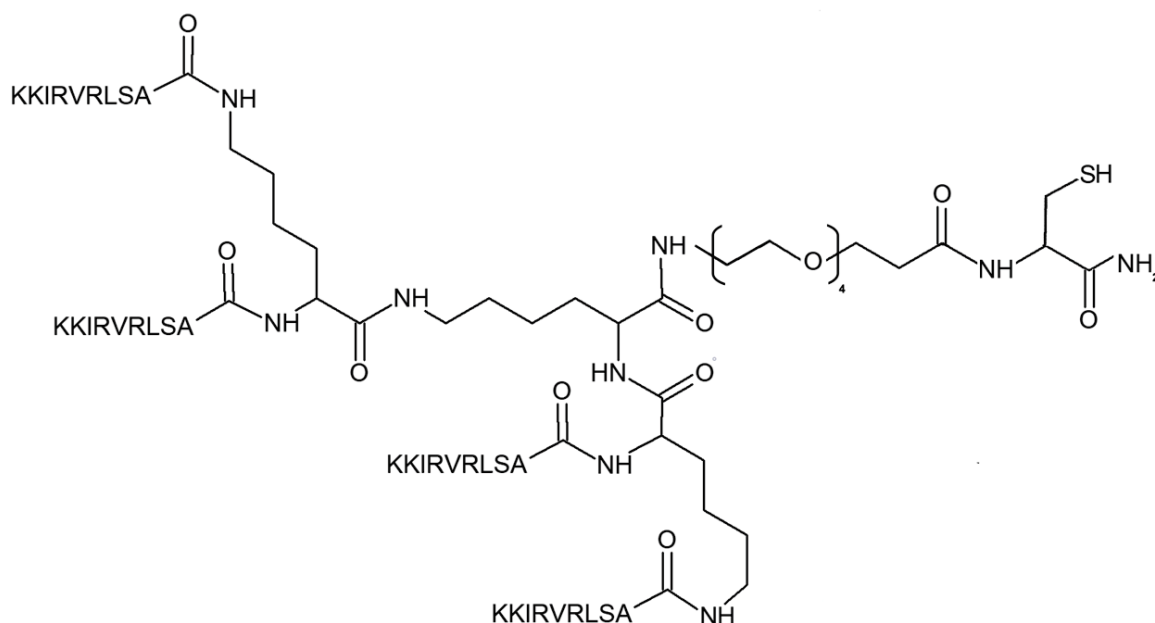


Figure 15 SET-M33 Cys-peptide

SET-M33-CYS conjugation on biocompatible matrix

Tetrabranched SET-M33 peptide has been conjugated to an agarose bead supports by using a Sulfolink Coupling Resin. This kind of resin allow the immobilization of sulfhydryl-containing peptides upon activation with iodoacetyl groups which specifically react with free sulfhydryls. A 12-atom spacer arm is used to reduce steric hindrance and ensure efficient binding interactions with the coupled molecule. The stability of the immobilized peptide is crucial to determine how frequently the column may be used without a reduction in binding power.

Briefly, Sulfolink coupling resin are equilibrate at room temperature and transfer in an empty column at an appropriate volume. Peptide were dissolved at a concentration of 1 mg/ml in coupling buffer (50 mM Tris, 5 mM EDTA-Na; pH 8.5) and added to the column to allow conjugation. The solution was incubated for 45 minutes. This passage was repeated for two rounds. A small amount of the peptide/resin mixture was collected to check the binding rate by measuring the free-peptide fraction present in the flow-through of the conjugation cycles. By using HPLC, the pre- and post- conjugation peptide peak area was evaluated. The column was then washed for 3 times with Wash solution (1.0 M NaCl, 0.05% NaN₃) and then with coupling buffer for two times. A solution of 50 mM L-cysteine-HCl was prepared in coupling buffer and incubate with the beads in the column for 45 minutes to saturate the non-specific sites. Finally, peptide-based agarose beads were equilibrated with PBS (0.05% sodium azide) and aliquoted into columns or devices of different sizes (1ml, 4ml) for the

storage at 4° C. sizes. Devices containing peptide conjugated with beads were washed with PBS before being used.

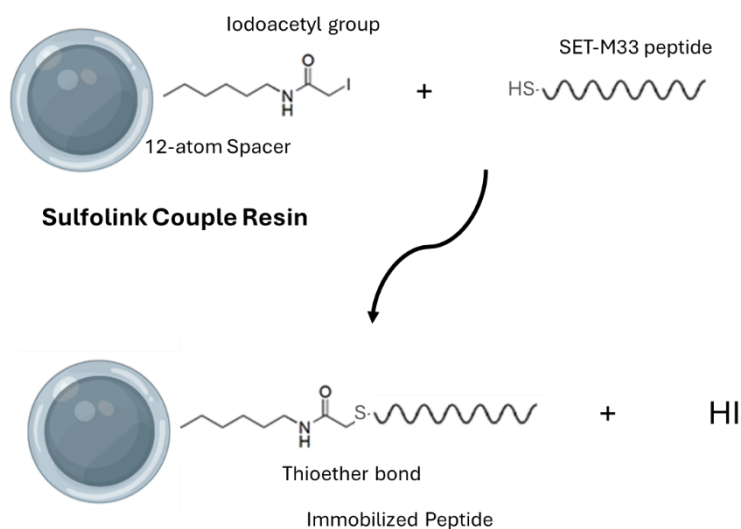


Figure 16 Mechanism of interaction and conjugation of the peptide with agarose beads.

Quantification of LPS by Limulus Amebocyte lysate test

The Pierce Chromogenic Endotoxin Quant Kit (ThermoFisher Scientific) is a quantitative and highly efficient assay which exploits amebocyte blood lysate of horseshoe crab (*Limulus polyphemus*) to quantify bacterial endotoxin (LPS) in protein, peptides, antibodies or nucleic acid samples. Upon the encounter of LPS, a series of cascade reactions are triggered and results in enzymatic activation of Factor C, B and pro-clotting enzymes, contained in Limulus amoebocyte lysate (LAL). The following addition of the colorless substrate Ac-Ile- Glu-Ala-Arg-pNA results in yellow color, for the cleavage of p-nitroaniline (pNA), catalyzed by activated enzymes. Amebocytes clotted in the presence of low levels of endotoxin. 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. A standard curve is prepared by diluting the endotoxin stock of *E. coli* O111:B4 (0.1-1.0 EU/mL). The 1.0 EU/ml dilution is prepared from a stock of 10 EU/ml.

Briefly, 50 µL of each sample, is added to the wells of the plate. Then 50 µL of reconstituted Limulus amebocyte lysate are added, keeping the plate at 37°C and gently mixing. After 12 minutes of incubation, 100 µL/well of pre-warmed chromogen substrate are added and incubated for 6 minutes. 50 µL/well of Stop Solution (25% acetic acid) is added. Absorbance values are obtained using the

plate reader (Gen5 software) at a wavelength of 405 nm. The optical density values are converted to EU/mL concentration based on the standard curve using the software CurveExpert.

Quantification of LTA by ELISA

The LTA presence in samples has been identified by using the commercial LTA ELISA kit (MyBioSource). This assay is suitable and highly specific to detect LTA in fluids and supernatants. It is based on a “double antibody sandwich technique” enzyme-linked immune-sorbent assay technology which allow to identify more than two epitopes of a target analyte.

To provide a reference point a standard curve (0.312-20 ng/mL) was prepared using a lipoteichoic acid from *S. aureus* (control standard) in Sample/Standard dilution buffer, and serially diluted.

Briefly, 100 µl of sample is added to micro-plate wells pre-coated with LTA antibody. After 90 minutes incubation at 37°C and plates are washed twice. 100 µl of LTA detection biotinylated antibody are added. Plates are now incubated for 60 minutes at 37°C and washed three times. 100µl streptavidin-horse radish peroxidase conjugate with Streptavidin (HRP-Streptavidin) are added followed by incubation (30minutes at 37°C in the dark). After 5 washing, 100 µl of chromogenic 3,3',5,5'-tetramethylbenzidine (TMB) solution are added and the sample is incubated at 37°C and protected from light. The HRP enzyme reaction is stopped within 30 minutes by addition of 100 µl of an acid stop solution. The intensity of color developed is proportional to the concentration of the lipoteichoic acids present in the sample. The readout is obtained at 450nm within 10 minutes using a plate reader. All sample was processed in duplicate.

Bacterial colony counting

Bacterial cells are usually quantified by colony forming unit (CFU) which provide a direct evaluation of viable cells. It relies on the number of single colonies that grow on a plate of selective media. Its standard unit measures the number of viable microorganisms present in 1 ml of culture and determined by serial dilution and following spread plating method.

Gram-negative (*E. coli* and *L. pneumophila*) 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. Briefly, viable bacteria were preliminary isolated from a saline solution and subsequently from sterile water. A pre-culture batch inoculum was incubated at 37°C 220 rpm

overnight. The batch inoculum was then diluted to 1:100 by transferring 1 mL of the previous dilution into a tube containing 9 mL of sterile medium. The liquid culture was incubated until O.D. 1 (600nm) was reached on the spectrophotometer. The sample was then diluted 1:25 and then incubated 2 h at 100 rpm in a column with the device derivatized with M33 peptide. The ability to inhibit bacterial growth was quantified by plating 100 μ L of the samples onto Petri dishes with agarized medium after a preliminary serial dilution 1/10 to 1/10¹⁰. Gram-negative bacteria (*E. coli* 0111:B4) and *L. pneumophila* (ATCC 33152) were plated onto Luria broth agar and BCYE agar Medium respectively while Gram-positive bacteria (*S.aureus* USA300) were plated in Mueller-Hinton agar.. The samples included: not incubated sterile water (negative ctrl), sterile water incubated in column with the device and without the device. After overnight incubation (72 hours for *L. pneumophila*) at 37°C, colony forming units were measured.

Statistical analysis

All statistical analyses were performed using GraphPad Prism version 6.01 for Windows (GraphPad Software, San Diego, CA, USA). Student's t test was used to examine geometric means between two different conditions.

RESULTS

SET-M33-CYS Synthesis, purification and characterization

SETM33 peptide was modified by the addition of a free sulfhydryl group. This residue is essential to increase the bond strength of the peptide on the agarose beads and to form a disulfide bridge which allows the conjugation to the agarose based biocompatible resin.

Subsequently, the peptide was purified and characterized using reversed-phase HPLC (Jupiter C18 Phenomenex) and mass spectrometer Q-Exactive Plus (ThermoFisher) respectively.

The final HPLC profile revealed a purity index higher than 92% with the retention time of peptide of 22.19 minutes (Figure 17).

The MS analysis shows a peak at molecular mass of 4960,2 Da, which is aligned to the expected molecular mass (C₂₂₀H₄₂₁N₇₇O₄₉S MW 4960.24 Da) (Figure 18).

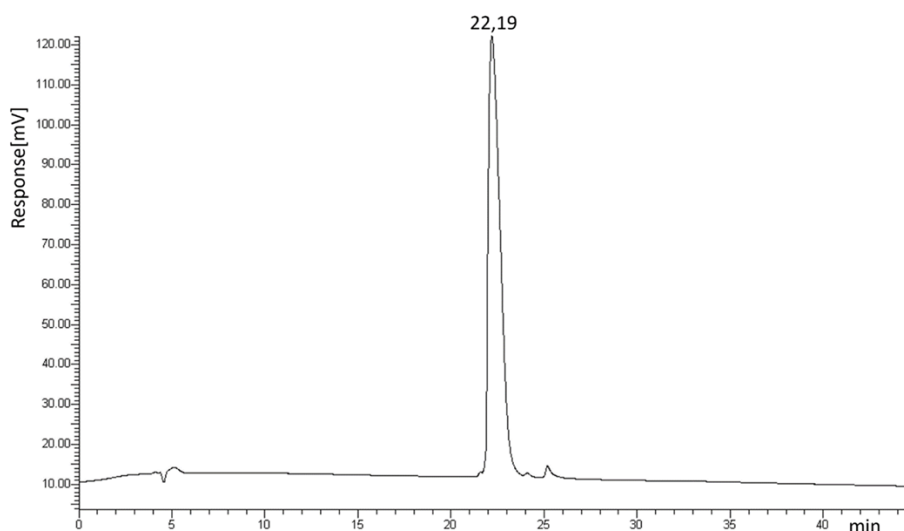


Figure 17 HPLC profile of SET-M33-Cys. The method was: Phenomenex analytical Column Jupiter C18 300Å 5µm 4.6 x 250mm, eluent A: 0.1%TFA / H₂O, eluent B Acetonitrile, gradient from 83:17 A/B to 70:30 A/B in 40min.

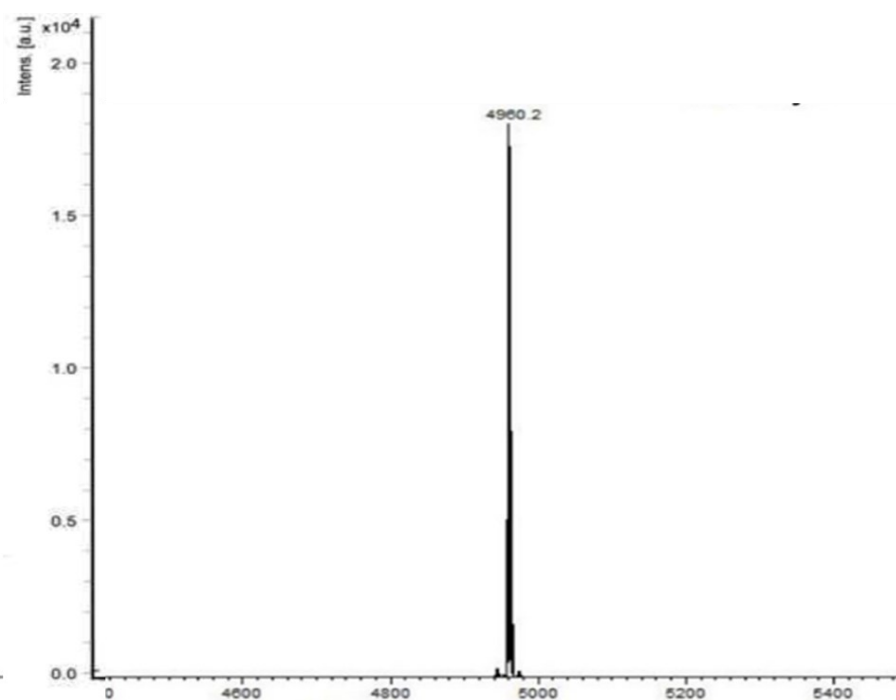


Figure 18. B. MALDI-TOF mass spectrometry profiles of SET-M33Cys

SET-M33-CYS conjugation with agarose beads

SET-M33-Cys peptide was coupled to the SulfoLink® Coupling resin with a covalent bond result of a stable thioether linkage (Figure 19). Indeed, this kind of agarose resin presents an iodoacetyl group with a 12-atom spacer (Figure 16) which at 7.5-9.0 pH undergoes a nucleophilic substitution of iodine with a sulfur atom from a sulfhydryl group (-SH) of the sidechain of cysteine in the peptide C-terminus.

The binding efficiency was then evaluated by HPLC test, comparing the flow-through collected during the conjugation steps of the peptide with and without the agarose beads. Results (not reported) confirm the successful binding of the peptide to the beads.

Then, peptide-conjugated agarose beads have been stored within a cartridge of various volumes (Figure 20).

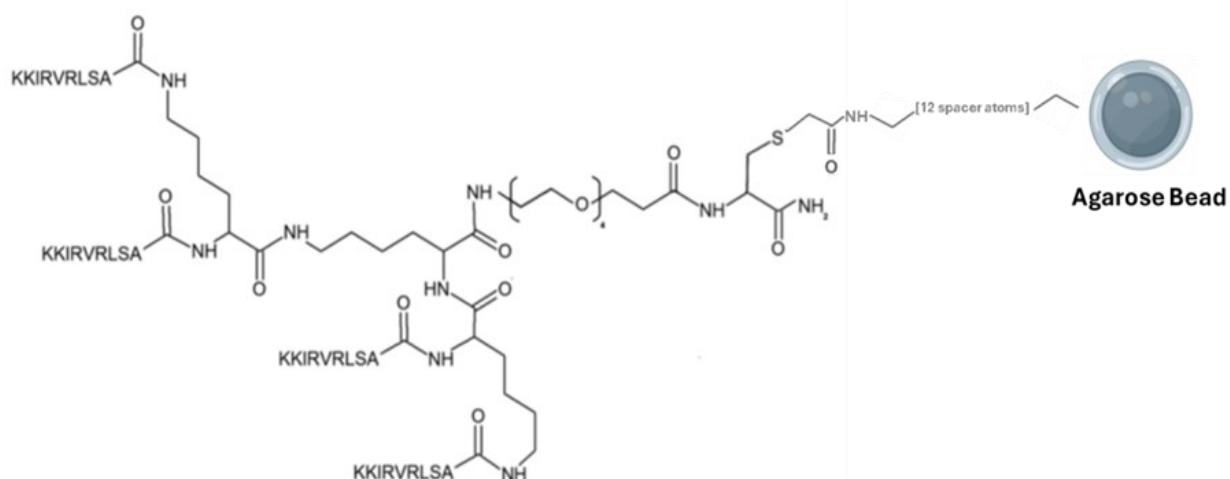


Figure 19 SET-M33-Cys peptide conjugated to agarose bead

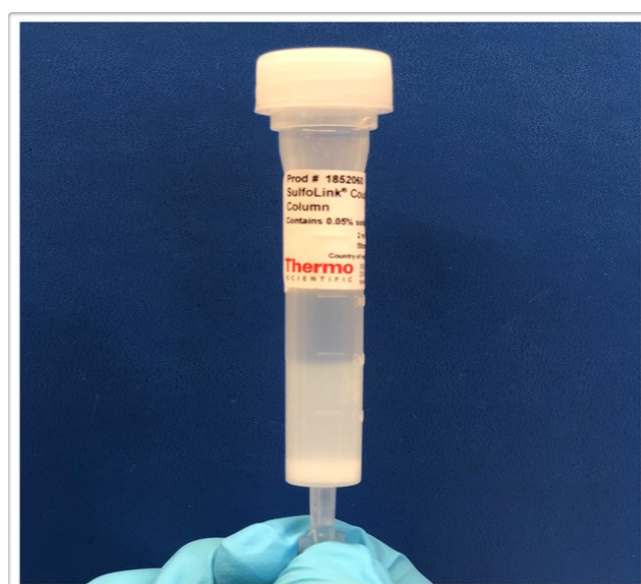


Figure 20 2 ml SulfoLink® Coupling resin cartridge

LPS removal from water samples

A 5 mL column, which contained 2 mL of resin (agarose beads) conjugated with 2 mg of SET-M33-Cys peptide was used. 2 ml of sterile drinking water were mixed with 75 Eu/mL of LPS from *E. Coli* O111:B4 (Sigma, St. Louis, MO) and incubated with the agarose resin in the cartridge at room temperature conditions for two hours in constant swirl. After that, the sample was collected and LPS content was determined by the LAL test.

The resin not derivatized with the peptide was used at the same concentration and condition as negative control. Moreover, a sterile water sample without LPS was used as second negative control.

As reported in Figure 21, the biodevice reduces up to 88% of LPS level compared to the resin not conjugated with SET-M33-Cys. The second negative control reported an intrinsic 0.5 % reduction of LPS.

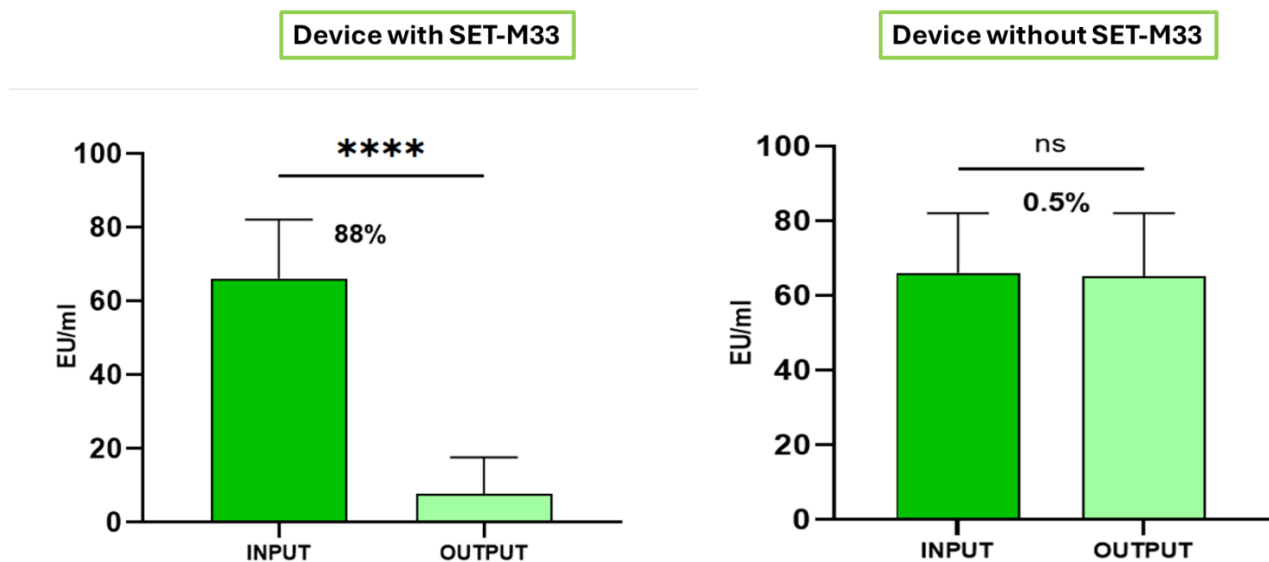


Figure 21 *E. coli* (O111:B4) LPS removal from sterile water. **A** The sample was passed through the resin cartridge coupled with SET-M33-Cys. **B** The sample was flown through the cartridge containing the resin without SET-M33-Cys. The Y axis displays the amount of endotoxin (LPS) per milliliter. The INPUT histograms represent the water containing LPS before the passage through the column. The OUTPUT histograms correspond to the same water sample after the passage through the column. Paired t-test was calculated for the output with respect to the input (****p-value ≤ 0.0001 , ns p-value > 0.05)

LTA removal from water

For this experiment, 2 ml of sterile drinking water mixed with LTA from *S. aureus* (MyBiosource) (500 pg/mL) were incubated at room temperature for two hours under constant rocking with a 5 ml cartridge containing 2ml of SET-M33-Cys-loaded resin. Upon the collection of the sample, LTA level was quantified by the Human LTA ELISA Kit, as described above.

The same amount of resin not loaded with SET-M33-Cys was incubated with water/LTA mixture and used as a negative control; LTA amount was measured as above. As for LPS removal, sterile drinking water only was used as further negative control.

As reported in Figure22, the conjugated resin removes up to the 92% of LTA from *S. aureus* whilst the unloaded resin has an intrinsic removal ability of 14%.

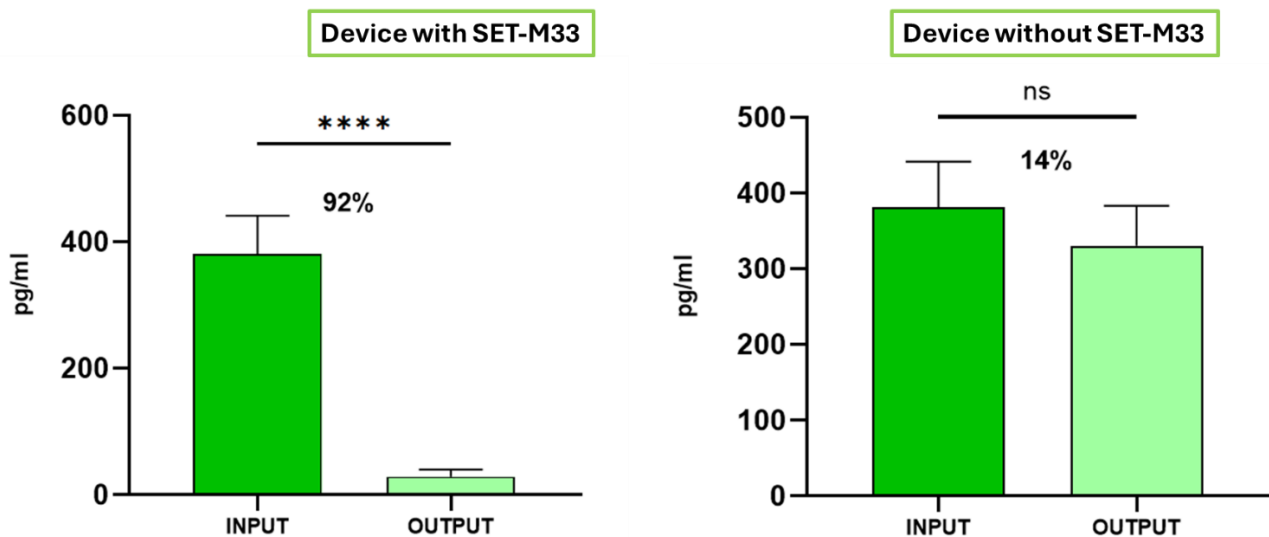


Figure 22 *S. aureus* LTA removal from water. **A** The sample was passed through the resin cartridge coupled with SET-M33-Cys. **B** The sample was flown through the cartridge containing the resin without SET-M33-Cys. The Y axis displays the amount of LTA per milliliter. The INPUT histograms represent the water containing LTA before the passage through the column. The OUTPUT histograms correspond to the same water sample after the passage through the column. Paired t-test was calculated for the output with respect to the input (****p-value ≤ 0.0001 , ns p-value > 0.05)

Removal of live Gram-negative and Gram-positive bacteria from water

The biodevice was also tested for its ability to remove live bacteria from fluids. Tests have been performed by using the device resin conjugated with SET-M33-Cys and non-functionalized with the peptide (CTRL). The presence of living cells has been analyzed before (INPUT) and after (OUTPUT) the loading of the sample onto the device. Several strains have been used, including *E. coli* ATCC 37780 (0111: B4), *S. aureus* USA300 and *L. pneumophila* ATCC 33152. The strains were preliminary isolated from a saline solution (not reported) and subsequently from sterile drinking water after an overnight incubation. After the passage through the cartridge 100 µl of solution has been plated in LB agar, MHA and BCYE agar plates respectively. The plates were incubated overnight at 37°C.

The data obtained reflect a relevant decrease of bacterial cells for the treatment with the column with the immobilized peptide both for Gram-negative and Gram-positive (Figure 23-24-25).

Water solutions incubated in the column with SET-M33-Cys peptide (M33) reported a significant lower number of bacteria when compared to the sterile water only both incubated (CTRL) and not incubated (INPUT) in the column.

For both *E. coli* a reduction of 99 % was observed while *S. aureus* was inhibited for 98 %. *L. pneumophila* growth was reduced of 99 %. In the controls (water incubated in the peptide-free columns) a small retention of bacteria was observed. This event may be explained by the entrapment of some bacteria in the agarose beads due to the unsterile conditions of the resin sedimentation steps. Particularly, a relevant number of colonies was highlighted in the CTRL of *S. aureus* probably due to the absence of the antimicrobial peptide (Figure 24).

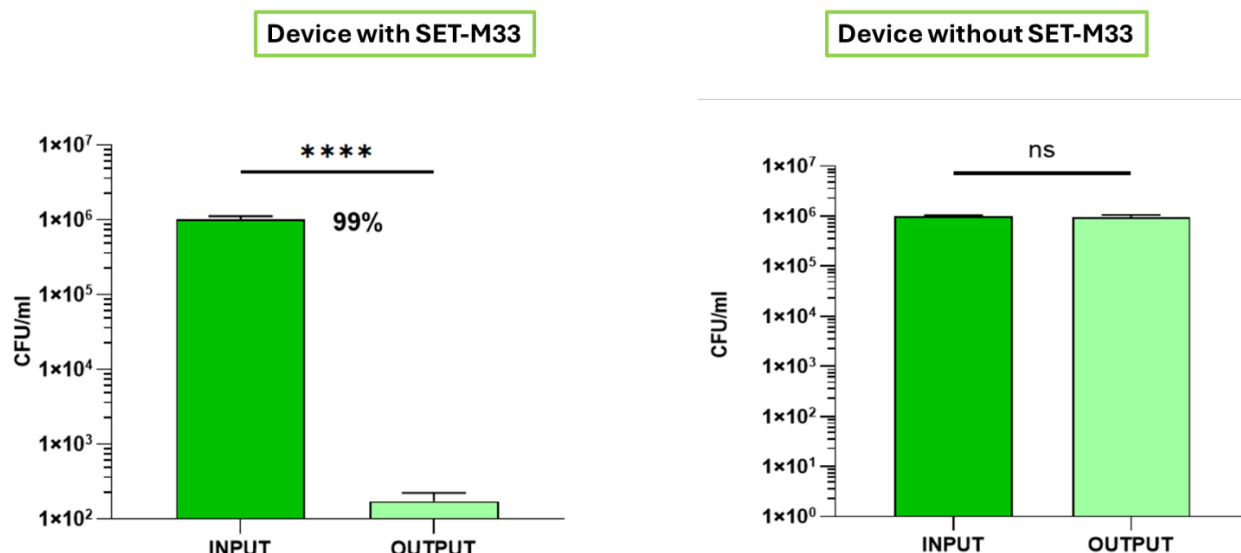


Figure 23 Removal of *E. coli* (O111:B4 serotype) from drinking water. The sample was passed through the resin cartridge coupled with SET-M33 Cys. **A** represents results for the devices with SET-M33, **B** represents results for the device without SET-M33. The Y axis displays CFU per milliliter. The INPUT histograms represent the water containing bacteria before the passage through the column. The OUTPUT histograms correspond to the same water sample after the passage through the column. Percentage is indicated as bars above the histograms. Paired t-test was calculated for the output with respect to the input (****p-value ≤ 0.0001 , ns p-value > 0.05)

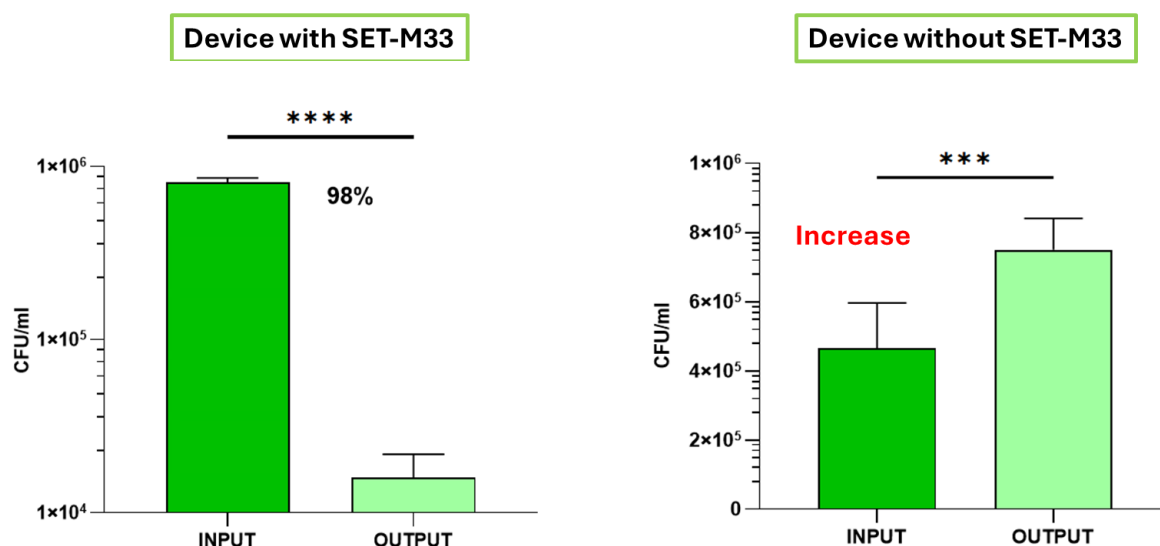


Figure 24 Removal of *S. aureus* (USA300) from drinking water. The sample was passed through the resin cartridge coupled with SET-M33 Cys. **A** represents results for the devices with SET-M33, **B** represents results for the device without SET-M33. The Y axis displays CFU per milliliter. The INPUT histograms represent the water containing bacteria before the passage through the column. The OUTPUT histograms correspond to the same water sample after the passage through the column. Percentage is indicated as bars above the histograms. Paired t-test was calculated for the output with respect to the input (****p-value ≤ 0.0001 , *** p-value ≤ 0.001)

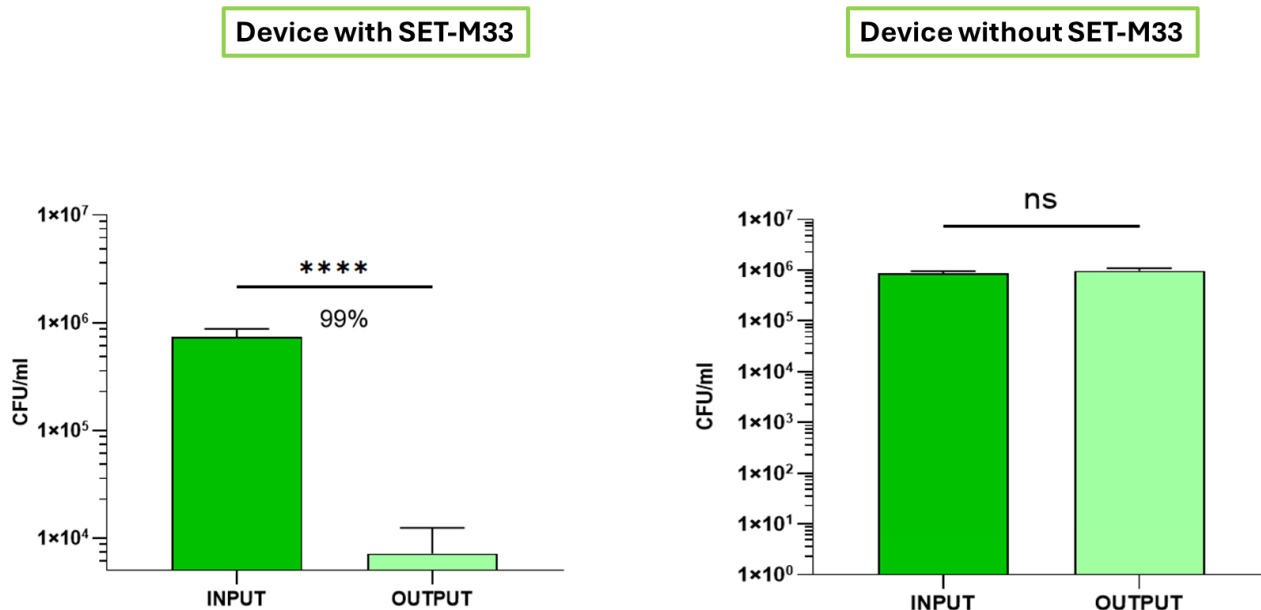


Figure 25 Removal of *L. pneumophila* (ATCC 33152) from drinking water. The sample was passed through the resin cartridge coupled with SET-M33 Cys. **A** represents results for the devices with SET-M33, **B** represents results for the device without SET-M33. The Y axis displays CFU per milliliter. The INPUT histograms represent the water containing bacteria before the passage through the column. The OUTPUT histograms correspond to the same water sample after the passage through the column. Percentage is indicated as bars above the histograms. Paired t-test was calculated for the output with respect to the input (****p-value ≤ 0.0001 , ns p-value > 0.05).

DISCUSSION

The world health organization (WHO) lists antibiotic resistance as one of the biggest global public health concerns, particularly because of the rapid spread of multidrug drug resistance (MDR) bacterial pathogens, which are able to evade standard antibiotic treatments and cause a growing variety of infections as well as hospital mortality. Additionally, the shortage of clean drinking water is listed as one main problems in poor countries due to the limit access to adequate safe water for drinking and for practicing basic hygiene at home, in schools and in health-care facilities.

The buildup of antibiotics in the environment and wastewater plays a significant role in antibiotic resistance genes dissemination. Because antibiotic-resistant bacteria are not eliminated by conventional water treatment, antibiotics and bacteria cannot be completely eradicated from the water, therefore they can spread along drinking water treatment plants.

In recent years, an increase in infections caused by multidrug-resistant bacteria has been observed. One of the main consequences of bacterial infections and particularly of AMR infection is sepsis which often manifest as a systemic syndrome and can result in organ failure and death. Sepsis has always been under attention for the complexity and the consequences for the health care systems. Therefore, it is more necessary than ever to find alternative solutions to counter this problem.

Modern water purification processes are only based on physiochemical characteristics of water, such as coagulation/flocculation, sedimentation, filtration, disinfection, but the removal of ARB and ARG still remains a challenge.

The effectiveness of a wide range of devices, including those based on antimicrobial peptides, cellulose, chitins, nanotubes, nanomembranes, and nanoparticles, is still being investigated. However, many drawbacks are reported such instability, issues of synthesis and manufacturing costs.

Antimicrobial peptides are very efficient molecules in part already employed in medical application as alternative or by enhancing classical antibiotic treatment. However, many drawbacks have been highlighted like side effects, low biocompatibility and bioavailability which limited their releases their commercial use.

Thus, it is necessary to explore new applications for AMPs like the development of devices both for medical use and environmental remediation to exploit their potential antimicrobial activity. Several antimicrobial based devices have been already released for clinical use; in water remediation field some examples are only reported in literature.

Due to their positive net charge, AMPs can be aspecifically attracted by negative net charge of bacterial membranes and other anionic surface structures such as LPS in Gram-negative bacteria and LTA in Gram-positive bacteria. By this interaction with bacterial components, bacterial lysis is induced, and it also provides immunomodulatory activity as it sequesters endotoxins not allowing them to stimulate the immune system.

SET-M33 is an antimicrobial peptide isolated by phage display technology against *E. coli*, which showed an excellent antimicrobial activity, anti-inflammatory activity through selective neutralization of LPS, low hemolytic activity, lack of immunogenicity, and the ability to eradicate biofilms. Therefore, it was selected as a potentially good candidate for the development of a peptide-based device to use in water remediation processes.

Preliminary results of the antimicrobial peptide SET-M33 immobilized to an agarose bead resin revealed:

- the peptide's capability to significantly reduce LPS from *E. coli* and LTA from *S. aureus*, showing a significant decrease in both bacterial toxins levels. SET-M33 was able to retain LPS and LTA in drinking water treated with bacterial toxins, with a binding percentage of 92% and 88%, respectively.
- the retention of living bacteria in drinking water contaminated with each bacterial suspension. Particularly, the device has been tested for *E. coli*, *S. aureus* and *L. pneumophila* showing the ability to inhibit bacterial growth up to 99%.

Based on the current results the next steps could analyze:

- the stability at different conditions (e.g. different temperature, pressure, waterflow) to guarantee good efficacy even after long periods after production.
- efficacy against other relevant bacterial strains mainly associated with water contamination and infections.
- scale-up of the device for its application on large scale for the purification of water based on the usage. For example, domestic use or wastewater purification from industry, hospitals or sewers.
- research for a new technology or material to develop as an alternative to agarose-based filters.
- analysis of its sustainability in terms of cost of production.

TASK 2

INTRODUCTION

Coronaviruses

Coronaviruses (CoVs) are clinically significant pathogens that infect livestock, mice, birds, and finally humans. They are included in the family *Coronaviridae*, under the order *Nidovirales*. CoVs are phylogenetically divided into four genera: the alpha, beta (SARS-CoV 1 and 2), gamma, and delta coronaviruses. Alpha and beta coronaviruses mostly have mammalian species as hosts, while gamma and delta coronaviruses primarily infect avian species (Corman *et al.*, 2018). The first clinically relevant CoVs epidemic since 2002 has been severe acute respiratory syndrome (SARS), caused by a new human coronavirus (SARS-CoV-1). Later on in 2012, MERS-CoV broke out in Saudi Arabia, causing Middle East Respiratory Syndrome (MERS). Finally, in December 2019 in Wuhan, the Hubei Province, China, a new pathogen, cause of a mysterious pneumonia, was identified and defined SARS-CoV-2, responsible of coronavirus disease 2019 (COVID-19). In March 2020, the rapid spread of the virus brought World Health Organization (WHO) to declare state of emergency and consider COVID-19 a pandemic. Latter hypotheses suggest that this CoV propagated from fruit bats to an intermediate host (e.g., the Himalayan palm civet for SARS-CoV-1 and the dromedary camel for MERS-CoV), and then spillover to humans. This thesis was supported by the fact that the greatest diversity of CoVs has been found in bats. (Alyami *et al.*, 2020).

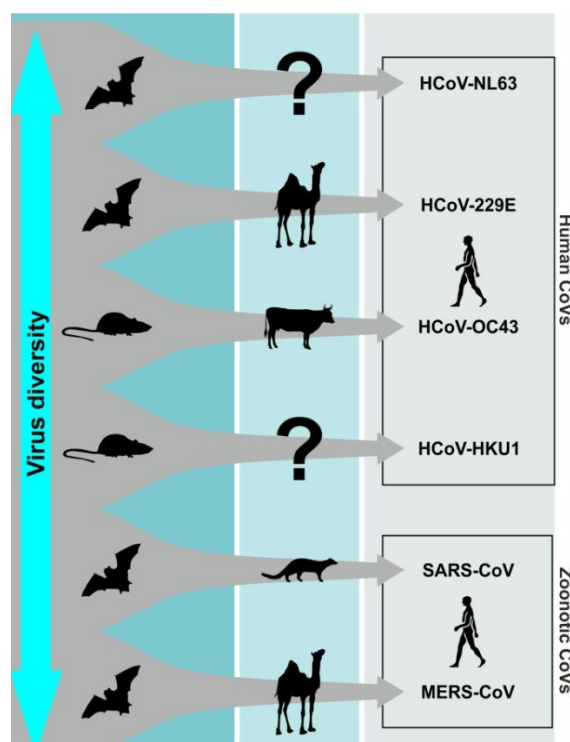


Figure 26 Summary diagram of the animal groups representing natural hosts and the putative intermediate hosts for the six CoVs found in humans.

Coronaviruses can cause localized infections in the respiratory and in the intestinal tract. Symptoms can range from mild to severe and may include fever, cough and difficulty breathing. Current evidence suggests that the virus spreads mainly between people who are in close contact with each other, for example at a conversational distance. The virus can spread from an infected person's mouth or nose in small liquid particles when they cough, sneeze, speak, sing or breathe. Another person can then contract the virus when infectious particles, that pass through the air, are inhaled at short range (this is often called short-range aerosol or short-range airborne transmission) or if infectious particles come into direct contact with the eyes, nose, or mouth (droplet transmission) (Kaye *et al.*, 2020).

All viruses in the Coronaviruses order are enveloped, non-segmented positive-sense RNA (+ssRNA) viruses, ranging from 80 to 160 nm in diameter. They all have very large genomes for RNA viruses, which contain from 27 to 33.5 kilobase (kb) and are the biggest genomes of mammalian viruses. The genome of CoVs contains 5'-cap structure and a 3'-poly-A tail; moreover, it encloses a 5'-leader-UTR-replicase-S (Spike), E (Envelope)-M(Membrane)-N (Nucleocapsid) and a 3'UTR-poly (A) tail (Pal). Usually at least six open reading frames (ORFs) are part of the CoV genomes. Two of these are ORF1a and ORF1b which overlap and represent two-thirds of the genome. Their translation produces two large polypeptides, which undergo co- and post-translational modifications and result in non-structural proteins (NSPs). The rest of the genome encodes for four main structural proteins: Spike (S), membrane (M), envelope (E), and nucleocapsid (N) proteins, necessary for virion assembly and infection. Other common features within the Nidovirales order include: (1) expression of downstream genes by synthesis of 3' nested sub-genomic mRNAs; (2) several unique or unusual enzymatic activities encoded within the large replicase–transcriptase polyprotein; (3) expression of many non-structural genes by ribosomal frameshifting; and (4) a highly conserved genomic organization, with a large replicase gene preceding structural and accessory genes (Yadav *et al.*, 2021). The major differences within the Nidovirus families are in the number, type, and sizes of the structural proteins. These differences cause significant alterations in the structure and morphology of the nucleocapsids and virions. Amino acid substitutions of some NSPs and S proteins contribute towards virulence and transmission (Angeletti *et al.*, 2020).

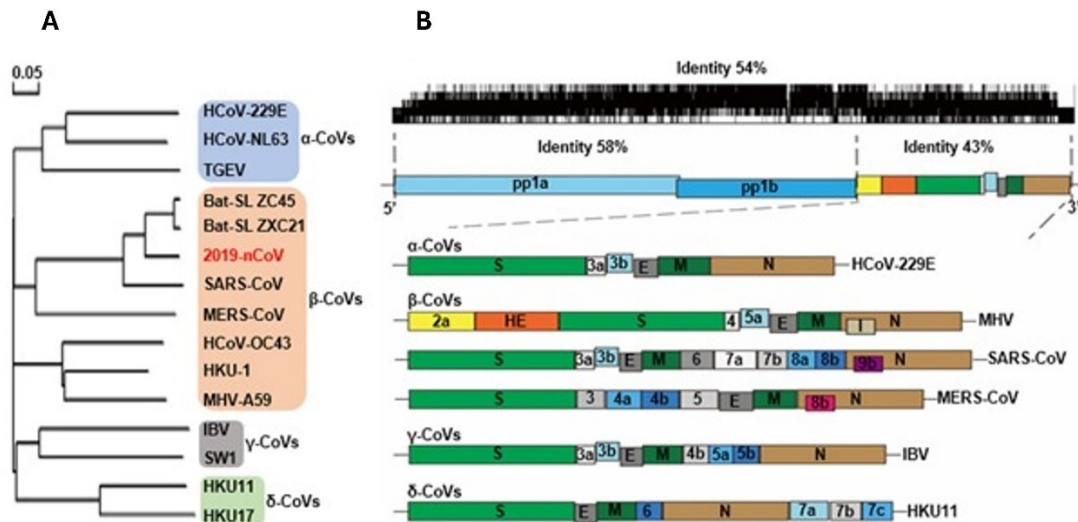


Figure 27 The genomic structure and phylogenetic tree of coronaviruses. A. The phylogenetic tree of representative CoVs, with the new coronavirus 2019-nCoV highlighted in red. B. The genome structure of four genera of coronaviruses. Pp1a and pp1b represent the two long polypeptides that are processed into 16 nonstructural proteins. S, E, M, and N indicate the four structural proteins Spike, envelope, membrane, and nucleocapsid. 2019-nCoV, 2019 novel coronavirus; CoVs, coronavirus; HE, hemagglutinin-esterase. Viral names: HKU, coronaviruses identified by Hong Kong University; HCoV, human coronavirus; IBV, infectious bronchitis virus; MHV, murine hepatitis virus; TGEV, transmissible gastroenteritis virus (Chen *et al.*, 2020)

COVID-19

In late 2019 a novel coronavirus designated SARS CoV-2 emerged in the city of Wuhan, China, and cause an outbreak of unusual viral pneumonia. Since the virus is highly transmissible, this novel coronavirus disease, also known as coronavirus disease 2019 (COVID-19), has rapidly spread throughout the world. Based on systematic reviews and meta-analyses in studies investigating infections with ancestral strains of SARS-CoV-2, the incubation period of COVID-19 is on average, five to six days, with most studies reporting a range of two to 14 days. The origin of this outbreak was associated with the Wuhan Wholesale Seafood Market, where not only seafood is traded, but also exotic fauna. This outbreak has overwhelmingly surpassed SARS and MERS in terms of both the number of infected people and the spatial range of epidemic areas. The outbreak of COVID-19 has posed an extraordinary threat to global public health. On May 2023 the WHO declared the ceasing of the emergency state for the COVID-19 pandemic (Zhu *et al.*, 2020) (Hu *et al.*, 2020) (Wang *et al.*, 2020).

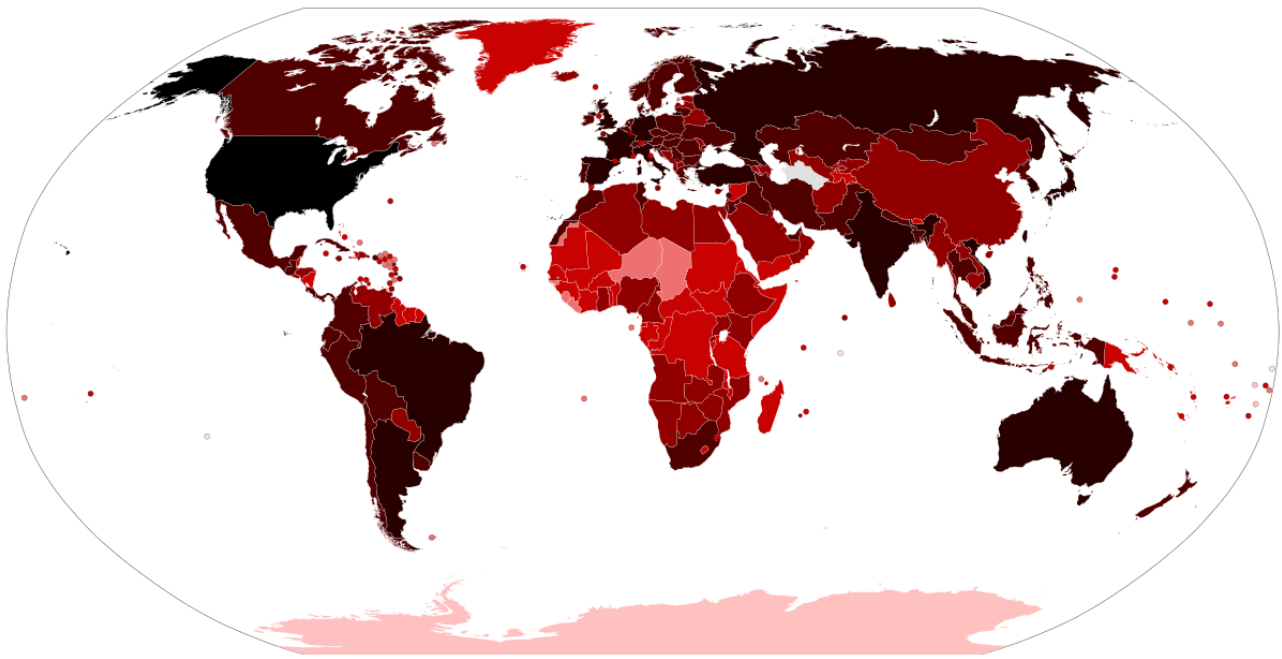


Figure 28 Map of the COVID-19 verified number of infected per capita as of 20 November 2022 (World Health Organization).

As a member of Coronaviruses family, the genome size of SARS-CoV-2 is approximately 29.9 kb (Lu *et al.*, 2020). It contains 14 ORFs and encodes 29 viral proteins. While structural and nonstructural genes remain relatively conserved between coronaviruses, the accessory genes vary (Agrahari *et al.*, 2020). Approximately two-third of the 5' end of the genome encodes two overlapping polyproteins: pp1a and pp1ab. These two polyproteins are digested by two viral proteases into 16 non-structural proteins, which are essential for viral replication and transcription.

The nonstructural proteins include: NSP1 mediates RNA processing and replication. NSP2 modulates the survival signaling pathway of host cells. NSP3 is believed to separate the translated protein. NSP4 contains transmembrane domain 2 (TM2) and modifies ER membranes. NSP5 participates in the process of polyprotein during replication. NSP6 is a presumptive transmembrane domain. The presence of NSP7 and NSP8 significantly increased the combination of NSP12 and template-primer RNA. NSP9 functions as an ssRNA-binding protein. NSP10 is critical for the cap methylation of viral mRNAs. NSP12 contains the RNA-dependent RNA polymerase (RdRp), which is a critical composition of coronavirus replication/transcription. NSP13 binds with ATP and the zinc-binding domain in NSP13 participates in the process of replication and transcription. NSP14 is a proofreading exoribonuclease domain. NSP15 has Mn (2+)-dependent endoribonuclease activity. NSP16 is a 2'-O-ribose methyltransferase (Low *et al.*, 2022). There is some evidence about NSP-mediated effects on splicing, translation, and protein trafficking to inhibit host defenses (Banerjee *et al.*, 2020). Upon

SARS-CoV-2 infection, NSP16 binds mRNA recognition domains of the U1 and U2 snRNAs to suppress mRNA splicing. NSP1 binds to 18S ribosomal RNA in the mRNA entry channel of the ribosome to interfere with the translation of mRNA. NSP8 and NSP9 binds to the 7SL RNA which locates at the Signal Recognition Particle to disrupt protein trafficking to the cell membrane (Banerjee *et al.*, 2020) (Yang *et al.*, 2021).

The four canonical structural proteins are encoded by four ORFs at the 3' terminus. These include nucleocapsid (N), Spike (S) protein, membrane (M) protein, envelope (E) protein which are responsible for virion assembly and for the suppression of the host immune response. Between these structural genes other accessory genes lie such as *orf3a* and *orf7a*, which encode for proteins involved in regulating viral infection but may not be incorporated into the virion. (Chen *et al.*, 2020) (Guo *et al.*, 2020).

Spike protein

The S protein is a very large (~150 kDa) transmembrane and heavily glycosylated protein that assembles into a homotrimer on the viral envelope, decorating the viral surface like a crown and mediates attachment and fusion to the host cell. It is particularly of interest for its role to mediate virus attachment to host receptors and in the binding with human ACE2 receptor. It belongs to the type I membrane-protein family. In the native state prefusion and postfusion conformations of S proteins can be traced simultaneously on the reconstructed virions (Walls *et al.*, 2020). SARS-CoV-2 S proteins are organized into two functional subunits, S1 and S2, which are responsible for mediating attachment to the host cell and membrane fusion respectively. These two subunits after the contact with the host receptor are cleaved but remain paired through non-covalent bonds. Particularly cryogenic-electron microscopy analysis (Cryo-EM) highlights how SD1 and SD2 (subdomains) of S1 wrap around an axis, covering the prefusion S2 subunit (Wrapp *et al.*, 2020).

In the prefusion state subunit S1 folds into receptor-binding domain (RBD), an N-terminal domain (NTD) and C-terminal domains (CTD1 and CTD2). RBD is packaged in a five-stranded antiparallel beta sheets core, flanked by a short helix. A receptor binding motif (RBM) extends out of this core, but it is not organized in a regular and specific secondary structure. On the other side the S2 subunit contains four conserved structural regions: (1) a transmembrane region, two heptad repeats (HR1 and HR2) and a fusion peptide. HR1 constitutes the main helical stalk while HR2 is the flexible conformation in the prefusion state. RBD can adopt two states: a “down” state in which a single RBD domain is closed, and it shields the central helical bundle of S2 and the two other RBDs; a “up” state which consents RBD to undergoes a joint-like conformational movement, to expose crucial residues

to human angiotensin converting enzyme (hACE-2) receptor on the host membrane. This interaction involves 17 residues of RBD and 20 residues of hACE-2 to form a network of hydrogen bonds and salt bridges. In the postfusion state, S1-S2 separate and HR1 and its helical stalk form an unusual long three-stranded coiled coil. This dramatic refolding can insert the fusion peptide into the target cell membrane (Polo-Megias *et al.*, 2022).

Briefly, following ACE2 engagement, two proteolytic cleavage steps occur. The first is localized to the S1-S2 boundary and it is mediated by furin-like proteases; after the S1-S2 boundary is cleaved, a cascade of refolding events forms a stable and elongated S2 subunit trimer. In the end the S2' site is cleaved either by TMPRSS2 or cathepsins to fully activate the fusion process. The ability to shift from a prefusion to a postfusion state consents the Spike protein to regulate the fusion of viral membrane with host cell membrane.

Anti-RBD antibodies have been shown to be the primary neutralizing immune response, therefore amino acid changes of the RBD can lead to immune escape.

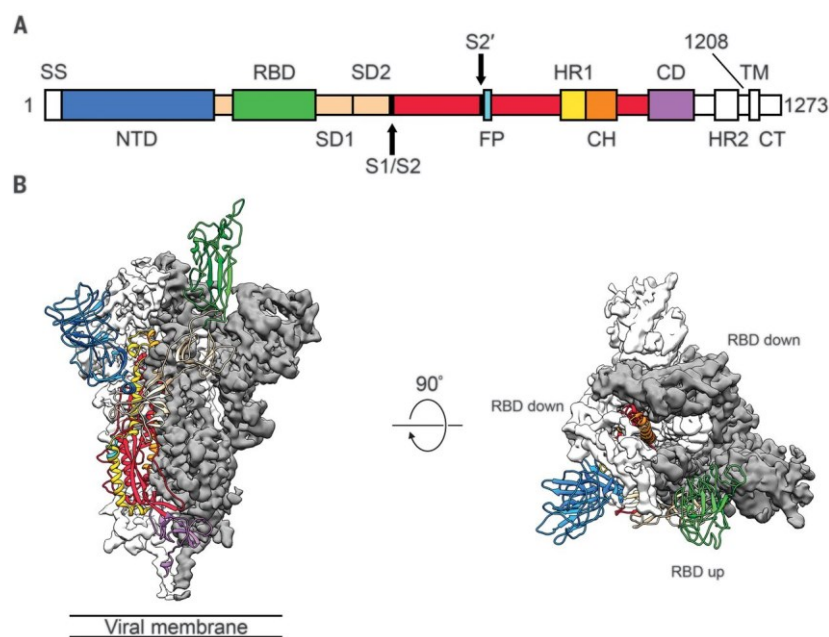


Figure 29 Schematic of 2019-nCoV S primary structure colored by domain. Domains that were excluded from the ectodomain expression construct or could not be visualized in the final map are colored white. SS, signal sequence; S2', S2' protease cleavage site; FP, fusion peptide; HR1, heptad repeat 1; CH, central helix; CD, connector domain; HR2, heptad repeat 2; TM, transmembrane domain; CT, cytoplasmic tail. Arrows denote protease cleavage sites. **(B)** Side and top views of the prefusion structure of the 2019-nCoV S protein with a single RBD in the up conformation. The two RBD down protomers are shown as cryo-EM density in either white or gray and the RBD up protomer is shown in ribbons colored corresponding to the schematic in (A) (Wrapp *et al.*, 2020)

Structural proteins

The M protein is the most abundant structural protein in the virion. It is a small (~25–30 kDa) multispinning membrane protein with a little N-terminal ectodomain and a cytoplasmic tail. Its role is to promote virion shape, by modification of flexure of membrane. Furthermore, it allows the link to the nucleocapsid.

Instead, the E protein is a small (~8–12 kDa) hydrophobic transmembrane protein that is involved in virus assembly, release and viral pathogenesis. After the viral entry this protein is responsible for cell lysis, viral genome release and subsequent viral budding at the level of endoplasmic reticulum and Golgi body membrane (Mariano *et al.*, 2020). Moreover, it is found to participate in activation of inflammasome cascade (Nieto-Torres *et al.*, 2015).

The N protein (43–50 kDa) is a phosphoprotein divided into three regions: an N-terminal domain, an RNA-binding domain and a C-terminal domain. It has the function to bind and protect from degradation by ribonucleases the RNA genome. It is also the main protein component of the nucleocapsid. During the first phases of infection, N is one the main antagonists of interferon (IFN) and immune response, thereby it is actively involved in viral replication. Recently, it has been identified its counter cellular RNAi-mediated antiviral activities through its binding with double-stranded RNA strings (Mu *et al.*, 2020).

The genome sequence of CoVs shows 58% identity on the NSP-coding region and 43% identity on the structural protein-coding region, suggesting that structural proteins are necessary in adaptation to new hosts. (Mariano *et al.*, 2020)

Viral entry

As we said, the first steps of SARS-CoV-2 infection involve the specific binding of S protein to ACE2 receptor by firstly the S1 subunit and then by the S2 subunit which promotes the fusion with host membrane. A final cleavage, operated by TMPRSS2 protease or by cathepsins, promotes the viral uptake. The expression and tissue distribution of entry receptors consequently influence viral tropism and pathogenicity. Different host cell tropism, replication kinetics and transmission of SARS-CoV-2 might be determined by S protein–ACE2 binding affinities.

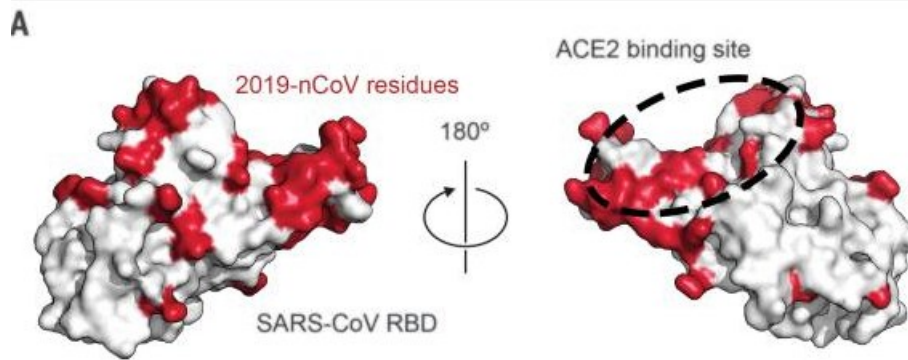


Figure 30 Antigenicity of the 2019-nCoV RBD. SARS-CoV RBD shown as a white molecular surface (PDB ID: 2AJF), with residues that vary in the 2019-nCoV RBD colored red. The ACE2-binding site is outlined with a black dashed line. (Wrapp *et al.*, 2020)

Following fusion and viral entry, genomic RNA is released and uncoated; two open reading frames, ORF1a and ORF1b, which encode for two large polyproteins pp1a and pp1ab are translated and then co-translationally and post-translationally processed into sixteen non-structural proteins (NSPs), upon proteolytic cleavage by two cysteine proteases. NSPs form the viral replication and transcription complex. In detail, proteolytic release of NSP1 occurs rapidly and enables NSP1 to target the host cell translation apparatus (Jackson *et al.*, 2020). NSP2 and NSP16 are organized into the viral replication/transcription complex (RTC) and determine the course of the replication cycle (V'kovski *et al.*, 2019). NSP11 is thought to provide support to the viral RTC, promoting the evasion of host immune response or providing cofactors for replication. NSP12 is an RNA-dependent RNA polymerase (RdRP), hence it is involved in RNA synthesis and modification. This enzyme works with two cofactors NSP7 and NSP8. Notably, NSP14 provides a 3'–5' exonuclease activity that assists RNA synthesis. Finally, the coronavirus capping machinery is represented by NSP10.

In parallel with the expression of NSPs, the biogenesis of viral replication organelles consisting of small open double-membrane spherules (DMSs), convoluted membranes (CMs) and perinuclear double-membrane vesicles (DMVs) generate a protective microenvironment for transcription of subgenomic mRNAs (sg mRNAs) and viral genomic RNA. A peculiar property of coronaviruses and most members of the order *Nidovirales* is the discontinuous mechanism of viral transcription process. During negative-strand RNA synthesis, the RTC interrupts transcription upon encountering transcription regulatory sequences (TRSs) located upstream to most ORFs. These TRS elements, or TRS 'body', arrest the synthesis of the negative-strand RNA. The process re-initiates at the TRS adjacent to a leader sequence (TRS-L). The discontinuous step of negative strand RNA synthesis results in the production of a set of negative-strand sgRNAs that are then used as templates to synthesize a characteristic set of positive-sense sg mRNAs, then translated into structural and accessory proteins. Definitive translated structural proteins translocate into endoplasmic reticulum

(ER) membranes and transit through the ER-to-Golgi intermediate compartment (ERGIC), where interaction with N-encapsidated, newly produced genomic RNA results in budding into the lumen of secretory vesicular compartments. Finally, virions are secreted from the infected cell by exocytosis (Sola *et al.*, 2015).

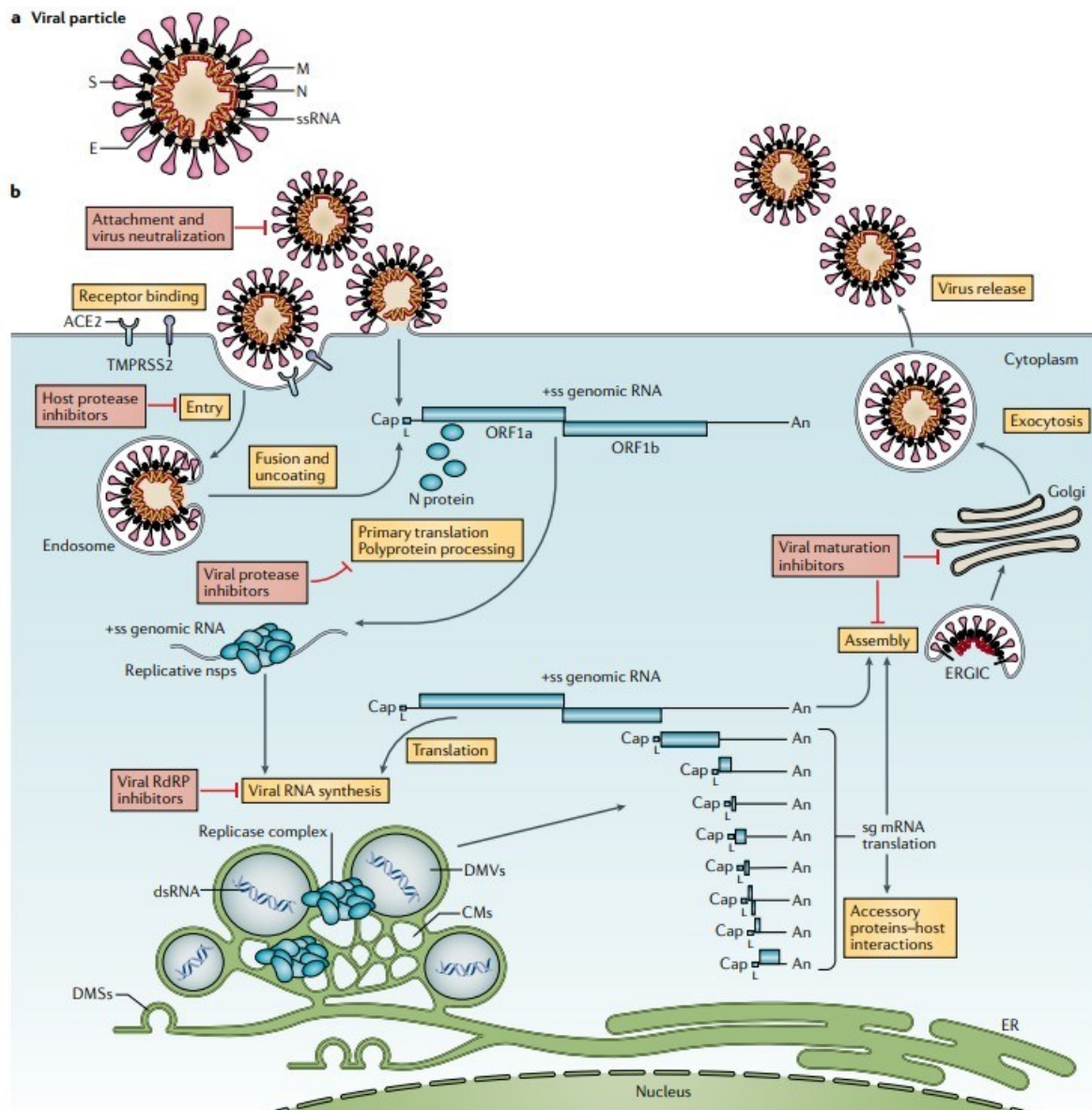


Figure 31 Coronavirus virion and lifecycle (V'kovski *et al.*, 2019)

Variants: VOCs, VOIs

Coronaviruses were considered to infect only animals until SARS pandemic emerged in 2002 and SARS CoV-2 in 2019. Since SARS-CoV-2 is an RNA virus, during the pandemic several significant variants have sorted out. New variants emerged due to mutations, deletions or insertions in crucial regions. The anticipated per-site per-year mutation rate of the human beta-coronavirus SARS Cov-2 has been estimated to be $\sim 1.12 \times 10^{-3} \text{ nt}^{-1} \text{ year}^{-1}$ (Koyama *et al.*, 2020). Some of these mutations are tracked and classified as VOC/VOI/VUM (variants of concern/variants of interest/variants being monitoring) by CDC (center for disease control), GISAID (Global Initiative on Sharing Avian Influenza Data), NEXTstrain and PANGO (Phylogenetic Assignment of Named Global Outbreak). The VOIs are variants resulted from genetic changes that affect transmissibility, pathogenicity and effectiveness of therapies. VOCs are VOIs with increasing virulence, transmission rate and immunity evasion, often associated with relevant public health concerns. This phenomenon occurs when a mutation promotes the evasion of the human immune system and causes infection and re-infection. It is also known as viral escape. Finally, VUM are described as variants that undergo unusual mutations. SARS CoV-2 variants have been originally categorized as lineage A and B and marked with numbers (i.e. A1, A2, B1, B1.1 etc). Subsequently, to avoid confusion and ease complexity, a new nomenclature based on Greek characters has been introduced (Choi *et al.*, 2021).

On spring 2023 WHO declared that only VOC would be associated to Greek letters, while VOI would be named with their scientific nomenclature. Among the variants. B.1.1.7 (Alpha), B.1.351 (Beta), P.1 (Gamma), B.1.617.2 (Delta), B.1.427/B.1.429 (Epsilon), B.1.1.529 (omicron) contain different and significant mutations in the Spike-glycoprotein and have been the most relevant VOCs. B.1.1.7 (Alpha) was first identified in UK and presents a critical mutation (N501Y) in RBD site which improves the accessibility to ACE-2 receptor. B.1.351 (Beta) variant came up in December 2020 in South Africa and present the alteration E484K in RBD site. Later, P.1 (Gamma) was detected in Brazil. For this virus several mutations were observed, including E484K and N501Y. The Indian variant B.1.617.2 (Delta) displayed noteworthy mutations such as L452R, T478K and P681R that were linked to a potential immune escape, increase in transmissibility and infection rate. Moreover, these mutations led to the block of the production of nAbs induced by vaccination as well as monoclonal antibodies (Mlcochova *et al.*, 2021). In November 2021, B.1.1.529 (omicron) appeared and was immediately classified as VOC for the number of mutations involved in receptor binding affinity, therefore in virus spreadability. Due to its rapid mutagenicity, many subtypes as BF.7, BA.4, BA.5 and BA.22 have been identified. Indeed, omicron variant displays more mutations than any other SARS-CoV-2 variant, with roughly 50 more compared to the Wuhan-Hu-1 or B variant. Compared to the original virus, the variant was identified by 30 amino acid changes, three minor

deletions, and one tiny insertion in the Spike protein, of which 15 are found in the receptor-binding domain (residues 319–541)

Other subvariants of omicron under WHO monitoring are BQ.1 (BA.5 sublineage), BA.2.7.5 (BA.2 sublineage) and XBB (recombinant of BA.2.10.1 and BA.2.75). In late 2023, JN.1 has become dominant replacing XBB variant. It evolved from BA.2.86 (also called Pirola) and has been characterized for its higher immune escape but mild severity. Analogously, KP.3.1.1 variants evolved from JN.1 and KP.2 in mid-2024 reporting a further reduced vaccine effectiveness due to the mutations F456L and Q493E. In early 2025, XEC variant became predominant.

In the same way, some consistent VOIs have circulated and regarded mutations such as R346T, Q183H, F456L K417T/N, E484K, L452R, N501Y, P681R and D614G. Such mutations have impacted on some prominent functions like virulence, antigenicity, transmissibility infectivity and re-infectivity and epidemiology. Epsilon, zeta, eta, iota, theta, kappa, lambda and mu were among the VOIs that have previously been in circulation (Ghildiyal *et al.*, 2024). One of the most studied mutations is the D614G in the Spike-glycoprotein, which was also noted in the early phase of the pandemic. It has been reported that this mutation might have been responsible for altered antigenicity, immunogenicity and mortality (Kim *et al.*, 2020) (Eaaswarkhanth *et al.*, 2020).

induced-IFN-I production and mtDNA release from adjacent endothelial cells. Increased pro-inflammatory factors may lead to the occurrence of cytokine storm (Wang *et al.*, 2020). Monocyte/macrophage activity is coupled with the dendritic cells (DC) and natural killer (NK) cells activity. The former are the main producers of IFN-I, a family of cytokines that promotes a positive feedback activity for DCs and contribute to macrophage-mediated cytokine storm in lungs. Particularly, they can mediate the transcriptional and epigenetic changes in macrophages, which facilitated their hyperactivation upon environmental stimuli (Fitzgerald-Bocarsly *et al.*, 2008). On the other hand, NK cells show a direct anti-viral activity, as indicated by the reduced SARS-CoV-2 protein levels in cocultured infected cells and can exhibit anti-fibrotic activity (Kramer *et al.*, 2021). Innate immune response provides three main functions: 1) restriction of viral replication within the infected site, 2) establishment of local antiviral conditions which include releasing of effectors and recruiting of effectors cells, 3) prime of adaptive immune response. SARS-CoV-2 is particularly effective at delaying intracellular innate immune responses associated with type I and type III IFNs (Blanco-Melo *et al.*, 2020). Indeed, those responses are crucial to block initial virus replication and to prime the adaptive immune responses.

Adaptive immune response

Antigen presenting cells (APCs) have the ability to phagocytose heterologous materials or pathogens. Upon this event, adaptive immune response arises. These cells present specific antigens to induce cellular and humoral immunity. Specifically, CD4⁺ T cells can be activated and differentiate into Th cells which promote the activation of macrophage-dependent virus clearance (Th1) via the production of macrophage activating cytokine (e.g. TNF- α , IFN γ) or activate B cells (Tfh) for the production of virus specific and neutralizing antibodies. CD4⁺ cells can also activate CD8⁺ T cells response which can differentiate into cytotoxic T lymphocyte (CTL) or specific B memory cells. The first have a direct cytotoxic activity by the secretion of granzyme B and perforins and are crucial in protecting from multiple severe viral infections.

CD4⁺ T cell response is associated with a primary SARS-Cov-2 infection. In acute COVID-19 disease they can be detected only as early as days 2–4 post-symptom onset and their rapid induction is associated with mild disease and accelerated viral clearance. These cells predominantly release IFN γ to accelerate virus clearance. Moreover, in this phase, T follicular helper (Tfh) specific for SARS-CoV-2 are generated to release neutralizing antibodies as well as to develop memory B cells. SARS-CoV-2-specific CD4⁺ T cells also express the chemokine receptor CCR6 to migrate to the mucosal tissues (Primorac *et al.*, 2022).

Following their activation by Tfh cells, B cells produce neutralizing antibodies for SARS-CoV-2 with a wide range of heavy chain and light chain V genes that mainly target the RBD of Spike protein, particularly those epitopes corresponding to the ACE2 receptor binding footprint. Neutralizing antibodies mostly prevent virus spread outside of the cells and their titer are often not correlated with lessened disease severity in primary infection. Indeed, the adaptive immune response is slow and many cells are already infected at the time of antibodies production while T cells monitor the primary phases of infection. For this reason, the cohabitation of cellular and humoral response is crucial.

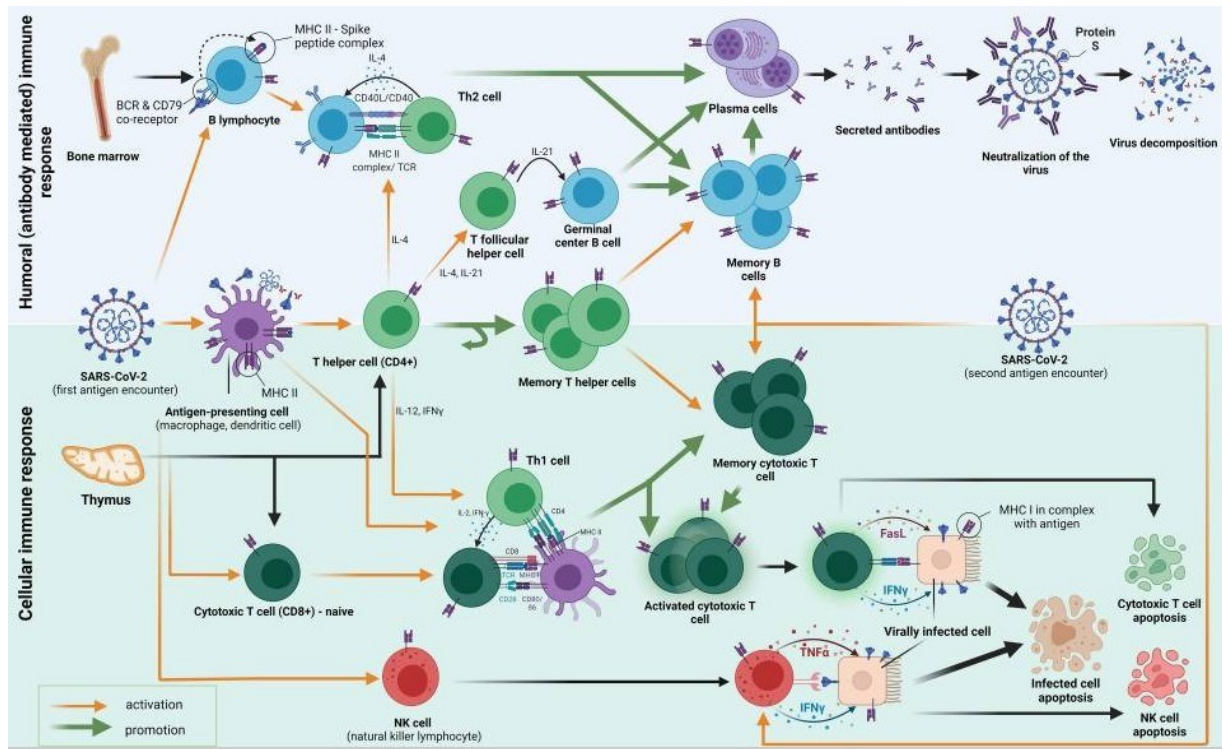


Figure 33 Adaptive immune to the first and second encounter with the SARS-CoV-2 antigen (spike protein) (Primorac *et al.*, 2022)

Vaccines

Along the SARS-CoV-2 pandemic, effective vaccination strategies have been studied and implemented to reduce public health pressure. Since the Spike glycoprotein is essential to penetrate into the cells and to induce the release of neutralizing antibodies, it has been studied to develop vaccines. Proposed and approved vaccination strategies and technologies include mucosal vaccines and intramuscular injection vaccines which are classified in nucleic acid, viral vectors, subunit, recombinant and virus-like particles vaccines.

Mucosal vaccines, are thought to offer two extra levels of protection in comparison to parenteral vaccination. Indeed, they are able to mimic natural infection without causing disease or virus

transmission and mainly elicit a local immune response, since they can stimulate the production of Secretory IgA (s-IgA), s-IgG (serum antibodies) and resident memory B and T cells in the respiratory mucosa. Particularly, the s-IgA antibodies serve as neutralizing antibodies (nAbs) to inhibit viral binding to the mucosa and, thus, protect it from the entry of the pathogen to cells, while tissue-resident memory B and T cells contribute to prevent viral replication at the time of infection as well as viral transmission, therefore, avoiding the transmission of the virus also from asymptomatic subjects. They can be classified into Nasal Vaccine, oral vaccine (tablet), sublingual vaccine, rectal route of vaccination. These kinds of vaccines are needle-free administered representing a minimal invasive delivery method; therefore, it is expected with higher production capacities and a more widely distribution. However, they are more difficult to produce and less stable. Moreover, they are mainly live virus-based vaccines (LAIV). Nowadays, the only mucosal SARS-CoV-2 vaccine to have been licensed is iNOVACC[®] vaccine BBV154 based on adenovirus vectors. It has only been approved for India.

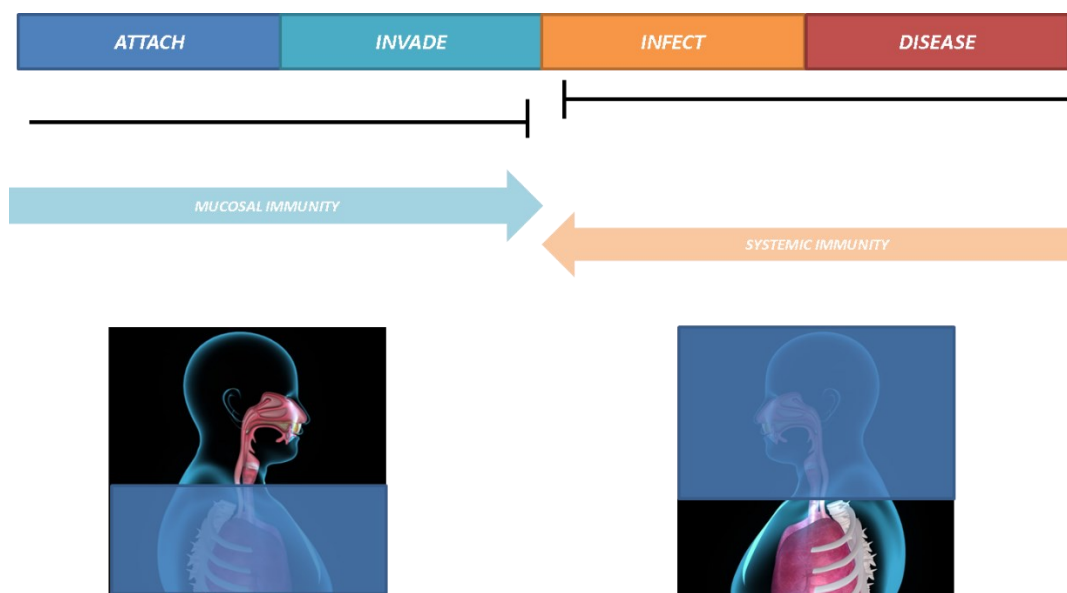


Figure 34 Nasal Vaccines vs. Systemic vaccines

On the other hand, intramuscular injection vaccines, or parental vaccines, are aimed at stimulate high levels of serum antibodies to neutralize the virus and prevent the disease, stimulate systemic immune response and contribute to decrease the severity of the disease; however, they cannot avoid viral replication in LRT/URT (Low/Upper respiratory tract), therefore they marginally prevent transmission of the virus. Moreover, systemic vaccines can decrease their effectiveness as the virus mutate. Due to the lower cost of their development and their easy scale-up, nowadays they remain the most administered and widespread.

The first vaccine to be approved was based on mRNA technologies. This technology relies on the ability of modified mRNA of SARS-CoV-2 Spike protein to penetrate and to be expressed in host cells, stimulating an immune response. The vaccine has firstly been studied by Moderna and it was named mRNA-1273. It showed an effectiveness rate of 94% and it was approved in December 2020. Meanwhile, Pfizer/BioNTech mRNA vaccine passed clinical trial and it was the first vaccine approved for application in the US. Later, in summer 2022, a boost vaccine was released against omicron variants by both companies.

A viral vector vaccine was firstly developed by Oxford-AstraZeneca and produced under the license by Serum Institute of India. This technology exploits a viral vector to deliver genetic material (DNA) that can be transcribed by the recipient's host cells as mRNA coding for a desired protein or antigen, to elicit an immune response. Viral vector-based vaccines can be replicating or non-replicating particles. Non-replicating particles are engineered as replication-deficient viral vectors and have the only capability to deliver genetic material of a particular antigen to the host cell. On the other hand, replicating vector vaccines can penetrate in host cells and release new viral particles which then enter more new cells which will also make the vaccine antigen. AstraZeneca developed its vaccine using a chimpanzee adenovirus vaccine vector made up of replication deficient chimpanzee adenoviral vector chAdOx1. It has been genetically engineered to match the SARS-CoV-2 Spike protein and is incorporated with the surface Spike protein genetic sequence of this surface Spike protein. Currently, other available adenovirus-based COVID-19 vaccines are produced by Janssen, Gamaleya Research Institute of Epidemiology and Microbiology and CanSino.

Other technologies are based on the production of subunit vaccines presenting SARS-CoV-2 S protein or SARS-COV-2 specific peptides without the introduction of whole pathogen particles. Sanofi-GSK vaccine has been developed using a baculovirus vector for the infection of insect Sf9 cells. Currently, Bimervax peptide-based vaccine has also been approved. This kind of vaccines is produced using a CHO cells platform for the expression of PHH-1V antigen, a recombinant protein based on the receptor binding domain (RBD) sequences of the Beta and Alpha variants of SARS-CoV-2 and elicits high and long-lasting levels of neutralizing antibodies against all studied variants, as well as a strong cellular immunity response, when used as heterologous booster in previously vaccinated individuals with mRNA and vector vaccines (Corominas *et al.*, 2023).

Additional type of vaccines is based on the production of virus-like particles. Novavax vaccine belongs to this category, and it is produced by engineering a baculovirus with a gene for a modified Spike protein. As for Sanofi-GSK vaccine, baculovirus is used to infect Sf9 moth cells, which can express and display modified S proteins on their cell membranes. Virus-like particles vaccines have

been also studied using plant-based platforms. This kind of technology uses weed or tobacco plants to grow nanoparticles expressing S protein. MedicaGo was a pioneer of this technology and its COVID-19 vaccine reach the Phase III of clinical trials until the shutdown of the company in winter 2023.

Nowadays, vaccination remains the most effective strategy to fight the spread of infectious diseases among population, despite current platforms display some drawbacks, including the low safety and immunogenicity and a high cost of production. Indeed, available vaccines are formulated following conventional and well-established platforms, such as live-attenuated or subunit vaccines, which can require multiple doses to attain effective and long-lasting immunity. Moreover, current vaccines cannot effectively target certain high-risk populations such as pregnant women, immunodeficient and chronically ill patients, children or elderly people and many diseases are still not fully preventable. For all these reasons, new vaccines strategies are required to prevent and fight infectious diseases. The unexpected global outbreak of COVID-19 has highlighted the urgent need of develop new innovative platforms allowing the rapid release of more cost-effective vaccines. Next-generation vaccines are considered a promising approach to fight future disease pandemic. Recent improvements in vaccine technologies have accelerated the development of efficacious vaccines during the COVID-19 outbreak and are being tested for other pathogens. However, there remain many challenges for next-generation vaccines, which should target conserved epitopes, induce durable protection, and inhibit transmission of the disease-causing agent.

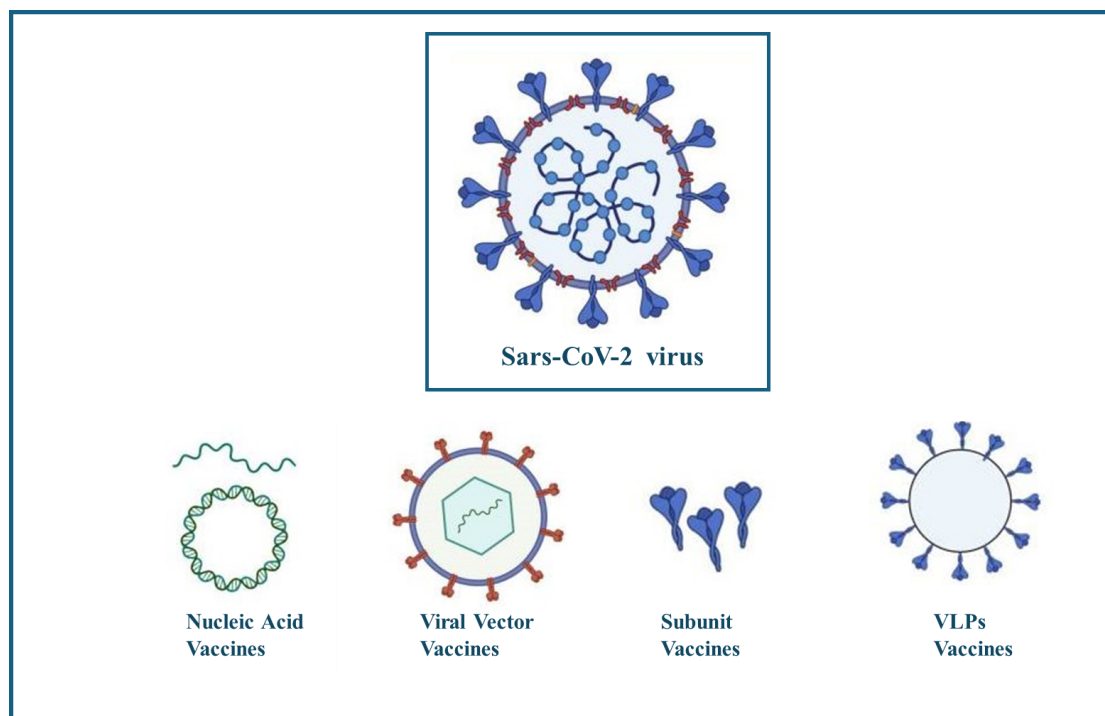


Figure 35 Current SARS-CoV-2 intradermal vaccines. The figure was generated with Biorender.com

Correlate of protection

Innate and adaptive immune responses to infections can protect from subsequent reinfection and are pathogen specific. Immunity can be also acquired upon vaccination, without a direct infection. In general, the vaccine-induced protection is assessed through clinical assays applied to blood sample from immunized patient. However, vaccine-induced immune responses are not easy to predict since they are often multi-faceted, even if in many cases they can be correlated to neutralizing antibody measurements. A correlate of protection (CoP) is a marker of immune function that statistically correlates with protection after vaccination. It may be either mechanistic (mCoP), which is a mechanistic cause of protection, or non-mechanistic (nCoP), which does not cause protection but nevertheless predicts protection through its (partial) correlation with another immune response(s) that mechanistically protects (Plotkin *et al.*, 2012). When more than one immune function is involved in protection, it is defined co-correlate. CoPs are specific for a viral disease or infection, for a specific population group (elderly vs children) and even for a specific type of vaccine (i.e. CoP for an inactivated vaccine may not necessarily be the same of that of an intra-nasal vaccine. In general, correlate of protection can be studied using different endpoints such as protection from infection/reinfection, mild disease, severe disease and death, despite the fact that can vary amongst the same virus depending on the phase of infection and the pathogen high degree of genetic variation. The establishment of a correlate is fundamental for vaccine development strategies, as it should meet certain criteria to be accepted by regulatory agencies.

In relation to correlates of protection, it is also crucial to distinguish between vaccine efficacy and vaccine effectiveness. Vaccine efficacy is the precise measurement of the ability of a vaccine to prevent disease in vaccinated individuals, with emphasis on the exact levels of vaccine-induced disease reduction. Vaccine effectiveness refers to how well a vaccine protects against a disease when routinely used in the community. It is established by observational studies and serological tests and represents the reduction of infection frequency in vaccinated individuals compared to those who have not been vaccinated (Trombetta *et al.*, 2014)

Immune protection from COVID-19 is highly correlated with neutralizing antibody levels as shown by several studies (Khoury *et al.*, 2020) (Earle *et al.*, 2021) (Manenti *et al.*, 2021). These results have also estimated the relationship between neutralizing titers and protection. However, they diverge in terms of estimation of the level of neutralizing antibodies required for protection. Particularly, Khoury *et al.*, found that study results converge on a consistent relationship between antibody levels and protection from COVID-19, by normalizing antibody titers. Although it is clear that neutralizing antibody levels are a good correlate of protection, there is a massive discussion in the identification of an applicable threshold for serologic assays. This debate is because of the presence of a gradient

of vaccine efficacy that increases with neutralization and is due only in part to the absence of a real threshold value. Furthermore, another significant question is raised to define a particular threshold at which a person's neutralization titer might have a high protection from COVID-19, since this value can be affected by the diversity of assays used to measure neutralization, the difficulty in translating neutralization levels between assays, the constant emergence of new and more escaped variants, and the uncertainties of estimating individual neutralization titers. For COVID-19 the term surrogate of protection is more appropriate, since there is not a particular threshold at which a neutralization titer is correlate to significant protection.

This finding can be useful for planning future vaccine use, determining population immunity, and reducing the global effects of the COVID-19 pandemic.

VLPs

Virus-like particles (VLPs) are viruses derived nanoparticles assembled upon expression of specific viral proteins. They present an external surface resembling that of their cognate virus and they have the ability to self-assemble, mimicking the form and size of a virus. Unlike viral particles, they do not present genetic material, so that they cannot infect host cells, nor they cannot replicate. VLPs have emerged as one of the principal vaccine platforms for their advantages in safety, immunogenicity, antigen stability, biocompatibility and manufacturing (ready scale-up, 1mg/g of plant), as VLPs combine the best features of whole-virus and subunit antigens for vaccine development. However, high production costs and difficulty in downstream processes are key challenges to overcome. Generation of VLPs can be experimentally performed in various living expression systems that include prokaryotic cells, yeast, insect cells lines, plants and mammalian cells. Moreover, chimeric VLPs can be expressed and produced by assembling structural proteins from different viruses. Due to their unique and organized structure and their underlying geometry, which presents repetitive antigenic epitopes on the surface, they resemble pathogen-associated structural patterns (PASP) and can efficiently stimulate both innate and adaptive immune responses favoring uptake by antigen presenting cells. Moreover, their suitable size, usually in the range between 20-60 nm, allows their diffusion also into the lymphatic system, through the pores in the walls of vessels. Thus, a longer and more efficient immune response is promoted (Nooraei *et al.*, 2021). The versatility of VLPs allows several functionalities, thus leading to a wide variety of applications (Tariq *et al.*, 2021).

Since their identification in 1955 in tobacco mosaic virus (TMV) and then in 1968 in the sera of patients with leukemia and hepatitis, VLPs have been established in therapeutics, immunologic and

diagnostic field as carriers for the delivery of bio- and nanomaterials, such as drugs, vaccines, genes, quantum dots and imaging substances. Indeed, they contain an internal cavity within their structure that can be used as efficient delivery vehicles. By the time, a wide variety of structural proteins from virus such as human immunodeficiency virus (HIV), adeno-associated virus, Arenavirus, Hepatitis B virus (HBV), Hepatitis C virus (HCV) and bacteriophages have been exploited to obtain VLPs, due to their different size. Moreover, their functionalities can be increased by changing external and internal surface features through the display of the above-mentioned heterologous epitopes or structural proteins, using genetic or biochemical strategies.

Nowadays, the VLP-based vaccines can potentially be used for the treatment of various infectious diseases, including HIV, influenza, hepatitis B (Spice *et al.*, 2020), hepatitis E (Li *et al.*, 2005), malaria (Janitzek *et al.*, 2019), Ebola virus (Tripathy *et al.*, 2021), SARSCoV-2 (Yang *et al.*, 2021), Zika virus, Dengue, and foot and mouth disease, among others (Balke and Zeltins, 2020). Moreover, several vaccines based on VLPs have already been licensed and are commercially available in markets including Engerix-B (GlaxoSmithKline) and Recombivax HB (Merck & Co) against HBV, Gardasil (Merck & Co) and Cervarix (GlaxoSmithKline) against HPV, Hecolin (Xiamen Innovax Biotech Co.) against HEV (Mohsen *et al.*, 2022), and Mosquirix (GlaxoSmithKline Inc.) against malaria (Laurenz *et al.*, 2020). To date, only two SARS-Cov-2 VLP vaccines have been authorized, while six are currently on clinical trials.

VLPs can form by spontaneous aggregation between one or more viral structural capsid proteins to form the final particle. Spontaneous polymerization can generate different geometrical symmetries, like spherical, or rod-like structures, depending on which virus from which they were derived. Based on their structural complexity and structural components, VLPs can usually be divided into different groups. A first classification is obtained based on capsid proteins organization. Capsid proteins can be organized in one, two or three layers that can contain more than one structural protein. Usually a single-protein VLP has a relatively simple structure, while multi-protein VLPs contain unique structural components such as presence of several distinct capsid layers.

A second classification is based on the presence or absence of lipid envelopes. VLPs such as those derived from influenza or SARS CoV-2 and HIV-1 virus, have a layer of lipid that contains viral surface antigens surrounding the capsid structure. This structure imitates the lipid envelope observed in natural infectious virus particles. Other enveloped VLPs contain a matrix protein located immediately inside the host-derived lipid membrane in which the viral glycoproteins are embedded (Mohsen *et al.*, 2022).

On the basis of the grade of complexity of the lipid envelope, capsid organization and the viral

proteins to express on surface, the choice of production system that can be used for their generation is extremely crucial.

Production and expression systems

Commercial VLP assembly has been obtained using several expression systems, such as *Escherichia coli*, Baculovirus based system, yeasts, mammalian cells, and plant cells. The structural immunogenicity and complexity are a crucial factor for the choice of the expression system.

Most of the bacterial systems are well studied on the industrial strains and vectors on *E. coli*. Their usage is very convenient in terms of costs and scalability but also as they are easy to manipulate, they have a high-yield and quite simple protein expression. However, they present several limitations that include inability of refold recombinant proteins following mammalian post-translational modifications (PTMs), scarce protein solubility, issues in obtain correct disulfide bonds, presence of bacterial contaminants like endotoxins and lipopolysaccharides and low or poor immunogenicity. Many VLPs based vaccines have been developed using *E. coli* expression system. Wang *et al.*, (Wang *et al.*, 2020) have expressed a full-length CP of type 2 porcine circovirus and VP2 protein of porcine parvovirus in *E. coli* strain B121 cells. In this case these proteins can be easily self-assembled into VLPs. Yazdani *et al.*, have analyzed the production of GFLV (grapevine fanleaf virus) VLPs and its use as carrier to present L2 epitope of HPV (Yazdani *et al.*, 2019). The first HEV vaccine has been released in 2012. It has been developed with the neutralizing and immunodominant epitopes from HEV genotype 1 on the surface of *E. coli*-derived p239 VLPs. Xiamen Inovax Biotech has used *E. coli* to produce a low-cost HPV vaccine based on L1 VLPs. L1 is the HPV major structural protein. A bivalent HPV vaccine (Types), based on these VLPs, has been developed and has been shown to be safe and highly immunogenic in preclinical studies.

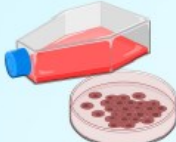
Expression Systems for VLP Production					
Expression systems	Bacteria	Yeast	Insect cells	Mammalian cells	Plant cells
					
Advantages	• Cost-effective • Scalable • Rapid growth • Easy manipulation • High-level expression • Genetic stability	• Low production cost • No endotoxins contamination • High-density fermentation • Support most protein folding	• Carry and deliver large amount of DNA • High protein expression • Support eukaryotic protein PTMs • Proper protein folding and assembly	• Perform proper folding, assembly, and PTMs of proteins	• Highly scalable • Cost-effective • Carry out N-glycosylation • High expression • Correct folding and assembly
Limitations	• Poor immunogenicity • Recombinant proteins lack PTMs • Protein solubility issues • Contamination by bacterial endotoxins • Inability to create di-sulfide bonds	• Vlp yield lower than E. coli • High mannose modification • Lack mammalian like PTMs	• Difficult to scale up • High production cost • Baculovirus contamination • Simpler N-glycosylation than mammalian cells • Incomplete modification of proteins	• Require large-scale production facilities • Lengthy expression time • Low yield • Contamination by mammalian pathogens	• Low yield than mammalian cells • Technical and regulatory issues

Figure 36 Advantages and limitations of different expression systems for the development of virus-like particles

Insect system

Baculovirus-insect expression system has resulted as a very effective tool to solve protein-based refolding issues. The ability of Baculoviruses to tolerate high amounts of heterologous DNA and to have a high yield of protein expression, have induced scientists to use this technology for several applications. Moreover, this system can bypass PTMs and proper protein folding issues.

The baculovirus expression vector system (BEVS) has been discovered in the early 1980s. These insect-specific pathogens were of great interest for their versatility as natural pest control agents, as alternative to chemical insecticides in agriculture. Only after the successful production of recombinant human IFN- β by using genetically modified *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) in lepidopteran cells, BEVS has been identified as a highly powerful and useful tool in biotechnology (Pidre ML *et al.*, 2023).

BVs are enveloped and coated by a class III glycoprotein, called GP64, responsible for the attachment and entry of the virus inside the host cell. Even though baculoviruses have a very limited host range in terms of their pathogenicity and replication, budded viruses can successfully enter multiple types of cells. In terms of insect cell lines, those derived from *Trichoplusia ni* and *Spodoptera frugiperda*

ovaries are the most widely employed. Commercially available cell lines include Sf9, Sf21. Insect cells differ from the eukaryote ones for the N-glycosylation patterns; indeed, insects produce quite simple N-glycans with terminal mannose residues, while mammals have a complex N-glycans with terminal sialic acid residues due to the wide variety of enzyme sets. Since, N-glycosylation can be of extreme importance for protein folding and functionality, cell lines have been genetically engineered to include the ability to add terminal N-acetylglucosamine, galactose, and sialic acid residues, but also cells to prevent the addition of fucose residues to N-glycans (Tariq *et al.*, 2022). Commercial bacmids include, PolyBac that allows multiple transgene insertions and glycosylated protein expression or SweetBac that provides the enzymes for the production of mammalian-like N-glycans and can be employed for the expression mammalian glycoproteins (Sari-Ak *et al.*, 2019).

Multiple commercially available systems for the production of recombinant baculoviruses are based on a strategy that involves homologous recombination inside insect cells. This event consists of a recombination between a baculovirus genome engineered to replicate in *E. coli* (a bacmid), which carries all the genetic information for the generation of the virus, and a transfer plasmid, which carries the gene of interest (GOI) sequence downstream of an insect or baculovirus promoter. This expression cassette is flanked by two regions that provide the recombination with the bacmid. When recombination occurs, the essential gene is reconstituted, restoring the ability of the baculovirus carrying the GOI to replicate in insect cells (Rodríguez-Hernández *et al.*, 2023).

More recently another strategy has been developed. This involves the site-specific transposition of Tn7 transposon into a bacmid. Briefly, this expression system relies on generation of recombinant baculovirus by site-specific transposition in *E. coli* DH10Bac cells rather than homologous recombination in insect cells using a transfer plasmid vector carrying the GOI under the control of a specific promoter flanked by Tn7 transposition sites. *E. coli* cells which contain both the bacmid and a helper plasmid encoding for the Tn7 transposase. After the transposition in bacterial cells, the bacmid is purified and transfected into insect cells to generate the recombinant baculovirus.

Recently, Multibac Bv system have demonstrated to be a very effective way to produce influenza virus vaccines, as well as egg and cell-based approach. Lai *et al.*, (2019) have developed a multigene expression platform and an effective upstream process for production of H7N9-TW VLPs (Hu *et al.*, 2019). Novavax COVID-19 and influenza vaccine has been developed using baculovirus technology.

Mammalian cells

For decades several mammalian cell lines have been used to produce recombinant proteins and VLPs. This system produces less production yield compared to other protein production systems, but it possesses the ability to produce more complex and accurate PTMs. Therefore, mammalian cells are typically used to produce complex enveloped VLPs composed of multiple structural proteins. Some of the widely used cell lines are Chinese Hamster Ovary (CHO) cell line and HEK293 cell line. The first one is not human derived so that it shows a lower risk of contamination by human viruses. CHO cells have already been used for the generation of hantavirus-like particles, which were able to induce a specific immune response in mice (Li *et al.*, 2010). The HEK293 cell line production platform has been tested for the production of many different types of VLPs, such as rabies, HIV, and influenza.

VLP production in mammalian cell cultures system can be performed in different ways. Stable cell line generation is the classical method in which the gene encoding the protein of interest is integrated in cell genome. The first phase of this process is the transfection of a cell culture followed by a single clone selection. This approach is useful when small quantities of different VLPs are needed, especially in initial research phases. It is also suitable when the wild-type antigen of interest undergoes frequent mutations, requiring the vaccine to be frequently modified, or when one of the VLP proteins is toxic for the producer cell line.

Plants

An alternative and innovative technology to construct VLPs is based on the utilization of plants. This kind of method is described with the term “molecular farming” and has been applied in several fields such as cosmeceutical, biopharmaceutical and vaccinology. Plant based vaccines technology is an interesting and emerging tool due to its potential to create a notable number of recombinant proteins with a minimal expense. Furthermore, the eucaryotic system consents to avoid misfolding and misassembly of proteins and to insert correctly post-translational modification (PTM), as N-glycosylation or high mannose modification (Ward *et al.*, 2014) (Castilho *et al.*, 2011). This type of system also does not necessitate to build duplicate facilities to scale-up production, nor does the request to invest in high expensive fermentation machineries to produce biomass (Kim *et al.*, 2014).

Plant expression systems use plant viral vectors engineered with proteins of interest and transient expression systems which enhance delivery, expression and assembly of VLPs. Vectors are delivered into plants exploiting *Agrobacterium* spp. which allow to obtain a high yield and fast specific and targeted expression of recombinant protein, accordingly with GMPs-complying industrial processes.

Most common used viral vectors are the ones of Tobacco Mosaic virus (TMV), the Cowpea Mosaic virus (CPMV), Potato virus X and Bean yellow dwarf geminivirus, especially for VLP vaccines against viruses that mutate their surface antigens rapidly, like coronaviruses or Flu (Hemmati *et al.*, 2021).

In the past, plant based VLPs vaccines were thought to have the drawback of reducing their efficacy and of arousing allergic adverse side effect in immunized individuals, due to minimal differences between plant and mammalian post-translation modification. An example is the different N-glycosylation which in plants involves specific beta-1,2- xylose and a core alfa-1,3-fucose residues instead of beta 1,4-gal and N-acetylneuraminic acid (Neu5Ac) residues typical of mammalian cells. Ward *et al.*, demonstrated that this kind of modification does not affect vaccine efficacy, safety and immunogenicity. Furthermore, current plant-based vaccines are produced using plant humanized lines, genetically engineered with mammalian specific PTMs systems (Jansing *et al.*, 2019), including high mannose, GnGn, G0–G2 galactose, bisected GlcNAc, fucosylated and nonfucosylated and terminal sialic acid addition with a high degree of glycan uniformity.

For more than three decades, plants have been used as hosts to develop biological drugs such as vaccines and monoclonal antibodies. Many of these have been evaluated in the clinical trials and have been approved, as reported in Table 1. In 2015, β -glucocerebrosidase and taliglucerase alfa have been approved for the treatment of Gaucher disease (Shaaltiel *et al.*, 2015) (Graboski *et al.*, 2014). It has been the first plant-based protein used in enzyme replacement therapy. In the same period, an HIV-neutralizing monoclonal antibody produced in transgenic tobacco (*Nicotiana tabacum*) plants has been successfully used in a phase I clinical trial in the field of HIV prevention (Ma *et al.*, 2015). In vaccine field, many approaches have been used to study and produce plant based VLPs in the prevention of several diseases, such as Human Papillomavirus (HPV), Ebola, Influenza or SARS-Cov-1. The first globally licenced plant-based vaccine has been a Newcastle disease virus (NDV) vaccine for poultry, approved by the United States Department of Agriculture (USDA) (Hu *et al.*, 2011). A plant-made single-chain fragment variable monoclonal antibody (scFv mAb) used in the production of a recombinant Hepatitis B virus (HBV) vaccine has been approved in Cuba. Medicago inc. turned out to be one of the most important realities in plant biotechnology due to its efforts in the study of plant-based Flu and SARS-Cov-1 vaccine. Particularly, its COVID-19 vaccine, COVIFENZ (Pillet, 2022), developed by transient expression in *Nicotiana benthamiana* plants, showed high efficacy and it has been successfully authorized and released for the use in humans by Health Canada. However, despite the huge success of this vaccine for the plant biotechnology field, in late 2022 the company announced to shut down its manufacturing due to management decisions in consideration of COVID-19 demand market changes. The success of COVIFENZ approach has pushed many

molecular farming companies to invest in the plant biotechnology industry. Among these the most relevant turn out to be Kentucky bioprocessing (USA), Fraunhofer (Germany), Protalix Biotherapeutics (Israel), Invitria (Usa).

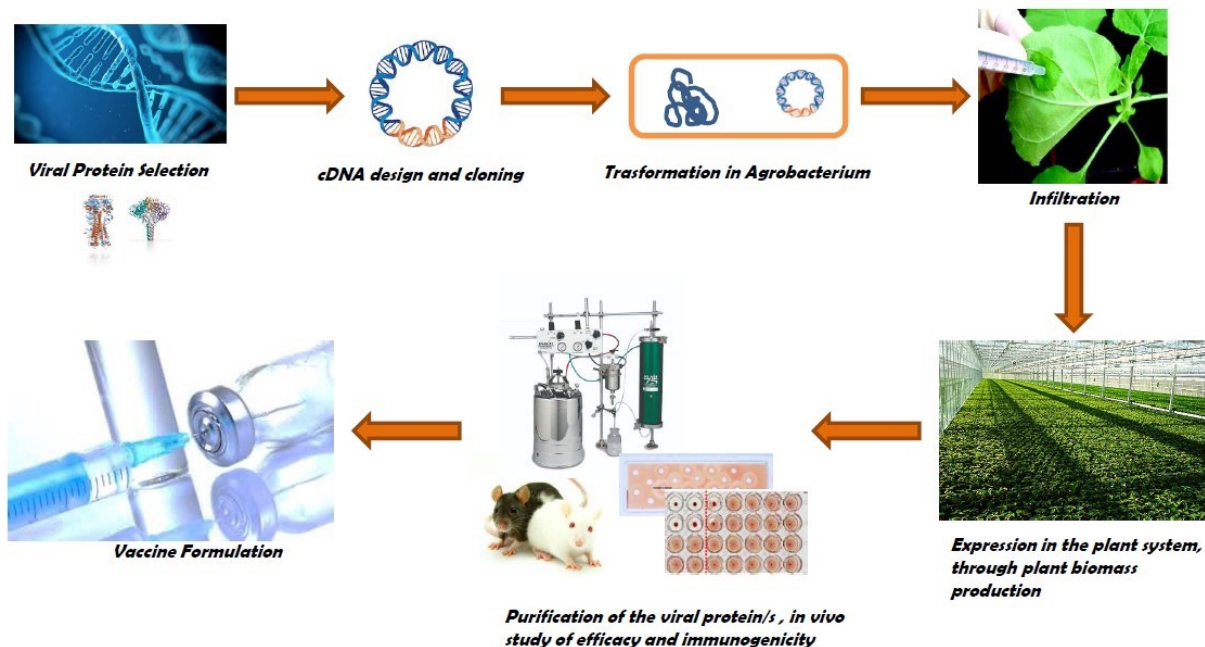


Figure 37 Plant-based vaccines production workflow

Serological assays to evaluate Sars-CoV-2 immune response

Regarding the impact of vaccination, it is crucial to detect the level of antibodies in the serum, to evaluate the effectiveness and the response of different kind of vaccine against VOCs. Serology assays are designed to detect antibodies in the serum within days to weeks following acute infection or vaccination. Moreover, serology tests can provide essential information about the ongoing response and surveillance of the outbreak and assessing the effect of non-pharmaceutical interventions at the population level (Winter *et al.*, 2020). Currently, serological tests can be divided into two main groups: tests aimed to detect all binding antibodies and tests to detect functional neutralizing antibodies. The first group includes solid phase immunoassays such as enzyme-linked immunosorbent assay (ELISA), Electrochemiluminescence Immuno-Assay (ECLIA), lateral flow immunoassays (LFIA) and fluorescence immunoassays (FIA). This kind of assays are easy, safe and cheap to perform and can be automatized maintaining a high throughput. However, they are not able to distinguish the functionality of the antibodies detected. On the other hand, functional neutralizing tests use the live viruses and need to be strictly performed into biosafety level 3 laboratories by trained personnel, therefore with higher costs. These tests are highly sensitive and specific and can detect a

wide range of functional, predominantly strain-specific neutralizing antibodies that inhibit virus attachment/entry in mammalian cells. The assay is conventionally based on the inhibition of the virus-induced cytopathic effect (CPE) on susceptible cells; however, it can be coupled to a faster and less variable downstream ELISA readout (VN-ELISA).

More recently, pseudotypes-based neutralization (PP-MN) assays have been developed and proposed to improve the throughput of classical VN assays and avoid the need of high biological containment. Indeed, pseudotypes are replication-defective viral particles composed of a structural and enzymatic core of one virus and an envelope of another virus to study. The core genome is depleted of viral replication factors and flanked by a reporter gene (luciferase or GFP) for the detection. The envelope is composed of proteins that allow the viral entry. Upon infection, the pseudotype genome integrates into the cell genome resulting in reporter gene expression. Therefore, the subsequent inhibitory effects of functional antibodies in serum can be quantified by the number of transduced cells accurately measured via a luminometer, a fluorescent microscope or Fluorescence-Activated Cell Sorting (FACS). This test has been shown to have a high sensitivity in detecting antibodies directed against Spike protein and to provide results in a wide range of neutralization titers. Pseudotypes bearing the protein of our interest can be used to detect antibodies and evaluate their neutralization activity together with well-characterized serological assays, such as Enzyme-linked immunoassay assay (ELISA) or Live virus Micro-neutralization assays (MN) (Hyseni I *et al.*, 2020).

MATERIALS AND METHODS

Microneutralization cytopathic effect-based assay

The MN-CPE (Microneutralization based on Cytopathic effect) method is a highly sensitive and specific technique used for the quantitation of virus-specific neutralizing antibodies (nAbs) to a given virus in mammalian samples. It is possible to test in this assay a lot of different matrix samples like serum or plasma samples from any species (human or animal), as well as monoclonal antibodies (mAbs). This assay is similar to a Plaque reduction assay, but it can be performed in a 96-well cell culture plate and for this reason it has a higher throughput compared to a standard Plaque reduction test.

The assay is based on the ability of the SARS-CoV-2 virus to infect the target cell through the interaction between the Spike glycoprotein and ACE-2 receptor on the cytoplasmic membrane, which leads to structural changes in the host cell, caused by viral infection; such capability results in the cytopathic effect, a phenomenon that can be observed through an optical microscope. If the serum contains anti-Spike antibodies that block this interaction, the cytopathic effect is inhibited. In particular, serial dilutions of treated serum are incubated with a standardized amount of virus, after which a fixed concentration of cells is added and the cytopathic effect determined, if anti-Spike antibodies are present, cytopathic effect will not be observed until the antibodies are sufficiently diluted. This test detects functional antibodies thus it is the most sensitive; in addition, since it is suitable for automation can be developed quickly upon recognition of a novel variant, even before that purified or recombinant viral proteins are available for other serological assays. Finally, MN-CPE seems to work better than other assays when serum antibody titers are low, especially with negative and negligible titers. However, the time-consuming and complex processes of traditional live virus-neutralizing antibody detection methods, including the need for laboratory personnel with a certain level of experience in virus plaque recognition and manual counting, as well as the use of different operators, represent huge drawbacks.

Titration of the SARS-CoV-2 virus for the MN-CPE assay

Titration experiments were performed to evaluate the amount of each SARS-CoV-2 virus strain needed for the MN-CPE assay. Each virus was serially 10-fold diluted in medium starting from 1:10, in a final volume of 200 µl. In particular, in a laminar flow cabinet, each well of a 96-well sterile flat-bottom microplate was filled with 180 µl of the dilution medium, except for the wells of column 11 and 12. 100 µl of medium were added to column 12, which was used as cell control in order to check the correct cell growth in absence of the SARS-CoV-2 virus. To the first column, 20 µl of virus were added, in order to achieve a 1:10 dilution of the virus. From the first column of the microplate, 20 µl

were transferred to the second column and this process was repeated until the column 10 was reached, discarding the last 20 µl. After performing the 10-fold serial dilutions of the virus, a volume of 100 µl was added to a plate containing confluent VERO E6 cells monolayer in each well of the microplate, except for column 11. Microplates were incubated in a CO₂ incubator for 3 days at 37°C ± 1°C. After the incubation period, each well was checked by using an optical microscope to evaluate the presence of CPE in the cell monolayer. Wells will be scored as positive for SARS-CoV-2 (i.e., CPE) or negative for SARS-CoV-2 (i.e., cells are alive and without CPE). Each titer was expressed as the tissue culture infective dose 50 % (TCID₅₀) in a volume of 100 µl, which represents the dose of the SARS-CoV-2 virus able to induce a cytopathic effect in 50 % of the cell culture. TCID₅₀ was calculated using the Spearman-Kärber formula, which considered the positive logarithm of the highest dilution of the SARS-CoV-2 virus at which all wells of a column showed CPE (X₀); doses distance in log (d, which was 1 in this procedure carried out with a log₁₀ dilution); the sum of the number of all wells showing CPE starting from X₀ ($\sum X_i$); and the number of repeats per dilution (n):

$$TCID/100 \mu l = X_0 - d/2 + d(\sum X_i/n)$$

SARS-CoV-2 virus titration for the MN-CPE assay was considered acceptable and reliable if cell control did not show any sign of CPE.

To obtain the dilution factor, the virus stock titre is divided for the titre that the viral working solution must have 500 TCID₅₀/1ml or 102.7. Since titres are exponential numbers, the results are obtained subtracting the exponents.

Ex . $105 / 103.3 = 5 - 3.3 = 1.7$, so 101.7 is the dilution factor, or 50.11.

The volume of the virus stock used to prepare the viral working solution must be ≥ 100 µl. If the volume used is lower the virus stock will be pre-diluted 1:10, 1:100 etc..etc.

Virus back titration

Virus back titration experiments were performed on the same day of each MN-CPE assay to check the working virus solution used for the MN-CPE test. Each working virus solution (200 TCID₅₀/100 µl of Sars-CoV-2 virus diluted in MN medium) was serially 10-fold diluted in MN medium, in a final volume of 200 µl. In particular, in a laminar flow cabinet, 2-12 columns of a 96-well sterile flat-bottom microplate were filled with 200 µl of the MN medium. To the first column, 292 µl of working virus solution were added. A 3,16-fold serial dilution (0.5log₁₀) by transferring 92 µl from column 1 to column 2, and up to column 11 was performed, discarding the last 92 µl. After performing the 10-fold serial dilutions of the virus, a volume of 100 µl was added to a plate containing confluent VERO

E6 cells monolayer in each well of the microplate, except for column. Microplates were incubated in a CO₂ incubator for 3 days at 37°C ± 1°C. After the incubation period, each well was checked by using an optical microscope to evaluate the presence of CPE in the cell monolayer, and TCID₅₀ was calculated using the same Spearman-Kärber formula used for the Sars-CoV-2 virus titration. Virus back titration for the MN-CPE assay was considered acceptable and reliable if cell control did not show any sign of CPE.

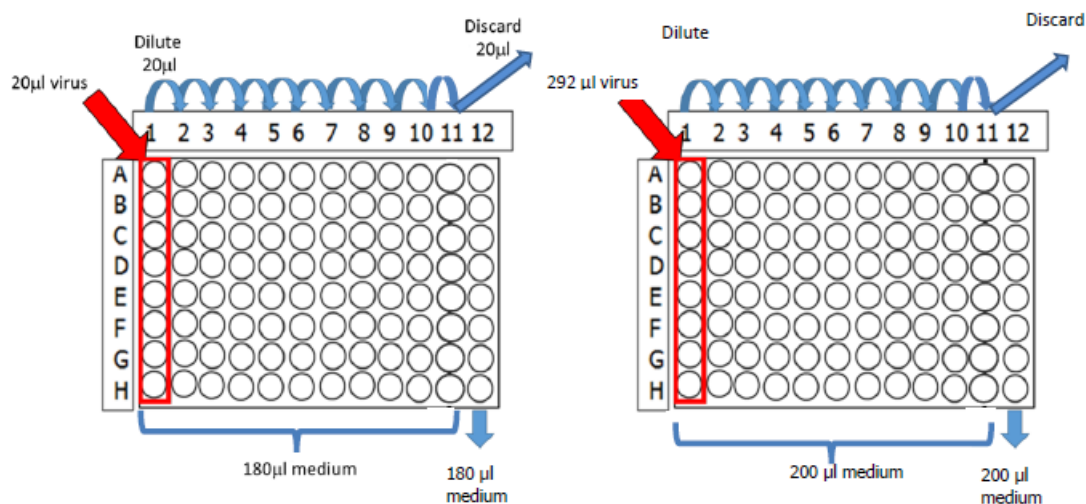


Figure 38 Virus Titration and back titration

Reagents and solution

The reagents and solutions used during the study were:

Medium: Medium to be used for the test (MN medium) is Dulbecco's Modified Eagle Medium (DMEM) with 2% heat-inactivated Foetal Bovine Serum (FBS), 1% L-glutamine, and 1% penicillin/streptomycin.

The medium used for cells growth (cell growth medium) is Dulbecco's Modified Eagle Medium (DMEM) with 10% heat-inactivated FBS, 1% L-glutamine, and 1% penicillin/streptomycin.

Viral working solution: The standard concentration of the virus to be used is usually, 500 TCID₅₀/1ml or 50 TCID₅₀/100 µl. For example: 500 TCID₅₀/1ml = 25 TCID₅₀/well

The volume of the virus stock used to prepare the viral working solution must be ≥ 100 µl. If the volume used is lower the virus stock will be pre-diluted 1:10, 1:100 ectetc..

All experiments with live SARS-CoV-2 viruses were performed inside the Biosecurity Level 3 laboratories of VisMederi Srl. Standardization of neutralizing titers was made following the guidelines of the NIBSC 20/136.

Microneutralization Assay procedure

Before starting the test, all the samples and controls were heat inactivated for 30 minutes $\pm$ 10 minutes at $56^{\circ}\text{C} \pm 1^{\circ}\text{C}$.

Samples were tested in duplicate in one session in two different flat-bottomed 96-well microtiter plates (Test 1A and Test 1B). The amounts of the reagents described in this procedure were indicative to perform one repetition per serum for plate.

The operative steps, are listed below:

1. All the wells of a dilution plate except column 1 and column 11 are filled with 60 μl of MN medium.
2. The column 1 is filled with 108 μl of MN medium. The column 11 of each plate (from A11 to H11) is generally used as cell control (CC), to check and monitor the cell growth of the standard lawn, and it is filled with 120 μl of MN medium.
3. Before the spotting, the serum samples are vortexed for at least 2 second using the vortex mixer. In case of microtubes, when the amount of sera is below 100 μl , the sample is mixed by pipetting 6 times.
4. 12 μl of heat inactivated serum sample is added to each well of column 1 in order to achieve a serum dilution of 1:10 (final dilution after the addition of the viral working solution).
5. 2-fold serial dilutions (1:2) are performed by transferring 60 μl progressively from column 1 to 10 and 60 μl after the last dilution step are discarded. In order to ensure proper homogenization, pipette at least 6 times for each dilution step.
6. 60 μl of viral working solution are added to each well excepted for column 11 (cell control).
7. The plates are incubated 1h at $37 \pm 1^{\circ}\text{C}$ and $5 \pm 1\%$ CO_2 .
8. At the end of the incubation time, 100 μl of virus-serum mixture is added to the 96-wells microtiter plate containing a healthy and sub confluent-to confluent cell lawn.
9. The plates are incubated at $37 \pm 1^{\circ}\text{C}$ and $5 \pm 1\%$ CO_2 for 3 days.

The Microneutralization titre (MNt) was defined as the reciprocal of the highest sample dilution that protects the cell monolayer from CPE. If no neutralization reaction was observed, it was assumed that MN titer was under the lower limit of detection, which was represented by the sample dilution at the first well, (e.g. in case 1:10, has to be reported as 5; in case 1:20, has to be reported as 10).

The test was considered valid only if all validity criteria were satisfied:

- The cell control (CC) shows a healthy cell monolayer and no evidence of 'CPE' (it is acceptable that only one well could be excluded)
- The virus control (VC) shows a cytopathic effect in a cell monolayer (it is acceptable that only one well could be excluded)
- The back titration of the viral working solution lies without the defined target range of 102.20 -103.20 TCID₅₀/ml or according to viral load used in the test
- The single neutralization titre of serum sample from a duplicate determination lies without a range of 1 titre step.

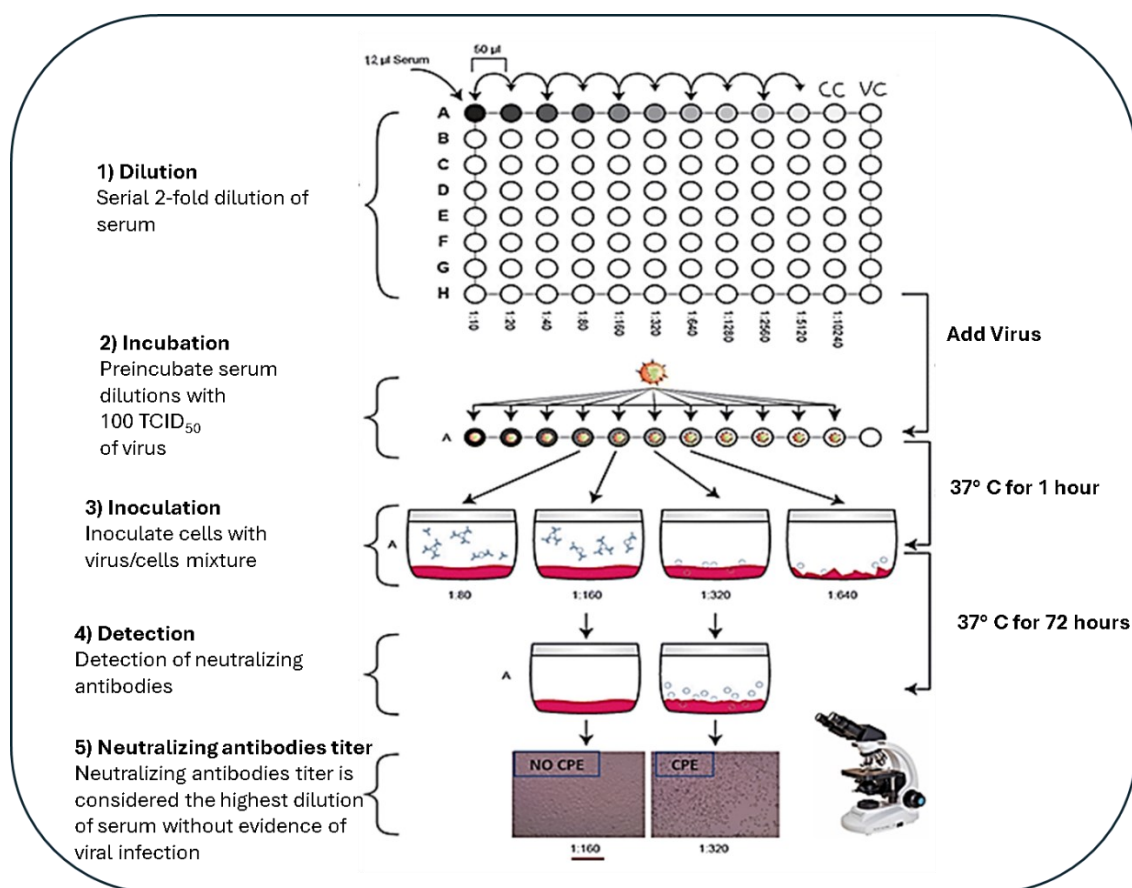


Figure 39 MN-CPE procedure

If only one of the first three criteria is not meet, testing is not acceptable, and the analysis must be repeated in the same conditions. If, even after a second test the results do not meet the criteria of acceptability, the test is repeated using new reagents.

Variant of concern used

For the initial screening of the panel of serum live ancestral SARS-CoV-2 virus (2019 nCoV ITALY/INMI1, provided by EVAg; Genebank: MT066156) was used.

For assessment of cross-reactivity against variants, the assay was conducted with live virus: Alpha (swab isolate 14484; mutations: N501Y, A570D, D614G, P678H, T716I, S982A, T572I, S735L, D69/70, D144Y), Beta (hCoV-19/Netherlands/NoordHolland_10159/2021), Gamma (human isolate PG_253 Clade Nexstrain 20 J/501Y.V3; Mutations: L18F, T20N, P26S, D138Y, R190T, K417T, E484K, N501Y, D614G, H655Y), Delta (sab isolate 31944, mutations: G142D, E156–158del, R158G, L452R, T478K, D614G, P681R, R582Q, D950N, V1061V), and Omicron (VMR_SARSCOV2_Omicron_C1, BA.1, Mutations: A67V, H69del, T95I, G142D, V143–145del, L212I, K417N, N440K, G446S, S477N, E484A, Q493R, G496S, Q498R, N501Y, Y505H, T547K, D614G, H655Y, N679K, P681H, N764K, D796Y, N856K, Q954H, N969K) variants.

Acceptance criteria and European criteria for the assessment of immunogenicity of COVID-19 vaccines

Each MN-CPE assay was considered acceptable and reliable if the cell control showed a healthy cell monolayer and no evidence of CPE; the back titration of the working virus solution lied between 2.75 and 3.75 TCID₅₀/ml; the neutralizing titer of the positive control lied within a target range defined by preliminary experiments; the same serum sample analysed in two different tests did not show two neutralizing titers with a difference greater than ± 1 titer step.

The EMA (European Medicines Agency) has formally standardized the EU requirements for the evaluation of SARS-CoV-2 vaccine immunogenicity in adults. These requirements include specified rates of specific criteria, in which four criteria have to be fulfilled, in particular:

- SARS-CoV-2 Spike (S) and RBD antibody quantification (D0, 28 days after first dose, 28 days after second dose, GMTs and GMFRs)
- Virus NAb assays against SARS-CoV-2 (D0, 28 days after first dose, 28 days after second dose, GMTs and GMFRs)
- Antibody seroconversion rate (≥ 4 -fold increase between D0 and D28) against SARS-CoV-2 S-protein, RBD and NAb
- Proportion with neutralizing titres ($> \text{LLOQ}$), Nab (data pertaining to this endpoint were not provided)

Seroconversion is defined as a ≥ 4 -fold rise in titres from the day of dosing baseline value to 28 days post each dose.

Cytopathic Effect

Historically in 1954 J.F.Enders proposed classifying viruses into groups of those that caused degeneration, those that cause formation of inclusion bodies and cell degeneration, those that cause formation of multinucleated cells (syncytia) and those that caused no cytopathic effect.

During the time that synthesis of viral components is occurring in the infected cell, the cell undergoes characteristic biochemical and morphological changes. Progression of these changes is most readily observed in cell culture, where infection of cells is more easily synchronized and where the cells can be observed and sampled frequently during the course of infection. All morphological changes occurring in cells and caused by viral infection are called cytopathic effects (CPE); the responsible virus is said to be cytopathogenic. The degree of visible damage/lysis to cells caused by viral infection varies with type of virus, type of host cells, multiplicity of infection (MOI), and other factors. Some viruses cause very little or no CPE in cells of their natural host.

Some CPE can be readily observed in unfixed unstained cells under low power (10X objective with 10X ocular for 100X magnification) of the light microscope, with the condenser down and the iris diaphragm partly closed to obtain the contrast needed for viewing translucent cells.

Design of a clinical trial

Preclinical studies

Every candidate drug, vaccine, medical device, or diagnostic assay have to be submitted to in vitro (test tube or cell culture) and in vivo (animal model) experiments using wide-ranging doses of the study agent to acquire preliminary efficacy, toxicity and pharmacokinetic information. These tests are included in the preclinical development and are crucial to evaluate whether a candidate drug can be studied for further development.

Following preclinical development, the effect of the candidate molecule can be evaluated in humans by clinical studies which include a phase 0, I, II and III.

Phase 0 trials are known as human microdosing studies since they are aimed to accelerate the development of promising drugs. Their designation allows to early evaluate the behavior of the examined drug in human subjects as expected from preclinical studies. It is often used as exploratory

study or as an extension of preclinical studies. Due to the too low dose to cause any therapeutic or adverse effect, phase 0 studies are not conducted to obtain data on safety and efficacy but to rank drug candidates to decide which has the best pharmacokinetic parameters in humans to take forward into further development.

Phase I

Phase I trials were formerly referred to as "first-in-man studies since they are the first stage of testing in human subjects. With these trials, safety, side effects, best dose, and formulation method for the drug are tested and evaluated. Normally in vaccine development, they are conducted on a small group of 20–100 healthy volunteers. These subjects are usually monitored until several half-lives of the drug have passed to assess the safety (pharmacovigilance), tolerability, pharmacokinetics, and pharmacodynamics.

The FDA must receive an Investigational New Drug application from the sponsor before a Phase I trial can start, outlining the drug's preliminary findings from animal and cellular model research.

Phase I trials can be further divided:

Phase Ia, called also Single ascending dose since a single dose of the drug is administered to a small group of people who are observed for a period of time to confirm safety. Typically, a small number of participants are submitted to a particular dose. If no adverse side effects are observed, and the pharmacokinetic data reflect the expected values, the dose is gradually increase.

This process continues until pre-calculated pharmacokinetic safety levels are reached, or intolerable side effects start showing up. It is assumed in this particular design that around one-third of participants will develop toxicities that are intolerable even at the highest dose that can be tolerated.

Phase Ib is based on a multiple ascending dose design which aim to investigate the pharmacokinetics and pharmacodynamics of multiple doses of the drug, supervising the safety and tolerability. Briefly, a group of patients is administered multiple low doses of the drug, whilst data on the metabolism of the drug in the body are obtained by collecting and analyzing samples (of blood and other fluids) at different intervals. The dose is subsequently escalated until a toxic effect is reached.

Phase II and III

The following steps aim to evaluate whether the drug has any biological activity or effect and are included in the phase II clinical trials. In this context tests are performed on larger groups (50–300 individuals) and are designed to assess the effectiveness, as well as to continue safety assessments in

a larger group of volunteers and patients. During the phase II trials the drug development fails if it is shown to appear hazardous side effects or less effect than expected. Phase II studies are sometimes divided into Phase IIa and Phase IIb.

Some Phase II trials are designed as case series to show safety and effectiveness in a selected group of people. Others are planned as randomized trials, wherein some patients receive the medication or equipment and others get normal care or a placebo.

Phase III trials have the goal to determine the effectiveness on a larger group of healthy people, therefore, to determine its application in clinical practice. In these trials randomization controlled studies on large patient groups (300–3,000 or more depending upon the disease/medical condition studied) are conducted to determine effectiveness of the drug compared current 'gold standard' treatment. Phase III trials are the most expensive, time-consuming and difficult trials to design and run. They are sometimes called the "pre-marketing phase" and they can continue during regulatory submission until the drug can be obtained by purchase and to obtain additional safety data. FDA (US), or the EMA (European Union) typically require at least two successful Phase III trials, to demonstrate drug safety and efficacy.

After Phase III trials, a large report including a complete description of the methods and results of human and animal studies, manufacturing procedures, formulation details, and shelf life is released. Based on that the submission to regulatory organs and authorities can be applied to obtain the approval to market the drug. Most drugs are marketed following country specific norms including recommendation and guidelines and are subjected to recall whether any unexpected side effect are observed.

In this work we report the results of the phase I clinical study of CoVLP, a virus-like particle (VLP) vaccine that is produced by transient transfection of *Nicotiana benthamiana* plants. These VLPs spontaneously assemble at the plant cell membrane and display SARS-COV-2 trimers of stabilized pre-fusion S protein on their surface. In the clinical study 180 healthy adults (18-64 years) subjects were recruited. Serum samples were withdrawn and analyzed at Day 0 (pre-vaccination phase), Day 21 post first immunization and Day 42 (21 days post second immunization). Serum samples from vaccinated subjects were also analyzed at Day 201 after second immunization (Figure 40).

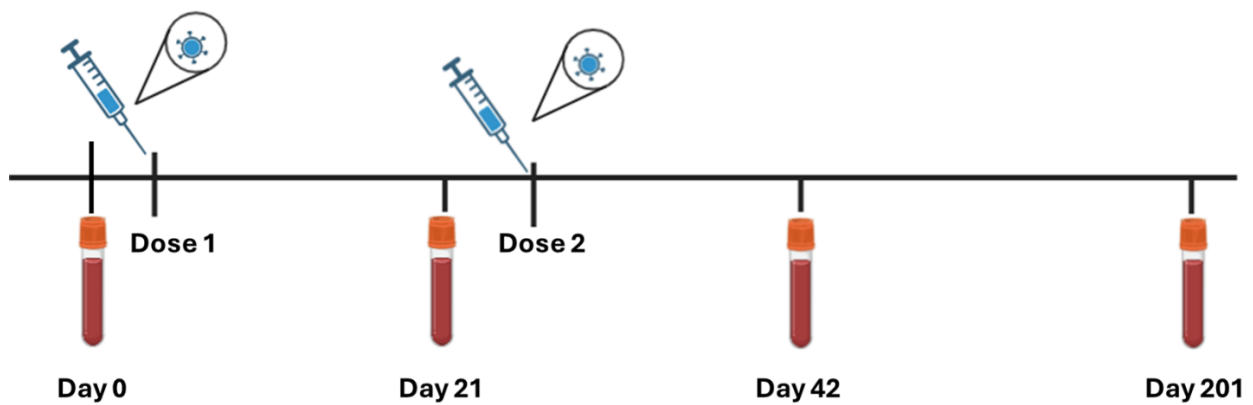


Figure 40 Study design of the phase I clinical study analyzed in this work. 720 serum samples were withdrawal in four phases. At Day 0 (baseline), Day 21 (visit 2), Day 42 (visit 3, 21 days after second dose) and Day 201 (visit 4).

Statistical analysis

All statistical analyses were performed using GraphPad Prism version 6.01 for Windows (GraphPad Software, San Diego, CA, USA). For each variant, the antibody levels were reported and statistically evaluated with respect to the wt strain using a paired t-tests.

RESULTS

Screening for detection neutralizing antibodies against SARS-CoV-2 original variant

A panel of total number of 540 human serum samples, which included day 0 (pre-first dose), day 21 (pre-second dose) and day 42 (21 days post second dose), were obtained from 180 subjects aged between 18 and 64 years and immunized in the context of a phase 1/2 clinical trial designed for the release of a *Nicotiana benthamiana*-based VLP vaccine.

Each serum sample was tested in two different MN-CPE tests, which were performed and read by two different high qualified MN-CPE operators, obtaining 4 results for each serum sample, for a total of 2160 results including day 0 (720 results), day 21 and day 42 (1440 results) for SARS-CoV-2 original strain. For each sample, the neutralizing titer was defined as the reciprocal dilution corresponding to the NT50. The samples below cut-off were attributed a value of 5 (half the minimum required dilution).

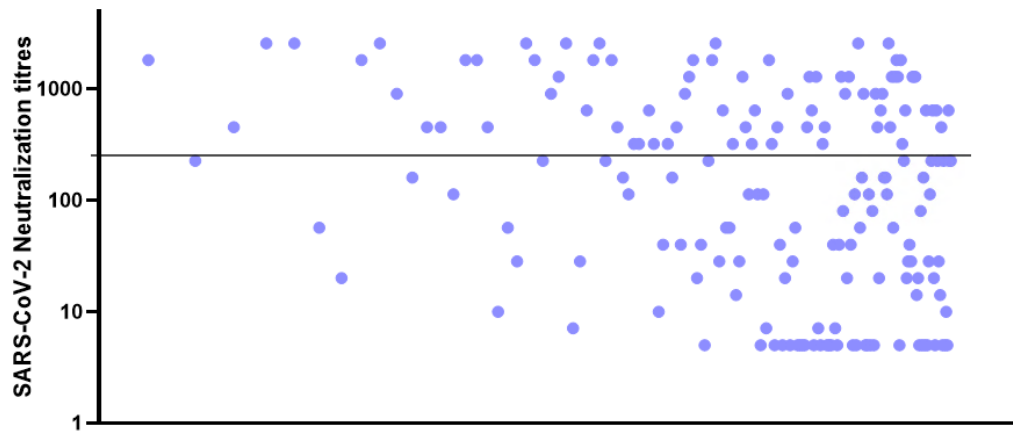


Figure 41 Screening of 180 sera. Points above the black line were chosen for a further analysis for their mid/high response against Sars-CoV-2 original variant. 57 subjects of 109 seropositive subjects show a mid/high response on day 42. These sera were chosen for a further analysis aimed at the evaluation of the effectiveness against the other VOCs.

Results obtained from visit 2 serum samples showed a geometric mean of the MN-CPE neutralizing titers (GMT) of 10.01, whilst results obtained from visit 3 serum samples showed a GMT of 120.36 (Figure 42). Among 180 subjects 71 (39.4%) reported a titer ≤ 4 -fold dilution compared to Day 0, even after second dose and were excluded from the screening.

57 subjects of the remaining 109 seropositive subjects show a mid/high response and reported a GMT of 987 on day 42 (Figure 42). These sera were chosen for a further analysis aimed at the evaluation of the effectiveness against the other VOCs B.1.1.7 Variant (Alpha variant), B.1.351 Variant (Beta

variant), P.1 Variant (Gamma variant), B.1.617.2 Variant (Delta Variant), B.1.1.529 Variant (Omicron Variant).

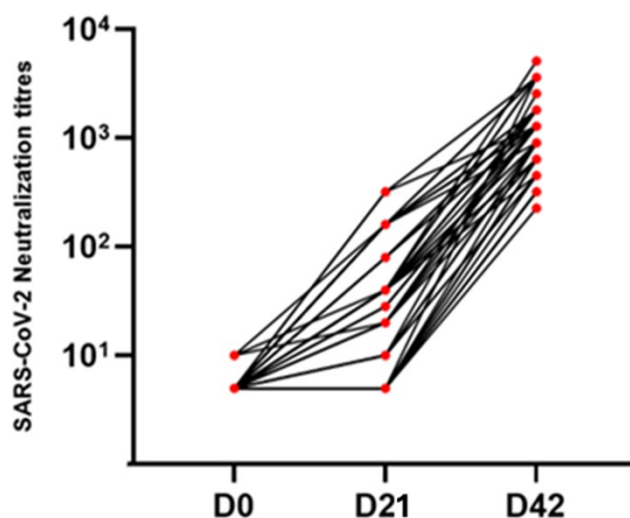


Figure 42 GMTs of Day 0, Day 21 and Day 42 serum samples obtained through the MN-CPE assay with the SARS-CoV-2 original strain. Individual values of serum samples are reported with black lines. Red dots represent GMTs at different clinical trial phases. Results for 57 subjects reported a mid/high response and reported a GMT of 987 on day 42.

Neutralizing antibodies against variants of concern

To better investigate neutralizing antibodies cross-reactivity to variants of concerns, both for visit 1, visit 2 (21 days after the first dose) and visit 3 (21 days after a second dose), a further analysis was carried out. A total number of 171 human serum samples obtained from 57 subjects screened with the previous experiments were analyzed.

Each serum sample was tested, as previously described, in two different MN-CPE tests, which were performed and read by two different high qualified MN-CPE operators, obtaining 4 results for each serum sample, for SARS-CoV-2 B.1.1.7 Variant (Alpha variant), B.1.351 Variant (Beta variant), P.1 Variant (Gamma variant), B.1.617.2 Variant (Delta Variant), B.1.1.529 Variant (Omicron BA.1 Variant) for a total of 3420 results including day 0 (1140 results), day 21 and day 42 (2280 results).

Alpha variant results obtained from visit 2 serum samples showed a geometric mean of the MN-CPE neutralizing titers (GMT) of 17.32, whilst results obtained from visit 3 serum samples showed a GMT of 380.54 (Figure 43). The MN-CPE neutralizing titers against B.1.1.7 variant reported at least 1/2-fold decrease in neutralizing antibody titer, compared to the results obtained for the original variant. Particularly, 4 serum samples displayed a titer $\leq$ 4-fold dilution over the LLOD (lower limit of

detection). A total reduction of 62 % in MN titer value was observed with a mean decrease of 1.5-fold.

Gamma variant results reported a geometric mean of the MN-CPE neutralizing titers (GMT) of 9.65 for day 21, while results obtained from visit 3 serum samples showed a GMT of 115.78 (Figure 43). The MN-CPE neutralizing titers against P.1 variant reported at least 3/4-fold decrease in neutralizing antibody titer, compared to the results obtained for the original variant. Titers were halved if compared to UK variant with 27 serum samples below the 4-fold dilutions compared to the baseline. A total reduction of 89 % in MN titer values was observed with a mean decrease of 3.3-fold.

Beta variant results obtained from visit 2 serum samples showed a geometric mean of the MN-CPE neutralizing titers (GMT) of 10.17, whilst results obtained from visit 3 serum samples showed a GMT of 129.21 (Figure 43). The MN-CPE neutralizing titers against B.1.351 variant were comparable to the P.1 one with a slight increase in neutralizing antibody titer GMT. A total reduction of 87 % in MN titer values was observed, overall.

For Delta variant results obtained from visit 2 serum samples showed a geometric mean of the MN-CPE neutralizing titers (GMT) of 17.6, while results obtained from visit 3 serum samples showed a GMT of 513.86 (Figure 43). The MN-CPE neutralizing titers gave back a result even higher than B.1.1.7 variant (14 % increased) ones but at least 1-fold decrease in neutralizing antibody titer, compared to the results obtained for the original variant (48 % of reduction). Only three results were considered below the canonical 4-fold rise in titres both from the day of dosing baseline and from day 21.

Lastly, Omicron variant results were obtained and analyzed from visit 2 serum samples showing a geometric mean of the MN-CPE neutralizing titers (GMT) below the LLOD and results from visit 3 serum samples with a GMT slightly positive, overall (Figure 43). Only two samples displayed a positive titer of 113 and 160, respectively, just above the threshold. These MN-CPE neutralizing titers confirmed the complete inhibition of vaccine effectiveness by Omicron variant.

Overall, cross-reactivity to Alpha and Delta variants was comparable to the ancestral strain while a modest decrease was observed for the more antigenically distant Beta and Gamma strains. Cross-reactivity to the antigenically distinct Omicron (BA.1) variant was extremely reduced.

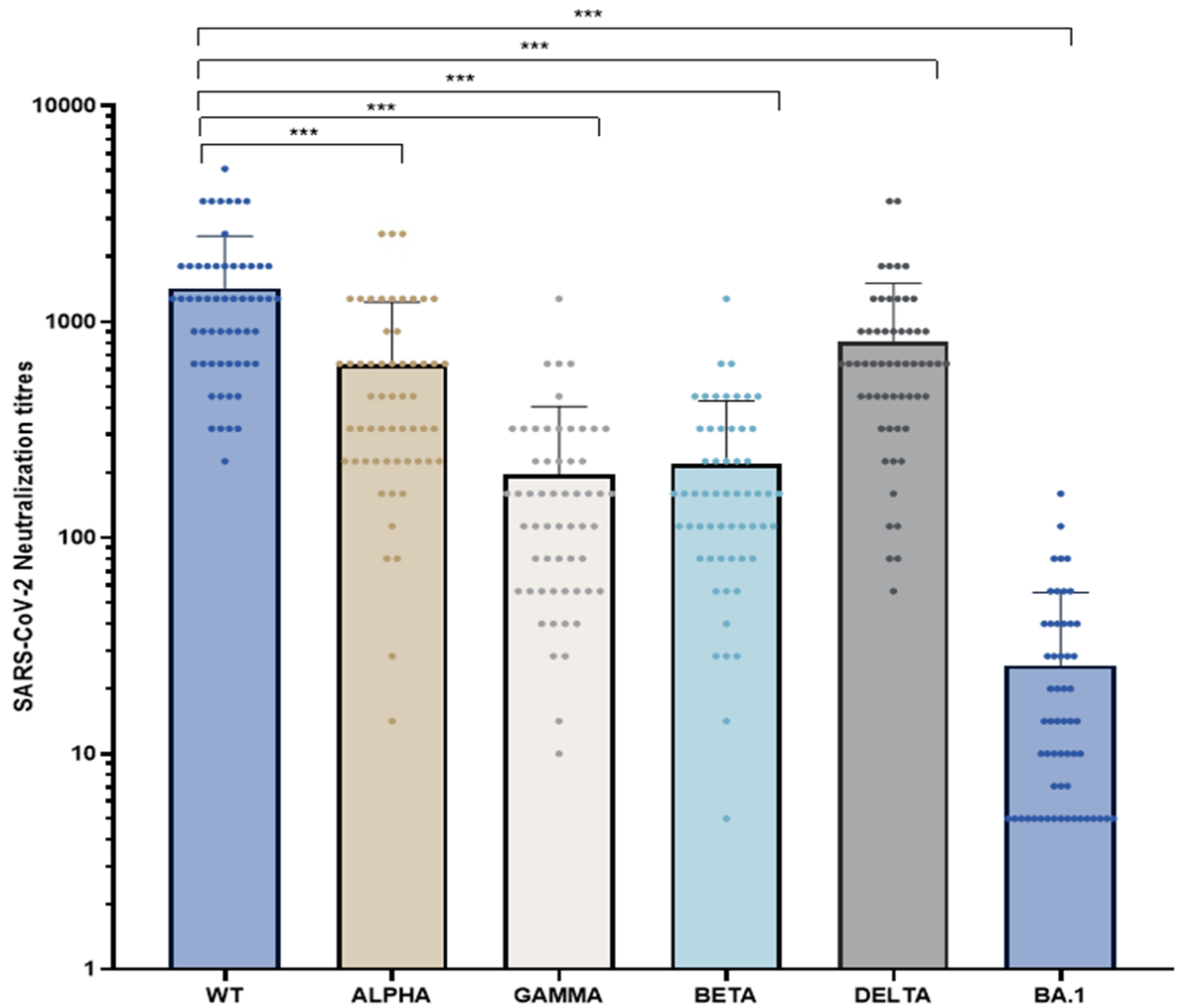
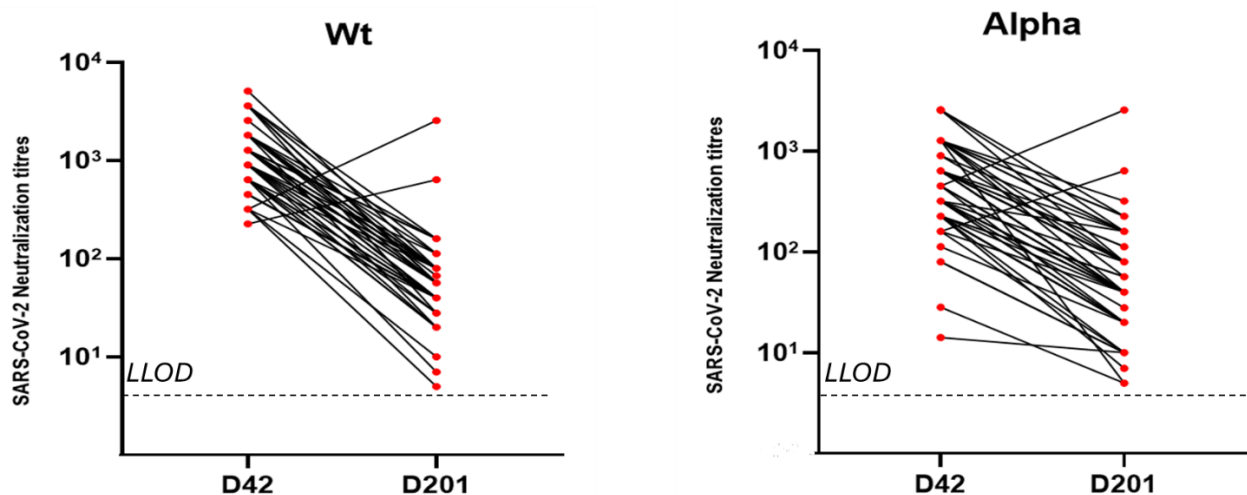


Figure 43 Results comparison of Day 42 serum samples. A gradual decrease in neutralizing titers was observed. Delta variant restored and even enhanced the titer of the first variant of concern (UK variant). Omicron variant showed a complete inhibition on vaccine inhibition. For each variant, VN titers were compared with respect to the wt strain using a paired t-test (**p-value $\leq$ 0.0001)

Neutralizing antibodies response after 201 days from second dose

Vaccine effectiveness was evaluated up to 201 days (six months) post second immunization for SARS-CoV-2 original variant. The experiments were performed as previously described by two different operators in two different tests to obtain 4 different results for a total of 228 results. As shown in Figure 44, on day 201 a persistent neutralizing reactivity was maintained for many serum samples, with a GMT of 57.8. Particularly, 23 of 57 (40.3 %) serum samples reported a titer ≥ 4 -fold from the baseline day 0 with 15 of these with a titer even 20-50x from LLOD. Surprisingly, 2 of 23 reported a titer even higher than Day 42 of 2/3-fold. Finally, 3 of 57 (5 %) serum samples were found completely negative or slightly positive with a titer of 5-10. Overall, a 4-fold decrease in titers compared to day 42 was observed, with a mean decrease of 4.4-fold, even though the decrease is not linear and predictable as for variant cross-neutralization.

Persistent cross-reactivity was observed in 23 of 57 (40.3 %) participants also for the Alpha and Delta variants, 8 of 57 (14 %) participants to the Beta variant, and 12 of 57 (21 %) participants to the Gamma variant. For the Omicron variant only titers below 4-fold compared to the baseline were reported, with 6 of 57 samples only showing a slight positive titer (Figure 44). For all VOCs an increase titer compared to Day 42 was confirmed for two participants as reported for ancestral variant. Finally, the rate of negative values was proportional to the distance of the variant from the ancestral strain. Overall, the D201 GMTs to Alpha, Gamma, Beta, Delta, and Omicron variants were 50.3, 21.9, 17.9, 52.5, and 7.24, respectively. Moreover, the trend of the titers reflects the results obtained at day 42 with a global mean decrease of 3/4-fold, confirming a certain predictability for variant cross-neutralization. Cross-reactivity to Alpha and Delta variants was comparable to the ancestral strain while a modest decrease was observed for the more antigenically distant Beta and Gamma strains. Cross-reactivity to the antigenically distinct Omicron (BA.1) variant was reduced.



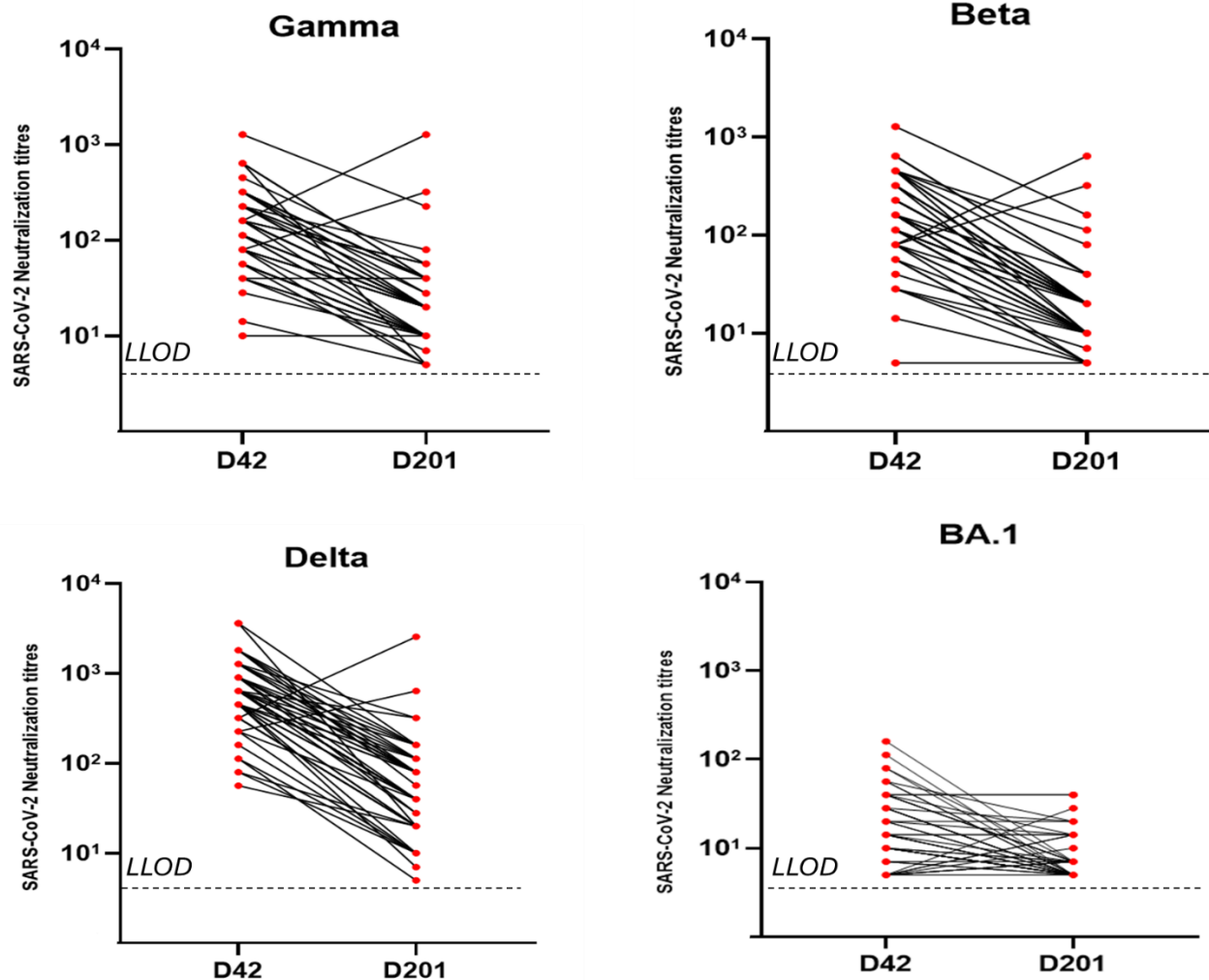


Figure 44 Neutralizing antibody cross-reactivity to SARS-CoV-2 variants of concern at Day 42 and Day 201 quantified through the MN-CPE assay. Individual values of serum samples are reported with black lines. Red dots represent GMTs on Day 42 and Day 201. The lower limit of detection (LLOD) is shown with a dotted black line.

Serum Neutralizing Activity against SARS-CoV-2 VOCs in Hospitalized COVID-19 Patients

Vaccine effectiveness of the candidate plant-based vaccine was compared to serum neutralizing activity in hospitalized COVID-19 patients. The study was carried out against Alpha, Beta, Gamma, Delta and Omicron BA.1 SARS-CoV-2 VOCs using 42 sera of COVID-19 patients positive for wild type variant, in collaboration with the University of Siena. Samples were recovered from hospitalized patients during the first epidemic wave that occurred in Italy between March and May 2020.

Serum neutralizing activity was evaluated at different time points: hospital admission (baseline), hospital stay (peak) and deceased/discharged. Serum samples was defined negative when below the LLOD (Lower Limit of Detection) (Figure 45).

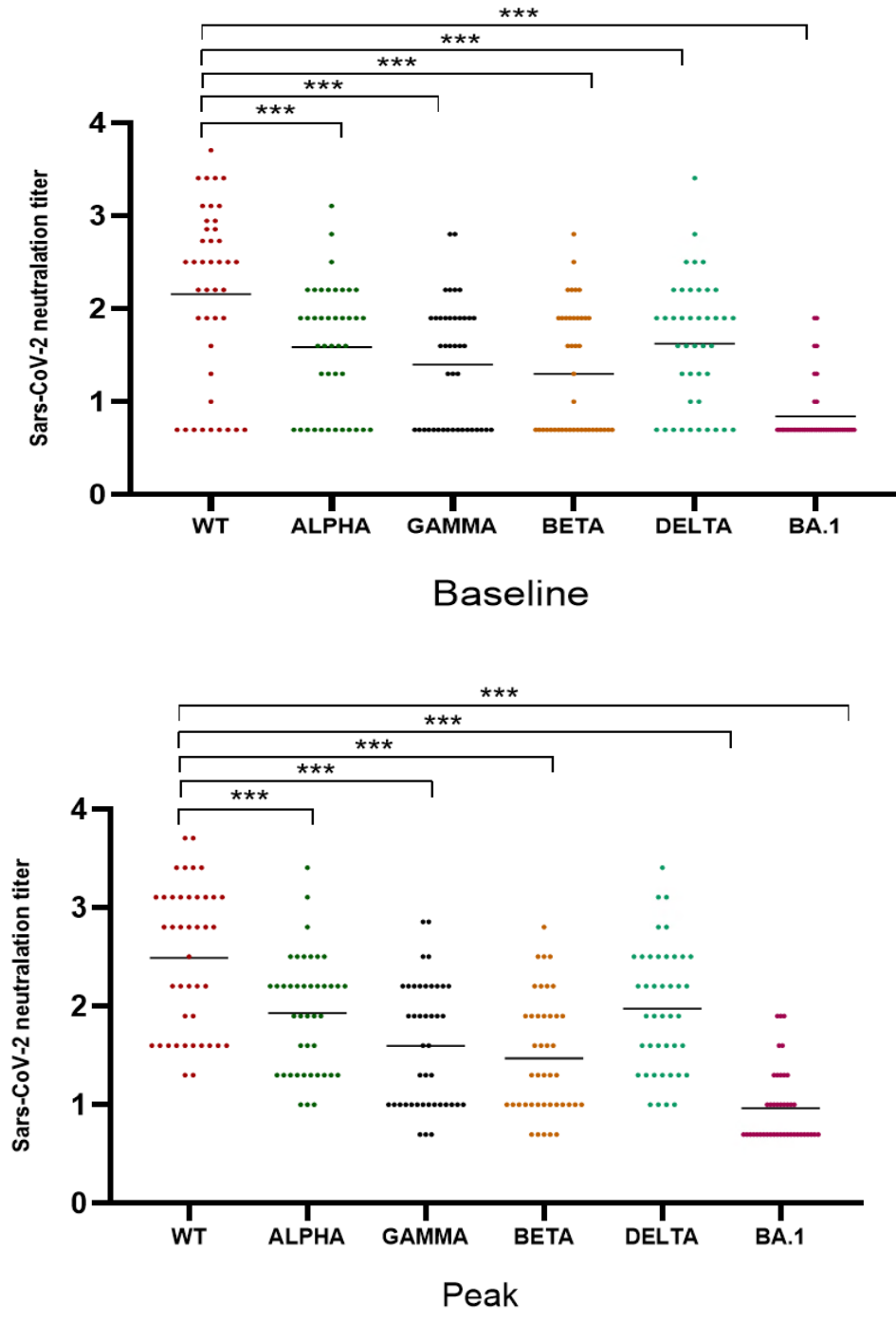
At baseline, 9 patients (21.4 %) were negative for neutralizing antibodies against the wt strain and all the other VOCs. 2 more patients (26.6 %) were negative for Alpha variant, while a total of 18 (42.8%), 25 (59.5%), 9 (21.4 %) and 37 (88%) patients were negative for Gamma, Beta, Delta and Omicron variants, respectively. Overall, 24 (57.4%), 30 (71.4%), 30 (71.4%), 25 (59.5%) and 42 (100%) patients showed a ≥ 2 -fold decrease in neutralizing antibody titer compared to wt against Alpha, Gamma and Beta, respectively.

At peak, all patients exhibited a neutralizing titer for the wt strain. 28 (66%) samples showed a ≥ 2 -fold decrease in neutralizing activity for Alpha variant, with a GMT of 85.46. Particularly, for 26 (92.8%) patients a 2/3-fold decrease was observed, while for 2 (7.1%) a ≥ 4 -fold decrease was reported. For Gamma variant, 36 reported a ≥ 2 -fold decrease; 20 (55.5%) showed a 2/3-fold decrease, 9 (25%) showed a 4-fold decrease, and 7 (19.4%) showed a >4 -fold decrease, with a GMT of 39.57. For Beta variant 19 (45.2%) showed a 2/3-fold decrease, 7 (16.6%) a 4-fold decrease, and 11 (26.1%) a ≥ 4 -fold decrease, with a total of 37 patients with a ≥ 2 -fold decrease and a GMT of 29.72. Delta variant reported 28 patients with a ≥ 2 -fold decrease, 18 with 2-fold decrease, 5 with 3-fold decrease and 2 $>$ of 4-fold decrease, with a GMT of 94.35. Omicron reported quite all the samples negatively below the LLOD with only 3 samples with an increase of 4-fold with respect to the baseline

Even at discharge/decease, all patients exhibited a neutralizing titer for the wt strain, although a reduction was detected. For Alpha variant, 25 (59.5%) patients reported a ≥ 2 -fold decrease, with one patient (2.4%) found negative, with a GMT of 48.76. For Gamma variant, 33 (78.5%) patients reported a ≥ 2 -fold decrease, with 7 patients (16.6%) found negative when tested and with a GMT of 25.62. 11 patients (26.1%) were found negative when tested against Beta variant, and a ≥ 2 -fold decrease was observed in 31 (73.8%) patients, with a GMT of 16.35. For Delta variant only 1 sample

was found negative, while 25 patients showed a ≥ 2 -fold decrease in neutralizing titer, with a GMT of 38.70. Omicron variant confirms the results obtained at peak phase with a maximum titer of 40.

Overall results obtain from these experiments revealed a close correlation between vaccine induced immunity and natural occurring immunity.



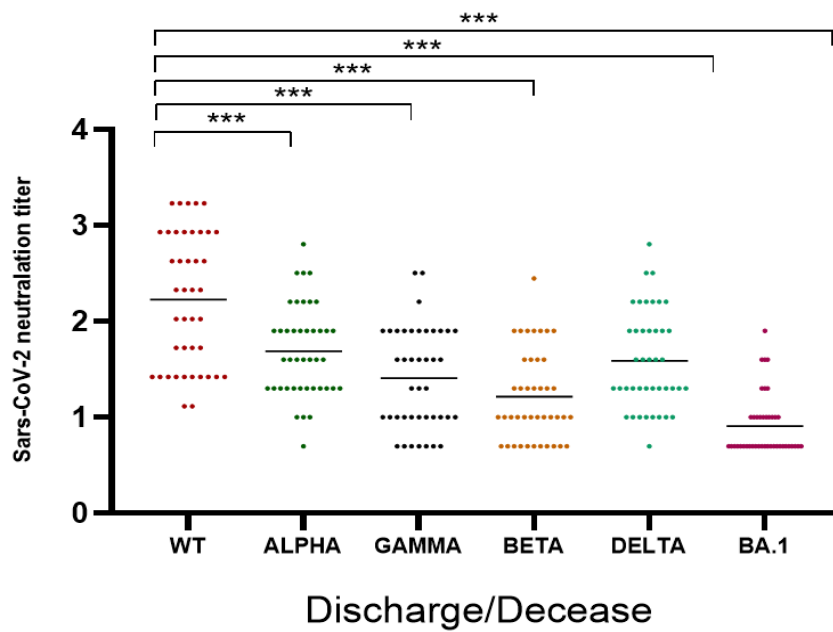


Figure 45 SARS-CoV-2 virus-neutralization (VN) titers of convalescent serum samples. For each variant, VN titers were reported at hospital admission (baseline), hospital stay (peak) and deceased/discharged. Overall, results reported an excellent response to wt variant as well to Alpha and Delta with a minor neutralization titer for Gamma and Beta and a strong decrease for Omicron. Data were compared with respect to the wt strain using a paired t-test (***) $p \leq 0.0001$.

DISCUSSION

Since it first appeared in Wuhan in December 2019, SARS-CoV-2 has led to over 700 million COVID-19 cases and over 7 million deaths by late 2024, according to WHO. SARS-CoV-2 belongs to the Severe acute respiratory syndrome-related coronavirus species, the only member of the Sarbecovirus subgenus, mainly present in horseshoe bats. However, its origin still seems to be unknown

SARS-CoV-2 is very effective at infecting humans and has the tropism for a broad range of host mammalian species, such as farmed mink, white-tailed deer (for which it represents a stable reservoir), and incidental infections of many other animal species.

Once entering humans, SARS-CoV-2 experienced minimal adaptation and phenotypic modifications during its initial months compared to its later development. In late 2020, these more highly mutated versions of SARS-CoV-2 showed increased transmission rates compared to earlier variants and were called 'variants of concern' (VOCs). Alpha, Beta, Gamma, Delta, and Omicron were designated as the VOCs and emerged separately from each other. Each variant quickly became dominant, either regionally or globally, surpassing previous variants.

The success of each VOC over the prior dominant variant was facilitated by changes in the virus's intrinsic functional traits (such as alterations in Furin cleavage site or interaction with the ACE-2 receptor) and, to varying extents, modifications to its antigenicity allowing it to evade a primed immune response.

The enhanced virus fitness linked to VOCs is due to a complex interaction between virus biology and evolving human immunity from vaccination and previous infection.

Even though small changes in antigens were found for Alpha and Delta, there was a noticeable evasion of vaccine-induced antibodies and recovered patients' sera in the case of Beta, Gamma, and Omicron during lab tests. This is due to the fact that while high-impact mutations that help the virus adapt and survive exist, they are less common than minor changes in amino acids with low or no effect. Hence, epidemiological and serological studies are necessary in order to assess the effectiveness of vaccines and factors that provide protection and to satisfy international standards and acceptability criteria. Additionally, manufacturers are getting ready for potential updates in vaccine sequences, highlighting the importance of monitoring genetic and antigenic changes in the worldwide virus population while conducting experiments to understand the effects of mutations.

Our work analyzed a phase I clinical study for the release of a VLP plant-based vaccine, which was represented by 180 subjects. Upon a first screening 57 subjects of 180 were selected for their positivity to SARS-CoV-2 wt at day 21 after second dose. Subsequently, the same 57 sera were tested for a further analysis against the VOCs Alpha, Beta, Gamma, Delta and Omicron. The response to

vaccination was also evaluated at day 201 after second dose for each variant of concern. Finally, these results have been compared to neutralization titer for each variant of concern of serum samples from naturally infected hospitalized patients.

Overall, for Alpha was observed a decrease of 1/2 fold compared to wild type with seroconversion observed for 53 serum samples at Day 42. This event can be attributable to some critical mutations like D614G or N501Y in RBD (receptor binding domain) site which would enhance Spike cleavage and improve the accessibility to ACE-2 receptor respectively, therefore favoring the entry of the virus in the cell by the so called “open” prefusion conformation state, even though without a significant implication for neutralizing activity (Wang *et al.*, 2021). This result reflected also the data obtained from the convalescent sera experiments, even though with an antibody titer globally lower than the one obtained from vaccinated subjects. Indeed, in the phase of peak 66% of samples displays ≥ 2 -fold decrease against the Alpha variant while in the phase of discharge a 2-fold decrease in neutralizing antibody titer was observed in $\geq 59.5\%$ of patients. In particular only 7% of these displays ≥ 4 -fold decrease at peak, whilst 20% exhibited at discharge. Conversely, different research has shown that samples from recovered or hospitalized patients can effectively neutralize the Alpha variant, with either maintaining comparable levels of neutralizing activity or showing a slight decrease in comparison to the original antibody concentration. (Rees-Spear *et al.*, 2021).

Results for Gamma and Beta variant are quite comparable since they showed a 3/4-fold decrease when related to the original variant with 27 and 31 samples below or equal to 4-fold dilution, respectively. This fact can be ascribable to the presence of some more key mutations like E484K/Q K417N/T, L452R and T478K, shared between the two variants and located in the Spike protein RBD with a great impact in the reduction of neutralizing antibodies. Particularly, it was reported that the E484 residue is crucial, when amino acid changes to K, Q or P, or the reduction of neutralization titers by more than an order of magnitude (Greaney *et al.*, 2021). In convalescent patients, the study found that 85.2% and 78.5% (respectively at peak and at discharge) showed a significantly decreased neutralizing activity (a ≥ 2 -fold decrease) due to the Gamma variant. The decrease in neutralizing ability was greater against the Beta variant, with 88% of patients experiencing a ≥ 2 -fold decrease, showing a slight reduction when compared to that seen against the Gamma variant. The results align with previous research by Wang *et al.*, 2021 showing that antibodies have lower neutralizing ability towards certain variants, but Gamma is better neutralized by convalescent and vaccinated sera compared to Beta.

The Delta variant showed a higher neutralizing titer, more similar to the original variant and the Alpha and a seroconversion approximately of 96 % of participants at Day 42. Indeed, data reported a

neutralizing activity reduced of 2-fold in the clinical study or even higher compared to Alpha in convalescent serum samples study, overall. The neutralizing titer was clearly higher of 2/3 fold when compared to variant Beta and Gamma. The results are also assimilable to the ones from natural infection for which 66.6% of the patients had a decrease (≥ 2 -fold) in neutralizing antibody titer at peak and 59.5% with only one patient was found negative, at discharge. This effect is probably linked to the absence of mutations as N501Y, E484K/Q and K417N which were reported to contribute to the increased infectivity, reduced antibody neutralization, and significant immune escape, but also resistance to vaccination for Beta and Gamma variant (Khan *et al.*, 2021). However, the presence of eight key mutations in S-protein, namely T19R, G142D, L452R, T478K, D960N, P681R, E156G and the deletion 157 and 158 can affect the antibody binding and lead to the evasion of immune response, therefore causing a more aggressive infection. Particularly several studies reported the importance of P681R (in furin cleavage site) and T478K (in RBD) mutations in the increase of pathogenicity and the infectivity (Plante *et al.*, 2021) (Saito *et al.*, 2022). Other mutations as T19R, G142D, E156G, L452R and del157/158 (at the N-terminal domain) seems to be crucial in the immune escape and evasion, particularly L452R in RBD's hydrophobic plaques in the S protein was able to enhance protein and ACE2 receptor interaction.

Omicron BA.1 variant displayed a huge decrease of neutralizing titers, compared to the other VOCs. Indeed, most of the sample showed a titer just above or equal to the LLOD. Overall, the results reflected the ones of convalescents analysis, highlighting the ability of this variant to easily escape immunization of the first phase of the pandemic. This event can be attributable to the nature of the variant considered widely distant from the original variant. Unlike the other SARS-CoV-2 variants, in fact, the Omicron variant is known to have around 60 mutations, with 15 alterations in the RBD and 35 alterations in the S protein; therefore, it has the most mutation sites of all known coronavirus variants. Particularly, these mutations regard 30 amino acid substitutions, three in-frame deletions, and two amino acids insertions. Regarding the receptor-binding domain of Omicron, substitutions such as G339D, S477N, Q493R, N501Y, N440K, S371L, S373P, S375F, Q498R, and T478K conferred higher binding affinity to ACE2, moreover 3 substitutions (N679K, H655Y, and P681H) close to the furin cleavage site conferred a higher evasion ability and an increase in transmissibility. Moreover due to these mutations the contribution of TMPRSS2 can be marginal. Despite for Omicron was reported a higher transmissibility, BA.1 displayed a less severe disease compared to the other VOCs (Willett *et al.*, 2022). This may be because of its faster growth rate and transmissibility. The large decrease in neutralization of live virus with Omicron Spike by sera from vaccine formulated on the original variant or from wt infection highlights the large antigenic distance with the previous variants.

Overall, this study revealed that a plant-based platform is an extremely powerful tool to rapidly design and release vaccines for the fighting and preparedness to future pandemic. Indeed, this kind of technology can be rapidly scaled up using vertical farming or greenhouse systems and it proves much faster and cheaper than traditional egg-based or mammalian cell-based systems with no need for sterile bioreactors systems. Moreover, it is an eco-friendlier technology compared to mammalian cell cultures for the reduction of water and energy use and the unneeded of antibiotics. However, challenges related to immunogenicity, regulatory approvals, and stability must be addressed to ensure their widespread use.

Results from this study reported an excellent response to wt variant as well to Alpha and Delta with a minor neutralization titer for Gamma and Beta and a strong decrease for Omicron. This decreasing power of neutralization against the different VOCs is due to the antigenic drift of the virus that occurs for the accumulation of mutations in the Spike protein, suggesting the need for a periodically update of the vaccine. This event is also more evident when compared to the neutralization titers of serum samples from convalescent subjects from the first wave of the pandemic. Indeed, the two studies revealed a quite similar behavior of the serum samples against the VOCs, even though with mean decrease of 2/3 fold, to prove the more powerful effect of an induced immunization compared to the natural infection. Moreover, as for other kind of vaccines, the plant-based tool showed a consistent decreasing in neutralizing titer, therefore in IgG level, at six months suggesting the need to administer more doses to obtain a significative vaccination coverage, even though the immune response induced by first doses of vaccine or natural infection may protect from severe disease. Furthermore, this study highlights the importance of evaluating SARS-CoV-2 pre-existing immunity against emerging variants as a tool to foresee the immune escape and the extent of vaccine efficacy.

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