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Evaluation of the efficacy of a peptide-based vaccine (PNV1) against multi-resistant gram-negative bacteria in a neonatal mice model

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ABSTRACT

According to WHO, bacterial infections are the leading cause of death among infants and newborns, with an estimated death of 4 million children worldwide. Although the large range of problems associated with this type of infection and the high incidence, neonatal sepsis is still difficult to diagnose and there are currently no adequate treatments.

Group B streptococcus (GBS) and *Staphylococcus aureus*, as gram-positive, and *Klebsiella pneumoniae* and *Escherichia coli*, as gram-negative, are the most prevalent pathogens in cases of neonatal bacterial infections.

Previous studies from our team demonstrated that the neonatal susceptibility to GBS infections is due to an early production of interleukin-10 (IL10), an immunosuppressive cytokine, which prevented an adequate response of the organism. This early production of IL10 prevents the recruitment of neutrophils to the site of infection. This allows bacterial proliferation and rapid dissemination throughout the body, leading to multi-organ failure and death. On the other hand, it was demonstrated by the group that this early production of IL10 was due to the activity of an extracellular bacterial protein, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) that functioned as a virulence factor of the bacteria. Due to the similarity of bacterial GAPDH to human GAPDH, the group developed a peptide-based vaccine (PNV1) composed by surface-exposed peptides of bacterial GAPDH that are completely absent from human GAPDH. Protective results were obtained in animals immunized with the vaccine against gram-positive infections, namely GBS, *S. aureus* and *Streptococcus pneumoniae*.

Taking these results into account, and considering the great similarity between GAPDH of gram-positive and gram-negative bacteria, the present study had as hypothesis the evaluation of the efficacy of this same vaccine in infections caused by gram-negative bacteria, such as *Klebsiella pneumoniae* and *Escherichia coli*.

Thus, in order to evaluate the efficacy of PNV1 in preventing neonatal infections caused by *Klebsiella pneumoniae* and *Escherichia coli* neonatal C57BL/6 mice were passively immunized with PNV1 IgG (purified from PNV1 immunized female adult mice) 12h before challenging infection with the referred bacteria. Passively immunized animals with PNV1 IgGs showed a significantly increase in the survival rate when compared to animals immunized with control IgG.

Besides the neonates, the individuals with type I diabetes are also at increased risk of contracting severe infections caused by these same bacteria. Thus, PNV1 efficacy was also tested in non-obese diabetic mice. The present results show that in these groups there was also an increase in the survival rate of the animals immunized with PNV1 IgG, that this may be a possible target for further intervention.

Finally, an optimization of the vaccine was also executed, by conjugation of the previously chosen peptides with a potent immunostimulatory molecule, keyhole limpet hemocyanin (KLH). This

conjugation led to a greater production of IgG antibodies in mice, suggesting that this conjugation allows an improvement of the product, thus allowing to advance later for clinical trials.

All these results shown that there is a common mechanism of virulence between infections caused by gram-positive and gram-negative bacteria and that PNV1 is a vaccine that can be a strong candidate in the fight against infections caused by this type of microorganisms.

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“The best way to predict the future is to create it.”

Abraham Lincoln

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ABBREVIATIONS AND SYMBOLS

2-ME	2-mercaptoetanol
AP	alkaline phosphatase
APC	antigen-presenting cell
BSA	bovine serum albumin
CD	cluster of differentiation
CDC	Centers for Disease control and Prevention
CEP	crude extracellular products
CFU	colony form units
CoNS	coagulase negative staphylococci
EOS	early-onset sepsis
FBS	fetal bovine serum
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GBS	Group B Streptococcus
HRP	Horseradish peroxidase
I.P.	Intraperitoneally
IAP	intrapartum antibiotic prophylaxis
Ig	immunoglobulin
IL	interleukin
IPTG	Isoprophyl-β-D-thiogalactopyranoside

IVIg	Intravenous administration of immunoglobulins
KLH	Keyhole Limpet Hemocyanin
LOS	late onset sepsis
LPS	lipopolysaccharide
MHC	major histocompatibility complex
MZ	marginal zone
NK	natural killer
NOD	non-obese diabetic
PAMPs	pathogen-associated molecular patterns
PBS	Phosphate Buffered Saline
PCR	polymerase chain reaction
PRRs	pattern recognition receptors
rGAPDH	recombinant GAPDH
ROS	reactive oxygen species
S.C.	Subcutaneously
TGF-β	Tumor growth factor beta
TLR	toll-like receptor
TLR2$^{-/-}$	TLR2 knockout
TMB	3,3`,5,5`-tetramethylbenzidine
TNF-α	Tumor necrosis factor alpha
T_{reg}	regulatory T cells
VLBW	very-low-birth weigh
WHO	World Health Organization
WT	wild-type

CHAPTER 1 – INTRODUCTION

1.1 NEONATAL SEPSIS

Bacterial infections are a major cause of death in infants and newborns (Lawn et al., 2005). According to the World Health Organization (WHO), nearly 4 million of infant deaths worldwide are caused by severe bacterial infections and nearly 25% of that occur in the neonatal period(World Health Organization, 2009). In addition to this high mortality, it is important to consider that there is a high morbidity among the survivors of these infections, with the prevalence of serious neurological sequelae (Schuchat et al., 1999).

Despite the high incidence, neonatal sepsis is not always easy to diagnose in due time, leading often to severe complications such as cyanosis and apnea, feeding difficulties, hypotonia, seizures, bleeding problems, abdominal distention and unexplained jaundice (Bonadio WA et al, 1993)(Gerdes, 1991) (Gerdes, 2004).

Neonatal bacterial sepsis is defined as a systemic inflammatory reaction caused by bacterial infection occurring within the first 28 days of life for a term baby and up to 4 weeks beyond the expected date of delivery in a preterm baby. According to the onset of the first clinical symptoms it is divided into early-onset sepsis (EOS) and late-onset sepsis (LOS) (Hornik et al., 2013). Clinical symptoms of EOS occur in the first 3-7 days of life and results almost exclusively from the vertical transmission of bacteria from the mother to the baby. This vertical transmission can occur during labor when the baby passes through a colonized birth canal. On the other hand, LOS infection occurs after the first week of life of the newborn and is considered to be mainly due to the acquisition of bacteria in a hospital environment (Stoll BJ, Gordon T, Korones SB, Shankaran S, Tyson JE, 1996). Pneumonia and bacteremia are more common in EOS, whereas meningitis is commonly observed in cases of LOS (Tsai et al., 2014).

According to a study performed in the United States between 2005 and 2008 and based on the incidence of EOS (Reis Machado et al., 2014), standardized by race and gestational age, it is possible to understand the epidemiology of these type of infections and the resulting outcomes. Among the responsible bacteria, Group B Streptococcus (GBS) (≈38%) was the most commonly reported pathogen followed by *Escherichia coli* (≈24%). Regarding its incidence, it was possible to conclude that it is higher in very low-weight-weight (VLBW) infants, which is often attributed to the immaturity of its immune system and its lack of competence to efficiently combat certain pathogens.

In neonates with a weight less than 1000g, the incidence is approximately 26 per 1000 live children and decrease for 8 per 1000 children if premature babies weight between 1000g and 1500g. On the other hand, black preterm infants showed the highest rate of infections (5.14 per 1000 births) and the highest mortality (24.4% of cases).

Concerning LOS, its incidence varies according to the weight at birth and gestational age. Thus, the incidence rate is 25-30% in very low birth weight (VLBW ≤ 1500g) infants (Stoll et al., 2015) and 6.2-7% in late-preterm (34-37 weeks) infants (Sohn et al., 2001). Boghossian et al. made a study using data from Eunice Kennedy Shriver National Institute of Child Health and Human Development Neonatal Research Network (NRN) for the comparison of the susceptibility to LOS in singletons and in multiple gestation, by comparing VLBW same-sex and unlike-sex twin pars (Boghossian et al., 2013). With this study, the authors found that the data was not significantly different: the overall rate of LOS was 25% (27.3% males; 22,8% females) among singletons and 22,6% (25,2% males; 20% females) among multiples. These similarities provided no evidence that susceptibility to LOS among VLBW infants is genetically determined. However, in the same study, it was possible to obtain significant information regarding the distribution of microorganisms, concluding that gram-positive bacteria are the most common causative agents. Among the bacteria responsible for the infection, *coagulase-negative staphylococci* (coNS) was responsible for more than 49% of gram-positive infections, while in the group of gram-negative bacteria, *Escherichia coli* and *Klebsiella pneumoniae* were the most common bacteria.

Thus, as a conclusion, it is possible to divide EOS and LOS according to the most prevalent microorganisms and associated risk factors. Shah et al. (Shah & Padbury, 2014), in a study based on information provided by Camacho-Gonzalez (Camacho-gonzalez et al., 2013), adapted this information to the following table:

Table 1. Microbial pathogens and risk factors in neonatal sepsis

Neonatal Sepsis	***Microbial pathogens***	***Risk Factors***
Early-Onset	• Group B streptococci • *Escherichia coli* • *Streptococcus viridians* • Enterococci • *Staphylococcus aureus* • *Pseudomonas aeruginosa* • Other gram-negative bacilli	• Maternal Group B streptococcal colonization • Chorioamnionitis • Premature rupture of membranes • Prolonged rupture of membranes (> 18 h)

		• Preterm birth (< 37 weeks) • Multiple gestation
Late-Onset	• Coagulase-negative Staphylococci • *Staphylococcus aureus* • *Candida albicans* • *Escherichia coli* • *Klebsiella pneumoniae* • *Enterococcus spp* • *Pseudomonas aeruginosa* • Group B streptococci	• Prematurity • Low birth weight • Prolonged indwelling catheter use • Invasive procedures • Ventilator-associated pneumonia • Prolonged antibiotics

1.2 PREVENTION VS. TREATMENT

Intrapartum antibiotic prophylaxis (IAP) is still the only method to prevent neonatal infections caused by gram-positive bacteria, in which intravenous ampicillin or penicillin is administered in mothers at risk of transmitting GBS to their babies. IAP proved to be highly effective in preventing invasive GBS disease in the first week of life (early-onset). In terms of effectiveness, this is similar between term and preterm infants (90%) when received for at least 4h before delivery. However, IAP had no impact at all on the incidence of late-onset disease caused by GBS (Schrag & Verani, 2013).

Current therapeutic approaches for neonatal sepsis consist on the administration of antibiotics. The most commonly used include ampicillin, gentamicin, cefotaxime, vancomycin, metronidazole, erythromycin and piperacillin. However, resistance to antibiotics remains a major problem associated with these pathogens (Edmond & Zaidi, 2010).

Because neonates have relatively low amounts of endogenous immunoglobulins, several studies have been done to evaluate if intravenous administration of immunoglobulins (IVIg) in cases of suspected or proven sepsis could be a way to reduce death rates. A systematic review of 19 trials showed that there was in fact a 3% reduction in the rate of late-onset infections, but there was no reduction in death rates and sepsis side effects (Ohlsson & Lacy, 2004). Another systematic study based on 7 trials involving 338 newborns of any gestational age showed that there were no significant differences in the mortality of these newborns (Alejandria, Lansang, Dans, & Mantaring III, 2002).

Finally, two other systematic reviews showed that adjuvant IVIg therapy reduced mortality (Jenson & Pollock, 1998; Ohlsson & Lacy, 2010) but, however, flaws in the evidence of one of the articles (Ohlsson & Lacy, 2010) did not adequately support the information.

1.3 IMMUNE RESPONSE TO BACTERIAL INFECTIONS

After an invasion by a microorganism, there is a cascade of reactions that initiates the first line of defense of the organism and many of these microorganisms are detected and destroyed within minutes or hours. This first line (or innate) response involves a very specialized type of receptors and secreted proteins, generically designated as Pattern Recognition Receptors (PRRs), that are germline encoded and endows our immune system of the ability to recognize molecular patterns among associated with microbes (Judy Owen, Jenni Punt, 2012). The leukocyte cells involved in this innate immune response are neutrophils, monocytes/macrophages, dendritic cells and some subsets of so called innate-like lymphocytes such as B1 cells, NKT cells or $\gamma\delta$ T cells. The adaptive immune response is not independent of the innate immune response but occurs later; the innate response is able to distinguish very effectively between cells of the host and the bacteria, providing an initial defense and contributing to the induction of an adaptive immune response. On the other hand, the later response, which involves the immune memory (which leads to the expansion of cell clones that are specific for certain antigens) involves a genetic rearrangement to generate a huge cellular repertoire of antigen-specific receptors that allows the recognition and distinction between molecules that are closely similar (Janeway, 1994).

Included also in innate immune system are cells of nonhematopoietic origin, such as epithelial cells; myeloid cells of hematopoietic origin (phagocytes and DCs) and the innate humoral defense, like the complement cascade, acute phase proteins and all the chemical mediators. The complement system is an enzymatic system of serum proteins (proteases) consisting of nine components (C1-C9) that are sequentially activated by proteolysis, resulting in a variety of antibacterial defenses. The complement system plays an important role in chemotaxis, opsonization and inflammatory response that culminates on the lysis of bacteria or other microorganisms. The initial response, acute inflammation, is the physiologic response to the microbial challenge to recruit adequate cells to sites of infection through the production of cytokines and chemokines (Janeway, 1994).

The chemical messengers that are released once the invading pathogen is recognized include histamine, leukotrienes and prostaglandins. Overall, they cause the blood vessels to dilate which

brings more white blood cells to the scene. They also cause fluid to leak out of the blood vessels - this contains proteins that wall off the injured area.

The first cells that are recruited to the site of infection are white blood cells called neutrophils (Willems, Vollstedt, & Suter, 2009). Once in contact with the microbial pathogen, Neutrophils release of ROS and antimicrobial agents contained within its cytoplasmic granules kill the pathogens. The primary granules release certain factors such as myeloperoxidase (MPO) and neutrophil elastase (NE). Secondary granules release others including peptidoglycan recognition protein (PRGP), M-ficolin and lactoferrin. Tertiary granules release matrix degrading proteins such as MMP-9. MPO and NE are responsible for antimicrobial activity. M-Ficolin and PRGP (from both secondary and tertiary granules) are responsible for specific bacterial and bactericidal activity (Smith, 1994).

The release of neutrophil intracellular content also leads to the formation of specific structures, designated as Neutrophil Extracellular Traps (NETs), that physically restrain the bacterial pathogens and prevent their dissemination. NETs are mainly composed of DNA (and DNA associated proteins such as histones) and elastase (Brinkmann & Zychlinsky, 2012).

While the innate immune response is tackling the invading pathogen, white blood cells called dendritic cells will engulf pieces of the invading organism and transport them to the secondary lymphoid organs, where cells from the adaptive immune system, namely $CD4^+$ T cells will start to trigger a coordinated and highly specific response against antigens of the invading pathogen (Judy Owen, Jenni Punt, 2012).

1.3.1 Immune recognition of danger signals

Phagocytes such as macrophages and neutrophils have surface receptors that recognize molecular patterns that are exclusively associated with microbes and, thus, not present on human cells (Zadeh, Nichols, & Miyasaki, 1999). These pattern recognition receptors, including the toll-like receptor (TLR) family, can identify non-host motifs (Rietschel & Brade, 1992). After recognition of microorganisms and foreign substances, chemokines and inflammatory mediators are secreted to attract phagocytes.

Dendritic cells express toll-like receptors, and different dendritic-cell subsets express distinct toll-like receptors that are associated with particular functions in innate responses and in the activation and differentiation of distinct T-cell subsets (Jarrossay, Napolitani, Colonna, Sallusto, & Lanzavecchia, 2001; Kadowaki et al., 2001). Toll-like receptors are also expressed on lymphocytes and osteoclast precursors, as well as on macrophages, osteoblasts and stromal and epithelial cells,

each of which has different toll-like-receptor expression profile (Hayashi et al., 2003; Kaisho & Akira, 2002). Toll-like receptors are unique receptors that recognize molecules that are broadly shared by microorganisms, but are distinguishable from host molecules; these are collectively referred to as 'pathogen-associated molecular patterns' (PAMPs). Toll-like receptors detect multiple PAMPs, including lipopolysaccharide, bacterial lipoproteins and lipoteichoic acids, flagellin, CpG DNA of bacteria and viruses, double-stranded RNA and single-stranded viral RNA (Iwasaki & Medzhitov, 2004). To date, 11 different toll-like-receptor molecules have been identified in human periodontal tissues, and their expression, distribution and ligand specificities have been characterized (Mahla, Reddy, Prasad, & Kumar, 2013).

Moreover, 4–7 days before the initial adaptive immune response takes effect, the innate immune response has a critical role in controlling infections during this period (Janeway, 1994).

After a contact of the bacteria with the organism, it will eventually be phagocytosed by dendritic cells, which will subsequently migrate to lymph nodes, thus initiating the so-called adaptive immune response.

One property of this immune response, is the ability to recognize and respond to them in a specific way, different from the initial response. This specificity is initiated by antigen presenting cells (APC) such B cells, macrophages and dendritic cells. These APCs have MHC Class II on their surface, coupled with the antigenic peptides. Although, in theory all the APCs possess the ability to induce TCR-specific $CD4^+$ T cells activation, the in vivo activation of naïve $CD4^+$ T cells occurs almost exclusively by antigen presentation by DCs. When MHC-peptide complex is presented to a $CD4^+$ T cell, it becomes activated and stimulates B cells to proliferate and to produce specific antibodies. After this activation, the B cells are also capable of producing memory cells and these cells have on their surface antibodies that are specific for antigens. In addition to living longer, these cells can, after a second contact with the same antigen, present a much stronger and more targeted response, thus allowing faster and more efficient elimination. This inherent characteristic of high affinity receptors and memory capacity of these cells is the basis of vaccination. Vaccination leads to a natural production of specific antibodies and so, once the antigen invades the body, it can respond quickly and specifically (Chaplin, 2010).

There are also other PRRs with known functions such as C-type lectin receptors (CLRs), NOD-like receptors (NLRs) and RIG-I-like receptors (RLRs). These receptors are divided, based on their location, in membrane-bound PRRs that include TLRs and CLRs and cytoplasmic receptors such as NLRs and RLRs. Despite its important function in the immune system, TLRs are the preponderant in this type of study of bacterial infections (Kawai & Akira, 2009).

1.4 NEONATAL SUSCEPTIBILITY TO BACTERIAL INFECTIONS

To a better understanding of the function of the newborn immune system, studies in neonatal mouse models and human umbilical cord blood cells were performed, which demonstrated similar responses comparing to adults in some aspects and clear differences in particular mechanisms. As previously mentioned, the incidence and severity of neonatal infections is often explained by the immaturity of the neonatal immune system, especially on preterm newborns. On the other hand, the presence of pro-inflammatory or anti-inflammatory cytokines at certain moments of the infection influences the fate of the infection. Depending on the kinetics of cytokine production, these molecules can have harmful roles and prevent the neonatal immune system from having an adequate response necessary to eliminate the pathogen.

Due to the fact that, during pregnancy, the fetus lives in a sterile and semi-allogeneic environment, its immune system is still not well-adapted to the antigenic-stimuli present in the "outside world". When it comes in contact with a microorganism-rich environment, completely different from uterus, the newborn is more susceptible to certain infections. An important line of defense of the neonates are the maternal antibodies transferred through the placenta or milk, that protect him at an early stage of the development. However, this passive protection is of short duration, lasting approximately 6-9 months (Waaijenborg et al., 2013). Thus, the newborn needs to mature its own immune system (Hodgins & Shewen, 2012; Siegrist, 2007).

The development of the immune system begins at an embryonic stage. Starting from the fetal liver and followed by the progenitors of hematopoietic stem cells from the bone marrow, lymphocytes and polymorphonuclear cells - neutrophils, eosinophils, basophils and mast cells - are formed. During gestation, T-cell progenitor cells expressing CD34 receptors migrate to the thymus and differentiate into subsets expressing CD4 or CD8 and αβ-T cell receptors (Aster & Bunn, 2017). A small portion of these progenitors will be able to express γδ TCRs. A study with human umbilical cord cells (Sanz et al., 2010) have shown that progenitors that are $CD34^{+}CD7^{+}$ and $CD34^{+}CD10^{+}CD19^{+}$ differentiate to form B cells. For the maturation of these B cells a somatic recombination is required, involving the activation and transcription of multiple factors, leading to the accumulation of IgM and IgD molecules on the surface of these B cells.

On the other hand, the consumption of breast milk influences the newborn defenses to microorganisms. Studies done by (Bryan, Hart, Forsyth, & Gibson, 2007) have shown that breast milk contains cytokines and immunomodulatory cells that protect children against some respiratory infections as well as against some allergies.

Due to limited exposure to antigens during pregnancy, newborns have an almost absent immune memory, being their defenses against infections carried out mainly by cells of the innate

immune system. Among these cells are macrophages, natural killer (NK) cells, granulocytes (mostly neutrophils), B1 lymphocytes and γδ-T cells (Belderbos, Levy, Meyaard, & Bont, 2013). Neutrophils are the leukocyte cells that are present in the greatest quantity in the blood and are responsible for the elimination of the pathogen during infection, as mentioned above. However, neutrophils in neonates have marked differences when compared to neutrophils in adults. In addition to reduced production and reduced storage pools of these cells, leading to a diminished response (Erdman SH, Christensen RD, Bradley PP, 1982; Lieschke GJ, 1995), neonates' neutrophils have decreased TLR4 expression, despite a similar expression of TLR2 (John Nicholas Melvan, Gregory J. Bagby, David A. Welsh, Steve Nelson, 2011). Another of the differences present in these cells is the low levels of expression of L-selectin and Mac-1 (CD11b / CD18) on the surface of the cells. These molecules are responsible for the adhesion to the endothelium and this lower expression on newborn's neutrophils may result in a reduction of about 50% in the neutrophil recruitment to the site of infection (Anderson et al., 1991; Donald C. Anderson, Robert Rothlein, Steven D. Marlin, Sharon S. Krater, 1900). Finally, there is also a reduced ability of these cells to form Neutrophil Extracellular Traps (NETs), a mechanism used to restrain extracellular bacteria (Yost et al., 2009). Coupled with its reduced phagocytosis capacity and reduced intracellular degradation of pathogens (Miller ME, 1979), all these conditions may increase newborn susceptibility to infections.

In addition to neutrophils, dendritic cells also have diminished functions due to decreased expression of certain TLRs, such as TLR4 (responsible for LPS recognition), although they have a normal expression of TLR2. On the other hand, the monocytes in circulation have a low expression of MHC class I molecules, decreasing the activity of these cells (Jones, Holloway, & Warner, 2002).

Regarding the receptors present in dendritic cells, the PRRs are very relevant in the response to infections. When compared with adults, neonates show a reduced TLR-induced response and their responses through cytokines are very different (Levy, Ofer, Zarember KA, Roy RM, Cywes C, Godowski PJ, 2004). TLR2 and TLR4 stimulation of preterm infants leads to the dominant production of an anti-inflammatory cytokine, interleukin (IL)-10, when compared to TLR stimulation of term infants whose levels of IL-6, IL-10 and IL-23 are comparable (Angelone et al., 2006). However, the increased secretion levels of these three cytokines decrease during the first year of life, being compensated in parallel with the production of pro-inflammatory cytokines such as IL-1β and Tumor Necrosis Factor- α (TNF-α) (Burl et al., 2011).

Innate-like B cells (ILBs) are a population of B cells with innate characteristics and responding properties and are present in increased numbers in newborns when compared with adults. B2 cells are responsible for immune responses against thymus-dependent antigens while B1 and marginal zone (MZ) cells are thymus-independent and rapidly respond and produce IgM antibodies with low specificity and broad reactivity. In these cells, the receptors are germline encoded with partial

diversity (Zhang, 2013). These cells, which are preferentially activated by PRRs, can produce a large number of natural antibodies thus allowing an initial and critical defense against certain infections and readily produce IL-10 upon stimulus.

On the other hand, B1 cells are essential for the removal of cellular debris and play an essential role in the defense of the host against microbial infections (Rothstein, Griffin, Holodick, Quach, & Kaku, 2013).

As described above, an antibody response is dependent on the interaction of B cells with T cells. T cells establish interactions between their receptors (TCR) and the engagement of co-receptors including CD28 and CD40 ligand on Th2 or follicular T helper cells with their corresponding binding partners HLA-peptide, CD80/86 and CD40 on antigen-specific B cells. However, neonatal B cells have low expression of these co-receptors and have low receptors for polysaccharide complexes. This cellular behavior implies that the immune response of the newborns is markedly inefficient in defense against T-cell dependent antigens and, therefore, the development of immunological memory is greatly impaired (Simon, Hollander, & McMichael, 2015).

T cells develop in the thymus, which is largest at birth and during the first years of life. Mature single $CD4^+$ and $CD8^+$ positive T cells are first detected in the thymus at week 15 and abundant in the periphery well before birth (Haddad et al., 2006; Zlotoff, Schwarz, & Bhandoola, 2008). However, neonatal T cells differ significantly from adult cells, reflecting the fetal life, where exposure to foreign antigens is largely restricted to non-inherited maternal alloantigens. The function of early-life T cells is different from adult T cells. For example, though fetal naive $CD4^+$ T cells respond strongly to alloantigens, they tend to develop towards $Foxp3^+$ $CD25^+$ regulatory T cells (T_{reg}) through the influence of TGF-β (Mold et al., 2010), and thus actively promote self-tolerance. Peripheral T_{reg} represent around 3% of total $CD4^+$ T cells at birth (Takahata et al., 2004) and these cells persist for an extended period of time (Burlingham et al., 1998), giving the early-life immune response an anti-inflammatory profile (Mackroth et al., 2011).

In addition to existing information, previous studies by the group have demonstrated that the neonatal susceptibility to GBS infections is related to a high production of IL-10 at a very early stage of infection (Madureira et al., 2011). Also, the group identified the extracellular form of bacterial glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as the main factor associated with the induction of high IL-10 production (Madureira et al., 2007, 2011). We also described that this IL-10-induced early immunosuppression blocks neutrophil recruitment to the infection site and allows bacterial proliferation and dissemination throughout the body (Madureira et al., 2011). It was also described by Elva et al. that in the absence of TLR2 signaling, neonatal mice are more resistant to

GBS-induced sepsis. This study also demonstrates a link between TLR2 signaling and early IL-10 production (Andrade et al., 2013).

By analyzing the current information on the immune system of adults and neonates, it is possible to suggest that the immaturity of the neonatal immune system per se is not responsible for the increased susceptibility to certain infections. The effector cells that lead to the clearance of the pathogen are not absent, they can only have a very strongly controlled function in the early life. In this way, the knowledge of these parameters becomes central.

1.5 VACCINATION - POSSIBLE TREATMENT APPROACH

Vaccination is an effective mean of providing protection against many bacterial infections, despite limited efficacy in the first months of life. Thus, a better understanding of differences in the immune system of newborns will define mechanisms of operation during this period and may lead to new prophylactic and therapeutic approaches designed specifically for this period.

After some research into the most effective method for protection against neonatal infections, several investigators have proposed the use of bacterial preserved protein antigens so that they could be detected by the first line of defense of the immune system in the newborn and way not to escape recognition (Johri et al., 2006).

Among vaccines currently used against bacterial infections, there are two types: pneumococcal polysaccharide vaccine (PPV) and pneumococcal conjugate vaccine (PCV). PCV is composed of capsule polysaccharides common to 13 identified serotypes of *Streptococcus pneumoniae* (Prevnar 13). This formulation is currently licensed for use in children under 5 years of age and is also immunogenic for the remaining age groups. On the other hand, PPV includes 23 polysaccharides from the capsule and is used in individuals from adulthood who are at risk of pneumococcal disease (World Health Organization, 2012). Although these formulations are based on the serotypes that most commonly invade the host, this is a condition that varies between countries (Jauneikaite, Jefferies, Hibberd, & Clarke, 2012), and a more in-depth study is needed to find a more effective method for this type of infection.

As previously mentioned, previous studies from the group identified GAPDH as a virulence factor in infections caused by GBS. Thus, given these results, emphasis was placed on this virulence factor and several tests were performed to prove their immunogenicity and the possibility of it use

either as a preventive method for neonatal infections or for treatment of the same infections, preventing neonatal sepsis. This prevention would be done through the mother's vaccination.

It was then proposed to use a vaccine, called PNV1, to act on this mechanism involving GAPDH and the exacerbated production of IL-10 as an effective mechanism of protection against infection by bacteria. Because GAPDH is a protein also present in humans, it has become important that the vaccine must be constituted by peptides which only existed in bacterial GAPDH and not to cause cross-linking with the human protein.

Thus, for the design of a vaccine with these characteristics, 3 peptides exposed on the surface of the bacterial GAPDH were chosen, which were conserved in the GAPDH of the various bacteria, and that were completely absent in the human GAPDH for no existing of reactivity.

The choice of the peptides was based on Multiple Alignment and Sequence Similarity percentages from the ClustalW2 server, after submitting bacterial GAPDH amino acid sequences (FASTA format). Peptides were synthesized at Biomatik (acetate salt, 95% purity in HPLC).

Previous studies of the group have demonstrated the immunogenicity of the chosen peptides against gram-positive bacteria. Thus, and having the rational based on previous results from the group and on the similarities between the infection mechanism of gram-positive and gram-negative bacteria, the objective of the present work is to evaluate the efficacy of a peptide-based vaccine against multi-resistant gram-negative bacteria in a neonatal mice model. In this way, the present work proposes the evaluation against the infection by *Klebsiella pneumoniae* e *Escherichia coli*, the most common gram-negative agents of infection.

CHAPTER 2 – AIM OF THE STUDY

The present study is based on previous work developed by the group that demonstrates that extracellular bacterial GAPDH is an important virulence factor that is associated with neonatal susceptibility to infections caused by GBS, *Streptococcus pneumoniae* and *Staphylococcus aureus*. In consequence, it was also demonstrated that the neutralization of extracellular bacterial GAPDH induced protection in new-born mice against infectious caused by these agents. It is also known that the similarity between GAPDH of gram-positive and gram-negative bacteria is high and, therefore, it was defined as the objective of this work:

- Evaluate the efficacy of a peptide-based vaccine targeting bacterial GAPDH (PNV1) in the prevention of neonatal sepsis caused by gram-negative bacteria - *Klebsiella pneumoniae* and *Escherichia coli*.

CHAPTER 3 – METHODOLOGY

3.1 ANIMALS

The animals used were six- to eight-week-old male and female BALB/c, C57BL/6 wild-type (WT), and a NOD/ShiLtJ strain BALB/c mice and were purchased from The Jackson Laboratory. Animals were kept at the animal facilities of the "Instituto de Investigação e Inovação em Saúde – Universidade do Porto". Adult mice weighed between 22 and 25 g at the time of first treatments. Mice were housed in Techniplast ventilated polycarbonate cages under positive pressure with hard-wood bedding and provided with Mucedola Diet and fresh tap water, ad libitum, throughout the study. All animals were housed in environmentally controlled cages with 40 air changes per hour. The temperature was maintained at 21–23 °C and the relative humidity at 55 ± 10% with a 12-h light/dark cycle. All animals were quarantined for a week prior to study initiation. Each study animal was assigned a unique number and identified by ear notches.

3.2 GROWN CONDITIONS OF BACTERIA

For these studies, the bacteria used are from clinical isolates obtained from "Centro Hospitalar do Porto". The Microbiology Department of Hospital Geral de Santo António provided all the bacterial strains: *Klebsiella pneumoniae* and *Escherichia coli*. These strains were grown in Todd-Hewitt broth or agar medium (Difco Laboratories). Bacteria were grown at 37ºC.

3.3 SYNTHESIS OF PEPTIDES

Peptides were synthesized at Biomatik (acetate salt, 95% purity in HPLC).

3.4 PREPARATION OF NEONATAL VACCINE

Neonatal Vaccine (PNV1) is composed of 3 surface-exposed peptides from different bacterial GAPDH that are completely absent in human GAPDH. The choice of the peptides was based on

Multiple Alignment and Sequence Similarity percentages from the ClustalW2 server, after submitting bacterial GAPDH amino acid sequences (FASTA format). Peptides were synthesized at Biomatik (acetate salt, 95% purity in HPLC).

Different doses of PNV1 were formulated prior to immunization with equal amounts of the different peptides (0,05 to 20 μg of each peptide). PNV1 was formulated in a 1:4 PBS-Alhydrogel suspension in a total volume of 200 μL. The sham-immunized control animals received 200 μL of PBS (Vehicle control without Alhydrogel) or a 1:4 PBS-Alhydrogel suspension (Vehicle control with Alhydrogel).

3.5 PREVENTIVE ANTIBODY TREATMENTS AND BACTERIAL INFECTIONS

Antibody treatments were performed in newborn BALB/c mice (<48h) 12 h prior to bacterial infection. For passive immunizations, pups were injected intraperitoneally (i.p.) with 100 μg of PNV1-elicited IgG antibodies. Control animals received the same amount of control IgG's. Challenging infections were performed subcutaneously (s.c.) with $2{,}5x10^5$ CFU of *Klebsiella pneumoniae* or *Escherichia coli*.

3.6 THERAPEUTIC ANTIBODY TREATMENTS

For the assessment of the therapeutic effect of anti-GAPDH IgG elicited with PNV1, newborn C57bl/6 mice (<48h) were immunized with 150μg of anti-GAPDH (PNV1) IgG, control IgG or 40 μL of saline solution. After 12h the animals were infected s.c. with 2.5×105 CFU of extended-spectrum β-lactamase producing strain (ESBL+) of *E. coli* or K. *pneumoniae*. Then a survival curve was performed.

3.7 PURIFICATION OF PNV1- IGG

Adult mice were immunized s.c. five times (with a 2-week interval between doses) with 25µg of PNV1 in a PBS/alum suspension. Sera were collected 10 days after the last immunization. Pooled serum samples were applied to a Protein G HP affinity column (HiTrap, GE Healthcare Bio-Sciences AB) and purified IgG antibodies were then passed through an affinity column with immobilized rGAPDH (Hitrap NHS-activated HP, GE Health-care Bio-Sciences AB). Control IgGs were obtained from sera of mice sham-immunized with a PBS/alum suspension and purified on a Protein G HP affinity column. Purified IgG antibody fractions were further equilibrated in PBS and stored at 4ºC in aliquots.

3.8 PURIFICATION OF THE BACTERIA RGAPDH

For production of rGAPDH protein from GBS, *Escherichia coli*, *Staphilococcus aureus*, *Streptococcus pneumoniae* and *Klebsiella pneumoniae*, *E. coli* BL21 (DE3) strains (Novagen) and the pET28a plasmid (Novagen) were used, as described previously [11]. *E. coli* was cultured on Luria-Bertani (LB) (Sigma Aldrich)) medium, containing 0.5 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) (Sigma-Aldrich), 16 h at 20 ^{o}C. After that, the pET28 derivative cells were harvested by centrifugation for 30 minutes at the maximum velocity and processed to disrupt the cell integrity (Emulsiflex). The soluble fraction was isolated by ultracentrifugation and applied to a His-Trap HP column (Amersham Biosciences). An imidazole (Sigma Aldrich) gradient (50 mM, 300 mM and 500 mM) was used and rGAPDH was eluated with 300 mM imidazole. A second purification was performed by size-exclusion chromatography (S200 26/60 GE Healthcare) using PBS as mobile phase. A Native-Page analysis was performed to confirm rGAPDH purification. The thermal stability was evaluated by Differential Scanning Calorimetry and enzymatic activity was studied using a specific kit – GAPDH Activity Assay (Abcam).

3.9 WESTERN BLOT

Extracellular proteins from culture supernatants of the *Klebsiella* and *E. coli* were separated by 10% SDS-page and analyzed by western-blot using anti-GAPDH IgG obtained from mice immunized with PNV1. rGAPDH was used as a positive control. After the separation, the proteins were transferred to a PVDF membrane. After this, the membrane was saturated for 1 hour with TBST buffer (0,01 M Tris (Merck Millipore), 0,15 M NaCl (VWR), 0,05% Tween 20 (VWR (pH 8.0)), containing 1 or 3% of BSA, and further incubation overnight at 4 ^{o}C (with mice anti-PNV1 IgG and control mice serum in TBST+3%BSA), or for 2 hours (with rat anti-PNV1 IgG in TBST+1%BSA) diluted 1:500. After that, it was incubated for 1 hour with appropriate secondary antibody diluted 1:10000 in TBST+3% BSA (HRP-coupled monoclonal goat anti-mouse IgG (Southern Biotech)) or 1:1000 in TBST+1% BSA (Mouse Anti-Rat IgG1-UNLB (Southern Biotech) and further incubation with Goat Anti-Mouse Ig (H+L)-AP (SouthernBiotech)). The detection was done using a solution of ECL, a luminol-based chemiluminescent substrate for the detection of HRP, with the chemiluminescent detection performed using the Chemidoc (Bio-Rad), or with nitro-blue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3`-indolyphosphate p-toluidine salt (BCIP) in alkaline phosphate (AP) buffer (0,01M Tris, 0,01M NaCl, 0,5mM MgCl2 (Merck Millipore) (pH 9.5)), as substrate.

3.10 QUANTIFICATION OF IGG

For the evaluation of the IgG production, an ELISA essay was performed. For this, microtiter plates (Nunc MaxiSorp 96 well plates) were coated with 5µg/mL of the protein in study (50µl per well for final volume) and remained overnight at 4ºC. After this, the plates were washed with PBS and blocked with 1% Bovine Serum Albumin (BSA) (VWR) in PBS for 1h, at room temperature. The samples were diluted in 1% BSA in PBS and incubated at room temperature during 2h. After this time, the plates were washed 5 times with PBS and incubated with the antibody Goat Anti-Mouse IgG Streptavidin-horseradish peroxidase (HRP) conjugated (Southern Biotech) for 1h, diluted in 1%BSA in PBS. After a final wash, 3,3',5,5'-Tetramethylbenzidine (TMB) was added and 1 M of sulfuric acid (H2SO4) were used to stop the reaction. Finally, the absorbance was read at 450 nm. For the IgG

titers, a curve was performed and titers were defined as the first value of sample dilution where the OD450≤ 0.1.

3.11 CRUDE EXTRACELLULAR PROTEINS (CEP)

Bacterial strains were grown in LB medium because TH medium naturally contains a high amount of proteins. A pre-grown was performed overnight at 37^{o}C and after that, bacteria were grown during 6h at 37^{o}C. The medium was centrifuged 20 min at 4^{o} C, 3500 g. The supernatant was filtered through a 1.2 µm, 0.45 µm and 0.2 µm pore size filter (Merck Millipore). The sample was concentrated by dialysis and reserved at 4^{o} C.

3.12 STATISTICAL ANALYSIS

All statistical analyses were performed in GraphPad Prism version 5.0 for Windows (GraphPad Software, San Diego, California). Student's T test was used to analyze the differences between groups. Survival studies were analyzed with the log-rank test.

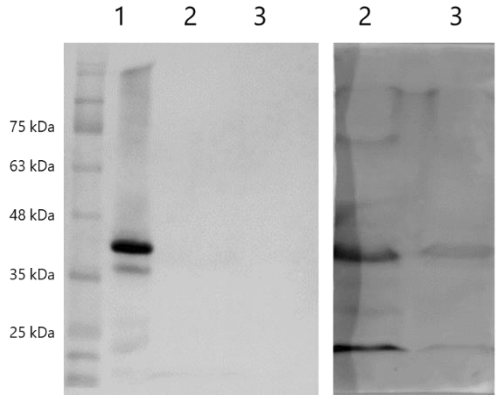
CHAPTER 4 – RESULTS

4.1 *ESCHERICHIA COLI* AND *KLEBSIELLA PNEUMONIAE* EXTRACELLULAR GAPDH

Previous work from the group demonstrated that different Gram-positive bacteria excrete GAPDH as a form to avoid host innate defences. In order to address if other relevant neonatal bacteria pathogens, as *Escherichia coli* and *Klebsiella pneumoniae*, also have an extracellular form of GAPDH we perform a western-blot (WB) of the of the supernatants of *Escherichia coli* and *Klebsiella pneumoniae* bacterial cultures. Purified IgG from mice C57Bl/6 immunised with recombinant GAPDH (rGAPDH) from E. coli were used as a primary antibody for the WB.

As observed in Figure 1, anti-GAPDH antibodies could recognize a band in the supernatants of the referred bacterial cultures, indicating that these bacteria also can excrete GAPDH.

A.



B.

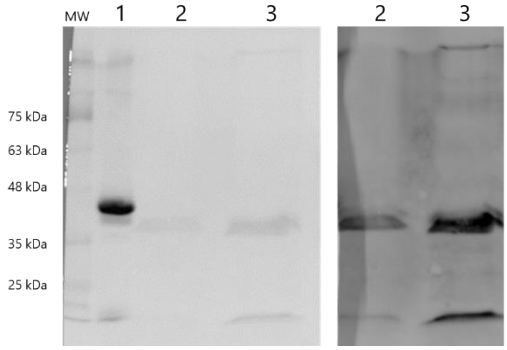


Figure 1. Western Blot analysis of Gram-negative extracellular GAPDH
Extracellular proteins from CEP of the indicated bacteria were separated by SDS-page and analysed by western-blot using anti-GAPDH antibodies obtained from PNV1-immunized mice. rGAPDH was used as a positive control. Western Blot analysis of blotted protein samples corresponding to *Klebsiella pneumoniae* GAPDH and *Escherichia coli* rGAPDH. A: Incubation with anti-*Escherichia coli* GAPDH antibodies (C = 4,43 mg/mL; dilution 1:500). 1 - rGAPDH E. coli; 2 – CEP E. coli; 3 – CEP K. pneumoniae B: Incubation with anti-*Klebsiella pneumoniae* GAPDH antibodies (C = 4,43 mg/mL; dilution 1:500). 1 - rGAPDH K. pneumoniae; 2 – CEP K. pneumoniae; 3 – CEP E. coli. Western Blot with reveals the presence of extracellular GAPDH from the extracellular products of *Klebsiella pneumoniae* and *Escherichia coli* bacterial cultures (CEP) as well as rGAPDH.

4.2 A PEPTIDE-BASED VACCINE - PNV1

In addition of being very conserved amongst bacteria, GAPDH is also presence in human cells. Thus, the use of the whole protein as a vaccine in humans wouldn't be possible. To overcome this issue, a peptide-based vaccine was developed, which is composed of surface-exposed peptides of bacterial GAPDH that are completely absent from human GAPDH. This vaccine, designated as PNV1, has demonstrated good immunogenicity for the GAPDH of Gram-positive bacteria, namely GBS, *S. aureus* and *S. pneumoniae* (Lemos, 2016; Nogueira, 2016).

PNV1 is composed of 3 surface-exposed peptides from different bacterial GAPDH that are completely absent in human GAPDH. The choice of the peptides was based on Multiple Alignment and Sequence Similarity percentages from the ClustalW2 server, after submitting bacterial GAPDH amino acid sequences (FASTA format). Depicted in figure 2 is the position of the peptides on the 3D structure of GAPDH from *Escherichia coli* obtained by PyMOL 2.0. In this figure, the *Escherichia coli* GAPDH is presented only in dimer since there is still no analyzed crystalline structure for the tetramer.

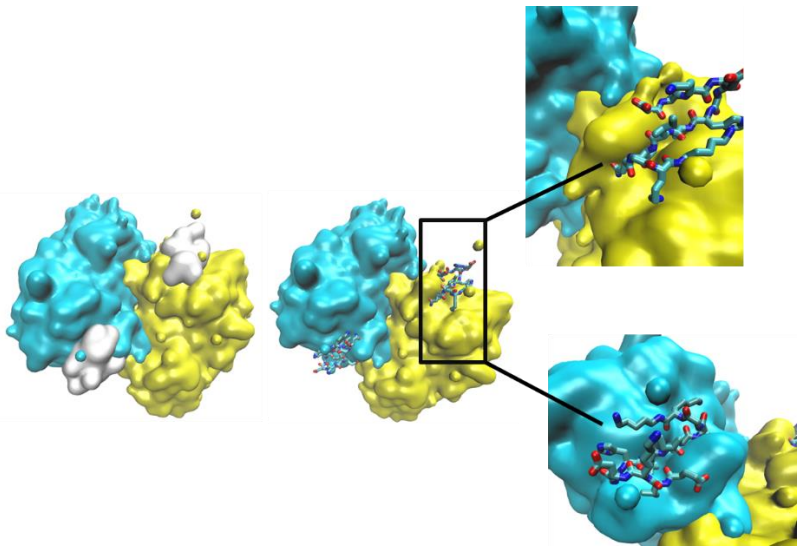


Figure 2. Localization of the peptide in Escherichia coli GAPDH. Structure PDB: 4QX6

The efficacy of a vaccine is demonstrated, initially, by its ability to induce an immunogenic response

in the host. This response is characterized by the production of specific antibodies against GAPDH and various concentrations were used and subsequently drawn a calibration curve.

To find the immunogenic dose for PNV1, 8-week old BALB/c female mice were immunized four times with a two-week interval between doses, with different amounts of peptide per vaccine (adjuvant and vehicle were kept at the same amounts throughout the different doses). Namely, three different doses of peptides per vaccine were used: 0.05, 0.10, 0.20, 0.50, 1.00, 2.00 and 20.00 µg of peptide.

The immunogenicity of PNV1 was assessed by the ability of inducing IgG antibodies specific for recombinant GAPDH from Gram-positive or Gram-negative bacteria.

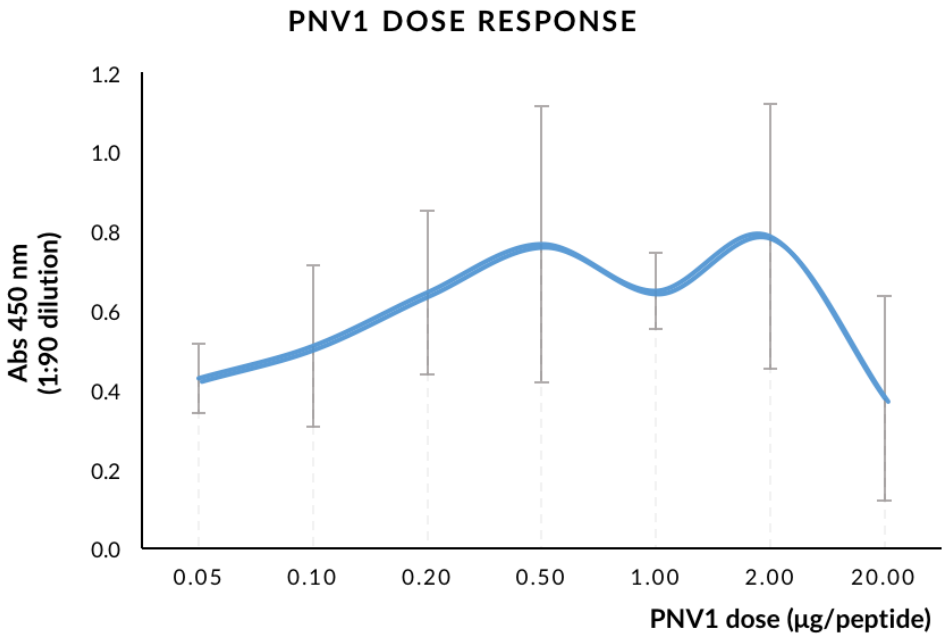


Figure 3. Immunogenicity of PNV1
Different doses of PNV1 were administered sc in 8-week old BALB/c female mice four times with a two-week interval between doses. Anti-GAPDH IgG titers were determined by ELISA in plates coated with purified recombinant GAPDH from *Escherichia coli*, GBS, *Streptococcus pneumoniae*, *Staphilococcus aureus*, *Klebsiella pneumoniae* or *Pseudomonas aeruginosa*. Depicted in the figure are the mean ±SD of the absorbance obtained in ELISA for a 1:90 dilution of the sera of all the different immunized animals for all the bacterial GAPDHs.

As depicted in figure 3 the dose corresponding to 0.2 µg of each peptide per vaccine formulation (A0.2) was the one that induced high IgG titers and the dose that presented better values of immunogenicity having as criterion the quantity-titles relation.

On the other hand, it became important to verify if the immunogenicity induced by the vaccine would be transversal to other species. For this, we immunized rabbits with the selected formulation (corresponding to 0.2 μg of each peptide) using the same methodology and schedule. As showed in figure 4, the same dose of PNV1 that proved to be immunogenic in rats also induced a robust IgG response in rabbit.

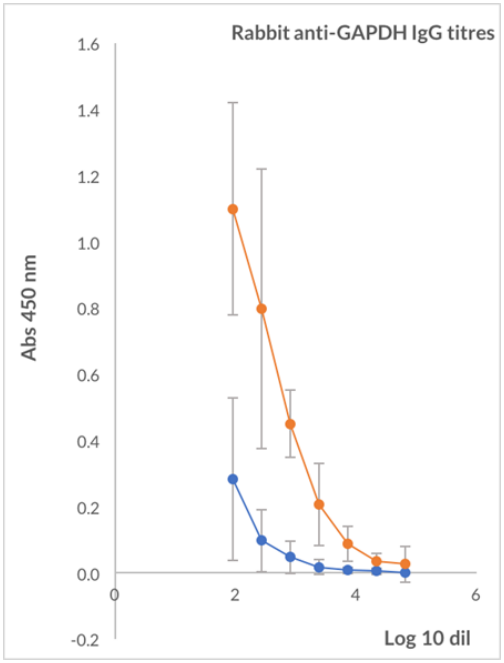


Figure 4. Immunogenicity of PNV1 in rabbits
A dose of PNV1 corresponding to 0.2μg of each peptide was administered intra-muscularly (IM) in rabbits four times with a two-week interval between doses. Anti-GAPDH IgG titres were determined by ELISA in plates coated with purified recombinant GAPDH from *Escherichia coli*, GBS, *Streptococcus pneumoniae*, *Staphilococcus aureus*, *Klebsiella pneumoniae* or *Pseudomonas aeruginosa*. Depicted in the figure are the mean ±SD of the ELISA curves for all the bacterial GAPDHs.

After verifying that there was an IgG production after administration of PNV1, it became necessary to check whether these antibodies would be specific for the interest groups, that is, if they recognized the extracellular GAPDH of the bacteria under study. So, it was necessary to verify if the PNV1-elicited IgG could recognize extracellular GAPDH from the supernatants of the different bacterial cell cultures. For this, a Western Blot analysis was performed.

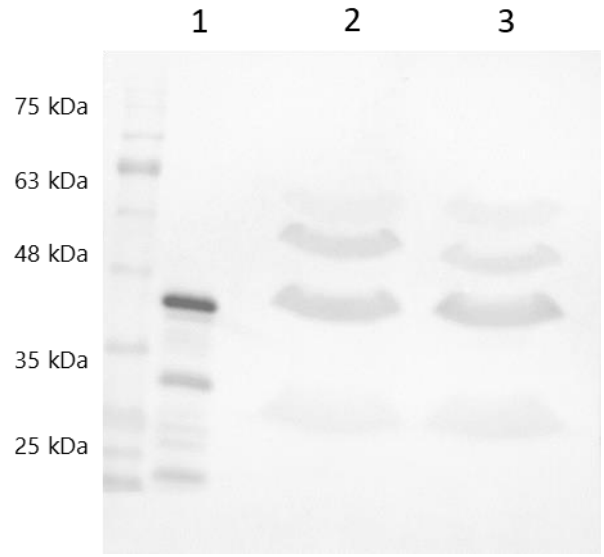


Figure 5. Western Blot with rGAPDH and CEP
Extracellular proteins from CEP of the indicated bacteria were separated by SDS-page and analysed by western-blot using PNV1-elicited antibodies obtained from PNV1-immunized mice. rGAPDH was used as a positive control. 1 - rGAPDH; 2- CEP E. coli; 3- CEP K. pneumoniae; Western Blot with reveals the presence of extracellular GAPDH from the extracellular products of Klebsiella pneumoniae and Escherichia coli bacterial cultures (CEP) as well as rGAPDH.

Western blot was performed with antibodies (IgG) purified from the serum of mice immunized with PNV1. Results shown in Figure 5 demonstrate that PNV1-elicited antibodies are able to recognize GAPDH from the supernatants of *Klebsiella pneumoniae* and *Escherichia coli*. The fact that other bacteria also possess extracellular GAPDH (Figure 1) is a strong indicator that *Escherichia coli* and *Klebsiella pneumoniae* also take profit from the propensity of neonates to produce high amounts of IL-10 in response to bacterial GAPDH. In addition, it also indicates that PNV1 could be used as a vaccine also to prevent infections caused by these Gram-negative organisms.

4.3 PROTECTION

As the main objective of this work was the induction of protection against bacterial infections in newborns, the next step was to verify this parameter. The survival curves of PNV1-IgG immunized newborns were analyzed for the evaluation of possible protection induced by passive immunization.

These animals were immunized 12h before the challenging bacterial infection and subsequently infected with 2.5×10^5 CFU of extended-spectrum β-lactamase producing strain (ESBL+)

of *Escherichia coli* or *Klebsiella pneumoniae*. Nine neonates were immunized and subsequently infected and 8 neonates were used as controls. These eight animals, received the same concentration of control IgG obtained from animals immunized with a PBS/alum and were infected with the same inoculum.

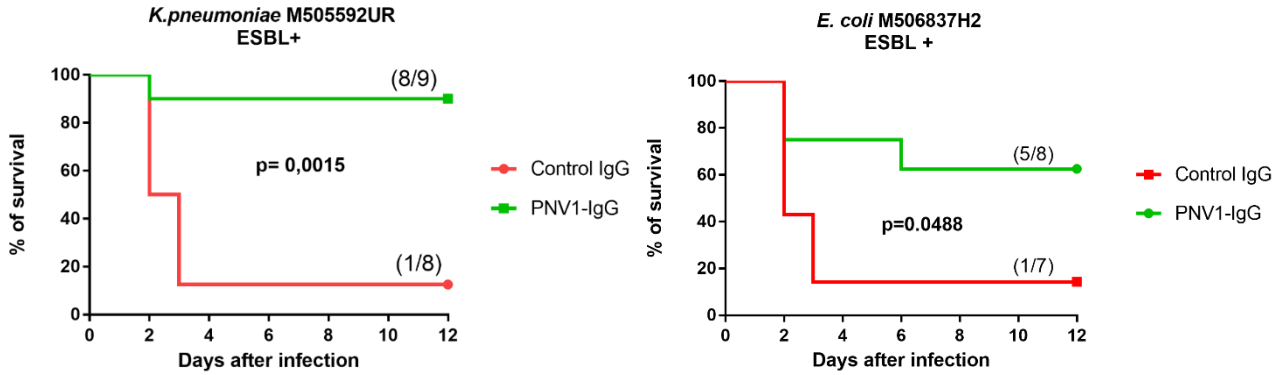


Figure 6. Passive immunization and infection of newborns
PNV1 IgG or control IgG (100 µg) were injected sc into mice pups 12 h before bacterial challenge. For bacterial challenge mice pups were infected sc with 2.5×10^5 CFU of extended-spectrum β-lactamase producing strain (ESBL+) of *Escherichia coli* or *Klebsiella pneumoniae*. The numbers between parentheses represent the number of animals that survived versus the total number of infected animals. Statistical differences (P values) are indicated.

After analyzing these data, it is possible to conclude that the administration of PNV1 IgG before infection significantly increased the survival rate of mice pups after infection with the different bacteria, when compared with pups that received control IgG.

For the *Klebsiella pneumoniae* group, the survival rate for the immunized animals was about 90%, whereas the control animals group was about 13%. For the *Escherichia coli* group, the survival rate of immunized animals was about 63%, while the control group had a survival rate of 14%.

These results demonstrate that antibody-mediated neutralization of bacterial GAPDH prevents neonatal infections caused by the most relevant sepsis-inducing bacteria. As shown in Figure 8, the use of anti-PNV1 IgG's elicited in passive immunizations of neonates significantly improve survival upon bacterial challenge.

Altogether, these results show that the new approach used to develop the PNV1 vaccine directs the immune system of neonates to a more robust and specific response towards sepsis-inducing agents.

4.4 TOXICITY

One of the problems in using any drug is the possible side effects in the body of the individual, commonly called toxicity. Although the product may have benefits for a pathology, if this means that there are other less favorable changes, its use is not acceptable. Thus, it became necessary to realize if the vaccine under study had undesired effects on humans. First, and in the case of a peptide-based vaccine based on a common protein to humans, reactivity analysis became dominant, i.e. it was important to verify that the antibodies produced in response to the vaccine would not react with human GAPDH. For this purpose, a ELISA was performed with a coating with human rGAPDH.

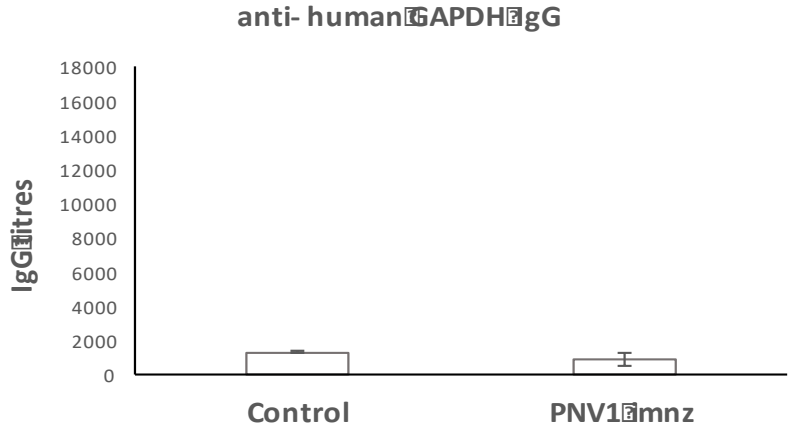


Figure 7. IgG titers for human GAPDH after PNV1 immunization
Different doses of PNV1 were administered sc in 8-week old BALB/c female mice four times with a two-week interval between doses. Anti- human GAPDH IgG titers were determined by ELISA in plates coated with purified recombinant human GAPDH. Depicted in the figure are the mean ±SD of the absorbance obtained in ELISA for a 1:90 dilution of the sera of all the different immunized animals for all the bacterial GAPDHs.

Another pertinent question, and given that the aim of the vaccine is to combat bacterial infections, is whether its administration causes changes in gut microbiome. It is known that the bacteria present in the gastrointestinal tract play a preponderant role in the homeostasis of the organism, even going through the fight against other bacteria. Thus, a change in the composition of this microbiome could imply a deregulation of the organism and the propensity for other types of problems. To evaluate if PNV1 has any effect on gut microbiome, a comparison was performed between the microbiome of immunized animals versus control animals.

The aim was to evaluate any possible acute or chronic effects caused by active immunization with PNV1 (0,2 μg/peptide in each dose) or with the passive immunization with PNV1 IgGs. In the active immunization group, mice received 4 doses of PNV1 with a 2-week interval between doses.

Controls received adjuvant alone or were left untreated. Gut microbiome composition was analyzed from the faeces of mice recovered 5 days or 1 month after the last dose. For the passively immunized group, we compare the gut microbiome of mice passively immunized with 120 mg/Kg of PNV1 IgG with mice that received the same amount of control IgG. Gut microbiome composition was analyzed from the faeces of mice recovered 5 days after weaning (26 days after birth) and 49 days after birth (7 weeks of age).

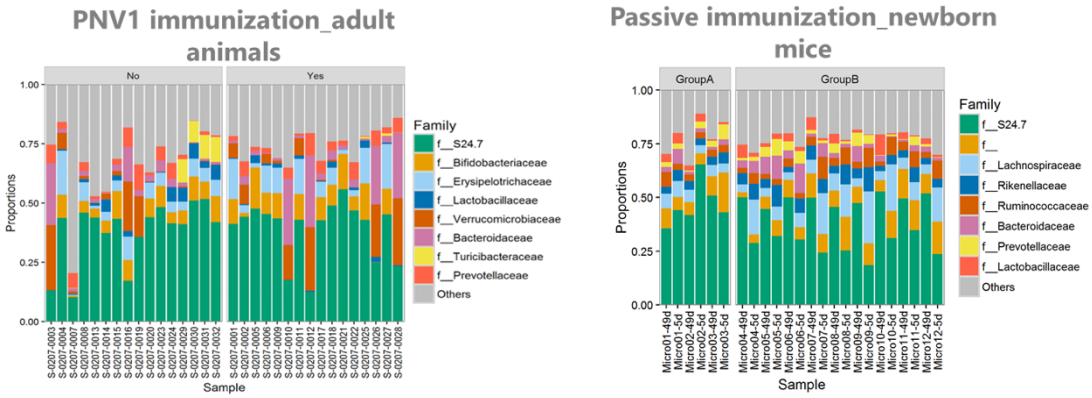


Figure 8. 16S rRNA gene V4 amplicons generated from mouse faecal samples on MiSeq

As it is possible to observe from Figure 7, immunization of mice had no discernible effect on gut microbial community composition.

4.5 OTHER INTERVENTION GROUPS

Along with newborns, there are other groups at risk for this type of infection. One of these groups is the existence of chronic diseases that makes these individuals as a group at risk for infections. One of the examples are those with type I diabetes who, due to the hyperglycemic environment, have a dysfunction of the immune system, as well as a decrease in gastrointestinal microbial activity. On the other hand, these are individuals with a greater need for medical care, which increases the probability of acquiring infections in a hospital environment.

In this way, another approach of the investigation was to verify if the vaccine was also able to induce protection in different risk groups from neonatal. For this, only for Klebsiella infections, the same test was done on elderly animals and on model animals for type I diabetes.

For the model animals for an immunodeficiency background, a NOD/ShiLtJ strain (commonly called NOD), a polygenic model for autoimmune type 1 diabetes, was used. A Diabetes in NOD mice is characterized by hyperglycemia and insulitis and it is the common choice in these cases. These animals were divided into 2 groups, both with four animals each: the first received 3 doses of rabbit control IgG and the other received 3 doses of PNV1. After 12h, the animals were infected with $7{,}4x10^6$ CFU of *Klebsiella pneumoniae* and a survival curve was performed.

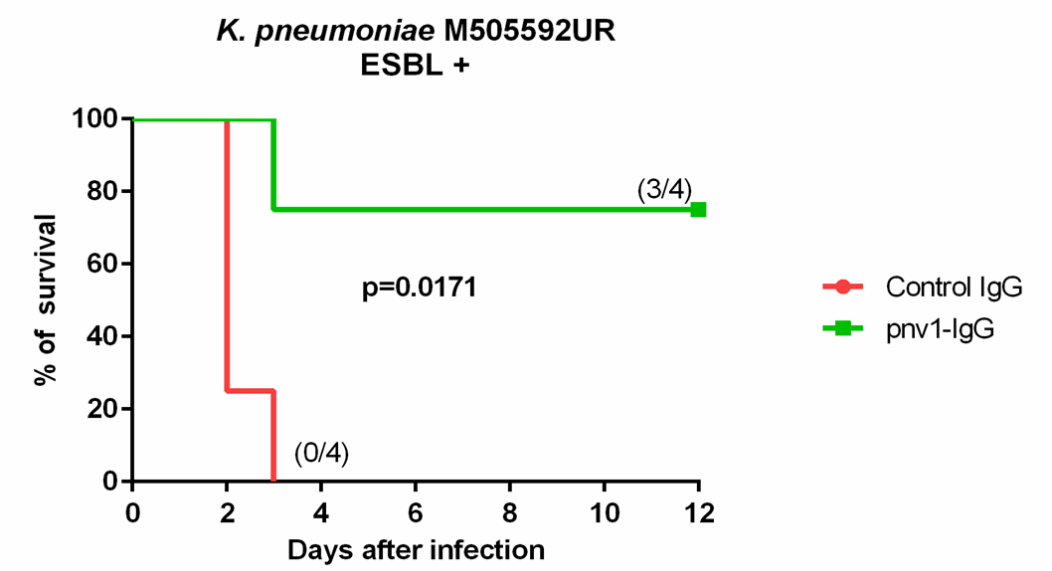


Figure 9. Passive immunization and infection of NOD animals

PNV1 IgG or control IgG (100 µg) were injected sc into NOD animals 12 h before bacterial challenge. For bacterial challenge NOD animals were infected sc with 7.4×10^6 CFU of extended-spectrum β-lactamase producing strain (ESBL+) of *Klebsiella pneumoniae*. The numbers between parentheses represent the number of animals that survived versus the total number of infected animals. Statistical differences (P values) are indicated.

This data allows to propose that the vaccine also induced protection on this animals since the survival rate of control animals was 0% (all died after 3 days) and the survival rate of the PNV1-immunized animals was 75%.

Although both results are for only one of the studied bacteria and are still preliminary results, it is possible to suggest that the vaccine under study also has the capacity to induce protection in other types of situations, even considering adults animals.

4.6 CONJUGATED-PNV

As indicated at the beginning of the study, the objective of this vaccine is for use in humans. Therefore, it is necessary for it to be optimized to advance to clinical trials after the finish of these studies. However, as described (Judy Owen, Jenni Punt, 2012), a peptide-based vaccine is poorly immunogenic. To be sure that the vaccine-induced response in humans is sufficiently robust and sufficient for protection, a different approach was performed, which consists in coupling a carrier protein to the peptides. Many times, the active immunotherapies are engineered to stimulate the body's own immune system. The intention of the vaccine modification passed for the choice of the Keyhole Limpet Hemocyanin (KLH), a copper-containing protein molecule derived from the haemolymph of the inedible mollusk, Megathura crenulate. This molecule is commonly used in immunological studies because of its great ability to induce a T-dependent immune response (Swaminathan, Lucas, Dear, & McMichael, 2014). Thus, to the developed peptides was then coupled another cysteine (which allowed the binding of the molecule of interest) and the conjugation of KLH. After this, 3 animals were immunized and a quantification of specific-IgG was performed.

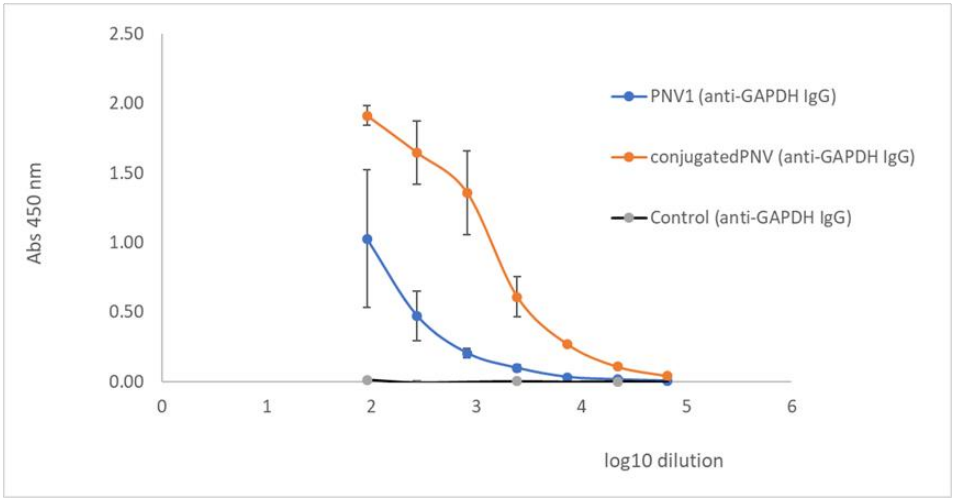


Figure 10. Immunogenicity of conjugated-PNV1
A dose of conjugated-PNV1 corresponding to 0.2µg of each peptide was administered intra-peritoneally (IP) in mice four times with a two-week interval between doses. Anti-GAPDH IgG titres were determined by ELISA in plates coated with purified recombinant GAPDH from *Klebsiella pneumoniae* or *Escherichia coli*. Depicted in the figure are the mean ±SD of the ELISA curves for all the bacterial GAPDHs.

Analyzing these values and comparing with the values obtained with the vaccine without the conjugation (Figures 3 and 4), it is possible to suggest that the response induced by conjugated-PNV1 is in fact more robust and the advance for clinical trials will be with a product optimized for the conditions under study.

On the other hand, there remained a need to verify if this new formulation did not continue to be reactive with human GAPDH. Thus, studies with cell line lysates from SW 982 cell line (synovium), HEK 293T cell line and erythrocytes were made. For this, purified IgG from mice C57Bl/6 immunized with conjugated-PNV were used as a primary antibody for the WB.

Firstly, the analysis of recognition of human GAPDH by anti-conjugated-PNV antibodies was performed.

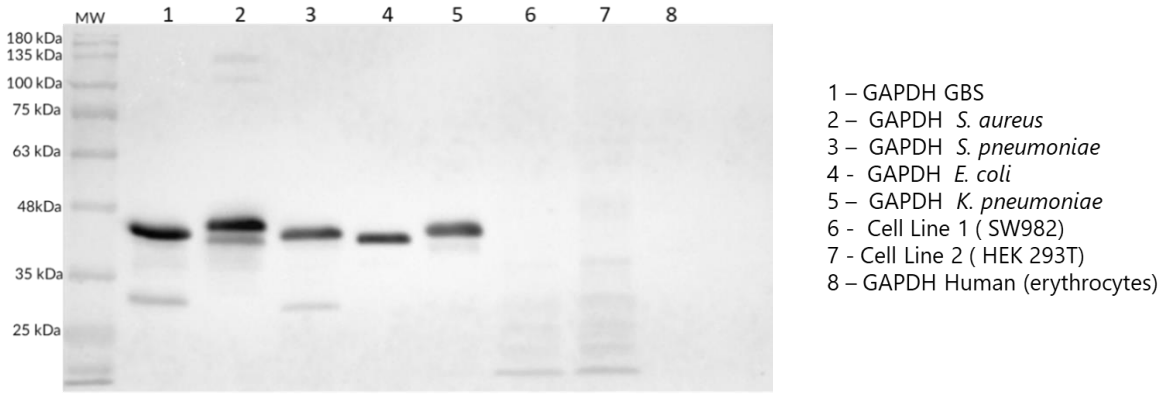


Figure 11. Western Blot with cell line lysates

Extracellular proteins from cells lysates were separated by SDS-page and analysed by western-blot using anti-human GAPDH antibodies. Western Blot with conjugated-PNV-elicited antibodies reveals that purified IgG from mice immunized with conjugated-PNV does not recognize the native GAPDH

This result shown that the conjugated-PNV vaccine does not induce the organism to produce anti-human GAPDH IgG. Thus, there are no cross-linking and the vaccine is safe for humans.

As control for the presence of GAPDH, the same membrane of WB was revealed with anti-human GAPDH antibodies.

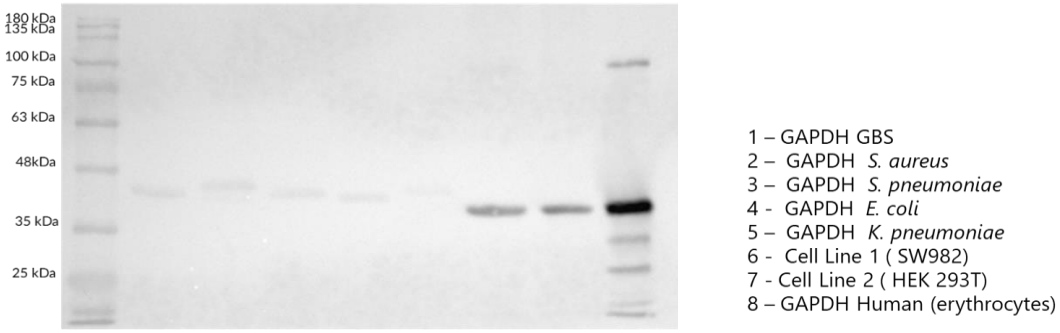


Figure 12. Western Blot with cell line lysates

Extracellular proteins from cells lysates were separated by SDS-page and analysed by western-blot using anti-human GAPDH antibodies. Western Blot with anti-human GAPDH antibodies reveals that GAPDH is present in the lysates.

As a final analysis, all these results indicate that PNV1 may be an effective vaccine against bacterial infections caused by *Klebsiella pneumoniae* and *Escherichia coli*.

CHAPTER 5 – DISCUSSION

Although neonatal sepsis presents a high incidence, its diagnosis is not easy and this implies that in many cases it leads to severe problems such as pneumonia, meningitis or even death. Nowadays, and without any efficient strategy to treat or prevent neonatal bacterial sepsis, it remains an unmet medical need (Schuchat et al., 1999; World Health Organization, 2009).

Neonatal bacterial sepsis is characterized by a systemic inflammatory reaction that can be defined depending on the detection of clinical symptoms such as early-onset sepsis (EOS) or late-onset sepsis (LOS) (Hornik et al., 2013). Among the bacteria responsible for these infections, current studies indicate that *coagulase-negative staphylococci* (coNS) infections are present in most cases of gram-positive bacteria, and *Klebsiella pneumoniae* and *Escherichia coli* in the case of gram-negative bacteria infections (Boghossian et al., 2013).

Despite being an emerging problem, the current methods of prevention or treatment of neonatal infections are not completely efficient. In cases of prevention, before pregnancy, there are vaccination methods that are based on maternal vaccination for subsequent passage of specific antibodies through the placenta during pregnancy (Barrow, 2012; Palmeira et al., 2012). There are currently some current therapies as well as model studies showing that maternal vaccination against GBS, for example, can prevent 60 to 70% of cases of infection by GBS (Sinha, Lieu, Paoletti, Weinstein, & Platt, 2005).

Nevertheless, current standard of care relies only on the administration of IAP. IAP is indicated in cases of risk such as for example having a GBS bacteriuria during the current gestation or presenting children of previous births who have suffered from infections caused by GBS. Despite the efficiency of this practice in EOS, the same is not true in cases of LOS. On the other hand, it was possible to verify, according to the study between the years of 1998 and 2000, although there was a decrease of infections caused by GBS, the rate of infections caused by gram-negative bacteria such as *Escherichia coli* increased significantly, becoming one of the most relevant agent of infections in neonates (Stoll et al., 2002). Thus, IAP, despite presenting an initial decrease in cases of neonatal infections, appears to be a currently ineffective strategy.

Finally, in cases of antibiotic administration after birth, the use of antibiotics such as penicillin, ampicillin and gentamicin is the most common (Edmond & Zaidi, 2010). Its use depends on the type of infection caused since ampicillin acts more strongly on gram-negative bacteria, whereas penincillin has better activity against group A and B streptococci and pneumococci and in case of treatment of meningitis (Darmstadt, Batra, & Zaidi, 2009). On the other hand, more recent studies are based on the administration of immunoglobulins in cases of suspicion or confirmation of sepsis.

However, the current information in this area does not become sufficient to define whether it is or not a completely effective method.

To understand the best strategies in these infections, the understanding of the immune system of the newborns in comparison with the adults has become preponderant, for the recognize of the best form of intervention.

It is known that in a host, after an invasion by a microorganism, there is the triggering of various physiological reactions such as the recruitment of cells and molecules with various functions to the site to combat the pathogen. Among these cells we have the recruitment of neutrophils, the first cells to be recruited and responsible for the engulf of external host material and other cells such like the macrophages, which present as a main characteristic the recognition of molecules that are in the surface of bacteria. This recognition is made through receptors called pattern recognition receptors (PRRs), as toll-like receptors (TLRs), leading to subsequent secretion of chemokines to attract other cell types (Murray et al., 2013).

However, in newborns, some of these functions seem to be compromised. Because during pregnancy the fetus is in a sterile environment, the organism of the fetus is unable to combat some microorganisms itself. Thus, soon after birth, the response triggered by antibodies transferred through the placenta by the mother provides an important line of defense against bacteria (Firth, Shewen, & Hodgins, 2005). In the first stages of life, the development of the immune system becomes preponderant to combat the invasion of the organism by microorganisms. Due to the lack of response through Th1, newborns are unable to develop immune memory and the body's response being mostly through the called innate immunity (Angelone et al., 2006; Chelvarajan et al., 2004; Vanden Eijnden, Goriely, De Wit, Goldman, & Willems, 2006). However, the innate immunity cells of the newborns exhibit different characteristics of the same cells in adults, both in terms of phenotype and in terms of number. For neutrophils, these cells are present in a smaller number and have a reduced expression of receptors like TLR4, although they present a normal number of other receptors like TLR2. On the other hand, these cells also have a recruitment capacity for the site of infection decreased in approximately 50%. Finally, the reduced capacity of phagocytosis of these cells makes the neonatal more susceptible to infections (Levy, 2007).

On the other hand, there are other cells that are in a large number in the neonatal period, such as B1 cells. These are lymphocyte cells and are part of innate-like B cells, a population of B cells that exhibit innate immunity characteristics. These cells, after activation through their receptors, are able to produce a large number of antibodies that although little specific are responsible for the initial combat to the microorganisms (Zhang, 2013).

Based on this information, several studies point the immaturity of the newborn's immune system as the cause for increased susceptibility to infections in this period.

As explained above, current therapies for neonatal bacterial sepsis essentially undergo administration of antibiotics. Thus, immunotherapies become crucial. Vaccination has been central as a possible approach to the protection of newborns. A formulation currently used is 13-valent PCP (Prevnar 13), used for the prevention of infections caused by *Streptococcus pneumoniae* in children, but does not present increased protection in newborns, neither present protection for all the identified serotypes of the bacteria, protecting only about 14% of the serotypes.

So, another approach that has emerged is the possibility of maternal vaccination for the transmission of antibodies during pregnancy and thus to protect the newborns in the early stages of life, both for EOS and LOS. For the development of these products, the understanding of the mechanisms of virulence of the bacteria becomes preponderant instead of the immune system of the newborns.

Many of the therapies are then based on a target in the bacteria that is considered the virulence factor. Previous studies from the group have identified a virulence factor in *Group B Streptococcus*. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), a moonlighting enzyme plays an important role in bacterial infections, including in processes of invasion of the immune system. In addition to this, previous studies from the group showed that GAPDH was responsible for the increased production of IL10, an immunosuppressive cytokine, in neonates following GBS infections through the TLR2 interaction (unpublished data). Early IL-10 production induced by bacterial GAPDH production was through the recognition of this protein by B1 cells and led to an impediment of the response by the organism, allowing the development of sepsis. Based on this, the hypothesis was to decrease the virulence capacity of these pathogens by neutralizing this virulence factor. For this, a vaccine was developed based on the structure of GAPDH, PNV1. However, given that GAPDH is a protein with high similarity in humans, the development of this vaccine was only based on protein peptides present in bacteria and sixteen amino acids of bacterial GAPDH were chosen. The objective of the vaccine was to produce antibodies to compensate for this lack in newborns.

The first results, developed against gram-positive bacteria, showed an effective protection of neonates against this type of infections. Passive immunization of neonates with anti-PNV1 antibodies induced an increase in the survival rate of the animals, suggesting that vaccination using PNV1 could be an effective approach.

Considering the high similarity between GAPDH of gram-positive and gram-negative bacteria, the present study aimed to verify the possibility of using PNV1 as a vaccine for infections by gram-negative bacteria, namely *Klebsiella pneumoniae* and *Escherichia coli*.

For this, we verified the presence of GAPDH in the supernatants of these bacteria and, in this way, could be a target of the therapy, which was verified. Subsequently, it would be necessary to understand if the PNV1 would be immunogenic. For this purpose, both rats and rabbits were tested

where it was found that the vaccine was immunogenic and that it would be possible to obtain anti-PNV1 antibodies for further protection verification.

In addition, an important aspect of anti-bacterial therapies is the need to verify the host microbiome's alteration. It is known that gut microbiome is crucial for homeostasis of the organism and a change in its normal constitution can present severe problems for the health of the individual. The aim of this part of the study was to evaluate the acute and chronic effects of active immunization with PNV1 or passive immunization with anti-PNV1 IgG. To that, the microbiome of immunized animals was compared to the control animal microbiome and no significant alteration was found. On the other hand, the verification of the existence of reactivity with the GAPDH was made and it is also possible to affirm that it does not exist, so there is not a reaction of the vaccine to host proteins.

Regarding vaccine-induced protection, tests were performed on newborns by passive immunization. When animals infected with either *Klebsiella pneumoniae* or *Escherichia coli* were analyzed, the group of immunized animals showed a significant increase in survival rate at the end of 12 days, showing that the neutralization of GAPDH mediated by anti-PNV1 antibodies was effective. These results demonstrated that, even with an infection, newborns can develop a robust and more specific response to infection.

In addition to the prevalence of this type of infection in neonates, it is known that are other groups at risk. It is known that the existence of chronic diseases such as type I diabetes can be considered a risk factor for the acquisition of infections (Casqueiro, Casqueiro, & Alves, 2012). The high frequency of infections in this type of patient is caused by the hyperglycemic environment that favors immune dysfunction, such as the degradation of neutrophil function, a decrease in the activity of the antioxidant system as well the decrease of the urinary and gastrointestinal antimicrobial activity. On the other hand, these patients require many medical care, increasing the possibility of acquisition of hospital infections. Thus, as another possible risk group, tests were done on type I diabetes animal models (the NOD/ShiLtJ strain, commonly called NOD) to check the protection induction. After analyzing the data, it is possible to verify that also in this risk group, the animals that were immunized with anti-PNV1 antibodies showed an increase in the survival rate, from 0 to 75%.

These results, although preliminary, suggest that there is strong evidence of a possible efficacy of the vaccine developed in other risk groups, in addition to neonates. Although further studies are needed, these results indicate that research in this area may involve parallel courses, thus increasing the area of application of the vaccine developed for bacterial infections.

Another issue raised during the development of the vaccine was its aim of the use in humans and the fact that peptide based therapies are known to be poorly immunogenic. Although the reason why peptides are not sufficiently immunogenic remains unclear (Judy Owen, Jenni Punt, 2012), some strategies have been used with the aim of increasing the immunogenicity of vaccines for a more

robust response by the immune system. One of them is the use of accessory molecules, conjugated with the vaccines under study. For this, the hypothesis raised by the group was the possibility of using one of these molecules in the developed vaccine. Thus, the chosen peptides were then conjugated with keyhole limpet hemocyanin (KLH), a molecule that has a strong ability to induce a response by the immune system. To that, the same initial immunogenicity tests were performed, this time with conjugated-PNV1. After comparing the data (Figures 3, 4 and 10), it shows that there was an increase in immunogenicity of the vaccine when conjugated to KLH. This result may be important for the advancement of clinical trials, which is an optimization of the vaccine to reinforce its importance and possible use in case of infections in humans.

Comparing all the results obtained with the literature, it is possible to suggest that the susceptibility of the newborns is not due essentially to the immaturity of their immune system but to the early production of IL10 induced by virulence factors as GAPDH. Thus, under conditions in which the immune system is not completely developed, after neutralization of extracellular bacterial GAPDH, it is possible that the immune system of the newborn responds appropriately to bacterial infections. Thus, and despite the immaturity of the newborn immune system, neonates are perfectly able to control infection if bacterial GAPDH is neutralized.

Despite the possible modifications that the vaccine may still undergo for optimization, a single vaccine can prevent infections caused by all different bacterial pathogens, structurally very different but that share the same mechanism of escaping host immune system.

Based on the results, the future work focuses essentially on the exact determination of the virulence mechanism of the bacteria under study. Despite the information already obtained for GBS supporting that GAPDH is responsible for an induction of IL-10 production, this has not been proven for gram-negative bacteria.

On the other hand, studies are still needed to evaluate the use of PNV1 in other at-risk groups such as elderly people and with chronic diseases such as type I diabetes. Despite the results, these are still very preliminary and require further study to support the hypothesis.

Finally, the study of conjugated-PNV1 also becomes important because of its increased ability to induce an immune response, and may be a better strategy for humans.

CHAPTER 7 – CONCLUSION

The sepsis is the leading cause of neonatal death and the treatments and prevention of these cases are a very important aspect of global health.

In addition to all the studies that have shown that the most prevalent bacteria are GBS and *Staphilococcus aureus*, gram-positive, *Klebsiella pneumoniae* and *Escherichia coli*, gram-negative bacteria, a need to combat the infection by these microorganisms has been emerging.

Previous studies of the group show that GAPDH was an important virulence factor in infections caused by GBS. Their presence induced an early production of IL-10, an immunosuppressive cytokine, which prevented the body from responding properly. Other studies of the group also showed that the use of a peptide-based vaccine (PNV1) was a good candidate for combating this type of infection, providing an effective protection against infections by GBS, *Staphilococcus aureus* and *Streptococcus pneumoniae*. This vaccine consists of peptides present on the surface of bacterial GAPDH and has no peptides common to human GAPDH to avoid a reaction to the host itself.

The present work was because of a great similarity between the GAPDH of gram-positive and gram-negative bacteria and proposed the use of the same vaccine to combat infections caused by gram-negative bacteria, *Klebsiella pneumoniae* and *Escherichia coli*. After analysis of the results, it can be concluded that there was in fact a protection induced by the PNV1 vaccine, resulting in an increase in the survival rates of the animals that were passively immunized with PNV1-IgG antibodies.

On the other hand, other tests were also performed considering other risk groups such as the people with chronic diseases such as type I diabetes. In this study, although the infection was only done with *Klebsiella pneumoniae*, it was possible to demonstrate that there was also some protection and the vaccine may be suggested as well as a possible vaccine.

Finally, the conjugation of the peptides with a molecule with a potent ability to induce an immune response (KLH) was suggested as an optimization of the study product to be able to proceed to clinical trials.

As future work was proposed to expand the study in the groups at risk as well as the conjugation of the vaccine.

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