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Development of a modular and multiplexed assay to predict the coverage of Bexsero™ against *Neisseria meningitidis* type B

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1. GLOSSARY

4CMenB, 4 Component protein-based meningococcal B vaccine

Ab, Antibody

Ag, Antigen

B, Blank

BSA, Bovine Serum Albumin

Capt, Capture

CI, Confidential Interval

Det, Detection

ELISA, Enzyme-Linked Immunosorbent Assay

EU, ELISA unit

fH, factor H

fHbp, factor H binding protein

GNA, genome-derived Neisseria Antigen

hSBA, Serum Bactericidal Assay using Human Complement

LD, Lysis Detergent

MATS, Meningococcal Antigen Typing System

MenB, serogroup B meningococcus

MenNZB, serogroup B meningococcus from New Zealand (NZ98/254 strain)

MFI, Median Fluorescence Intensity

MFI-B, Median Fluorescence Intensity minus Blank signal

MH, Mueller Hinton

NadA, Neisseria adesin A

NHBA, Neisseria Heparin Binding Antigen

NZ, New Zealand

OD, Optical Density

OMVs, Outer Membrane Vesicles

OMVnz, Outer Membrane Vesicles from NZ98/254 strain

pAb, polyclonal Antibody

PBT, Positive Bactericidal Threshold

PE, Phycoeritrin

PI, Pre-Immune

PNAS, Proceedings of the National Academy of Sciences of U.S.A.

PorA, Porine A

QC, Quality Control

RP, Relative Potency

rSBA, Serum Bactericidal Assay using Rabbit Complement

ρ, Spearman's coefficient (rho)

Sub-var., Sub-variant

UK, United Kingdom

Var., Variant

VR, Variable Region

WHO, World Health Organization

xMAP, Multi-Analyte Profiling by Luminex Technology

2. SUMMARY

Bexsero[TM] vaccine against *Neisseria meningitidis* serogroup B (MenB) strains has been approved in several countries. It is composed by three sub-capsular antigens, selected by reverse vaccinology approach: factor H binding protein (fHbp) variant 1.1, Neisseria Heparin Binding Antigen (NHBA) peptide 2 and Neisseria adesin A (NadA) variant 3. Bexsero[TM] vaccine includes also Outer Membrane Vescicles (OMVs) derived from the New Zealand strain NZ98/254, expressing PorA serosubtype P1.4. Evaluation of antigen expression by circulating *N. meningitidis* strains is a very critical step in order to predict the vaccine coverage and, a specific test has been set up for this purpose (Boccadifuoco et al., 2012).

The Meningococcal Antigen Typing System (MATS) was designed to measure immunologic cross-reactivity and quantify antigen content of target MenB strains. MATS results from a combination of three sandwich Enzyme-Linked Immunosorbent Assay (ELISA) assays, one for each vaccine antigen, plus sequencing of Porin A (PorA) P1.4 (Serruto et al., 2012). The readout of single meningococcal strains is expressed as Relative Potency (RP) for fHbp, NHBA, and NadA. RPs are calculated comparing serial dilution curves of tested strains with those of reference strains by a variance-weighted regression method. In order to determine RPs cut-off values able to predict strain susceptibility by human Serum Bactericidal Assay (hSBA), MATS RPs have been related to hSBA titers of 13-months-old children pooled sera, immunized with Bexsero[TM] at 2, 4, 6, and 12 months of age (Donnelly et. al., 2010). This correlation has defined a Positive Bactericidal Threshold (PBT) for each vaccine antigen above which the majority of strains are killed in hSBA.

Although working, conventional ELISA makes immunogenicity evaluation of a multi-component vaccine difficult, laborious, time-consuming and expensive, since only one immunogen per assay run can be tested.

xMap Luminex Technology allows the development of multiplex immunoassays where, multiple antibody types can be determined simultaneously in one assay run.

Taking the case of MATS-ELISA assay and Bexsero[TM] vaccine as reference, this PhD project aim to: (i) develop a flexible multiplex and quantitative sandwich assay (on Luminex platform) allowing the simultaneous measurement of all vaccine antigens expressed by bacterial strains, in order to predict the coverage of Bexsero[TM]; (ii) qualify the multiplex assay performance

(repeatability, sensitivity, precision, intra/inter assay variability); (iii) evaluate comparability of the new assay with the currently accepted ones, taking also into account results of the Serum Bactericidal Assay (SBA), the only accepted reference test for functional antibodies directed to meningococcus.

A 4-plex assay based on Luminex Technology able to simultaneously quantify fHbp, NadA and NHBA content of serogroup B menigococcal isolates was developed. The possibility to multiplex, allows measuring also the OMVs content and, consequently, eliminating PorA sequencing. This step shows the way forward to speculating on a possible role of other OMVs-components (not only PorA-correlated) in the coverage of Bexsero[TM].

We tested a 28-strains panel on optimized Luminex 4-plex assay in order to investigate the correlation with MATS data. These 28 strains are a sub-set of a 57-strains panel selected in 2010 by Donnelly et al. to correlate MATS results with hSBA titers.

Statistical analysis showed that, Luminex 4-plex assay is close correlated with MATS assay in terms of Relative Potencies (RPs) and has RPs strain rank highly correlated for each antigen.

After validation and further investigation, Luminex 4-plex assay could represent a promising test to obtain information about Bexsero[TM] coverage in easy, fast, cheap and more reproducible way. Furthermore, due to the high flexibility of this technology it will be possible to increase the antigens panel (from 4 up to 100 microspheres in the single well) and detect other vaccine antigens expressed on bacterial strain to predict the coverage of a new generation multi-component vaccine.

3. PhD PROJECT RATIONALE AND AIMS

Multiplex assays can offer significant benefits for routine serological testing through reduced staff and reagent costs and streamlined laboratory operation (Pickering et al., 2002).

xMap Luminex Technology allows the development of multiplex immunoassays where, multiple antibody types from a single serum, can be determined simultaneously in one assay run.

Bexsero[TM] vaccine against *Neisseria meningitidis* serogroup B has been approved in several countries. It is composed by three sub-capsular antigens, selected by reverse vaccinology approach: fHbp variant 1.1, NHBA peptide 2 and NadA variant 3. Bexsero[TM] vaccine includes also Outer Membrane Vescicles (OMVs) derived from the New Zealand strain NZ98/254, expressing PorA serosubtype P1.4. However, it remains critical to evaluate whether circulating *N. meningitidis* strains express these antigens in order to predict the vaccine coverage (Boccadifuoco et al, 2012).

The Meningococcal Antigen Typing System (MATS) was designed to measure immunologic cross-reactivity and quantity of vaccine antigens in target strains of *N. meningitidis B*. In particular, it is a combination of three sandwich Enzyme-Linked Immunosorbent Assay (ELISA) assays, one for each vaccine antigen, and the sequencing of PorA P1.4 (Serruto et al., 2012). However, a classical ELISA method could make multi-component vaccine immunogenicity evaluation difficult, laborious, time-consuming and expensive due to only one immunogen per assay run can be tested.

Taking the case of MATS-ELISA assay and Bexsero[TM] vaccine as reference, the PhD project aims are: (i) to develop a flexible multiplex and quantitative sandwich assay (on Luminex platform) allowing the simultaneous measurement of all vaccine antigens expressed by bacterial strains in order to predict the coverage of a multi-component based vaccine; (ii) to qualify the multiplex assay performances (reproducibility, sensitivity, accuracy, precision, intra/inter assay variability); (iii) to demonstrate the comparability of the new assay with previous data by using the relevant technical and statistical tools and bridge results with Serum Bactericidal Activity (SBA) of immune sera.

4. INTRODUCTION

4.1 <u>Vaccinology</u>

Along with improved sanitation and antibiotics' discovery and use, vaccination is considered a fundamental milestone for healthcare, providing life-long protection against a wide variety of infections. Vaccines also represent the most cost-effective method of improving health and saving lives (Levine and Lagos, 2004).

In 1796 Edward Jenner, following the path opened by the ancients who had used the smallpox virus itself in the practice of variolation, demonstrated that it was possible to protect people against smallpox through inoculation with cowpox (*Variolae Vaccinae,* from the Latin word *vacca*, cow). Although Jenner's discovery was crucial, in the early 1800s the work of Louis Pasteur (figure 1) on chicken cholera, opened the way to vaccine development in the laboratory and set up the basis of vaccinology era (Plotkin, 2003), leading to identification of microorganisms as causing agents of infectious diseases. In fact, by accidentally leaving a bacterial culture without nutrients, he observed the presence of microorganisms showing attenuated virulence and able to confer immunity against disease. The French microbiologist referred to these attenuated bacteria as "vaccines" in honour of Edward Jenner's discovery.

Since then, the principle of "isolate, inactivate and inject" the causative agent of disease proposed by Pasteur, led the vaccine development throughout the 20th century (Plotkin, 2009). This empirical strategy has been applied for most of the vaccines licensed to date, that can be classified into three categories: inactivated microorganisms, live-attenuated agents and subunit based vaccines (composed by infectious agent purified portions;
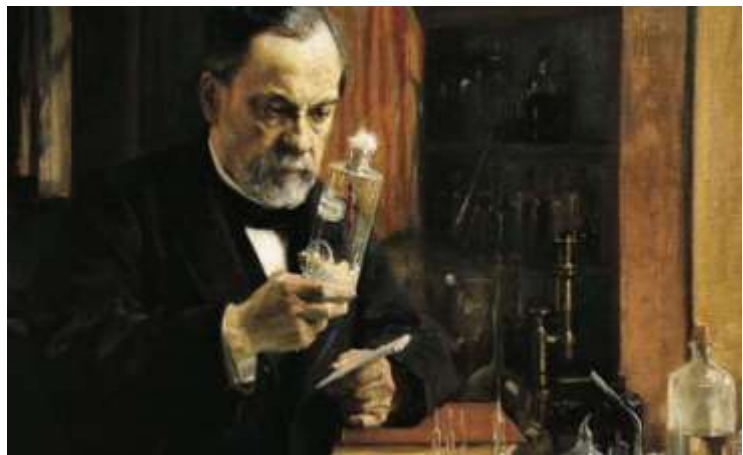

Figure 1. Louis Pasteur, founder of modern microbiology who discovered the principles of vaccination

Moylett and Hanson, 2003).

The main targets of these conventional vaccines are microorganisms that have invariant antigens or whose related disease results in natural immunity to reinfection (antibody-mediated responses; Rappuoli, 2007).

Moreover, the difficulty to cultivate some pathogens in laboratory and the lack of suitable animal models of infection, constitute other major limitations to vaccine development. However, vaccines development for many pathogens remains elusive, and there is a growing requirement for the fast development of effective vaccines for emerging diseases (Morens, Folkers, et al., 2008).

During the last three decades, the vaccine field has been transformed by new technologies, such as recombinant DNA and chemical conjugation of proteins to polysaccharides. More recently, new antigen-discovery, new design methods and investigation of vaccine responses have been applied, including structural biology and systems biology. (Rinaudo, Telford, et al., 2009). Genome-based technologies have enabled functionally blind selection of vaccine candidates, and have not only led to the discovery of novel protective antigens, but have also revealed new virulence factors of several pathogens. Consequently, the pathogenesis-to-vaccine paradigm has been reversed in several situations, and vaccine development frequently leads to a better understanding of pathogenesis, which has in turn led to novel approaches in studying not only the organism itself, but also the strategies for the design of more successful vaccines. The genome era, in fact, has revolutionized vaccinology with the first complete genome sequenced of *Haemophilus influenza* in 1995 (Fleischmann, Adams, et al., 1995).

The combination of sequencing technology with bioinformatics tools have resulted in the concept of "reverse vaccinology" (Rappuoli, 2000). The basic idea behind this strategy is the complete screening of the entire pathogenic genome by using computational analysis in order to identify interesting genes, encoding potential vaccine targets. Among them, there are virulence factors identified through sequence similarity to known pathogenic proteins, secreted or membrane-associated proteins containing signal peptides or anchoring motifs, lipoproteins and integral membrane proteins that could be involved in the recognition and interaction with host structures. The selected interesting ORFs are expressed and purified in *Escherichia coli* heterologous system, and then they undergo the normal laboratory testing for immune response. Only the antigens that provide high level of protection in animal model are selected for further

characterization before entering in clinical trials (Rappuoli, 2001). Reverse vaccinology approach was successfully applied for the first time in the identification of vaccine candidates against *Neisseria meningitidis* serogroup B (MenB), leading to a vaccine named Bexsero[TM] that has been approved for use in more than thirty countries, especially in Europe and Canada.

MenB, the major cause of meningococcal disease in the developed world, is an optimal example for which the conventional vaccines approaches failed. The success of reverse vaccinology for meningococcus catalyzed a paradigm shift in vaccine development, and paved the way to a variety of other genome-based vaccine discovery projects such as *Streptococcus agalactiae*, *Streptococcus pneumoniae*, *Chlamydia pneumoniae*, *Bacillus anthracis*, *Porphyromonas gingivalis*, *Mycobacterium tuberculosis*, *Helicobacter pylori* and others (Chakravarti, Fiske, et al., 2000; Ross, Czajkowski, et al., 2001; Wizemann, Heinrichs, et al., 2001; Ariel, Zvi, et al., 2002; Betts, 2002; Montigiani, Falugi, et al., 2002).

4.2 *Neisseria meningitidis* and current meningitis vaccines

N. meningitidis is a major cause of meningitis and sepsis, two devastating diseases that can kill children and young adults within hours, despite the availability of effective antibiotics. Meningococcus is a human-specific Gram-negative bacterium adapted to colonize the upper nasopharynx and directly spread from host to host, without requiring a reservoir outside humans. Carriage rates are very variable among human population, depending on different factors such as age, concurrent respiratory tract infections and frequency of social contacts. In Europe and United States, point-prevalence carriage rates have been estimated to range from 10% to 35% in young adults (Claus, Maiden, et al., 2005; Stephens, 2007).

In a small but significant number of infections, the bacterium overcomes the respiratory tract epithelium and reaches the bloodstream, causing septicemia. From the blood, meningococcus is also able to cross the blood-brain barrier and infect the meninges, causing meningitis. The ability to colonize and cause disease results from meningococcus specific mechanisms to evade the human immune system (Lo, Tang, et al., 2009).

A capsule made up of complex polysaccharides surrounds all currently known disease-causing meningococci and, is one of the essential meningococcal attributes for pathogenesis. The capsular polysaccharide (CPS) inhibits bacterial adhesion by masking the action of meningococcal adhesins and is critical for bacterial survival in the blood (Schneider, et al., 2007). *N. meningitidis* can be classified in 12 serogroups on the basis of CPS chemical composition, but, more than 95% of total cases of invasive disease are caused by five major serogroups: A, B, C, Y and W135. The distribution of the serogroups varies globally. There are an estimated 1.2 million cases of meningococcal infection per year, with a death toll of almost 135,000 worldwide. Serogroup B meningococci, which are generally absent in Sub-Saharan Africa, are the primary concern in industrialized countries; however, changes in serogroup circulation are unpredictable and can occur very quickly (Khatami, 2010). Taking into account all these observations, it is widely recognized that vaccines conferring broad protection against *N. meningitidis* are a priority for of global health.

Vaccines against serogroups A, C, Y and W135 were developed in the 1960s by using purified CPS as antigen. Since then more effective, conjugated vaccines have been introduced, in which CPS components are conjugated to carrier proteins such as CRM197, a non-toxic mutant of the

diphtheria toxin (Costantino, Rappuoli, et al., 2011). The first conjugate vaccines targeting group C *N. meningitidis*, that were developed to control the ongoing hyperendemic level of disease in infants and children, showed immunogenicity and safety in all age groups studied. Following the success of MenC vaccines quadrivalent meningococcal conjugate vaccines, containing the polysaccharide from serogroups A, C, Y and W135 conjugated to a carrier protein, have been introduced. These vaccines offered the potential to broaden protection against meningococcal disease beyond that offered by monovalent MenC conjugate vaccines: MenACWY-CRM, has been shown to be immunogenic in all age groups, including infants (Snape, Perrett, et al., 2008). Development of a broadly protective vaccine against MenB has been a difficult and unmet challenge for decades. The principal reason is that the MenB capsular polysaccharide, a homopolymer of $\alpha(2\text{-}8)$-linked polysialic acid, is poorly immunogenic as it is antigenically similar to the human foetal neural cell adhesion molecule. This led to the concern that a MenB polysaccharide or glycoconjugate vaccine might induce autoantibodies (Finne, Leinonen et al., 1983). Therefore, the search for a vaccine focused on non-capsular target antigens and, over the last 40 years, great efforts have been directed towards the identification of meningococcus B antigens as the basis of a new vaccine. However, the high sequence variability of these proteins among different MenB strains represents a serious obstacle to production of a globally effective anti-MenB vaccine (Bai, et al., 2010; Sadarangani, et al., 2010; Tan, Carlone, et al., 2010).

Until recently licensed vaccines against serogroup B disease made use of outer membrane vesicles (OMVs), produced by detergent extraction of the bacterial outer membrane. A variety of 'tailor-made' MenB OMV vaccines have been developed and licensed to control regional epidemics that, on the contrary of endemic disease, tend to be caused by a single clone of *N. meningitidis*. OMVs were able to induce protective antibodies against the homologous strain (*i.e.* the strain causing the epidemic and used to prepare the OMVs) in all age groups, and have proved to be successful in controlling epidemic disease in the onset's countries (Holst, et al, 2013). The main limitation of OMV vaccines is that they are strain-specific and do not provide protection against heterologous strains, due to high variability across different isolates of PorA, which is the major protective antigen of the OMV-based vaccines, and the most abundant integral outer membrane protein. As a consequence, there were no effective licensed vaccines available for the prevention of MenB disease, which is responsible for one third of meningococcal disease in the USA, and up to 80% of cases in Europe. A second generation of

OMV vaccines has been developed in order to broaden strain coverage. Meningococcal strains expressing six different PorA variants have been genetically engineered in order to produce the hexavalent PorA OMV vaccines (Claassen, Meylis, et al., 1996). However, since the use of multivalent OMV vaccines does not promise a simple universal solution, alternative approaches based on surface-exposed proteins were sought.

4.3 Discovery of MenB vaccine antigens by reverse vaccinology and development of Bexsero^{TM} vaccine

The availability of whole genome sequences, in the genomic era, has radically changed the approach to vaccine development. The genome represents a virtual database of all protein antigens that the pathogen can express at any time, so that it is possible to identify potentially surface-exposed proteins in a reverse manner, starting from the genome rather than from cultures of the microorganism (Rappuoli, 2001). The concept of reverse vaccinology was first successfully used by Novartis Vaccines and Diagnostics (now Gsk Vaccines) to develop a novel MenB vaccine, by using the genome of MenB strain MC58 (figure 2; Pizza, Scarlato, et al., 2000; Giuliani, Adu-Bobie, et al., 2006).

The sequence of MenB virulent strain MC58 was determined by the shotgun strategy, revealing over 2000 predicted proteins (Open reading Frames, ORFs) (Tettelin, Saunders, et al., 2000). All the bacterial encoded proteins were screened for their potential localization on the bacterial surface by using bioinformatics algorithms, in order to identify novel vaccine antigens.
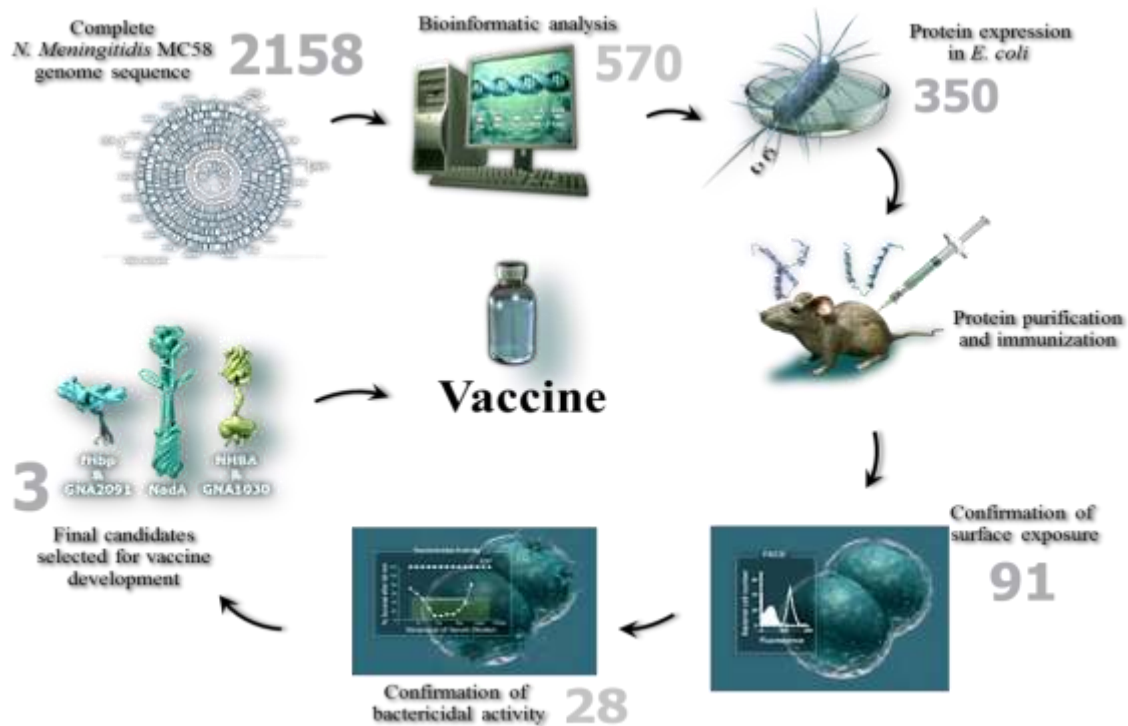

Figure 2. Schematic representation of Bexsero^{TM} development by reverse vaccinology

Those ORFs predicted to be surface-exposed or secreted, were expressed in *E. coli* as recombinant proteins, purified and then, tested for their potential to induce bactericidal antibodies (Pizza, Scarlato, et al., 2000).

Each purified recombinant protein was used to immunize mice and antibody response was analyzed by Western blot analysis, using both total cell extracts and purified outer membrane proteins to verify protein expression. Surface localization of target protein was confirmed by Enzyme-Linked Immunosorbent Assay (ELISA) and flow cytometry on intact, whole bacteria.

Since the main protective response against *N. meningitidis* relies on bactericidal circulating antibodies, complement-mediated bactericidal activity, measured by serum bactericidal activity assay (SBA) using human complement, is the accepted correlated for *in vivo* protection, and has been adopted in clinical trials of meningococcal vaccines as the surrogate for protection (Borrow, Carlone, et al., 2006). SBA was used to evaluate the complement-mediated killing activity of the antibodies. Among the 91 proteins found to be positive in at least one of these assays, 28 were able to induce antibodies with bactericidal activity.

The antigens selected by reverse vaccinology were prioritized based on their ability to induce broad protection (*i.e.* ability to kill a diverse collection of strains) as inferred by SBA, or the

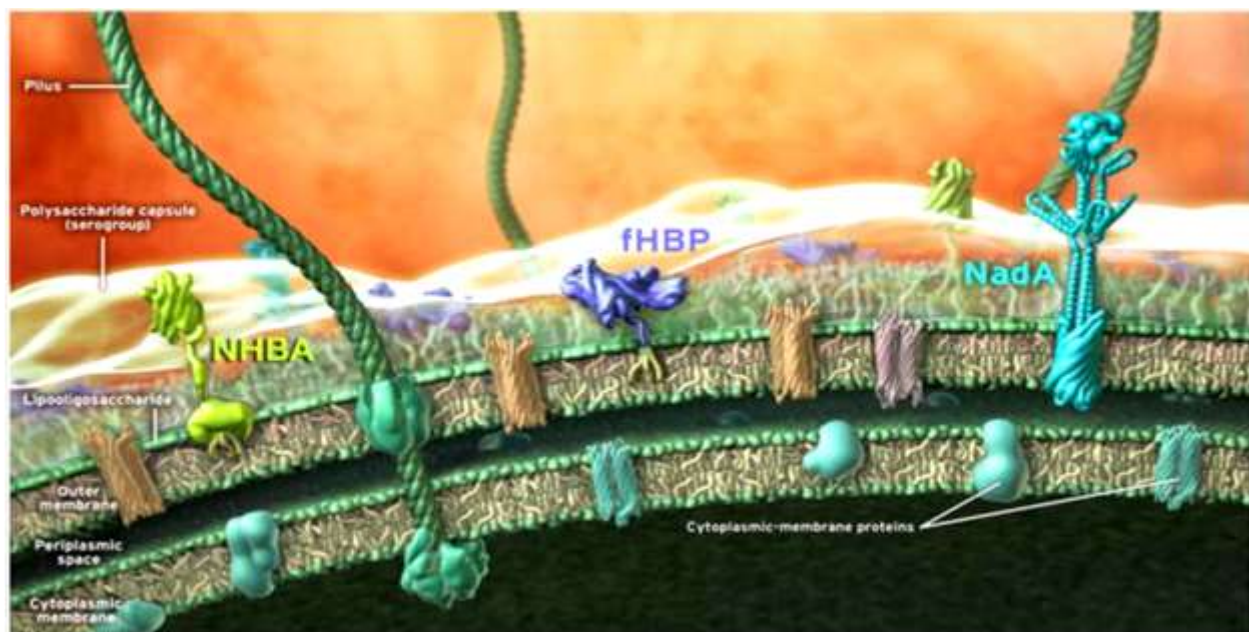**Figure 3.** Schematic representation of MenB vaccine antigens (NHBA, fHbp and NadA) on bacterial surface with the different bacterial compartments depicted. NHBA and fHbp's structures are derived from the Nuclear magnetic resonance (NMR) structural data available and reported as cartoon, while NadA is a model based on the structural homology with other protein members of the Oca family (Serruto et al., 2012)

ability of specific antibodies to confer passive protection in the infant rat or mouse models (Giuliani, Adu-Bobie et al., 2006). The proteins that met these prioritization criteria (see figure 3 on the previous page) were selected and called Genome-derived Neisseria Antigens (GNA) 2132 (Neisseria Heparin Binding Antigen, or NHBA), GNA1870 (factor H binding protein, or fHbp) and GNA1994 (Neisseria adhesin A, or NadA).

Two additional antigens, GNA2091 and GNA1030, were also selected due to their capability to induce protective immunity in some assays. In order to facilitate large-scale manufacturing of the vaccine, four of the selected antigens were combined as two fusion proteins. Among the several protein-protein fusions generated, the best performing combinations in terms of production and immunogenicity were NHBA plus GNA1030, and GNA2091 with fHbp. NadA did not perform well when fused to other proteins, probably due to the disruption of its trimeric organization. These two fusion proteins were formulated with NadA to produce a novel recombinant MenB vaccine (rMenB). To fully assess whether the vaccine formulation developed was able to induce protection against a wide proportion of MenB strains, a large panel of clinical isolates representing as much as possible the diversity of the bacterial population was collected. Sera obtained by immunizing mice with the vaccine were tested in a bactericidal assay against a panel of 85 meningococcal strains. Preclinical characterization showed that the vaccine induced bactericidal antibodies against 78% of the strains (Giuliani, Adu-Bobie, et al., 2006).

To investigate ways of increasing immunogenicity, rMenB was also formulated with OMVs in a novel vaccine named 4CMenB (4 Components against MenB vaccine, figure 4). OMVs were produced in *N. meningitidis* strain NZ98/254 (expressing PorA serosubtype P1.4). The addition of MenNZB component was driven by the positive results obtained with the OMV-based
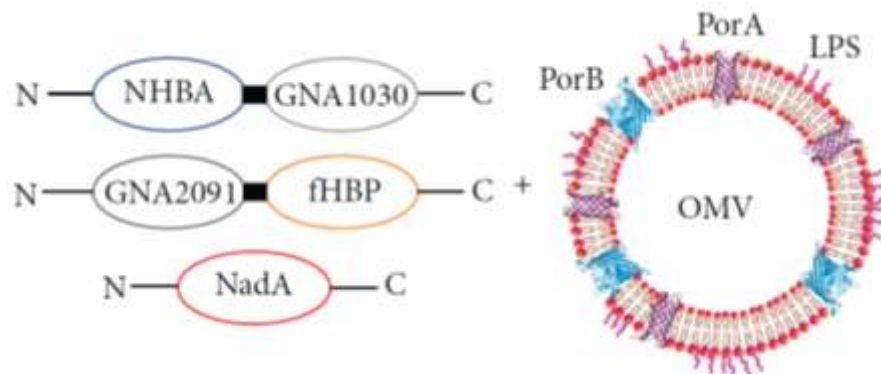
Figure 4. 4CMenB vaccine composition (Domnich et. al., 2015)

vaccine, which was shown to be safe and efficacious in the control of the clonal meningococcal serogroup B epidemic in New Zealand (Oster, Lennon, et al., 2005).

The final vaccine formulation, which was submitted in December 2010 to the European Medicines Agency for a marketing authorisation, is composed by rMenB+OMVnz and has been assigned the trade name of Bexsero[TM]. Bexsero[TM] was approved by the European Commission on January 2013 as a vaccine formulated with aluminum hydroxide, to be administered by intramuscular injection (Findlow, et al., 2010; Snape. et al., 2010).

4.4 Components of Bexsero^{TM} vaccine

4.4.1 Factor H binding Protein (FHbp)

Factor H binding protein (fHbp or GNA1870) is an ubiquitous menigococcal surface-exposed lipoprotein (Masignani, et al., 2003) that binds human factor H (fH), an inhibitor of the alternative complement pathway. Evasion of the human complement system is critical for meningococci ability to cause invasive disease.

Three-dimensional (3D) structure of the 28-kDa fHbp has been determined by both Nuclear magnetic resonance (NMR) spectroscopy (Cantini, et al., 2009; Mascioni, et al., 2010), and X-ray crystallography (Cendron, Veggi, et al., 2011). Both structures reveal that fHbp is composed of two domains: a N-terminal domain of 10 anti-parallel β-strands forming a highly curved anti-parallel β-sheet (approximating a β-barrel) and a C-terminal domain that is a well-defined β-barrel comprising 8 anti-parallel β-strands.

FHbp can be classified into three genetic and immunogenic variants: fHbp var.1, 2 and 3 (figure 5, Masignani, et al., 2003), which are not cross-protective, and can be further divided into sub-variants 1.x, 2.x and 3.x. Sequence conservation within each variant ranges from 92% to 100%, while between the variants the conservation can be as low as 63%.

This diversity has an important impact on the immunological properties of fHbp, since members of each variant induce a strong protective immune response against meningococcal strains carrying homologous alleles, but are ineffective against strains that express distantly related
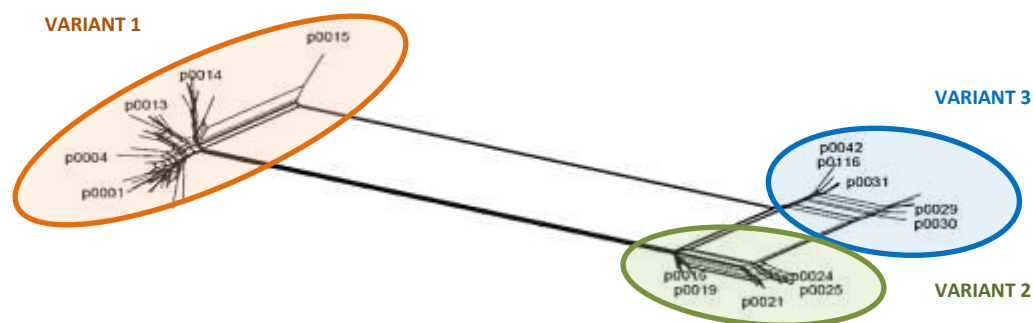

Figure 5. Phylogenetic tree showing phylogenetic relations between variants 1 (orange circle), 2 (green circle) and 3 (light blue circle) of fHbp (Masignani, 2014)

variants (Masignani. et al., 2003). In a different nomenclature scheme based on genetic information, the variants have been grouped into family A (var.2 and var.3) and family B (var.1) (Fletcher, et al., 2004).

4.4.2 Neisseria adesin A (NadA)

NadA (*Neisseria* adhesin A or GNA1994) is 38-kDa protein which belongs to the class of trimeric auto-transporter adhesins (TAAs) (Pizza, et al., 2000; Pillai, et al., 2005). TAAs mediate adhesion through interaction with extracellular matrix proteins, and are involved in invasion of target cells (Plested, et al., 2008). Structurally, they are obligate homotrimers, and accordingly the recombinant NadA-3 (the variant included in Bexsero[TM] vaccine), lacking of the C-terminal membrane anchor region, forms soluble, stable trimers (Rosenqvist, et al., 1995). TAAs are generally formed by a conserved C-terminal integral membrane β-barrel, which anchors the proteins to the outer membrane, and an N-terminal "passenger" domain responsible for adhesion. The TAA passenger domain typically is made of a central α-helical domain (stalk) forming coiled-coil structures and a distinct N-terminal domain (head) that is mainly responsible for binding to host cellular receptors.

Two main genetically distinct groups of NadA have been identified that share overall amino acid sequence identities of 45–50%. Group I includes the three most common variants (NadA-1, NadA-2, and NadA-3), which share ~95% sequence identity and are immunologically cross-reactive (Comanducci, et al., 2004). Group II includes three rarer variants, sharing ~90% sequence identity (Bambini et al., 2014): NadA-4, primarily associated with carriage strains (48. Comanducci et al., 2004), NadA-5, mainly found in strains of clonal complex 213 (Lucidarme, et al., 2009; Bambini, et al., 2014), and NadA6. Vaccine variant NadA-3 is highly immunologically cross-reactive with NadA-1, NadA-2, and other NadA-3 subvariants but poorly cross-reactive with NadA-4 (Comanducci, et al., 2004).

4.4.3 Neisseria Heparin Binding Antigen (NHBA)

NHBA (Neisseria Heparin Binding Antigen, or GNA2132) is a 51-kDa surface-exposed lipoprotein, target of both meningococcal and human proteases, which binds to heparin *in vitro*

through an arginine-rich region (Serruto, et al., 2010). Upon binding heparin, which is often used as a surrogate for host polyanions in *in vitro* assays, unencapsulated bacteria showed increased survival in human serum (Serruto, et al., 2010). *In vivo*, NHBA binds glysoaminoglycans (such as heparan sulfate), which are present in mucosal secretions. In this context, NHBA contributes to the interaction of meningococcus with the host cells (Vacca, et. al., 2016). These data may point to a role for NHBA in protection of unencapsulated meningococci (as found in the nasopharynx) against complement. The interactions between heparin and the complement system are complex and involve several of its component proteins (Sahu and Pangburn, 1993), including complement inhibitors such as fH, C4b-binding protein and vitronectin. It is conceivable that the establishment of an NHBA-heparin complex on the meningococcus cell surface could recruit complement inhibitors, which in turn act to prevent complement activation. Analysis of gene sequences from genetically diverse serogroup B strains reveals the existence of more than 400 distinct variants, named 'peptides', for which each is assigned a numerical identifier. The NHBA variant included in Bexsero[TM] is the one which contains the amino acid sequence of the peptide 2.

4.4.4 Outer Membrane Vesicles (OMVs)

Outer Membrane Vesicles (OMVs) are spherical bi-layered membrane structures with a diameter in the range of 20–250 nm, that are pinched off from the outer membrane of Gram-negative bacteria. In natural OMVs of *Neisseria meningitidis,* a total of 155 proteins were identified: outer membrane proteins (such as porines), lipopolysacchharide (LPS), opacity-associated protein C (Opc), periplasmic constituents (van de Waterbeemda, et al., 2010), and some Bexsero[TM] component as porine A (PorA), fHbp, NadA (van der Pol, et al., 2015).

PorA protein was identified as the major protective antigen in OMVs, but is highly variable between the circulating serogroup B strains. The OMV component of Bexsero[TM] contains the PorA protein of subtype P1.7-2,4. The PorA Variable Region 2 (VR2) P1.4 epitope is the major PorA target for the immune response elicited by Bexsero[TM] vaccine (Martin, et al., 2006). There were 28 *porA* subtypes (VR1 and VR2 combinations) present among the isolates, 24 of which were identified in just one (17 subtypes), two (5 subtypes), or three (2 subtypes) isolates each.

The four major subtypes (representing 62% of the isolates in total) were P1.7-2,4 (20%), P1.22,9 (18%), P1.22,14 (17%), and P1.19-1,15-11 (7%) (Lucidarme, et al., 2010).

4.5 Serum Bactericidal Activity assay (SBA): the only correlate of protection

The serum bactericidal assay with human complement (hSBA) is a universally accepted correlate of protection against meningococcal disease that quantifies the complement-mediated killing of bacteria by functional antibodies present in sera from vaccinees (Vogel et al., 2010).

In general, an hSBA titer $\geq 1:4$ is considered to be a correlate of protection. Despite its strengths, hSBA has some shortcomings. First of all, hSBA is a labor-intensive technique and testing a large number of single circulating strains would produce logistical difficulties. In the other hand, it requires collecting considerable amounts of sera from immunized individuals, which would be ethically debatable, especially in pediatric studies. Furthermore, the standardization of hSBA across numerous strains and complement sources is also burdensome (Donnely, et al., 2010; Boccadifuoco et al., 2012). While hSBA is able to assess the effectiveness of a vaccine by measuring bactericidal antibody titers, it does not provide information on the contribution of each vaccine component. Indeed, the surface-exposed proteins fHbp, NHBA, and NadA display considerable sequence and expression variability, as well as different degrees of cross-reactivity among variants of a protein antigen to the antibodies induced by the vaccine (Beemink, et al., 2009; Lucidarme, et al., 2010). For these reasons, an assay that can reliably assess the expression of those specific antigen variants, predicted to be targeted by bactericidal antibodies elicited by the vaccine on different bacterial isolates, is needed.

4.6 <u>Meningococcal Antigen Typing System (MATS)</u>

To overcome the aforementioned limitations of SBA, a novel approach, named Meningococcal Antigen Typing System (MATS) has been developed. MATS, is an assay able to predict susceptibility of MenB isolates to vaccine-elicited bactericidal killing, measuring both antigenic reactivity and expression level of antigens on a given meningococcal strain (Donnelly, et al., 2010).

Basically, MATS combines 3 modified sandwich Enzyme-Linked Immunosorbent Assay (ELISA) assays, to measure antigenic cross-reactivity and expression of fHbp, NadA and NHBA on bacterial lysates, plus sequencing of dominant OMV immunogen. A strain that matches PorA serosubtype (PorA 1.4) is considered covered by Bexsero^{TM} vaccine. To evaluate whether the antigen content measured by MATS correlates with bactericidal activity, 57 serogroup B isolates, with known antigen genotypes and MATS values, have been tested by hSBA using pooled sera from infants immunized with 4CMenB vaccine. In details, MATS measures fHbp, NadA and NHBA content of serogroup B menigococcal (MenB) isolates relative to a reference strain in order to provide a "Relative Potency" (RP). By comparing MATS RP values with SBA results, threshold values were defined for each of the three antigens (Positive Bactericidal Thresholds, PBTs), specifically 2.1, 29.4 and 0.9% for fHbp, NHBA and NadA respectively. A strain with a relative potency above the PBT for at least one of the 3 antigens is predicted to be killed in SBA at a $\geq 80\%$ probability (Donnelly, et al., 2010).

Intrinsically, MATS is a conservative predictor since it leads to an underestimation of Men B coverage when compared to hSBA (Frosi, et al., 2013).

4.7 <u>Principles of Luminex Technology: ELISA vs Luminex</u>

Immunogenicity is a crucial point of vaccine protective activity, and ELISA assay is the most common test to determine antibody titer against an immunogen. However, a classical ELISA method could make multi-component vaccine immunogenicity evaluation difficult, laborious, time-consuming and expensive due to only one immunogen per assay run can be tested. Another disadvantage is the assay limited dynamic range, such that repeated testing with further test sample dilutions may be necessary. In addition, ELISA is based on no covalent interactions of a molecule to a functionalized polystyrene plate (coating). As a consequence, the coating conditions setup could be difficult since it depends on many factors such as temperature, type of plate, immunogen chemical nature and concentration.

Beside to the traditional ELISA approach, a possible alternative to test antibody response to a multi-component vaccine is represented by xMAP Luminex technology. This technology is mainly known for the possibility to manage multiple analytes within a single assay run.

Due to robust multiplexing, xMAP technology potentially delivers more data in less time than other bioassay products, with comparable results to ELISA and microarray. The technology offers several other distinct advantages over traditional methods: a. speed/high-throughput (a large number of different bioassays can be performed and analyzed simultaneously thanks to the unique spectral signature of each beads); b. versatility (a single xMAP technology-based system can perform bioassays in several different formats); c. flexibility (the technology can be customized for the user's specific needs or periodically updated by attaching a specific probe to a uniquely colored microsphere; d. accuracy (possibility to perform real-time analysis and accurate quantification of biological interactions); e. reproducibility (high-volume production of xMAP microspheres within a single lot allows a grade of assay standardization not provided by solid-phased planar arrays (Luminex corp website, last update 2016, 8th Sept)).

Luminex approach relies on 6.2 μ diameter magnetic polystyrene microspheres (or beads), named MagPlex®, internally dyed with different intensities of red and infrared fluorophores. As a consequence, 100 distinct bead regions, based on dye mixture, have their unique spectral signature. Due to capacity of xMAP to uniquely detect and identify beads, simultaneous multiple measurements are possible (potentially up to 100 analytes/assay run).

Bead surface can be variously functionalized, e.g. carboxylated, allowing covalent coupling of analytes and bio-analytical reactions to take place over them. Inside the Luminex system, beads are lined up by fluidics in a single-line and pass through a detection chamber where a red laser excites their internal dyes allowing beads identification, and a green laser quantifies the bio-molecular interaction occurring at bead surface. Software analysis sorts registered events by side scattering, thus events larger or smaller than a single microsphere are excluded, as well as aggregated microspheres (figure 6).

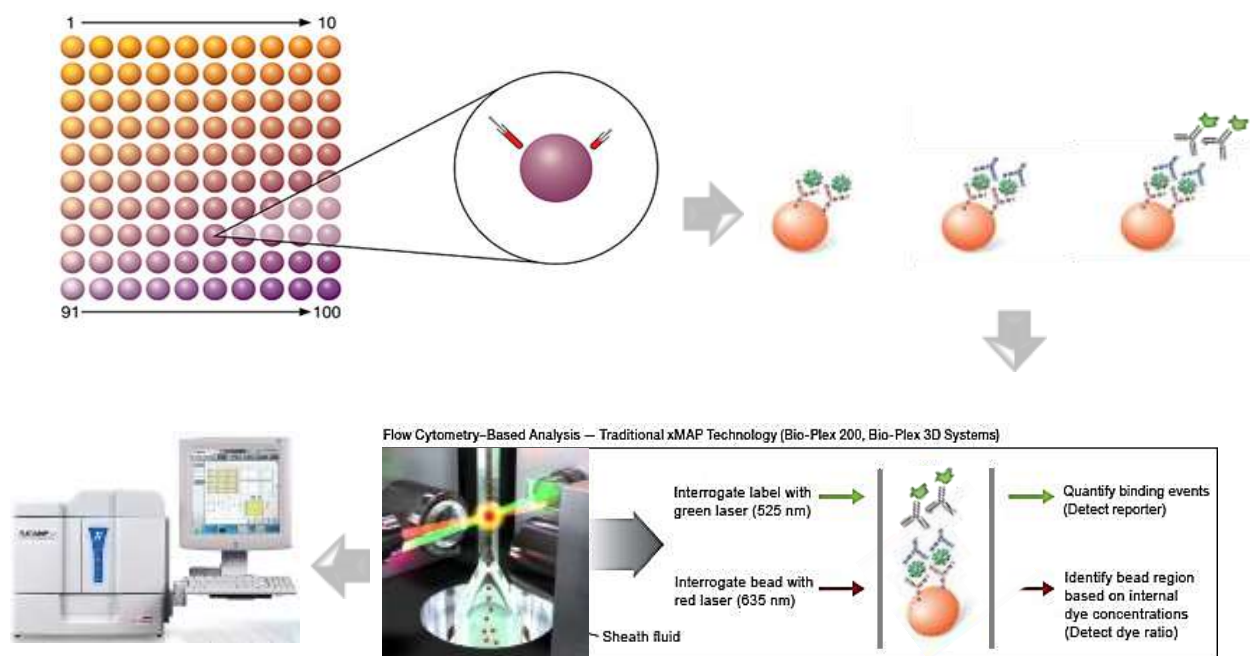**Figure 6.** Luminex technology: 100 distinct bead regions based on red/infrared dye mixture are available. Selected bead region is conjugated by COOH groups to NH_2 of the analyte. Coupled beads react with the sample and bind detection Ab and a dye-labeled secondary Ab (e.g. sandwich assay). Sheath fluid aligns beads in single file through a cytometric flow. Assay results are expressed in Median Fluorescent Intensity, MFI

4.8 4-plex sandwich assay development on Luminex platform to predict the coverage of a protein-based vaccine: the Bexsero[TM] example

During my PhD, I have developed and qualified a flexible 4-plex sandwich assay on Luminex platform in order to predict the coverage of a multiple protein-based vaccine. In particular, I have focused my analyses on the coverage of Bexsero[TM] vaccine versus circulating Men B strain. The first part of my work was devoted to selection of an optimal assay format, ending with two promising formats to be investigated: MATS-like format, which uses the same rabbit vaccine antigen-specific antibodies as capture and detection and NEW Format, where the capture Ab was substituted by a mouse vaccine Ag-specific Abs.

The setup started on single-plex assay (one analyte/well). Both MATS-like and NEW format were investigated in order to study single-plex assay sensitivity and specificity by using recombinant vaccine antigens as a first step and, finally, testing Men B lysates. In detail, the performed steps were the following: a. identification of optimal capture and detection antibodies concentration to obtain a specific and linear signal with the best sensitivity and dynamic range; b. analysis of cross-reactivity between each vaccine Ags to obtain more information necessary to implement multiplex assay, c. investigation of assay reproducibility and d. matrix effect study.

Preliminary results on MATS-like format on Luminex platform have shown that, although different growing detection Ab concentrations were tested, it was no possible to reach the instrumental working range. This evidence has drawn attention to a possible risk to be not sensitive against heterologous Ag-expressing strains and has led to the selection of New Format as the best format candidate. The second part of my PhD project was focused on optimization of NEW Format assay by using *Neisseria meningitidis* serotype B as samples, in order to confirm reproducibility, specificity and sensitivity of single-plex format. In detail, I performed the analyses on a larger panel of MenB lysates normally used as quality control for MATS assay. This preliminary screening demonstrated that single-plex and multiplex assay show high correlation (R^2>0.9 for each vaccine Ag between the two assays) and MFI profiles agree with antigen-expression and MATS data (data not shown). By using the software Softmax, I produced a relative potencies for MenB strains tested on multiplex assay, demonstrating that Luminex's RPs are comparable with MATS's RPs, since they are included in the 95% Confidence Interval of MATS' RPs (data not shown).

Last part of my work was focused on qualification of optimized multiplex assay. In particular, I performed incubation times, specificity, sensitivity and reproducibility analyses, investigated precision of the assay and defined the assay cut-off following bioanalytical method validation guidance available in literature. Finally, I have screened a broad and heterogeneous panel of 28 MenB lysates (a subset of the 57 tested to evaluate the performance of MATS (Donnelly et al., 2010) and compared the results with SBA data.

5. MATERIALS AND METHODS

5.1 Enzyme-Linked ImmunoSorbent Assay (ELISA)

ELISA assay was used to assess immunogenicity of mouse and rabbit sera before the purification of Bexsero^{TM} antigen specific IgG to be used for MATS and Luminex assay.

5.1.1 ELISA equipment and softwares

The following reagents and equipments are needed for the ELISA assay: Nunc Maxisorp 96-well microtiter plates (Cod. 442404); saturation buffer 2.7% polyvinylpyrrolidone 15 (PVP) in bi-distillated water; washing buffer for GNA2091-fHbp, NadA, NHBA-GNA1030 0.05% Tween20+PBS 0.074M (PBT), washing buffer for OMVs PBS 0.5X+Tween 20 0.05%; dilution buffer 1% BSA+0.05% Tween-20+PBS 0.074 M; alkaline phosphatase-conjugated secondary anti-species antibodies (Sigma Cod. A3687); substrate p-nitrophenyl phosphate (pNPP, Sigma cod. P7998); antigen dilution buffer 0,148 M (Na_2HPO_4 1,15 g, KCl 0,2 g, KH_2PO_4 0,2 g, NaCl 8,0 g, pH 7,4 ± 0,1 in milliQ H_2O filter-sterilized to 1 litre), 1.0 µg/ml of GNA2091-fHbp, NadA and NHBA-GNA1030 solutions, 5.0 µg/ml of OMVs solution, plate reader EPOCH, BioTek and plate washer BioTek ELx405.

5.1.2 Experimental procedure

ELISA for mouse sera

96 well-plates are coated with 100 µl/well of 0.015 µM Bexsero^{TM} antigen solution and incubated overnight at 4°C (except for OMVs: plates are incubated 2.5 hours at 30°C). After the coating, plates are washed three times with washing buffer; 250 µl/well of saturation buffer is added and the plates are incubated for 2 hours at 37°C (1 hour at room temperature for OMVs). After 3-time washing with washing buffer, 1:40000 diluted mouse sera are dispensed (100 µl/well) and diluted two-fold along the strips. Standard serum is represented by a pool of iper-immune mouse sera and, as a positive control, a pool of medium/low immune responsive sera

was selected (both are dispensed at adequate dilution). Plates are then incubated for 2 hours at 37°C, and wells are washed 3 times with PBT before addition of alkaline phosphatase-conjugated secondary antibodies (100 µl/well, 1:2000 diluted). After 90-minutes incubation at 37°C and 3-time washing with PBT, p-nitrophenyl phosphate is added (100 µl/well) and plates are incubated at room temperature for 30 minutes. Reaction is stopped by addition of of 4N NaOH (100µl/well) and OD 405/620-630 nm is measured. Antibody titers are expressed as ELISA units per millilitre (EU/ml o IU/ml) and are quantified via interpolation against a reference standard curve by using the software Combistats (version 4.0, EDQM).

ELISA for rabbit sera

The experimental procedure for rabbit sera follows the same steps and needs the same reagents used for mouse sera. The only exception is represented by the dilution factors of rabbit sera and relative secondary antibodies, which are respectively 1:10000 and 1:5000.

5.2 <u>Serum Bactericidal Activity (SBA) assay</u>

Serum bactericidal activity against *N. meningitidis* strains was evaluated by using pooled baby rabbit serum (CedarLane) as complement source. Serum bactericidal titers were defined as the serum dilution resulting in 50% decrease in CFU/ml after 60 min incubation of bacteria with reaction mixture, compared with control CFU/ml at time 0. Typically, bacteria incubated with the negative control antibody in the presence of complement showed a 150 to 200% increment in CFU/ml during the 60 min incubation.

5.2.1 Equipment

A specialized equipment is required to study the serum bactericidal activity against *N. meningitidis*: a biosafety level 2 safety cabinet to manipulate the pathogens, a spectrophotometer to measure bacterial culture OD_{600} and incubators allowing optimal growth conditions (+37°C and 5% CO_2).

For menigococcal growth, the following media are needed: chocolate round agar plates (Biomerieux # 43101, stored at 4˚C) for overnight culture; Mueller Hinton Broth (DIFCO#275730, Becton Dickson Cat. no. 0757, stored at room temperature) for liquid culture and 25% w/v glucose (Sigma Cat. no. G7528, or equivalent) in milliQ H_20 (filter-sterilized) To perform the assay the following materials are required:

N. meningitidis test strains (collected and stored at -80° C) in Mueller Hinton Broth plus 7% w/v Glycerol ; rabbit or mouse test sera (usually kept at 20/-80° for storage, stored at 4°C during the analysis and no longer than 1 month); positive controls (either serum samples or monoclonal antibodies); baby rabbit complement (CedarLane); Dulbecco's saline phosphate buffer pH 7.4 + 1% BSA (Bovine Serum Albumin,) + 0,1% glucose (filter-sterilized, and stored at +4°C); 96-well tissue culture U-bottom plates; Mueller Hinton agar square plates (stored at 4°C); .

Rabbit serum used as a source of complement, is stored at -80° C; once thawed, an aliquot is heat inactivated at 56°C for 30 min and used as a source of inactivated complement.

5.2.2 Assay protocol

N.meningitidis strains are plated onto chocolate agar plates and grown overnight at 37°C in a humidified chamber (5% CO_2). The day after, bacterial colonies are collected and inoculated into 7 ml of MHB containing 0.25% (v/v) glucose. Bacterial growth at 37°C 5% CO_2 is followed for 1 hour, starting from an OD_{600} of 0.05. The optimal OD_{600} to be reached is 0.23-0.24 and, it usually takes 1,5-2 hours to reach the desired OD, depending on the single strain.

The assay is performed on 96 well plates in a final volume of 50 µl/well (refer to fig. 7 for the assay layout). Dulbecco's PBS containing 1% (w/v) BSA and 0.1% (w/v) glucose is added to each well: 25 µl columns 1 to 11; 20 µl column 12 . Serial 2 fold dilutions of test sera are prepared dispensing 25 µl of pre-diluted sample (a 1:8 pre-dilution in assay buffer will result in a 1:16 dilution in the first well of assay plate) in column 1, passing 25 µl from column 1 through column10 and discarding the last 25 µl.

The SBA assay format has two kind of internal controls, that are used to calculate the average of colonies at time zero (T_0).

Complement Dependent Control (CDC), Column 11 (25µl of buffer; 12,5µl of active complement; 12,5 µl of bacteria; no serum), represents bacterial killing due to complement alone, in the absence of antibodies. Complement Independent Control (CIC), Column 12 (5 µl of pre-diluted serum; 12,5 µl of heat-inactivated complement and 20 µl of buffer), accounts for killing due to serum alone in the presence of heat inactivated complement. The 50% count of T_0 is the number of colonies considered to define the higher bactericidal serum dilution.

Following bacteria preparation steps must be conducted in the Biosafety Level 3 laboratories. Bacterial culture is diluted 1/10000 by serial
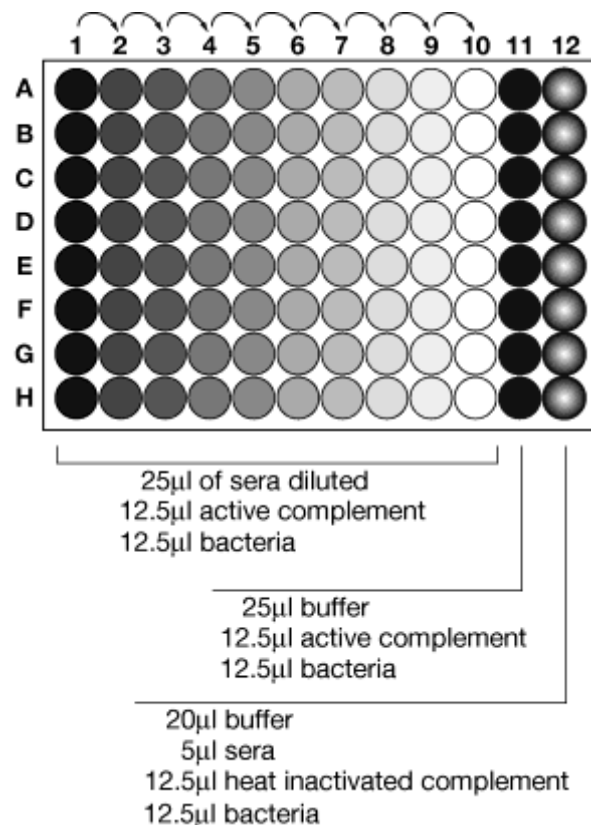

25µl of sera diluted
12.5µl active complement
12.5µl bacteria

25µl buffer
12.5µl active complement
12.5µl bacteria

20µl buffer
5µl sera
12.5µl heat inactivated complement
12.5µl bacteria

Figure 7. Plate layout

dilution and 12,5 µl of bacterial culture are added to each well. 12,5 µl/well of active complement (obtained by reconstitution of lyophilized baby rabbit complement in 1ml of ice cold water) is then added in columns 1 to 11, according to plate template (see fig. 7 on the previous page)., Bacteria are plated out at T_0 by pipetting up and down the well content 3 times and then placing 10 µl from 2-4 wells of controls column 11 (CDC) and 12 (CIC) to the top of a square MH agar plate. Tilting the plate allows bacterial inoculum to run down to the plate bottom in a straight line. This will cause streak lines to form, leading to single colonies growth, that will simplify colony counting. Subsequently test plate is closed by lid, parafilm sealed and incubated for 1h at 37°C 5% CO_2 under soft orbital shaking. At t=60 min, the contents of each well is mixed by pipetting up and down 3 times, and then 7 µl of each well are spotted on square MH agar plates as previously described for controls. After tilting, plate is incubated overnight at 37˚C , 5% CO_2.The day after, colony forming units (CFU) generated by single spots within each of the seeded plates are counted and recorded. All wells are plated in duplicate and duplicate counts must be recorded.

Bactericidal titer is defined as the reciprocal of serum dilution that gives a 50% CFU decrease after 60 min incubation in the reaction mixture, compared with the mean CFU number in the control reactions at t=0. T_0 average of both controls (column 11, CDC, and column 12, CIC), must be between 30 and 100 CFU. Killing by serum alone in presence of inactivated complement (CIC), should be minimal. In the case of meningococcal SBA, these values have to be less than 30% of T_0 for the experiment to be considered valid.

5.3 <u>Meningococcal Antigen Typing System (MATS)</u>

5.3.1 Equipment and softwares

To perform a MATS assay the following materials are required, all of them to be prepared following Grifols Diagnostic Solutions's procedures:

test and reference strains (collected and stored at -80°C); anti-fHbp, anti-NHBA-GNA1030 and anti-NadA coated 96 microwell plates (to be stored between 2°C and 8°C); anti-fHbp, anti-NHBA and anti-NadA biotinylated antibodies (to be stored between 2°C and 8°C); HRP conjugated streptavidin (to be stored between 2°C and 8°C); substrate buffer, (to be stored between 2°C and 8°C); properly diluted recombinant antigens (storage: PBS 1X); 5% Empigen BB lysis buffer detergent (Sigma Cat. 30326, to be stored at room temperature), ; 20X wash buffer (phosphate buffered saline, to be stored at room temperature); chocolate agar plates (Biomerieux cat. No. 43101); dehydrated Mueller-Hinton Broth (MHB) (Beckton Dickinson DIFCO cat. No. 275730); 95-97% sulfuric acid (H_2SO_4, Sigma-Aldrich Cat. No. 84720 or equivalent); sample buffer preparation (MHB+1/11 5% Empigen BB lysis buffer detergent), OPD tablets (Sigma P8287).

The lab equipment needed for MATS is the following: incubator for bacterial cell culture (set-point: 5% CO2, 37°C, 95% RH); laminar flow workbench (or safety cabinet); water bath; Ultrospec 10 Classic spectrophotometer (Amersham Biosciences distributed by GE Healthcare, Product No.80- 2116-30); TECAN Power Washer 384 or equivalent plate washer; dry incubator for ELISA plates (Set-point: 37°C), Molecular Devices Spectramax 340PC384 (Molecular Devices) or equivalent microplate reader with SoftMax Pro Data Acquisition and Analysis Software.

5.3.2 Assay protocol

MATS assay (see figure 8 on page 35) starts with overnight (16 hours) bacterial growth on chocolate agar plates (37°C, 95% of relative humidity, and 5% of CO_2). The day after, bacteria

are resuspended in MHB to OD_{600} 0.4 and lysed with 5% Empigen BB detergent (final volume of 1:11). Lysates are then inactivated at 45°C for 1 hour in a water bath.

Duplicate two-fold serial dilutions of bacterial lysates are incubated in three different ELISA microwell plates (100 μl/well), each coated with rabbit polyclonal antibodies against fHbp, NHBA, and NadA, respectively. Plates are incubated for 1 hour at 37°C and washed with PBS 1X/0.05% Tween20, after which detection of bound antigen is performed by addition of biotynilated rabbit IgG (100 μl/well), specific for each vaccine antigen, and 1 hour incubation at 37°C. Finally, plates are washed and incubated with streptavidin-horseradish peroxidase (100 μl/well) for 30 min at 37°C, followed by 20 min at room temperature with ortho-phenylene diamine substrate (100 μl/well). The reaction is stopped by adding 50 μl/well of 4 N sulfuric acid solution and OD 492nm is read by an ELISA reader (Molecular Devices Spectramax 340PC384 or equivalent manufactured by Molecular Devices with SoftMax Pro Data Acquisition and Analysis Software).

MATS readout of single meningococcal strains is expressed as Relative Potency (RP) for fHbp, NHBA, and NadA; RPs are calculated comparing serial dilution curves of tested strains with those of reference strains, whose RPs have been assigned the arbitrary value of 1 (or 100%), by a variance-weighted regression method. Reference strains are H44/76 for fHbp, NGH38 for NHBA, and 5/99 for NadA.

In order to determine RPs cut-off values able to predict strain susceptibility by hSBA, MATS RPs have been related to hSBA titers of 13-months-old children pooled sera, immunized with 4CMenB at 2, 4, 6, and 12 months of age. On this basis, the Positive Bactericidal Threshold (PBT) has been defined for each vaccine antigen component as the MATS RP point estimate above which the majority of strains are killed in hSBA. PBT values are 0.021 (2.1%) for fHbp, 0.294 (29.4%) for NHBA, and 0.009 (0.9%) for NadA respectively (Donnelly, et al, 2010).
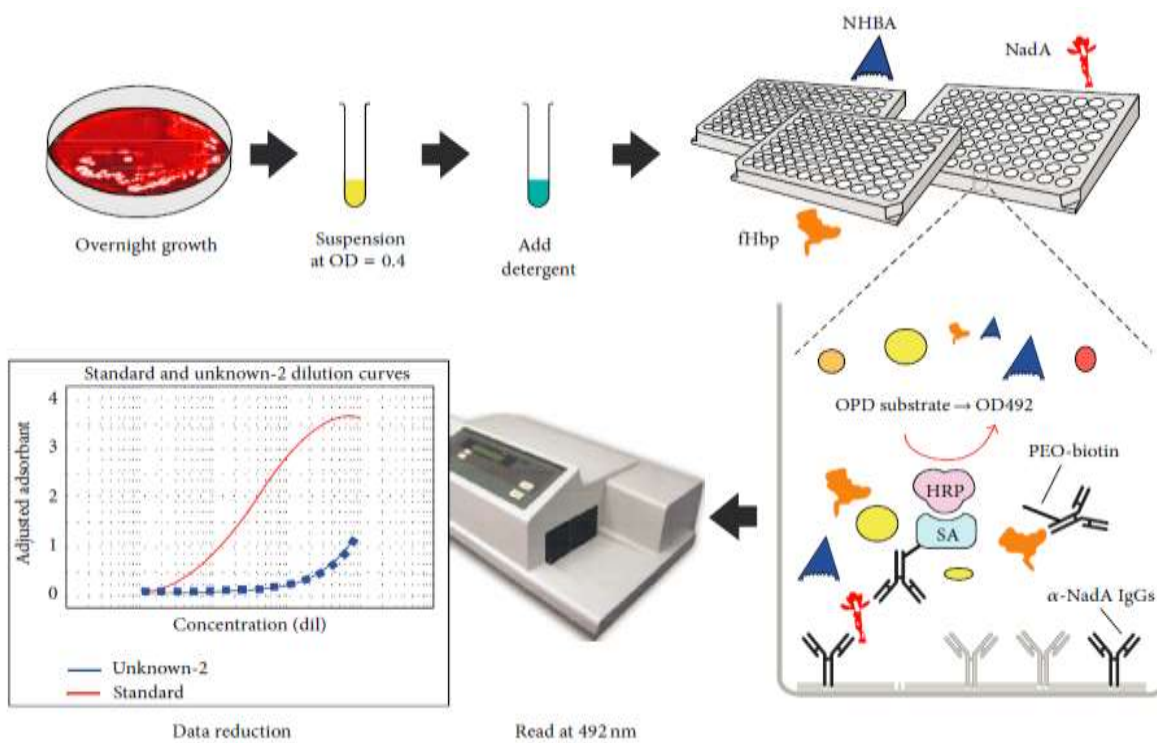
Figure 8. Schematic representation of MATS method

5.4   __Luminex assay__

Luminex Technology (Luminex Corp., Austin, TX) is based on microscopic magnetic polystyrene particles (microspheres, diameter 6.2 μm) exposing on the surface multiple carboxyl groups as sites for covalent ligand attachment, and internally labeled with two different fluorophores. Each microsphere is identified by a unique ratio of the two different dyes obtaining up to 100 different fluorescent profiles (figure 9).

As soon as a microsphere passes through the reader, the flow cell is interrogated by two different lasers. When beads are excited by a 635 nm laser, the internal fluorophores emit at a characteristic wavelength which uniquely identified the microsphere. Simultaneously, a 523 nm laser quantifies the amount of analyte bound to the microsphere by detecting the PE-labeled reporter molecule, usually conjugated to the detection antibody, resulting in a signal expressed as median fluorescence intensity (MFI).
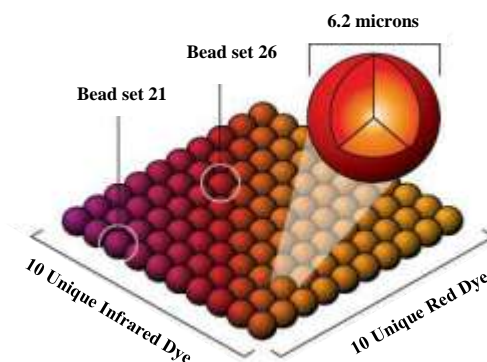

Figure 9. Principle of Luminex Technology: the unique ratio of red and infrared dye allows to obtain up to 100 different bead regions.

5.4.1   Equipment, reading instrument and software

A Luminex 200 instrument (Luminex Corporation) was used for plate reading. Median fluorescence intensity (MFI) was evaluated by using Bio-Plex manager 6.0 software (Bio-Rad, Hercules, CA).

The equipment required for Luminex analyses developed in this work is the following: MagPlex® COOH microspheres (one single region for each specific Ab or Ag to be coupled); MenB lysates to be tested and antibody/antigen to be coupled; assay/dilution buffer (PBS 1X); detection antibody specific for the target antigen (either unlabeled or biotin-labeled); R-Phycoerythrin AffiniPure F(ab')$_2$ Fragment Goat Anti-Rabbit IgG (H+L) (Jackson Immuno Research; Cat. N. 111-116-144); R-Phycoerythrin AffiniPure F(ab')$_2$ Fragment Goat Anti-Mouse

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IgG (H+L) (Jackson Immuno Research; Cat. N: 115-116-146); assay buffer (Mueller-Hinton Broth+5% Empigen BB detergent); storage buffer (also used as reading volume to reduce aggregation; PBS 1X, 0.5% BSA, 0.02% Tween20), beads dilution buffer (PBS 1X+ 0.02% Tween 20), Low Bind tubes (Eppendorf; Cat. N.022431081), streptavidin-R-phycoerythrin (SAPE, Moss SAPE-001G75, Life Technologies S-866 or equivalent). Sample and beads loading are performed in 96-well Multiscreen HTS filters Plates (Millipore, Bedford, MA).

For coupling methods the following reagents are needed: 1-Ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC, Pierce cat. N. 77149), Sulfo-NHS (Pierce cat N.24510 or 24520), activation buffer (0.1 M NaH_2PO_4 pH 6.2; Sigma Cat. N.S3139), coupling buffer (50 mM N-morpholinoethane sulfonate (MES) pH 5.0, Sigma M2933), distilled deionized water (ddH_2O).

Microspheres should be protected from prolonged exposure to light throughout all steps.

Washes are assessed by using an automatized magnetic washer (HydroSpeed 96i, Tecan, Männerdorf, Switzerland). During incubation steps, plates are placed on a titer plate shaker.

5.4.2 Coupling: equipment and manual/automated methods

There are approximately 100 million carboxyl groups on each MagPlex® COOH microspheres. Antibodies/antigens are coupled by two-step carbodiimide procedure during which microsphere carboxyl groups are first activated with EDC reagent in the presence of Sulfo-NHS to form a sulfo-NHS-ester intermediate. The reactive intermediate is then replaced by reaction with the primary amine of the target molecule (antibody, or peptide) to form a covalent amide bond (figure 10).
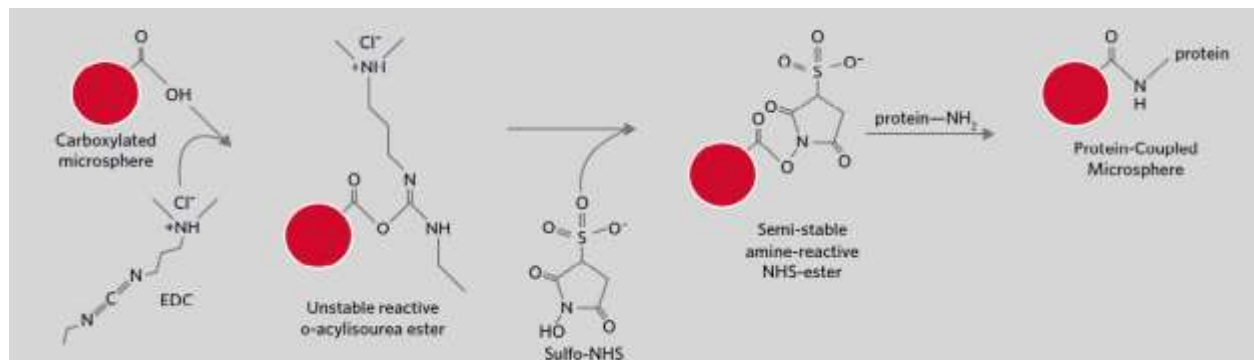

Figure 10. Schematic representation of coupling reaction

Manual coupling

For sandwich assay, 5 μg of each purified mouse IgG anti-fHbp, anti-NadA, anti-NHBA-GNA1030 and anti-OMVs were coupled to 4 different spectrally unique MagPlex® microspheres (Luminex Corporation, Austin, TX). For the indirect assay, beads were coupled with 20 ug of each recombinant antigen (fHbp, NadA, NHBA, OMVs). .

Beads activation: after resuspension, 1.5×10^6 of stock uncoupled microspheres are transferred to a 1.5 ml LowBind eppendorf tube, that is placed for 30 to 60 seconds into a magnetic separator to allow beads separation and supernatant removal. The tube is then removed from the magnetic separator and the microspheres are resuspended in 100 μl of ddH$_2$O by vortex and sonication for approximately 20 seconds. After a second separation and supernatant removal, taking care not to disturb the microspheres, beads are resuspended in 80 μl of 100 mM Monobasic Sodium Phosphate, pH 6.2 by vortex and sonication for approximately 20 seconds. Without washing, 10 μl of 50 mg/ml Sulfo-NHS (diluted in dH$_2$0) are added to the microspheres and gently mixed by vortex, followed by addition of 10 μl of 50 mg/ml EDC (diluted in dH$_2$0) and mixing by vortex. Beads are then incubated for 20 minutes at room temperature with gentle mixing by vortex at 10 minute intervals and finally, positioned into magnetic separator for 30 to 60 seconds allowing separation. After the removal of the supernatant, microspheres are resuspended in 250 μl of 50 mM MES, pH 5.0 by vortex and sonication for approximately 20 seconds. After a second MES washing and separation step the activated and washed microspheres are resuspended in 100 μl of 50 mM MES, pH 5.0 by vortex and sonication for approximately 20 seconds. At this stage, the optimal amount of Ab/Ag is added to the resuspended microspheres and total volume is brought to 500 μl with 50 mM MES, pH 5.0 and the coupling reaction is incubated for 2 hours with mixing (by rotation) at room temperature. After removal of supernatant into magnetic separator coupled microspheres are resuspended in 500 μl of PBS-TBN by vortex and sonication for approximately 20 seconds. Two washes with 1 ml of PBS-TBN are performed. Removing the tube from the magnetic separator the coupled and washed microspheres are resuspended in 250-1000 μl of storage buffer (PBS-TBN-BSA) in order to saturate with BSA activated carboxylic group eventually not conjugate to beads surfaces.

Count is assessed by hemacytometer (total microspheres = count (1 corner of 4 x 4 section) x (1 x 10^4) x (dilution factor) x (resuspension volume in ml)). Coupled microspheres should be stored at 2-8°C in the dark.

Automated coupling

In order to optimize and standardize the coupling reaction an automated coupling method was used. The method was developed, and subsequently applied according to the steps described for manual coupling, by using an automated liquid handling workstation (Hamilton –Microlab STAR IVD).

5.4.3   Coupling confirmation

Proteins and antibodies are typically coupled in random orientation as they have many lysine groups available for coupling. Functional testing is also critical during assay development. Once antibodies have been coupled to xMAP® beads, assessment of coupling efficiency before proceeding to assay development is strongly recommended. For this purpose, coupled and opportunely diluted microspheres react with 2-fold dilutions of PE-labeled anti-species secondary antibody (starting from 1:200; phycobiliprotein final concentration: 2.5 µg/ml - determined by absorption = 82.0 at 566 nm for a 1% solution for only those R-PE molecules to which at least one molecule of active antibody is bound) for 30 minutes at room temperature/dark room on a plate shaker. After three washes with PBS 1X, reading buffer is added (PBS 1X-Tween20 0.02%-BSA 0.5%) and samples flow through the fluidics of a Luminex® instrument. Coupling is confirmed if the signal obtained is linear respect to the dilution step of the detection antibody.

If beads are coupled with an antigen, coupling confirmation is obtained by an indirect assay, where an Ab raised against the antigen target reacts with coupled beads for 30 minutes on a plate shaker at room temperature/dark room. After 3 washes with PBS 1X, a PE-labeled anti-species antibody reacts with the immune-complex per 15 minutes on a plate shaker at room temperature/dark room. After the plate washing, the reading volume is added and samples MFI signals are recorded by the instrument.

5.4.4 Inhibition assay

An inhibition assay was developed in order to add information about assay specificity. MenB lysates reference strains were incubated with detection rabbit Abs mix, both at fixed concentration, followed by addition of 2-fold serial dilutions of capture mouse Ab.

Not inhibited signal obtained from Ag-detection rabbit was quantified by the 4-plex assay using a PE-labeled anti-rabbit secondary Ab. Assay specificity was assessed by complete signal inhibition respect to control sample (no inhibitor).

5.4.5 Multiplex sandwich assay (4-plex)

A capture sandwich 4-plex immunoassay was developed to predict the coverage of a multi-component based vaccine. Sandwich assay is used to detect an antigen (target) with the use of a capture antibody attached to the surface of a microsphere and a detection antibody bound to a secondary anti-species Ab that incorporates a fluorescent label (figure 11).
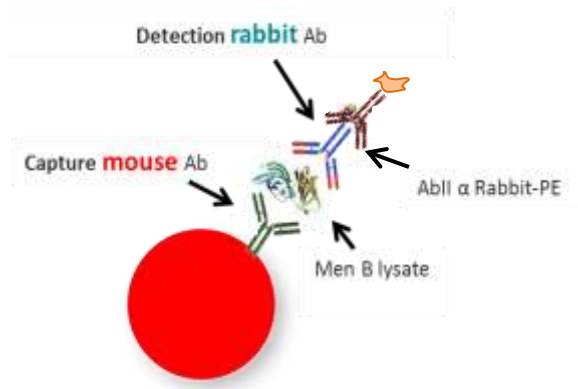

Figure 11. Schematic representation of sandwich assay

After the selection of the appropriate mouse antibody-coupled microsphere sets, beads are resuspended by vortex and sonicated for approximately 20 seconds. This step should be performed under a biosafety level 2 safety cabinet because the samples are bacterial lysates. 100 μl/well of samples are dispensed in duplicate and 2-fold serial diluted in assay buffer (50 μl/well). Four different coupled-bead sets (pAb anti-fHbp, anti-NadA, anti-NHBA-GNA1030 and anti-OMVs) mixed at 1:4 dilution, are added to the pre-diluted samples (10 μl/well).

Positive control is represented by a mix of recombinant of fHbp, NadA, NHBA and OMVs (final Ags concentrations: 0.25 μg/ml) and is added to the plate in duplicate as a single data-point. Standards curve is represented by a 2-fold serial diluted curve of each reference strain (H44/76 for fHbp, 5/99 for NadA, NGH38 for NHBA and NZ98/254 for OMVs). Samples and beads are incubated for one hour (see table 1 on the next page for template layout). Plates are then washed

and 50 µl of detection rabbit antibody mix are added to wells. The mix is composed by pAbs anti-fHbp, anti-NadA, anti-NHBA and anti-OMVs with the final concentration of 3 µg/ml, 2.7 µg/ml, 18 µg/ml and 13 µg/ml, respectively. After an incubation of one hour and plate washing, 50 µl/well of phycoerythrin-conjugated anti-rabbit secondary antibody (starting from 1:200; phycobiliprotein final concentration: 2.5 µg/ml - determined by absorption = 82.0 at 566 nm for a 1% solution for only those R-PE molecules to which at least one molecule of active antibody is bound) is added and incubated for 15 min. The resulting immune-complexes are washed and resuspended in 1X PBS to be analyzed on the Luminex LX-200 system.

All reaction steps were carried out at room temperature, in the dark and under agitation motion using a horizontal shaker; after each incubation step, plates were washed in 1X PBS by an automatized magnetic washer HydroSpeed 96i (Tecan, Männerdorf, Switzerland).

piastra 1	1	2	3	4	5	6	7	8	9	10	11	12
A	Ref 1	Ref 1	Ref 2	Ref 2	Ref 3	Ref 3	Ref 4	Ref 4	Ukn 1	Ukn 1	Ukn 2	Ukn 2
B	Ref 1	Ref 1	Ref 2	Ref 2	Ref 3	Ref 3	Ref 4	Ref 4	Ukn 1	Ukn 1	Ukn 2	Ukn 2
C	Ref 1	Ref 1	Ref 2	Ref 2	Ref 3	Ref 3	Ref 4	Ref 4	Ukn 1	Ukn 1	Ukn 2	Ukn 2
D	Ref 1	Ref 1	Ref 2	Ref 2	Ref 3	Ref 3	Ref 4	Ref 4	Ukn 1	Ukn 1	Ukn 2	Ukn 2
E	Ref 1	Ref 1	Ref 2	Ref 2	Ref 3	Ref 3	Ref 4	Ref 4	Ukn 1	Ukn 1	Ukn 2	Ukn 2
F	Ref 1	Ref 1	Ref 2	Ref 2	Ref 3	Ref 3	Ref 4	Ref 4	Ukn 1	Ukn 1	Ukn 2	Ukn 2
G	Ref 1	Ref 1	Ref 2	Ref 2	Ref 3	Ref 3	Ref 4	Ref 4	Ukn 1	Ukn 1	Ukn 2	Ukn 2
H	Ref 1	Ref 1	Ref 2	Ref 2	Ref 3	Ref 3	Ref 4	Ref 4	B	B	C+	C+

Table 1. Template layout of 4-plex assay

5.5   Recombinant vaccine antigens

Antigens coupled to the Luminex microspheres (Luminex Corp.) and used as analytes for preliminary Luminex analyses were obtained from private sources (Gsk Vaccine Biochemistry Unit).

5.6   Rabbit and mouse sera

To perform each immunoassay described in this work, sera positive for each Bexsero™ antigen were obtained by rabbit/mouse immunization with each antigen separately. Only sera with optimal Ab-titers were selected and used for the IgG purification.

Animal treatments were performed in compliance with Italian laws and approved by the institutional rewiew board (Animal Ethical Committee) of Gsk Vaccine, Siena, Italy.

For mouse sera, female CD1 mice (6-8 weeks aged) were immunized three times with intraperitoneal injection with 20 µg of fHbp, NadA and NHBA-GNA1030 and 10 µg of OMVs adjuvanted with 600 µg of Alum. Second and third immunizations were performed 21 days and 35 days after the first dose, respectively. Pre-immune, post 2 (14 days after second dose) and post 3 (14 days after third dose) bleeding were collected for each mouse. Individual and group – pooled sera were used for total and functional Abs detection.

For rabbit sera, female New Zealand rabbit (weight:2-2.5 kg) were immunized four times with subcutaneous injection with 50 µg of fHbp, NadA and NHBA-GNA1030 and 25 µg of OMVs adjuvanted with 1500 µg of Alum. Second, third and fourth immunizations were performed 21 days, 35 days and 49 days after the first dose, respectively. Pre-immune, post 3 (14 days after third dose), post 4 (16 days after fourth dose) bleeding were collected for each rabbit. Individual and group –pooled sera were used for total and functional Abs detection (see the tables 2 and 3 on the next page for immunization schemes nomenclature).

RABBIT Abs	anti fHbp rab-pool
	anti NadA rab-pool
	anti NHBA rab-pool
	anti OMVs rab-pool

Table 2. Rabbit immunization schemes nomenclature

MOUSE Abs	anti fHbp mouse-pool 1
	anti fHbp mouse-pool 2
	anti NadA mouse-pool 1
	anti NadA mouse-pool 2
	anti GNA1030-NHBA mouse-pool 1
	anti GNA1030-NHBA mouse-pool 2
	anti OMVs mouse-pool

Table 3. Mouse immunization schemes nomenclature

5.7 Selection of polyclonal detection and capture antibodies (sandwich immunoassay)

The selection of rabbit (detection) and mouse (capture) antibodies was performed based on ELISA results and by indirect Luminex assay as reported in section 6.1 ('Results' chapter). Both rabbit and mouse sera selected show bactericidal activity (see in paragraph 6.2 – 'Results' chapter). The total IgG fraction of iper-immune mouse and rabbit sera were purified by using Protein G HP SpinTrap and Protein A HP SpinTrap, respectively. Rabbit and mouse specific IgG were also obtained from private sources (Gsk Vaccine Biochemistry Unit) and, in this case, IgG were titred by indirect Luminex assay.

5.8   Serogroup B strains panel selection

To explore the relationship between Luminex MFI obtained with the qualified 4-plex assay, bactericidal titers and MATS ELISA relative potencies for each individual antigen, 28 serogroup B strains were selected (table 4). In particular, these 28 strains are included in a 57-strains panel selected in 2010 by Donnelly et al. to evaluate the performance of MATS (in turns, a subset of 124-strains panel tested on hSBA by using pooled sera from 141 infants who had received three immunizations or three immunizations plus one booster of 4CMenB).

Strain Isolate	Year	Country	Serogroup	Serotype	ST	PorA VR1	PorA VR2	fHbp variant	fHbp sub-variant	NHBA peptide	NadA variant	NadA sub-variant
961-5945	1996	AUS	B	2b	153	21	16	2	16	20	2	3
5/99	1999	NOR	B	2b	1349	5	2	2	23	20	2	3
M01-0240364	2001	GBR	B	2a	11	5	2	3	31	28	2	5
H44/76	1976	NOR	B	15	32	7	16	1	1	3	NA	NA
M01239	1994	USA	B	14	437	23	14	3	28	1	NA	NA
NZ98/254	1998	NZL	B	4	42	7-2	4	1	14	2	NA	NA
NGH38	1988	NOR	B	NT	36		3	2	24	2	NA	NA
M03369	1997	USA	B	10	1576	19	15	3	31	16	NA	NA
NM117	1998	GBR	B	21	1195		9	1	15	21	NA	NA
M01-0240988	2001	GBR	B	1	213	22	14	3	30	10	5	12
M10837	2003	USA	B	NA	409	18-1	34-2	2	19	2	NA	NA
M10994	2003	USA	B	NA	44	21	16	2	19	29	NA	NA
M11003	2003	USA	B	NA	5097	7-2	4	1	4	2	NA	NA
M11048	2003	USA	B	NA	60	5-1	2-2	1	13	24	NA	NA
M12425	2004	USA	B	NA	44	7-1	1	1	83	29	NA	NA
M12886	2004	USA	B	NA	6147	22-15	28-2	1	4	10	NA	NA
M12898	2004	USA	B	NA	457	5-1	2-2	2	16	143	NA	NA
M14459	2005	USA	B	NA	2048	22	9	1	180	19	NA	NA
M14882	2006	USA	B	NA	44	7-1	1	2	19	29	NA	NA
M15564	2006	USA	B	NA	32	7	16	1	1	5	1	NA
M16019	2007	USA	B	NA	32	7	16	1	1	5	1	NA
M16405	2007	USA	B	NA	136	7-2	13-1	2	218	10	NA	NA
M16686	2007	USA	B	NA	2487	7-1	1	1	13	29	NA	NA
M18483	2008	USA	B	NA	2808	7-2	4	1	12	222	NA	NA
M01-240500	2001	GBR	B	NT	269	7	4	1	15	21	NA	NA
M01-240660	2001	GBR	B	NT	1049	19	15	1	15	21	NA	NA
M01-240200	2001	GBR	B	NT	275	22	9	1	13	17	NA	NA

Table 4. 28-sergroup B panel selected to be tested on new 4-plex Luminex assay

6. RESULTS

6.1 Immunochemical characterization of animal sera used for capture/detection steps

In order to generate antibody tools for Luminex assay, groups of rabbits (n= 3-15 /group) and mice (n= 8/group) were immunized with recombinant NadA, fHbp, NHBA and OMVs single antigens adsorbed to Alum Hydroxide as described in section 5.6 ('Materials and Methods' chapter). Two weeks after the third injection, antisera were collected and individually analyzed by ELISA or Luminex indirect assay to evaluate the level of antibodies induced by each Bexsero^{TM} vaccine component.

6.1.1 Rabbit Sera

All individual sera from each immunization group were able to elicit high antibody titers vs recombinant proteins and Outer Membrane Vesicles as well (panel 1).

Upon ELISA titer evaluation antisera were also tested by SBA to assess presence of functional antibodies.
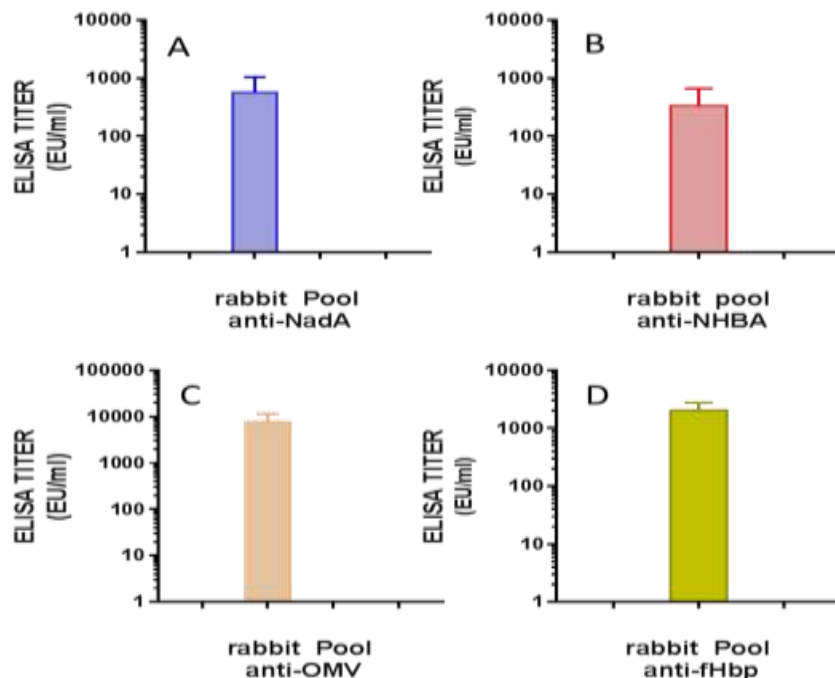
Panel 1. Total IgG titers (GMT ± 95% CI) of rabbit sera raised against Bexsero^{TM} antigen components, as measured by ELISA on immobilized purified recombinant proteins NadA (A), NHBA (B) , OMV (C) and fHbp (D), respectively

6.1.2 Mouse sera

Mice antisera were collected two weeks after the third injection of individual Bexsero[TM] components, pooled and tested by Luminex for immunogenicity assessment. Anti OMVs mouse-pool and anti fHbp mouse-pool 1 were titrated by 2-fold serial dilutions (starting point 1:5000) by using the indirect monoplex assay (beads only coupled with each corresponding vaccine Ag). Immuno-complexes were detected by a PE-labeled secondary anti-mouse Ab (starting from 1:200 dilution, panels 2 and 3 - see on the next page).

The same indirect assay was used for titration of purified IgG from anti NBHA-GNA1030 mouse pool 1 and 2, anti fHbp mouse pool 1, anti NadA mouse pool 1 and 2, respectively.
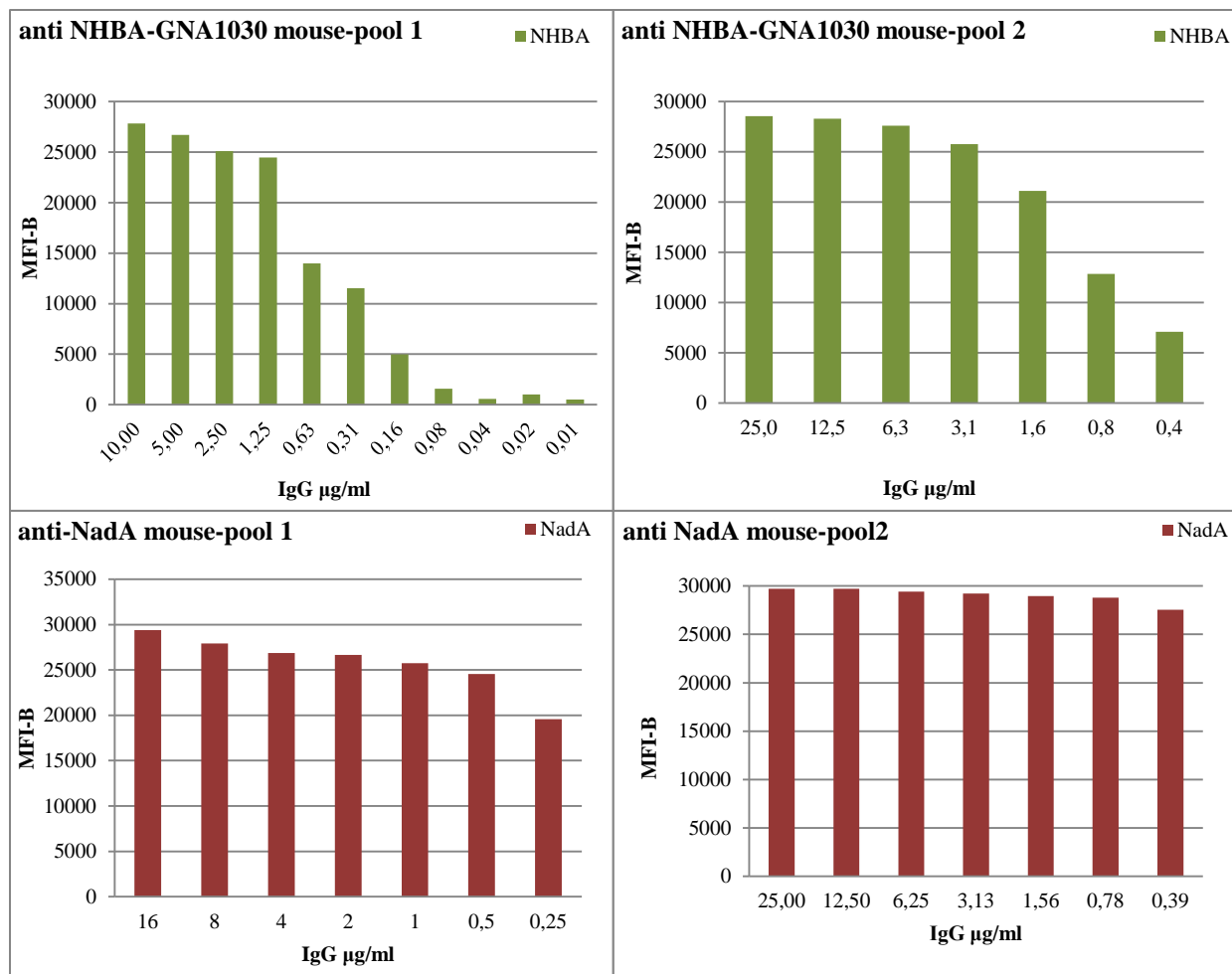
Panel 2. **Titration of mouse purified IgG by Luminex indirect assay.** IgG anti NBHA-GNA1030 mouse pool1 and pool 2 titration started from 10 and 25 µg/ml of each purified Abs, respectively. Anti NadA mouse pool 1 and pool 2 were titrated starting from 16 and 25 µg/ml, respectively
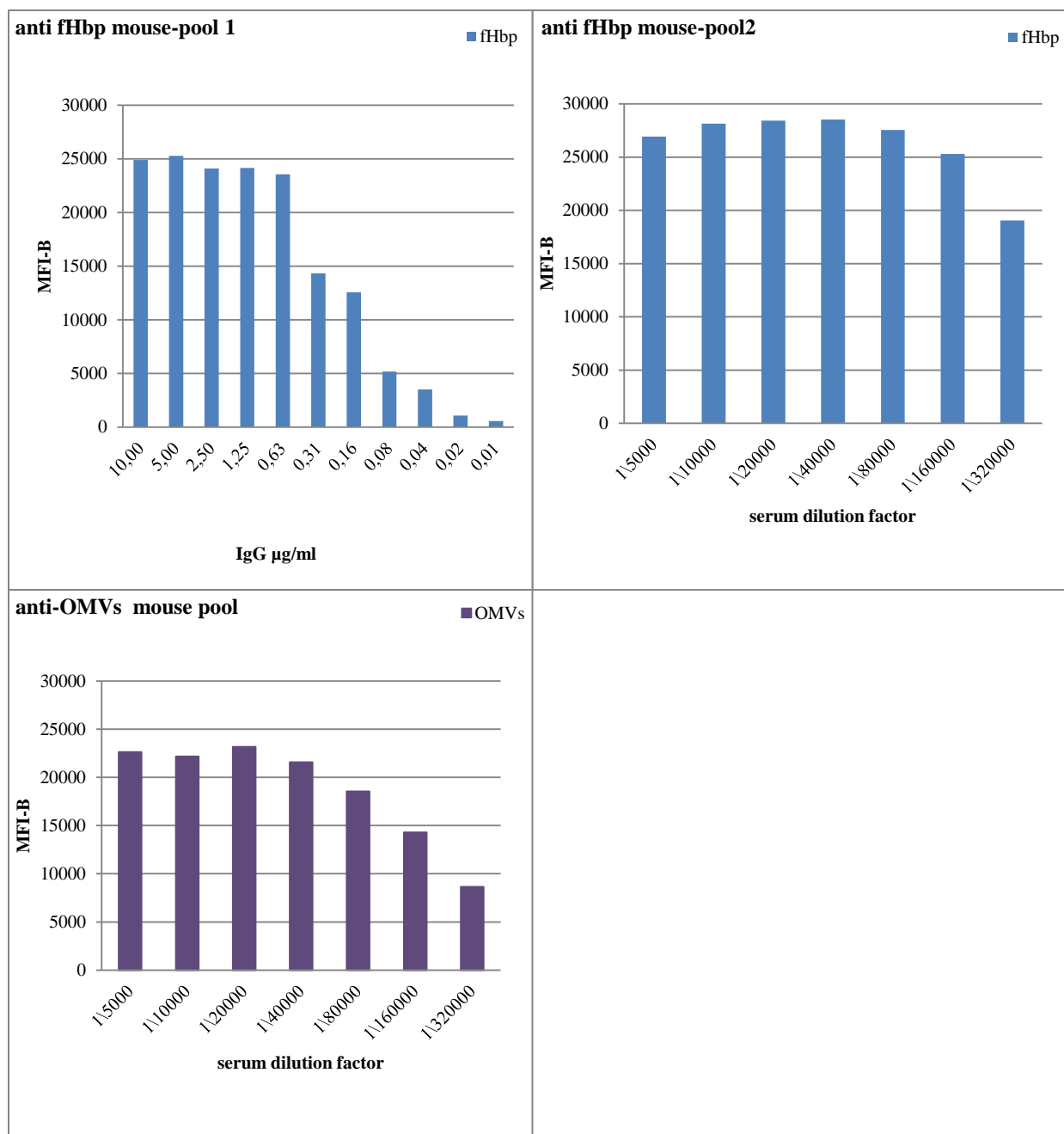
Panel 3. Titration of mouse purified IgG by Luminex indirect assay. anti fHbp mouse-pool 1 IgG were titrated starting from 10 µg/ml. Titration started from a 1:5000 dilution for anti fHbp mouse-pool 2 and anti-OMVs mouse-pool sera

6.2 Bactericidal activity assessment of selected rabbit and mouse sera

The serum bactericidal assay (SBA) was used for antibody functionality evaluation.

SBA measures the amount of serum antibodies required for bacterial killing by the classical pathway of complement activation. Since SBA titers are worldwide accepted as correlate of protection against *N. meningitidis,* they are the "gold standard" for evaluation of functional antibodies and, therefore, predominantly used during meningococcal vaccines development (Feavers and Walker, 2010).

The preferred complement source for SBA is human serum. Alternatively, serum from different species (rabbit, rat) free of cross-reactive bactericidal antibodies can be used, since interfering endogenous antibodies to meningococcal antigens can be present in human sera due to a high frequency of meningococcal carriage among human population. To ensure an adequate supply of complement commercially available sources are recommended (Giuliani, 2006) and, therefore, commercial rabbit complement was used in SBA for screening of antisera.

SBA titers are defined as the reciprocal of the highest serum dilution at which 50% of bacterial killing is observed.

6.2.1 SBA Analysis of rabbit sera

Individual rabbit antisera raised against OMVs, NHBA NadA and fHbp were tested for the presence of bactericidal activity against the individual homologous *N. meningiditis* strains: NZ98/254 for OMVs, M4407 for NHBA, 5/99 for NadA and H44/76 for fHbp. As showed in figure 12 (see on the next page), all antisera tested showed protective antibody titers vs each homologous strain.

Since the individual SBA titers measured were comparable within each immunization group, samples were pooled and rabbit IgG purified as described in section 5.7 ('Materials and Methods' chapter).

Purified antibodies were used as detection reagents for the Luminex assay.
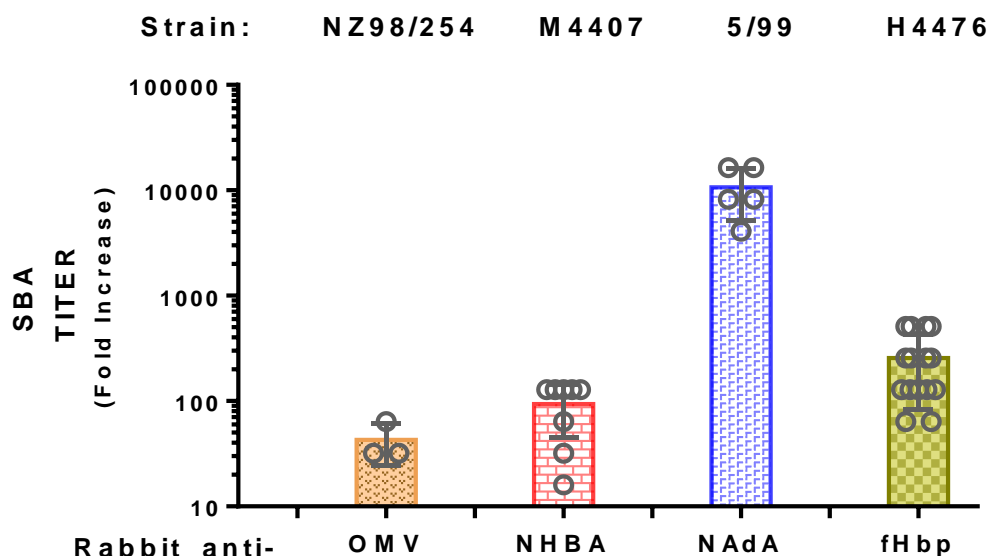
Figure 12. rSBA titers vs antigen-homologous *N. meningiditis* strains. Individual titers are reported (dots) with the mean and SD. Titers are expressed as Fold Increase of post 3rd dose serum vs pre-immune one

6.2.2 SBA Analysis of mouse sera

Pooled murine antisera obtained from intraperitoneal immunization with OMVs, NHBA NadA and fHbp (as described in Section 5.6, 'Materials and Methods' chapter) were tested for the presence of bactericidal activity against the individual homologous *N. meningiditis* strains: NZ98/254 for OMVs, M4407 for NHBA, 5/99 for NadA and H44/76 for fHbp. SBA titers of pooled mouse sera are reported in table 5 (see on the next page).

For fHbp, NadA and NHBA two mouse pools were tested by SBA, and their titers were very high and comparable for the same antigen. A high SBA titer was also observed for the sera anti OMV mouse pool tested on a NZ98/254 homologous strain.

IgG specific for each Bexsero[TM] antigen component were purified from mouse pooled sera as described in section 5.7 ('Materials and Methods' chapter) and used as capture reagents for Luminex assay.

POOLED MOUSE SERA	SERUM BACTERICIDAL TITERS AGAINST			
Specificity	**H44/76**	**M4407**	**5/99**	**NZ98/254**
Anti-fHbp pool 1	32768			
Anti-fHbp pool 2	65534			
Anti-NadA pool 1			8192	
Anti-NadA pool 2			16384	
Anti –NHBA pool 1		16384		
Anti-NHBA pool 2		65536		
Anti-OMVs pool				65536

Table 5. rSBA titers of pooled mouse sera vs homologous *N.meningiditis* strains

6.3   Single-plex assay: design and optimization

6.3.1 Coupling

5µg of each vaccine Ag-specific mouse pAb (NEW format) and anti fHbp rab-pool (MATS format) was coupled to the carboxyl groups of 2.5×10^6 MagPlex microspheres (Luminex Corporation, Austin, TX) following manufacturer's instructions. Each Ab was coupled to a microsphere set, identifiable through its unique spectral signature.

Once antibodies have been coupled to the beads, coupling confirmation was assessed in order to achieve the best sandwich assay sensitivity and dynamic range. Antibody coupling is confirmed by testing the coupled microspheres with serial dilutions of a phycoerythrin (PE)-labeled anti-species IgG antibody.

A dose-response increase in terms of MFI should be observed over increasing concentration of anti-species Ab. In general, an antibody coupling should yield at least 10,000 MFI at saturation for optimal use in immunoassays. Results are reported in the following panels 4 and 5 (see on the next page).
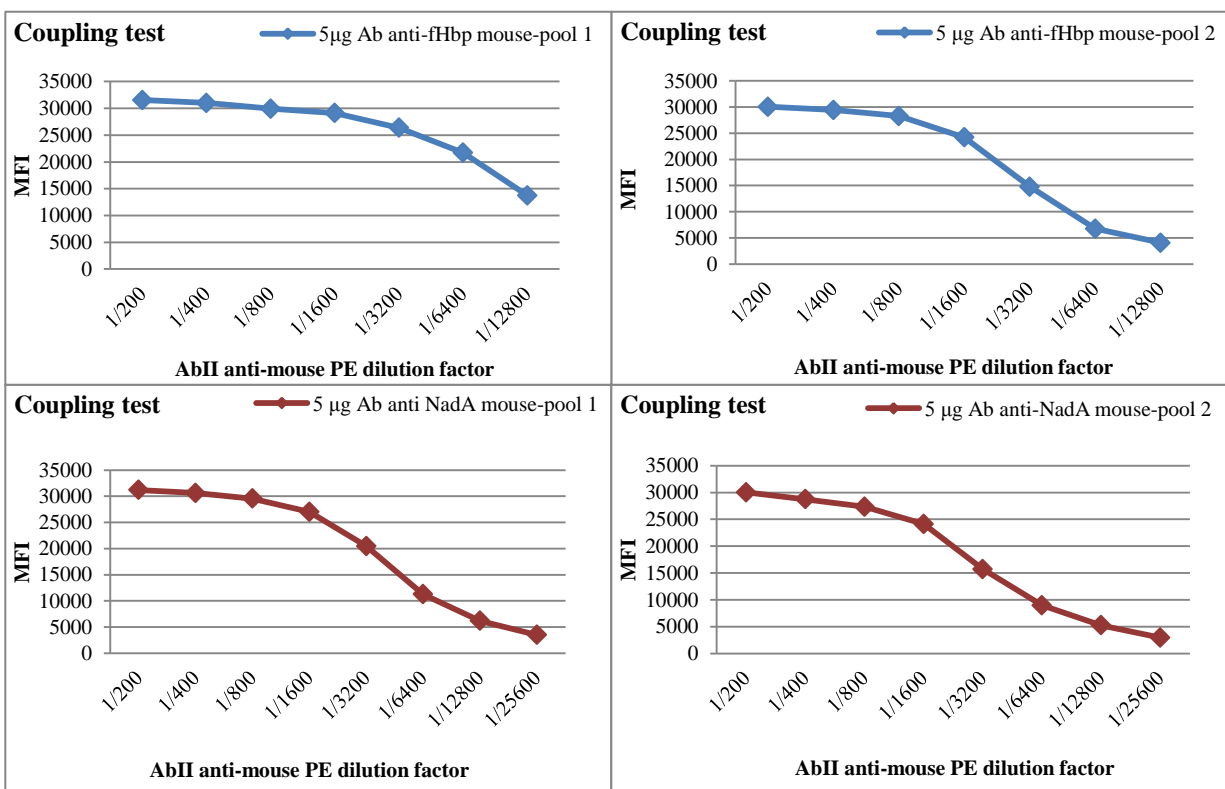
Panel 4. Coupling efficiency assessment for beads coupled with anti fHbp mouse pool 1 and 2 and anti NadA mouse pool 1 and 2 pAbs
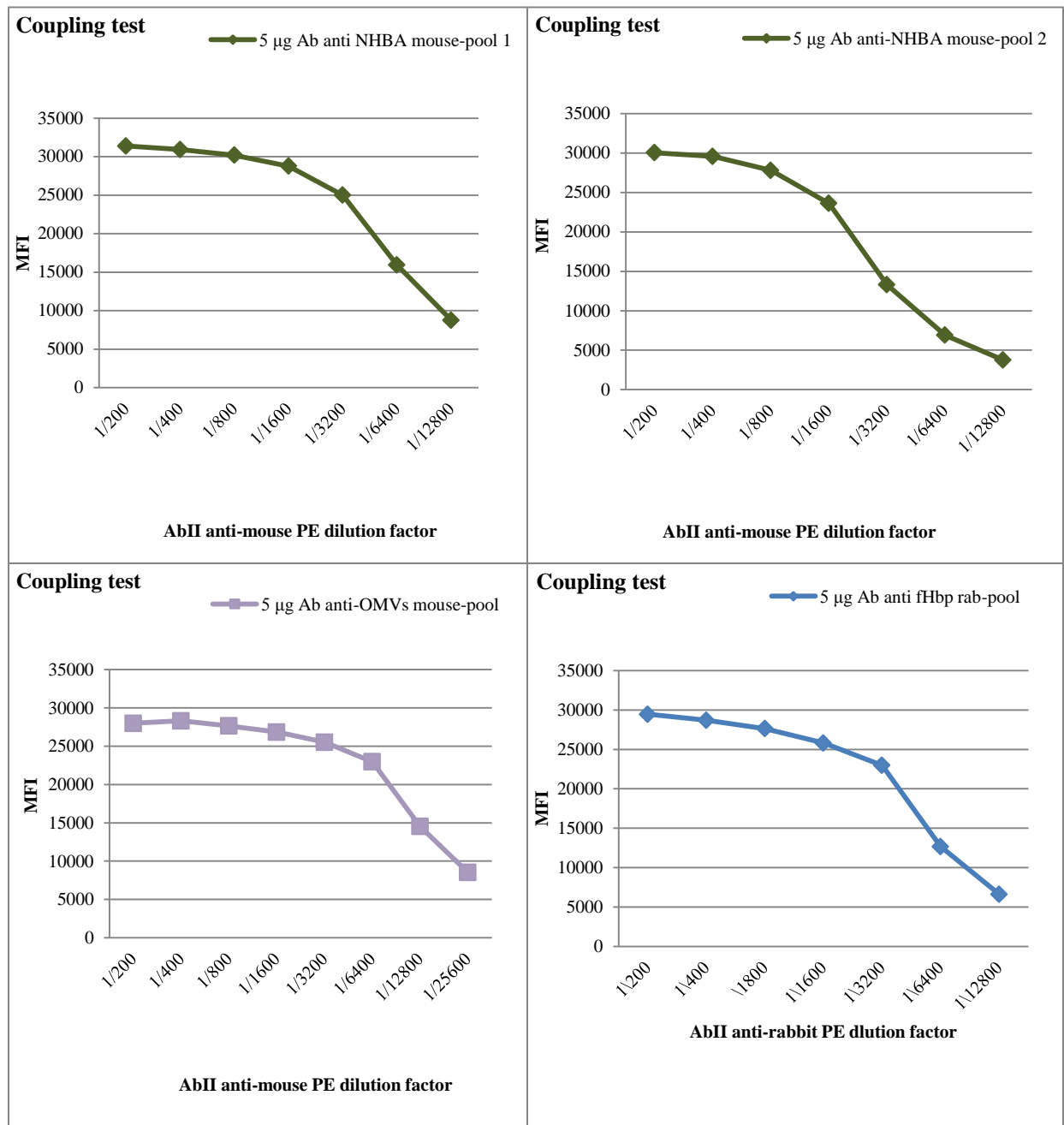
Panel 5. Coupling efficiency assessment for beads coupled with anti NHBA mouse pool 1 and 2, anti OMVs mouse pool and anti fHbp rab-pool pAbs

6.3.2 Selection of sandwich assay format: comparison between Luminex MATS-like format and NEW format for rfHbp antigen

Following Luminex's guidelines for multiplex assay development a Luminex single-plex sandwich immunoassay was initially developed as a first step, in order to identify the optimal capture and detection antibodies concentration to obtain a specific and linear signal with the best sensitivity and dynamic range. Each bead set was prepared following the procedure reported in the paragraphs 5.4.2 and 5.4.2 ('Materials and Methods' chapter).

The first format tested on Luminex platform was the MATS-like (see fig. 13 on the next page, reference assay), characterized by the usage of same rabbit polyclonal antibody specific for the vaccine antigens both as capture and detection reagent; detection antibody is biotin-labeled and MFI signal is revealed by extravidin-PE.

Sensitivity was the first parameter evaluated for single-plex. For this purpose, recombinant fHbp variant 1.1 (vaccine antigen variant) was used as analyte. Despite the high Ab concentration used (15 μg/ml of biotinylated anti fHbp rab-pool and starting from 1000 ng/ml of rfHbp 2-fold diluted - data not shown), MATS-like format achieved only half of the instrumental working range, and linearity was not as expected (five linear data-points: R^2=0.71 - data not shown). For this reason a different format, named NEW, was tested.

NEW format microspheres are coupled with mouse polyclonal antibody (pAb) raised against rfHbp 1.1 (immunization schemes: anti-fHbp mouse-pool1 and pool2), while detection relies on the same anti-fHbp rab-pool pAb used for MATS-like format (see figure 14 on the following page). In this case, biotinylation of detection antibody was no necessary and MFI signal was detected by a PE-labeled anti-rabbit secondary Ab, a feature that may reduce the variability due to random biotinylation of detection Ab.

Graph 1 (see on the next page) shows that NEW format achieved instrumental working range, obtaining optimal linear MFI signal (five linear data-points: R^2=0.97), with lower detection antibody and recombinant antigen concentrations (1.5 μg/ml of not biotinylated anti-fHbp rab-pool and starting from 15.6 ng/ml, respectively). This experiment demonstrates that changing species of capture antibody is possible to enhance the sensitivity of the assay, probably due to reduced competition between capture and detection Abs. In fact, when the same antibody is used as capture and detection, competition is generally increased in three-dimensional Luminex liquid kinetics assay compared to a traditional planar ELISA assay.
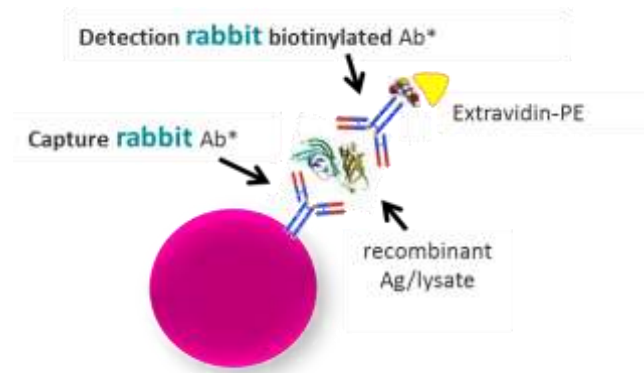
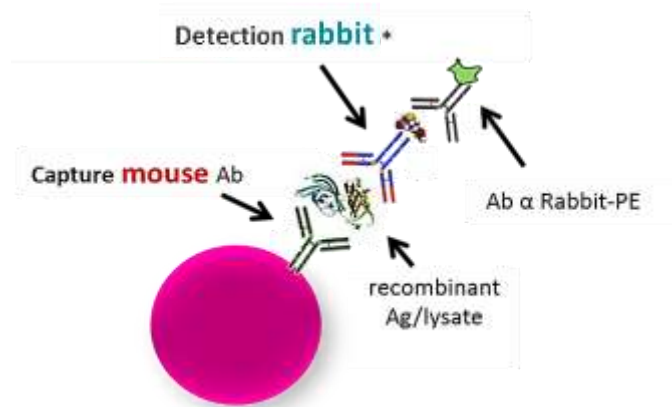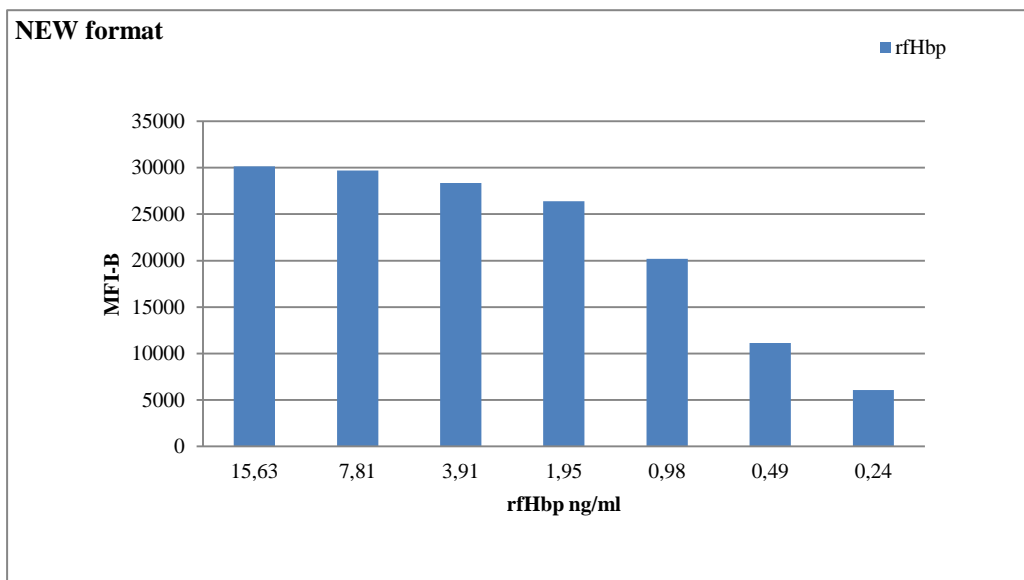**Figure 13.** MATS-like format for Luminex assay

**Figure 14.** New format for Luminex assay

Graph 1. New format for Luminex assay

6.3.3 Further investigation on selected formats: sensitivity and specificity assay assessment on fHbp sub-variants

Assay specificity was tested for both formats, in order to define which one was the best to be selected. Recombinant fHbp variant 1.4 and 1.15 were used as analytes.

The aim was to understand if both formats were able to discriminate between two fHbp sub-variants phylogenetically distant from the vaccine homologous one (for which capture/detection pAbs are specific).

Also in this case, MATS-like format needed higher detection Ab concentration (15 μg/ml, graph 2) to obtain specific and linear MFI signal compared to NEW format (we used 5 μg/ml in order to avoid not to detect the two sub-variants 1.x, graph 3). In both formats rfHbp variant 1.1 was better recognized than 1.4 and 1.15 (1.1>1.4>1.15) on Luminex single-plex assay, according to phylogenetic sequence distance and MATS data (Domnich et al. 2015), thus confirming preliminary specificity of the two formats.
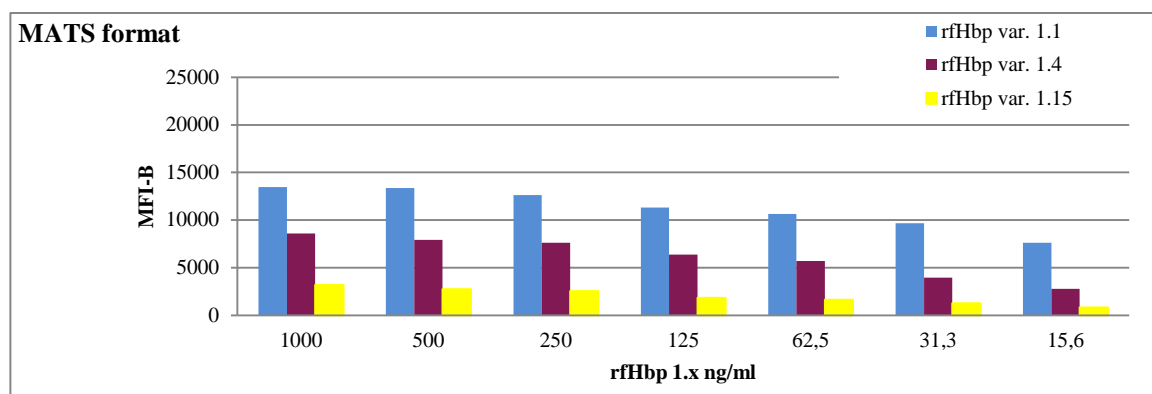
Graph 2. MATS format on rfHbp 1.x sub-variants
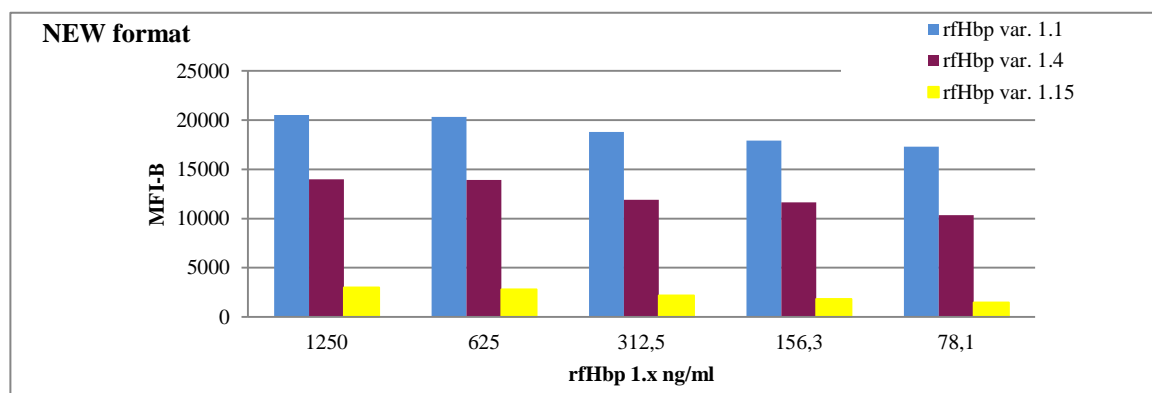
Graph 3. New format on rfHbp 1.x sub-variants

6.3.4 Matrix interference study on binding Ag-Ab

The next goal was to determine the matrix effect on binding between recombinant Ags and Abs on Luminex assay and instrumental fluidics. The sample to be used for the multiplex sandwich assay is a bacterial lysate, whose matrix is represented by Mueller Hinton broth + 5% Empigen Lysis Detergent. MATS format was tested using a detection anti-fHbp Ab concentration of 15 µg/ml. The two matrices tested were PBS 1X, usually used as matrix for Luminex assay, and MHB+LD (MATS assay matrix). As reported in the graph 4 on the next page, there is no significant different between the two matrices. Each experiment showed in the next pages was performed by using Mueller Hinton + Lysis Detergent as matrix.
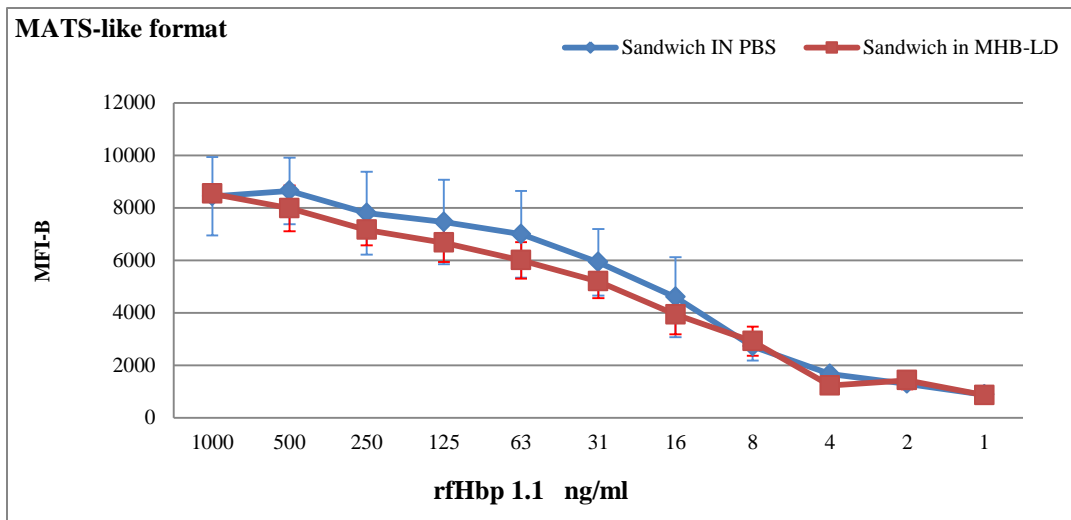
Graph 4. MATS matrix effect on Ab-Ag binding

6.3.5 Specificity investigation of fHbp-Luminex sandwich assay on MenB lysates

Both formats were tested on a H44/76 lysate, the Men B strain used as fHbp reference in the MATS assay. The aim was to define the optimal anti-fHbp Ab concentration for detection of bacterial lysates, maintaining the linearity and sensitivity observed by using rfHbp as analyte. Each experiment was repeated 3 times (in duplicate) in order to calculate the standard deviation.

As showed in graph 5 (see on the next page), MATS-like format noticeably lost assay sensitivity (i.e. at 1:32 dilution) and, it was not possible to enhance the MFI signal, even increasing the detection Ab concentration. On the other hand, NEW format preserved both assay sensitivity and linearity when applied to H44/76 strain lysate, also reducing detection Ab concentration (see graph 6 on the next page). To confirm this data, two additional Men B strains were selected: M16019, expressing the homologous vaccine variant of fHbp, (medium fHbp expression) and UK293, which expresses fHbp variant 2.19 (sub-variant not recognized by Bexsero™-induced antibodies, Domnich, et al., 2010), as a negative control.

Strains were tested by using the detection Ab concentration showing the best performance for each format (11 µg/ml for MATS-like format and 5 µg/ml for NEW format). In both formats (see graphs 7 and 8 on the next page), according to fHbp expression, detection Ab better recognized H44/76 var. 1.1 (reference strain) than M16019 (var. 1.1), while UK293 (var 2.19) strain was not recognized, as reported for MATS assay (Domnich et al., 2010), according with phylogenetic distance.

In order to optimize the detection anti-fHbp Ab concentration for NEW format, an additional titration at 3 and 1.5 µg/ml was performed on the reference strain H44/76 (data not shown). 1.5 µg/ml was selected as optimal concentration and was tested on additional strains to confirm that specificity was preserved (the experiment was repeated 3 times). According with phylogenetic distance (see figure 5 on page 19), strains expressing fHbp variant 1.x were recognized by detection Ab, while variant 2.x-expressing strains were not detected (see graph 9 on page 60). The optimal concentration of detection Ab for fHbp was set on 1.5 µg/ml, for NEW format, and 11 µg/ml, for MATS-like format (not biotinylated and biotinylated anti fHbp rab-pool, respectively).
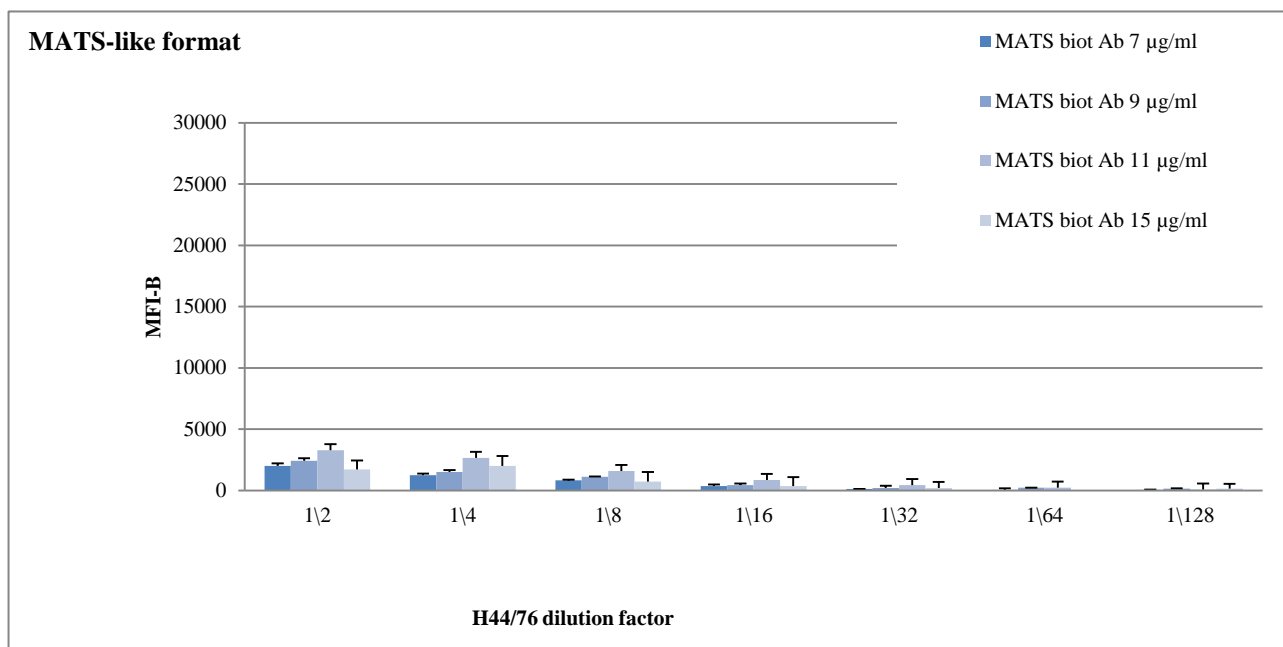
**Graph 5.** Detection Ab titration on MATS format
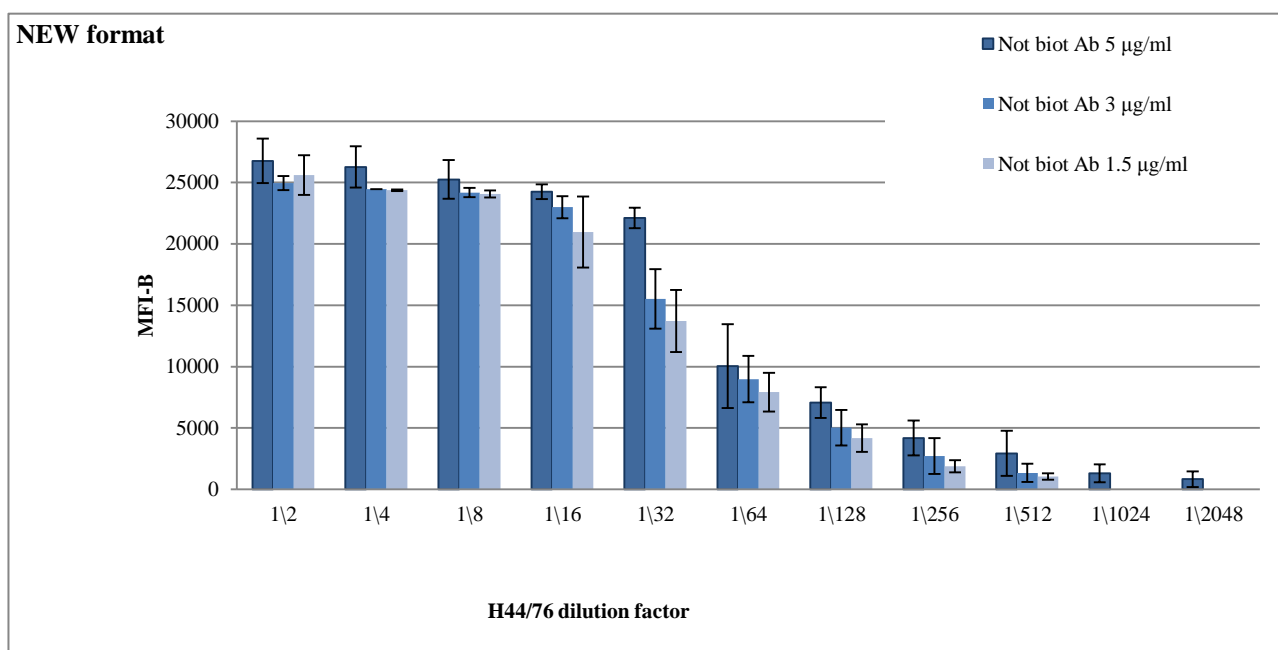
Graph 6. Detection Ab titration on New format
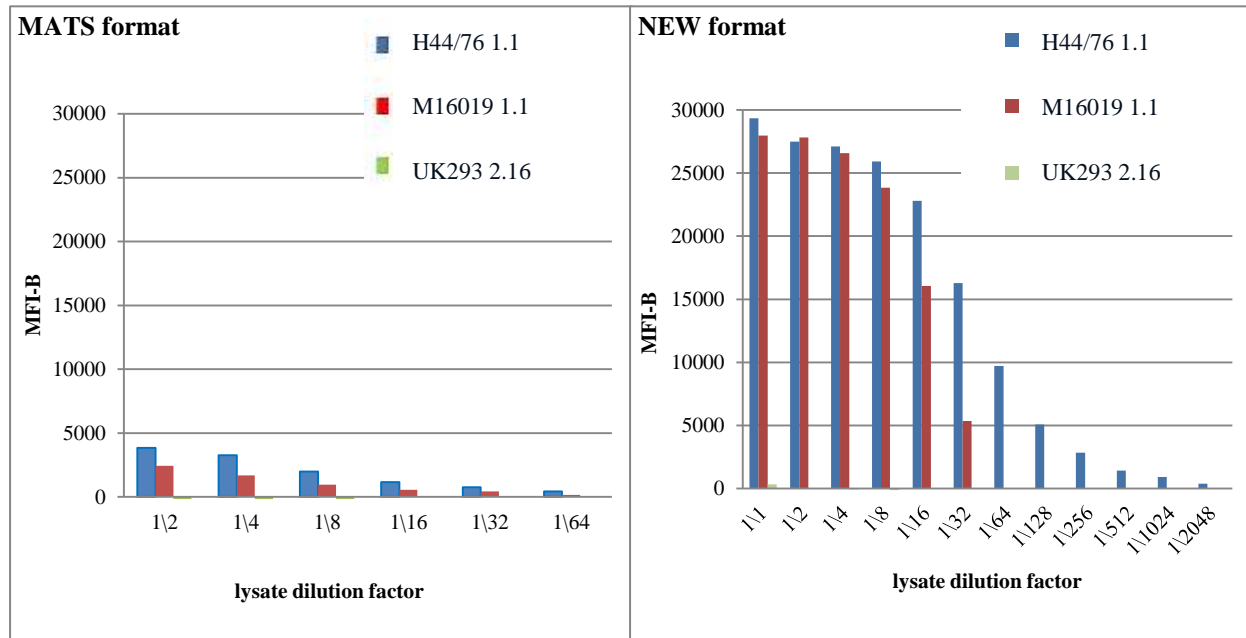
Graph 7. MATS-like format on 3 different fHbp-expressing strains by using 11 μg/ml of biotinylated anti-fHbp rab-pool pAb. Experiment was repeated three times

Graph 8. NEW format on 3 different fHbp-expressing strains by using 5 μg/ml of anti-fHbp rab-pool pAb. Experiment was repeated three times
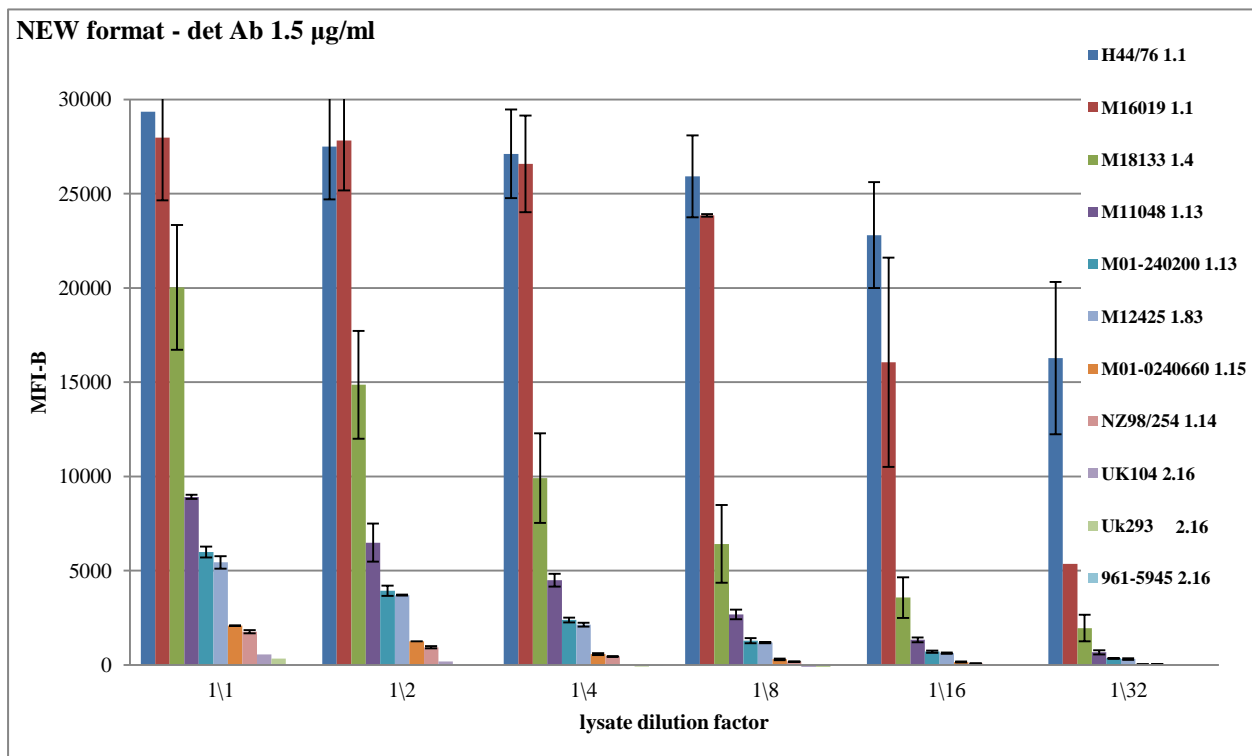
Graph 9. NEW format assay on different fHbp-expressing strains

6.3.6 Set-up of format conditions for NadA and NHBA

Identification of optimal capture and detection antibody concentration to achieve the best sensitivity and dynamic range on both the MATS-like and New Format was performed for NadA and NHBA antigens.

For both NadA and NHBA antigens, beads were coupled with anti-NadA mouse-pool 1 and anti NHBA mouse-pool 1 capture pAbs, respectively (according to paragraphs 5.4.2 and 5.4.3 of 'Matherials and Methods' chapter).

Detection NadA-specific rab-pool Ab was titrated at the concentration of 1.35, 0.8, 0.45 µg/ml by starting from 250 ng/ml of antigen (2-fold serial dilutions). The optimal detection Ab concentration was set to 1.35 µg/ml (see graph 10).

Detection NHBA-specific rab-pool Ab was titrated at the concentration of 4, 2, 1 µg/ml by starting from 2000 ng/ml of antigen (2-fold serial dilutions) and the optimal concentration of detection Ab was set to 4 µg/ml (see graph 11 on the next page).

Testing the two selected concentrations on reference Men B lysates (5/99 for NadA and NGH38 for NHBA), the optimal concentrations with best dynamic range were 1.35 µg/ml for anti NadA rab-pool Ab and 8 µg/ml for anti NHBA rab-pool Ab (data not shown).

The results obtained on the MATS format were similar to those reported for rfHbp antigen: a higher concentration of detection Ab was needed for both NadA and NHBA antigens (data not shown), and this was no sufficient to reach the instrumental working range.

Thus, in order to avoid the risk of being not sensitive enough for heterologous variants, NEW format was selected as the best one for the 4-plex assay.
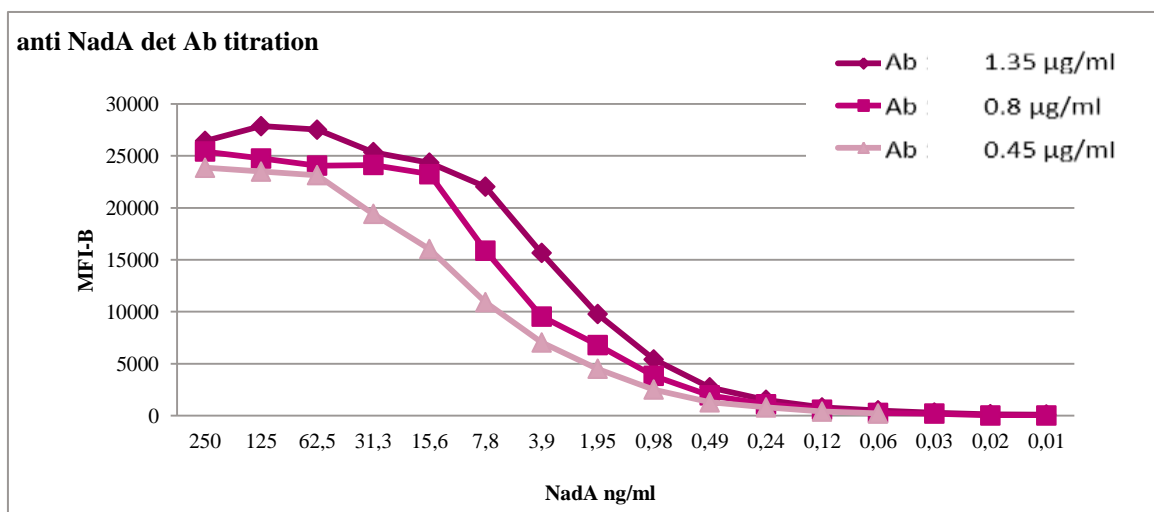
Graph 10. NadA-specific rab-pool Ab titration on NEW format
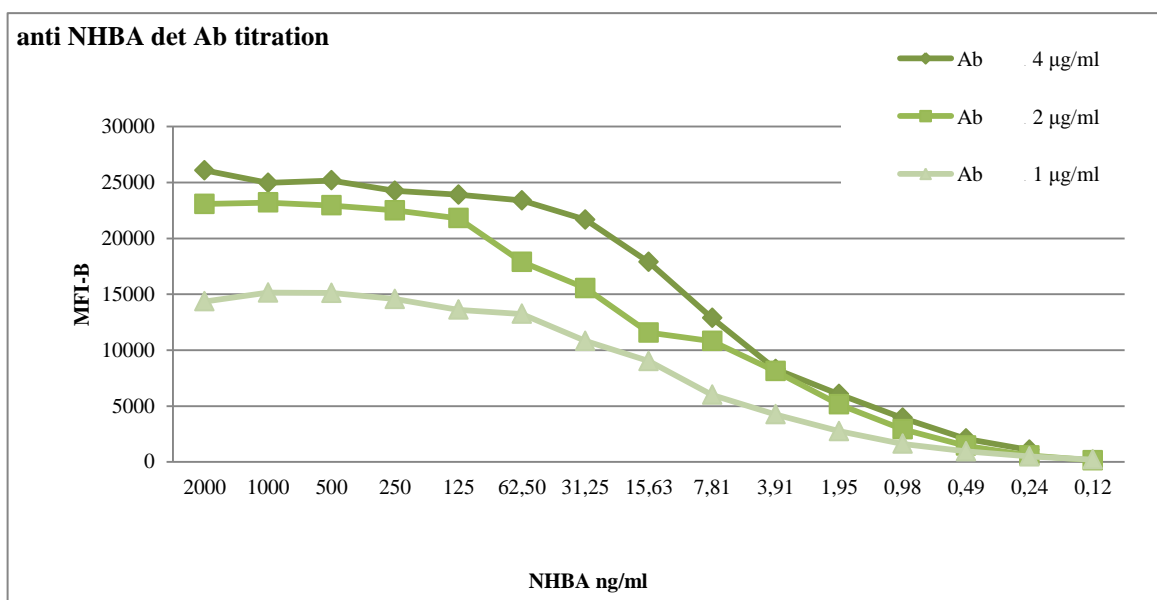
Graph 11. NHBA-specific rab-pool Ab titration on NEW format

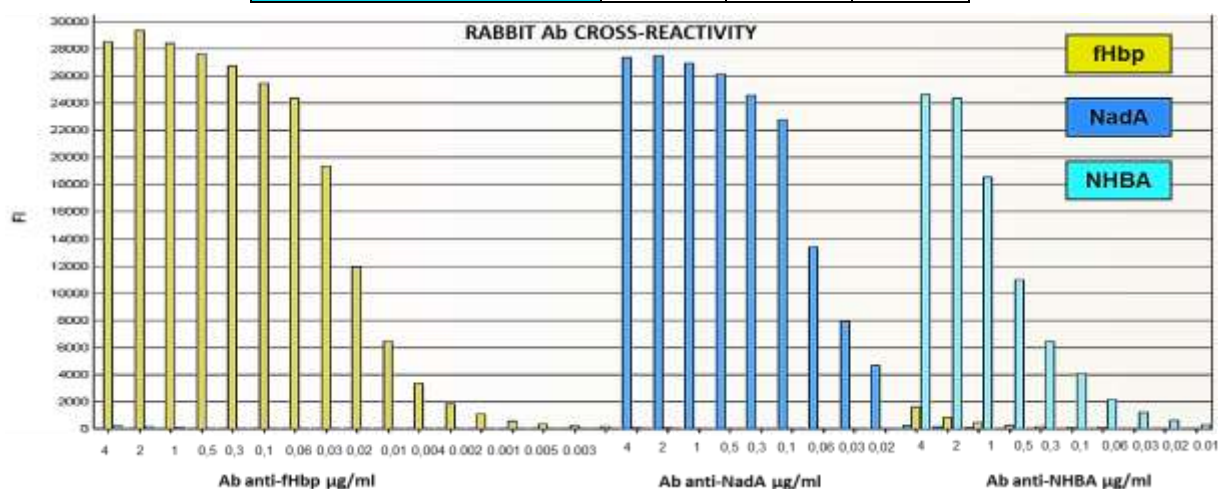### 6.3.7 Cross-reactivity study

Cross-reactivity is defined as the ability of an antibody to react with similar antigenic sites on the surface of unrelated proteins, thus providing non-specific responses.

The possible non-specific interactions were evaluated by using a 3-plex indirect assay for both mouse (capture) and rabbit (detection) Abs.

Each Ab was tested starting from 4 µg/ml (2-fold serial dilutions) by using a mix of 3 different bead regions coupled with fHbp, NadA and NHBA, respectively.

This analysis showed a weak non-specific interaction of anti-fHbp and anti-NHBA Abs with NHBA and fHbp, respectively (for both mouse and rabbit Abs, see panel 4 e 5 - on the next page). This evidence can be explained by a degree of sequence homology between the two antigens, due to their evolution from a common ancestor (Esposito et al., 2011). Anti NadA mouse-pool1 Ab showed a weak non-specific interaction with fHbp. This non-specific signal is detectable only at the highest Ab concentrations tested and so it is possible to avoid it increasing Abs dilutions.

	MFI		
	fHbp	NadA	NHBA
Ab anti fHbp rab-pool	27511.3	68.5	318
Ab anti NadA rab-pool	85.6	26962	56.5
Ab anti NHBA rab-pool	2005.6	341.5	24432

Panel 4. Cross-reactivity study on rabbit Abs by indirect assay

	MFI		
	fHbp	NadA	NHBA
Ab anti fHbp mouse-pool	26024.5	127.5	1024.3
Ab anti NadA mouse-pool	726.5	28125.5	173.8
Ab anti NHBA mouse-pool	234.5	132	26839.3
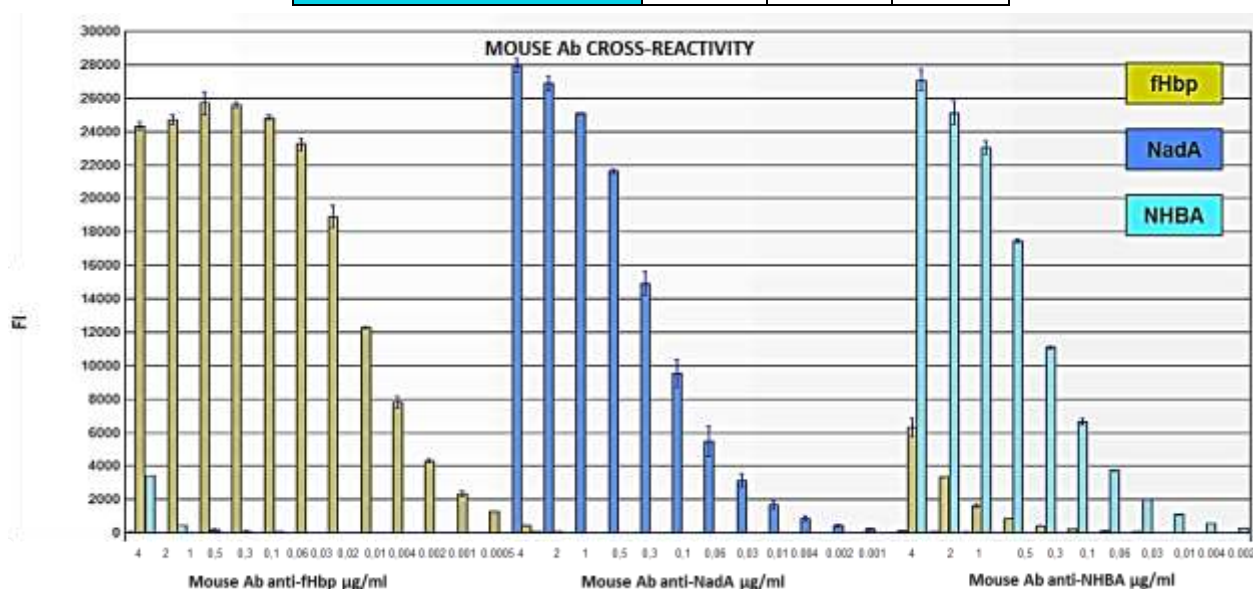
Panel 5. Cross-reactivity study on mouse by indirect assay

6.3.8 Set-up of format conditions for OMVs

Detection OMV-specific rab-pool Ab was titrated at the concentration of 3 and 1 µg/ml by starting from 15 µg/ml of antigen (2-fold serial dilutions) and the optimal concentration of detection Ab was set to 3 µg/ml (graph 12). However, testing the selected concentration on reference Men B lysate (NZ98/254 for NHBA), the optimal concentration with best dynamic range were 9 µg/ml (data not shown).
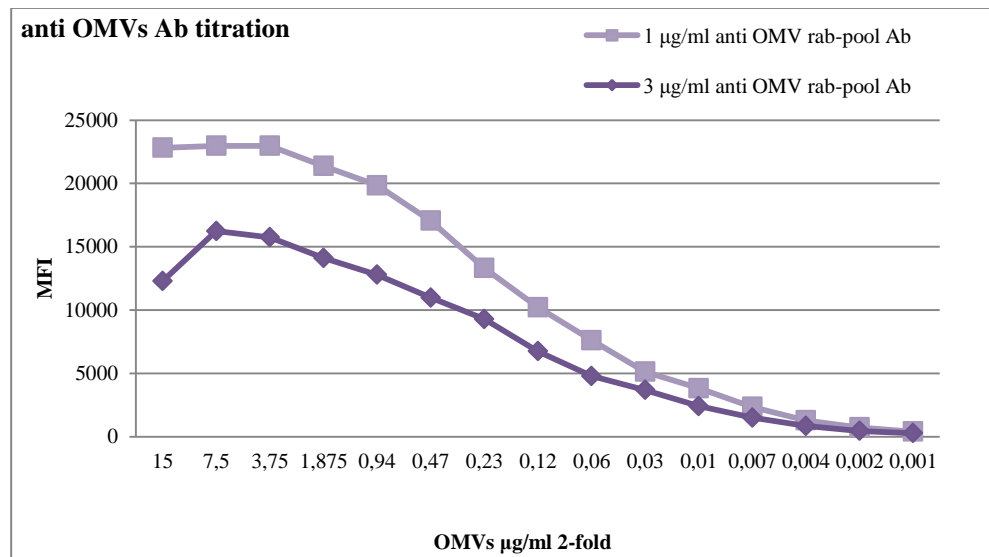
Graph 12. Detection Ab anti OMV titration on recombinant OMVs

6.4   Incubation times

Bexsero[TM] Men B reference strains were used as analytes in order to optimize incubation times performance of the 4-plex assay.

Sandwich assay protocol consists of three incubations: i) capture Ab-coupled beads/analyte reaction, ii) detection Ab binding to the analyte captured by coupled-beads and iii) recognition of detection Ab by a PE-labeled anti-species secondary Ab.

The following incubation time combinations were tested: A) i). 60 minutes;ii). 60 minutes; iii) 15 minutes; B) i) 90 minutes; ii) 60 minutes; iii) 15 minutes; C) i) 60 minutes; ii) 90 minutes; iii) 15 minutes; D) i) 90 minutes, ii) 90 minutes; iii) 15 minutes.

The last incubation time was not changed since it involved a commercial secondary Ab used according to manufacturer's instructions for optimal performance. Panel 6 and 7 (see on the next page) show that the combination A) could be considered the best one for fHbp antigen. Furthermore, there were no significant differences for NHBA and NadA antigens between the four combinations, while OMVs-MFI signals seemed to lose linearity with combination B), C), and D).

Based on this observation, combination A) was selected, in order to save time and preserve assay linearity.
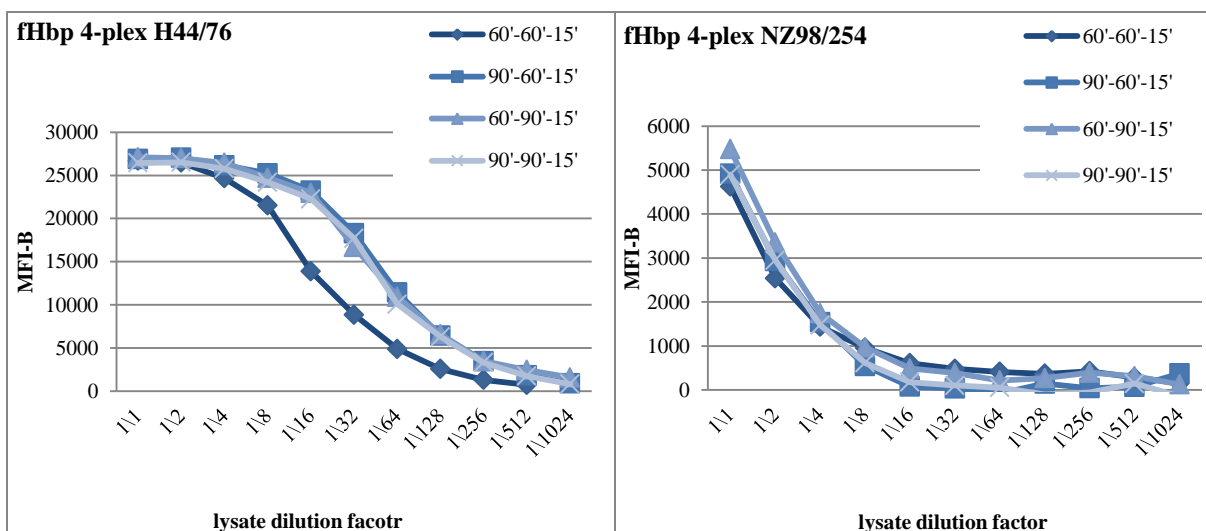

Panel 6. Incubation time study for fHbp antigen

Panel 7. Incubation time study for OMVs, NHBA and NadA antigens
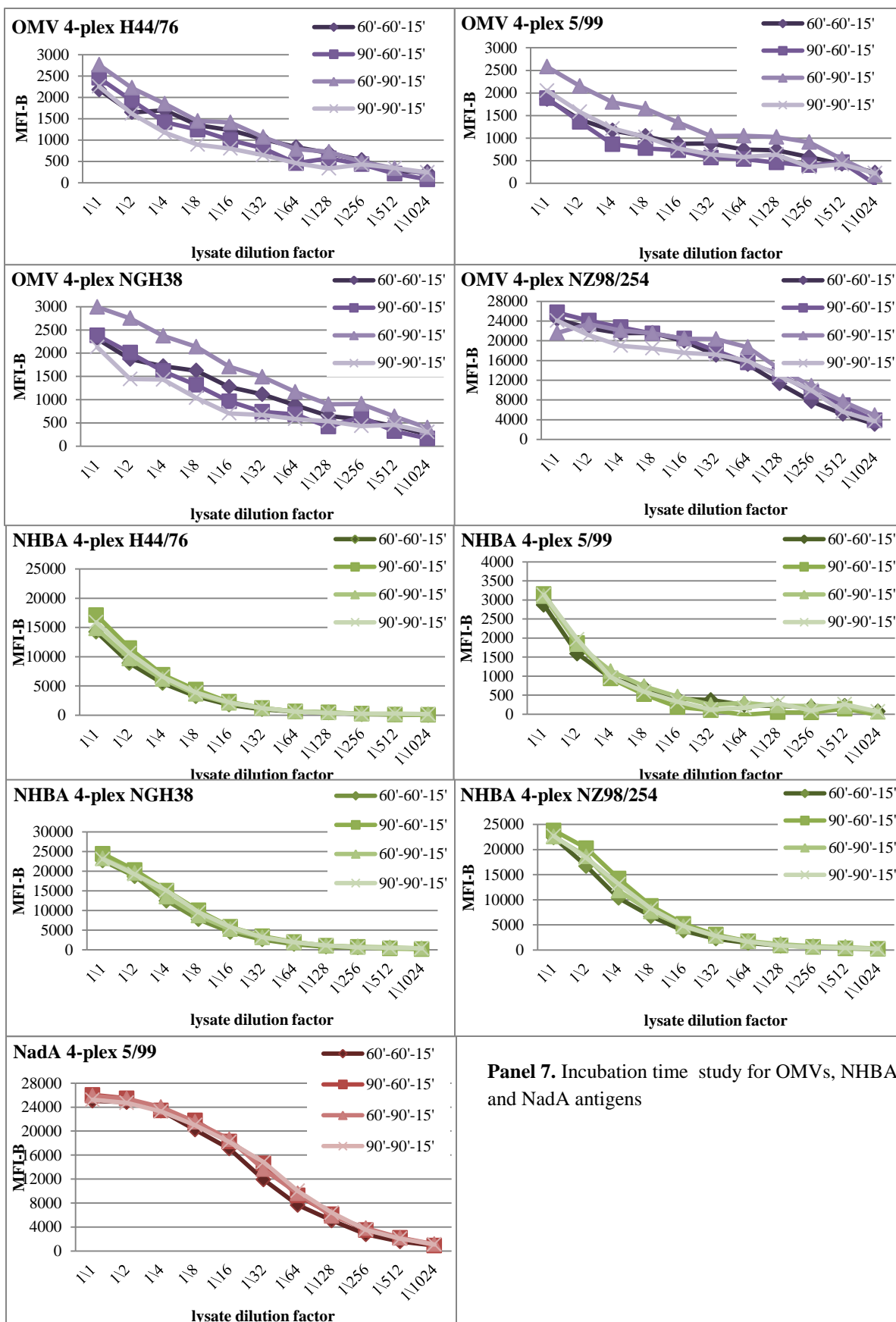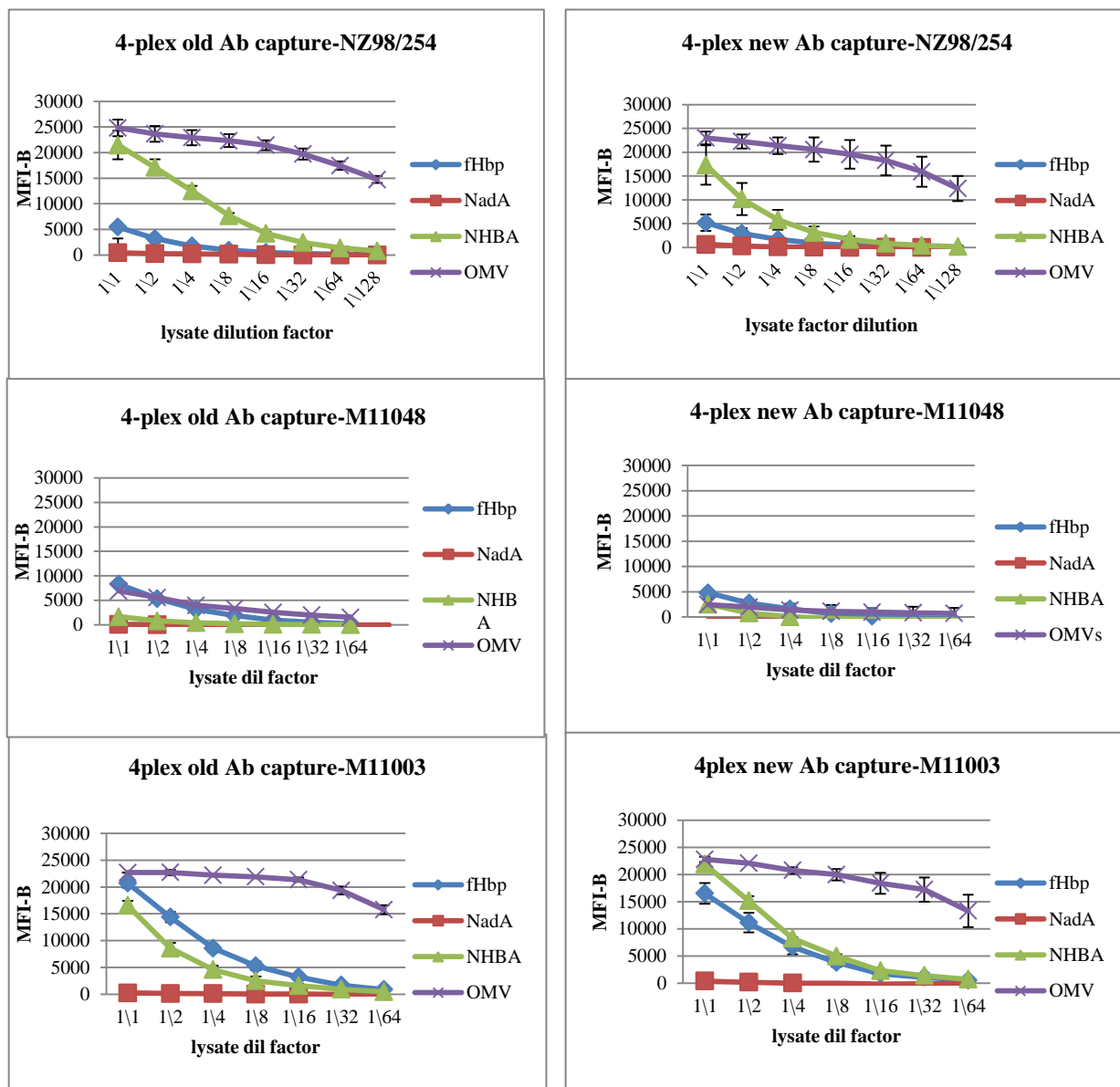
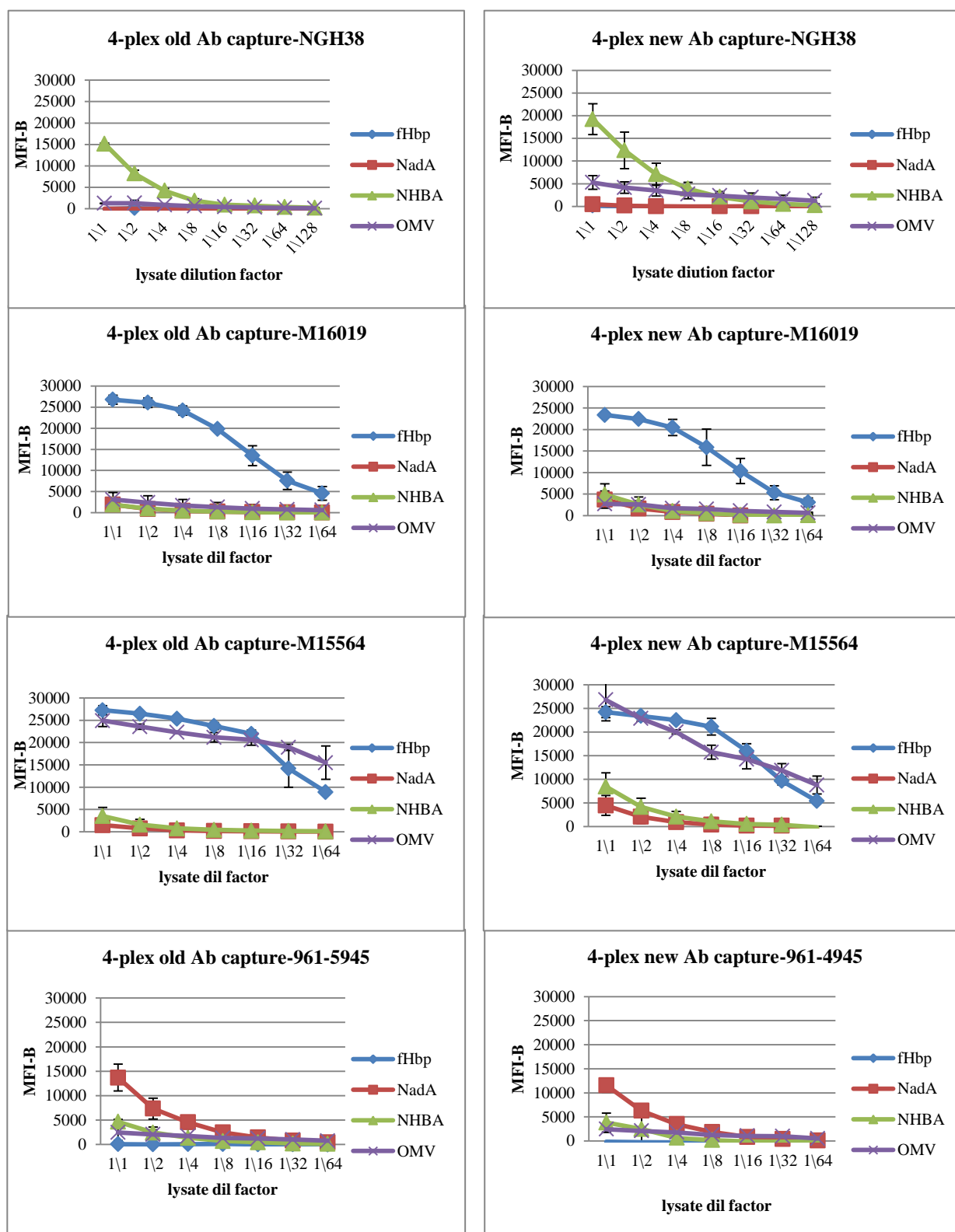## 6.5   4-plex assay: correlation with single-plex

Detection rabbit pAbs were titrated again when a new lot of mouse capture pAbs was used (immunization scheme anti fHbp mouse-pool1, anti NadA mouse-pool1, anti NHBA-GNA1030 mouse-pool1, and anti OMVs mouse pool). Each reference and Quality Control (QC, see table 6 on page 72) MATS strain was tested in order to bridge results obtained by using the old capture mouse pAb with the new one. Each experiment was repeated at least 3 times and the standard deviation was calculated. Panels 8, 9 (see on the following page) and 10 (see on page 69) show that there was no significant different between the two capture Ab lots. New detection rabbit pAb concentrations were set to 3 μg/ml for anti fHbp Ab, 2.7 μg/ml for anti NadA Ab, 18 μg/ml for anti NHBA and 13 μg/ml for anti OMVs Ab respectively. MFI signals detected showed the expected profiles for each strain, according to antigen-expression and MATS data (data not shown).
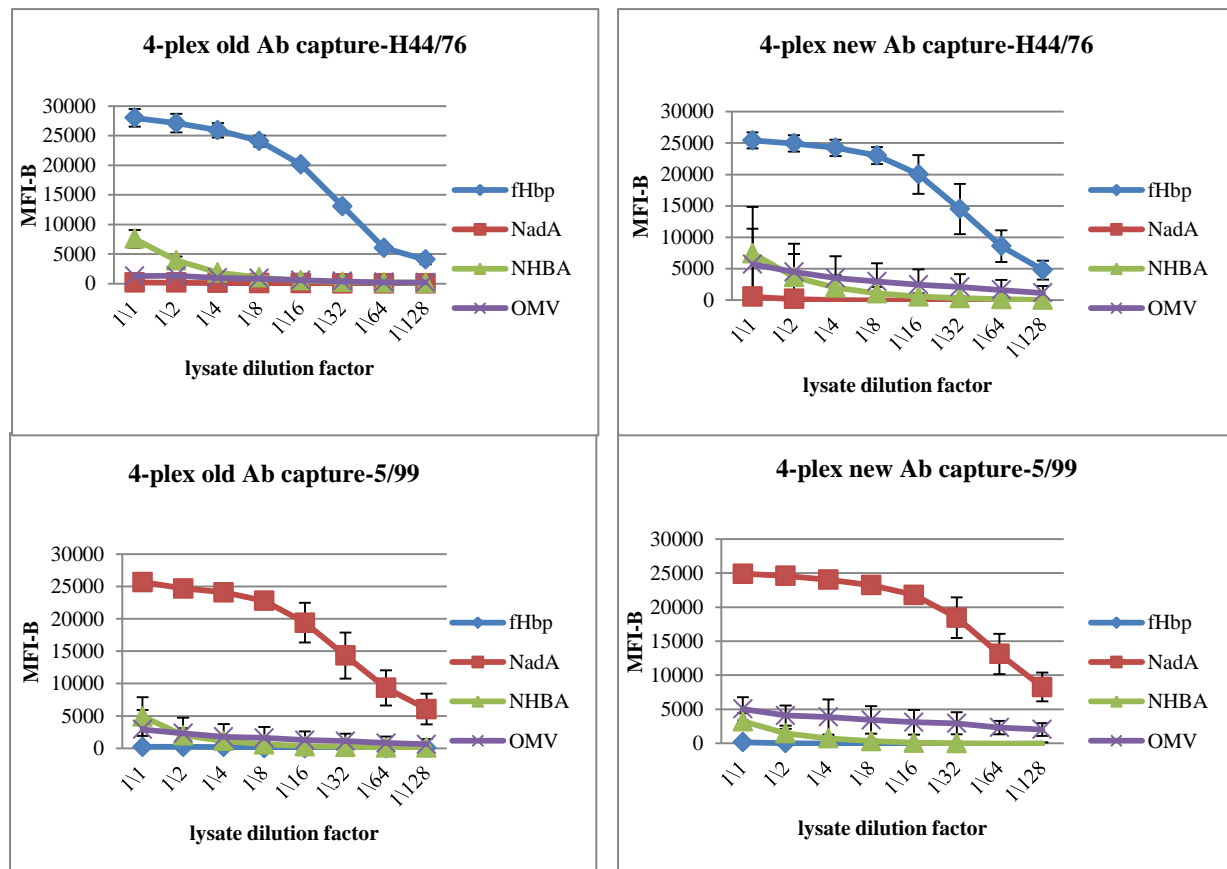
**Panel 8.** Old vs new capture mouse pAb correlation on NEW format (reference strains)

Panel 9. Old vs new capture mouse pAb correlation on NEW format (QC MATS strains)

Panel 10. Old vs new capture mouse pAb correlation on NEW format (QC MATS strains)

Furthermore, single-plex and multiplex assay on reference and QC MATS strains have shown a good correlation with $R^2 > 0.9$ for each vaccine Ag (panel 11 and 12 on the next page).
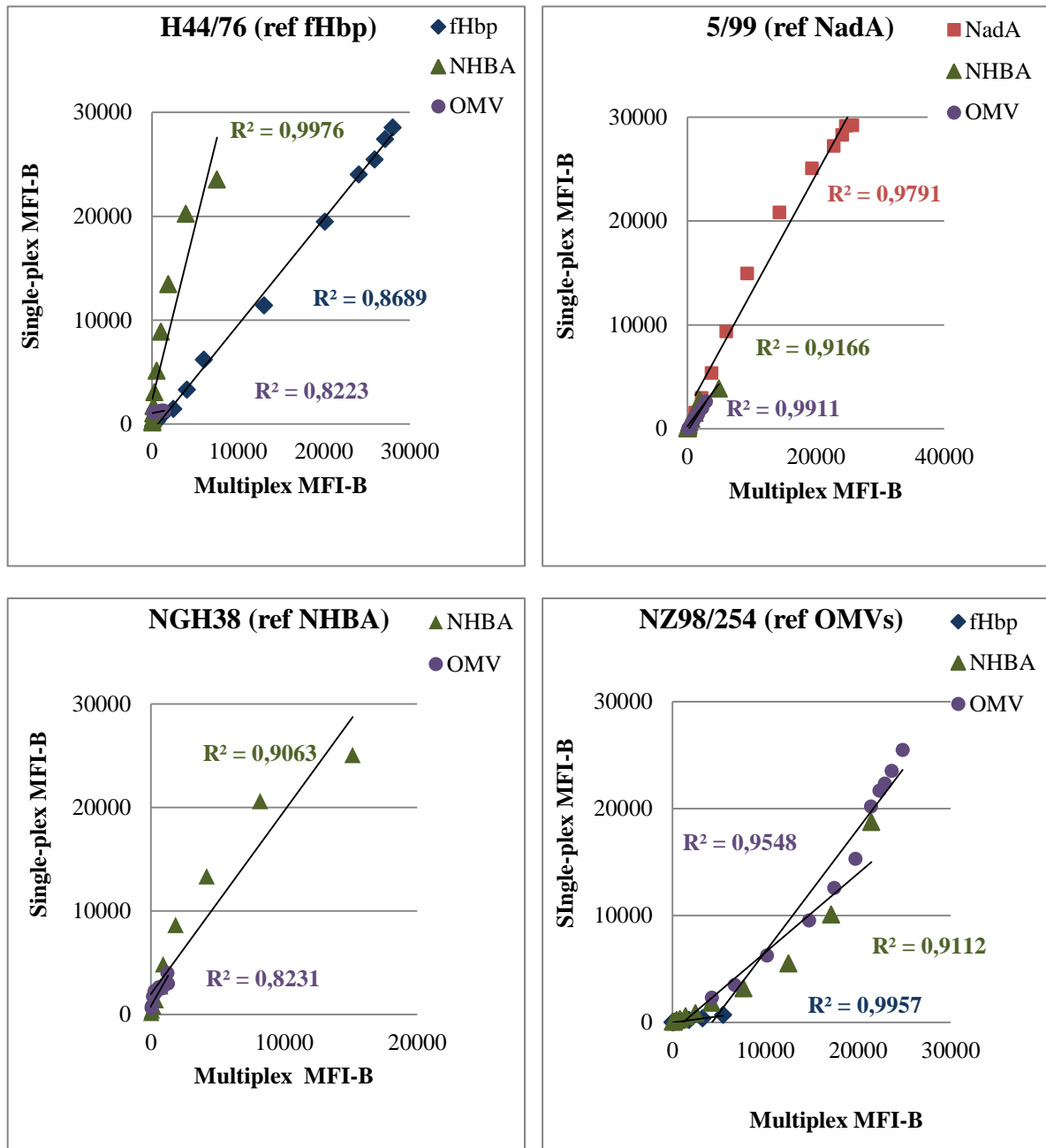
Panel 11. Single-plex vs 4-plex correlation for vaccine reference MenB strains. See details on Ag-espression on the table 6 on page 72
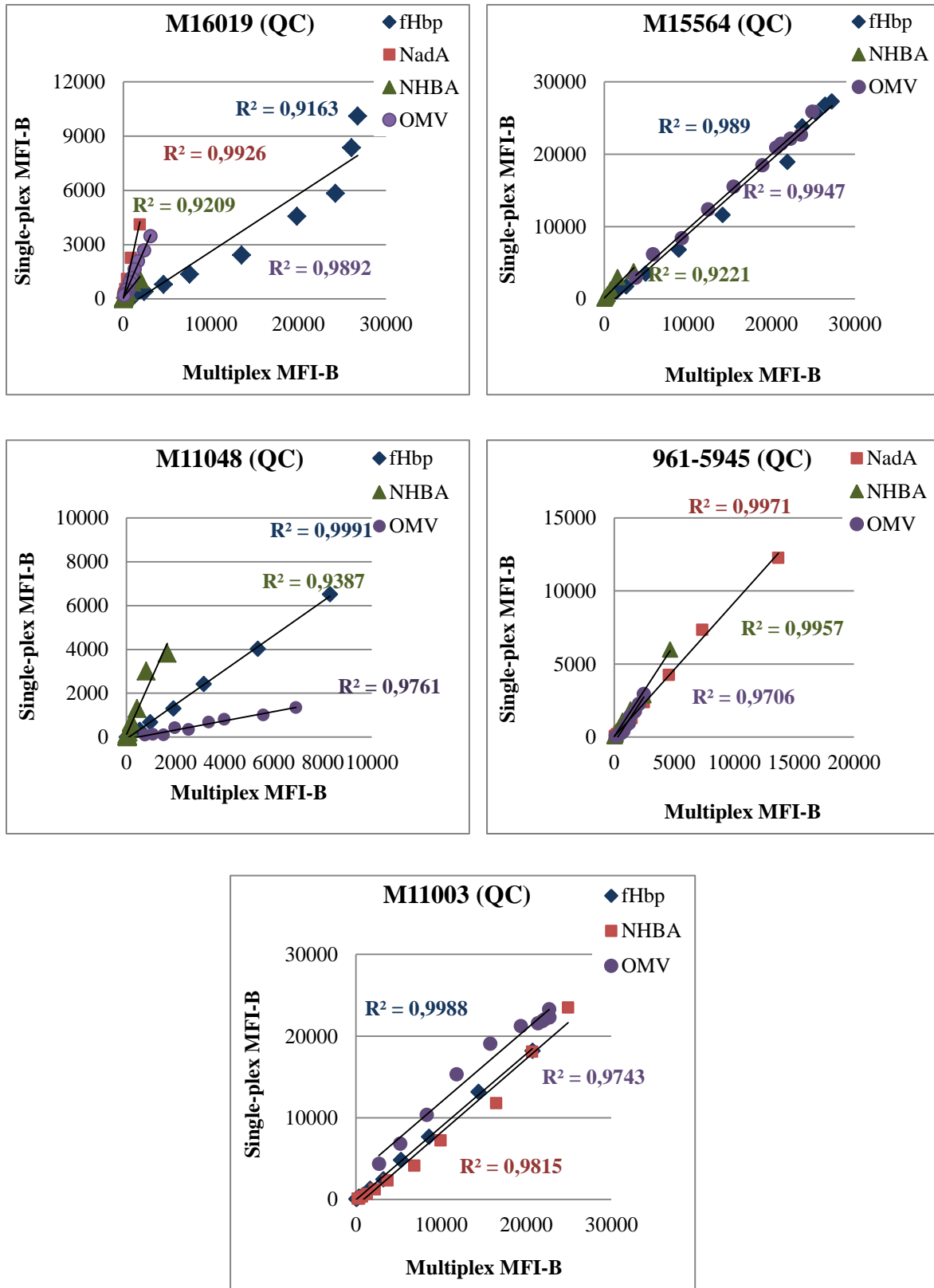
Panel 12. Single-plex vs 4-plex correlation for MATS QC MenB strains. See details on Ag-espression on the table 6 on the next page

	fHbp var./ subvar.	NHBA peptide	NadA var.	PorA VR1-VR2
H44/76	1.1 (ref)	3	No gene	P1.7,2.16
5/99	2.23	20	3 (ref)	P1.5-2.2
NGH38	2.24	2 (ref)	no gene	P2.3
NZ98/254	1.14	2	no gene	P1.7-2,4 (ref)
M11003	1.4	2	No gene	P 1.7-2,4
M11048	1.13	24	No gene	P1.5-1,2.2-2
961-5945	2.16	2	test	P1.21,16
M16019	1.1	5	1	P1.7,16
M15564	1.1	5	1	P1.7, 2.16

Table 6. Summary of reference and MATS QC strains vaccine antigen expression

6.6 MATS assay vs 4-PLEX assay correlation

Relative Potency (see section 5.3 of 'Materials and Methods' chapter for further information) values were calculated for MATS Quality Control and reference strains by using SoftMax ProData software for both assays and are reported in panel 13. Preliminary correlation results indicated that the 4-plex assay could be a promising candidate test to investigate coverage of Bexsero[TM] vaccine.
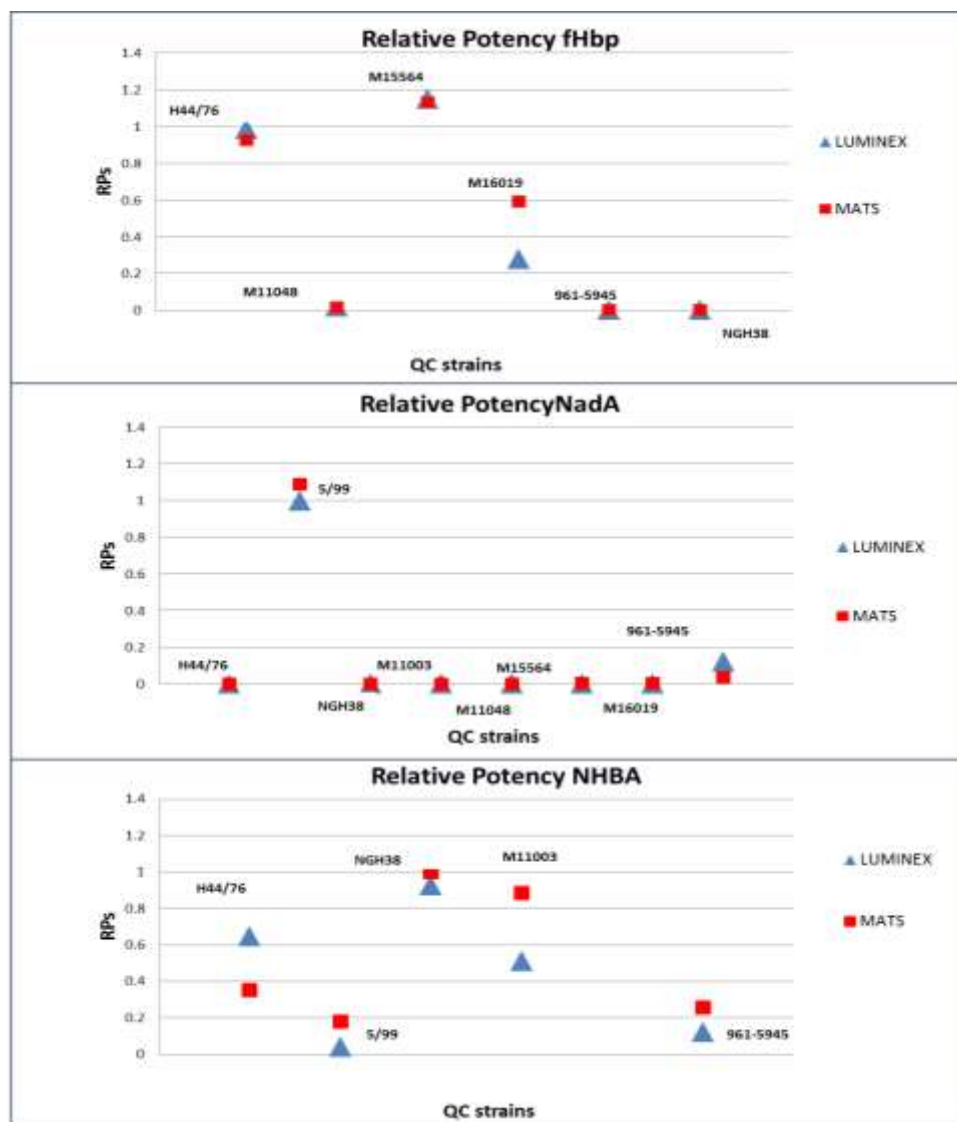
Panel 13. Correlation MATS vs Luminex in terms of Relative Potencies. Data are expressed in average (n=3) for Luminex 4-plex assay and in geometric mean (n=18) for MATS assay. Luminex assay RP values were within the 95% Confidence Interval of MATS assay (data not shown). See details on Ag-expression on the table 6 on the previous page

6.7   <u>4-plex sandwich qualification</u>

6.7.1   Specificity by inhibition assay

To gain additional information on assay specificity an inhibition assay for our sandwich assay was developed. MenB lysates reference strains were incubated with detection rabbit Abs mix, both at fixed concentration, followed by addition of 2-fold serial dilutions of capture mouse Ab. Not inhibited signal obtained from Ag-detection rabbit was quantified by the 4-plex assay using a PE-labeled anti-rabbit secondary Ab. Assay specificity was assessed by complete signal inhibition respect to control sample (no inhibitor). These experiments confirm that each mouse antibodies used as inhibitor (the same coupled to the microspheres as capture) is appropriate and do not compete with lysate binding from rabbit detection antibodies. Only for NHBA we observed a competition that may be referable to the sequence homology between fHbp and NHBA. In fact, anti NHBA mouse-pool 1 could have bound a part of fHbp in the 4-plex assay, lightly enhancing fHbp inhibition and, consequently reducing NBHA inhibition.
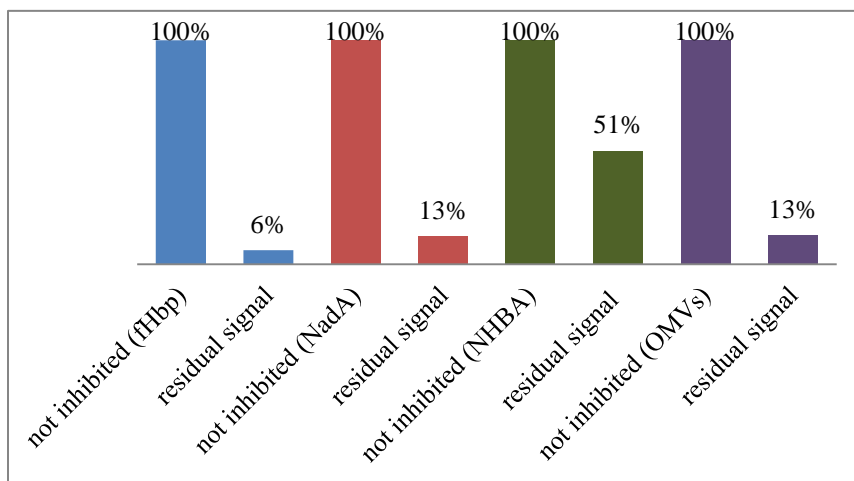
Inhibition percentage was calculated by using the following formula:

$$100 - \left(\frac{\text{inhibited MFI signal}}{\text{not inhibited MFI signal}} \right) * 100$$

Details on inhibition percentages and residual MFI signal for each Ag are reported on the table 7 and graph 13, respectively.

Ags	Inhibition percentage (%)
fHbp	93.7
NadA	87.5
NHBA	51.6
OMVs	87.2

Table 7. Inhibition percentage for each vaccine Ag

Graph 13. Residual inhibition percentage for each vaccine Ag

6.7.2 Assay cut-off: LLOD and LLOQ

The Lower Limit of Detection (LLOD) defines the smallest concentration of analyte that can be detected by an assay, with no guarantee about the bias or imprecision of the result.

The LOD for 4-plex assay (see table 8 on the next page) was set at the highest point of dilution of Men B samples, with CV% intra-assay <15%, selected among the 28-panel strain tested and following the instructions below:

- for fHbp, variant 2 and 3 expressing strains that are not recognized by Bexsero^{TM} -induced Abs;

- for NadA, strains without NadA gene;

- for NHBA, reference strain (because a NHBA-negative strain was not available within the panel tested);

- for OMVs, hSBA negative strains.

The Lower Limit of Quantification (LLOQ) is defined as the lowest concentration of analyte in a sample that can be consistently detected and quantitatively measured with suitable precision and accuracy (or linearity as surrogate of accuracy) under assay conditions. This corresponds to the quantifiable concentration threshold of the assay.

The 4-plex assay LLOQ for each antigen was set at the highest value among the Lower Limit of Lineary (LLOL), the Lower Limit of Precision (LLOP) and the LLOD, which were calculated by considering 25 indipendent experiments conducted on each Bexsero^{TM} antigen reference strain, respectively (see table 8 on the following page).

Contrary to the validation guidelines (LOD= blank samples average+3SD blank samples), LOD was calculated from not diluted negative samples. In fact, the assay matrix (MHB+LD) without strain component induced a background noise higher than the blank signal. This phenomenon may be explained by a possible saturating effect (like BSA) of bacterial lysate proteins that decreases the nonspecific binding in this immunoassay.

	Ags	LLOD	LLOQ
Antigens	**fHbp**	**1064.8**	**1101.5**
	NadA	**1602.5**	**1602.5**
	NHBA	**3711.8**	**4189.4**
	OMVs	**5926.1**	**5926.1**

Table 8. LLOD and LLOQ of 4-plex assay expressed in terms of Median Fluorescent Intensity (MFI)

6.7.3 Precision: repeatability, CV% intra and inter-assay

Repeatability of the assay was assessed for each antigen by determination of intra and inter-assay variation. A set of 25 indipendent experiments with 4-plex assay performed in different days under the same operating conditions was used (table 9).

Ags	CV% intra-assay	CV% inter-assay
fHbp	**2,8**	**14,3**
NadA	**2,3**	**10,44**
NHBA	**4,02**	**24,4**
OMVs	**3,3**	**12,6**

Table 9. CV% intra and inter-assay of 4-plex assay

6.8   Screening of a broad and heterogeneous meningococcal B panel

The 28-strain panel reported in the paragraph 5.8 (chapter 5. Materials and Methods) were tested on 4-plex optimized assay in order to investigate the correlation with MATS data. These 28 strains are a sub-set of a 57-strain panel selected in 2010 by Donnelly et al. to correlate MATS results with hSBA.

6.9   Statistical analyses

We produced the Relative Potencies for each Men B strain tested by 4-plex assay in order to correlate 4-plex assay results with MATS data. For this purpose, we used a GSK validated spreadsheet which compares serial dilution curves of tested strains with those of reference strains, whose RPs have been assigned the arbitrary value of 1 (or 100%), by a variance-weighted regression method. Each LLOQ was converted from Median Fluorescence Intensity in Relative Potency: 0.0001 for fHbp, 0.0816 for NHBA, 0.0009 for NadA and 0.0033 for OMVs. Statistical analysis on Luminex and MATS RPs dataset was performed by evaluation of correlation coefficient for each vaccine antigen by using Spearman method. The Spearman's rank-order correlation is the nonparametric version of the Pearson product-moment correlation. Spearman's correlation coefficient, (ρ) measures the strength and direction of association between two ranked variables.

Spearman's test showed that MATS RPs highly correlate with Luminex 4-plex assay RPs for NadA (ρ=0.99, spearman's p value=0.0003, on the top of the panel 14, page 79) and, sensibility of 4-plex assay is the same of MATS assay on the 28-strain panel tested.

We obtained a close correlation for NHBA as well (ρ=0.7, spearman's p value=0.0002, on the bottom of the panel 14, page 79). However, five strains detected by MATS were just below the Luminex LLOQ. The calculation of NHBA LLOQ for Luminex, in fact, is highly conservative and underestimate the sensitivity of the assay. To optimized the LLOQ calculation for NHBA we need to test more NHBA expressing strains with low expression of this antigen.

The correlation for fHbp was weak (ρ=0.62, spearmna's p value<0.05, top-graph in the panel 15, page 80) when we compared Luminex RPs with MATS RPs calculated for the MATS standardization in 2009. However, parameters for fHbp on MATS assay have been second time standardized on 2014 (data under publication) and, comparing Luminex RPs with the new calculated MATS RPs for this antigen, we observed a high correlation (ρ=0.85, spearman's p value<0.0001, middle-graph in the panel 15, page 80). Moreover, 4-plex assay has a light higher sensitivity for fHbp, in fact it can detect one strain more respect to MATS assay (both 2009 and 2014 standardizations).

Looking at the RPs strain rank between the two assays, we observed that there is a high correlation for each antigen (see panels 17 and 18 on pages 82 and 83, respectively).

In addition, we were able to obtain RPs values for OMVs (panel 16 on page 81). 4-plex assay measured, for the first time, the contribute of all OMVs components (not only PorA-correlated) in Bexsero^TM immune-response and, testing additional strains we may avoid MATS underestimation adding information on OMVs role in the vaccine coverage.
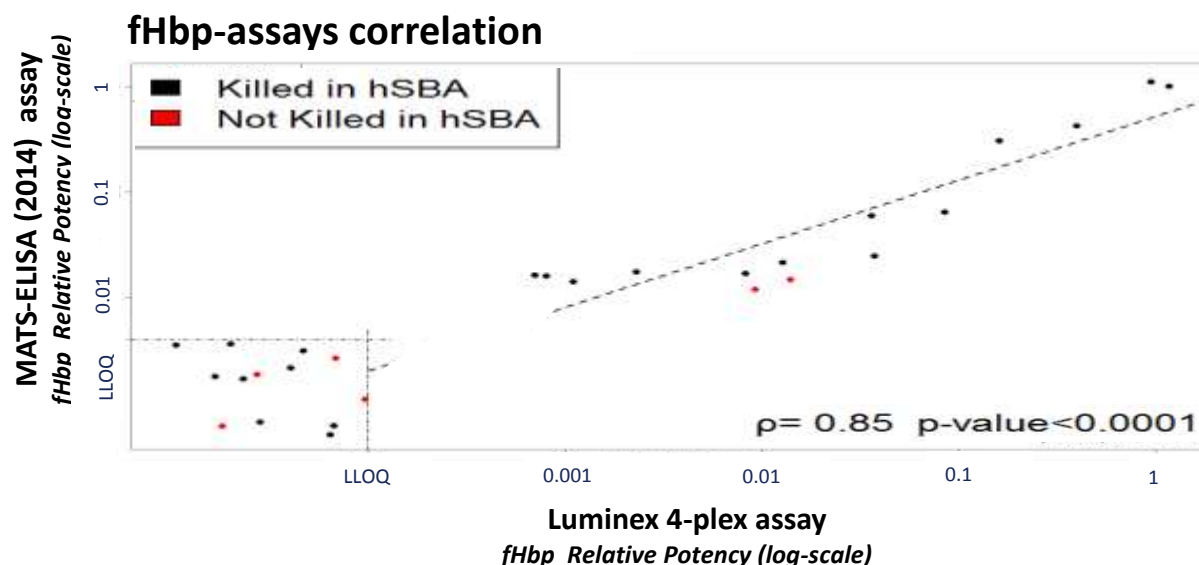
NadA-assays correlation
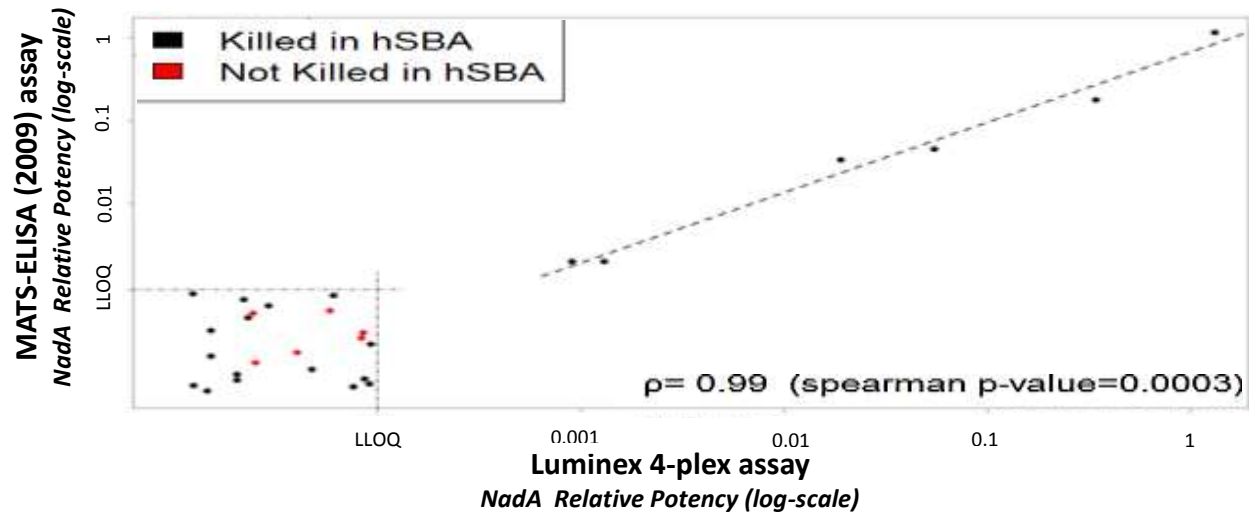
	Not Measurable	Measurable
Luminex 4-plex assay	22	6
MATS-ELISA(2009)	22	6

NHBA-assays correlation
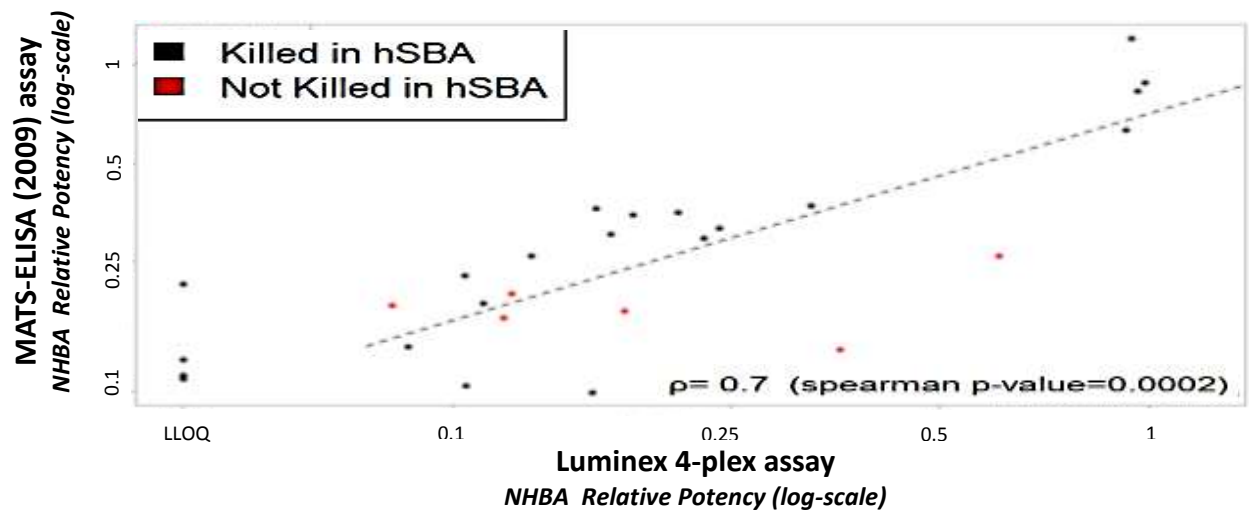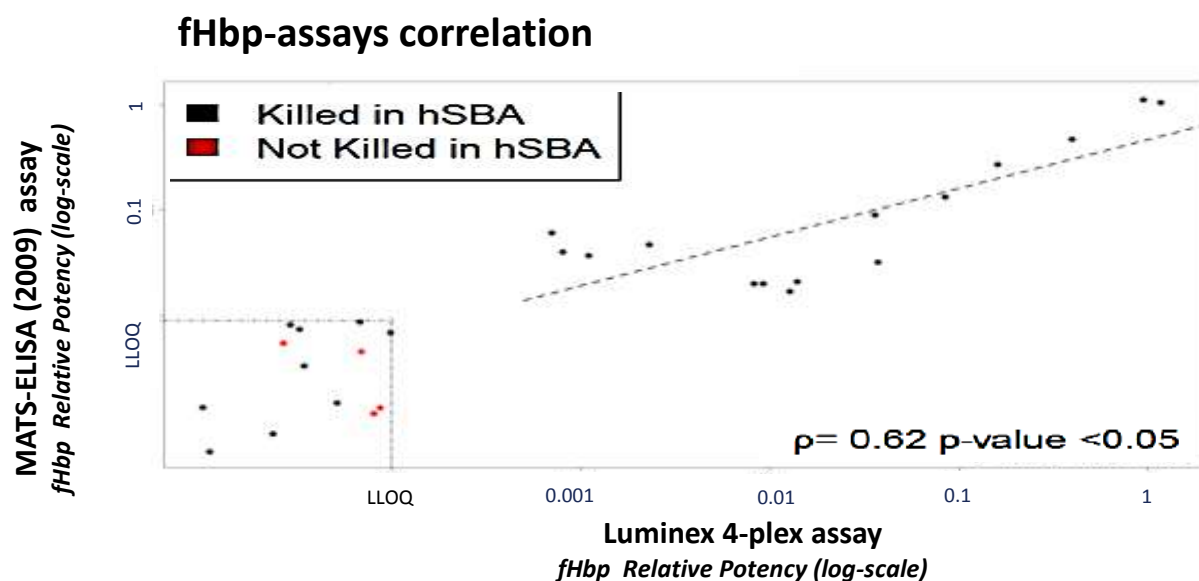

	Not Measurable	Measurable
Luminex 4-plex assay	5	23
MATS-ELISA(2009)	0	28

Panel 14. Spearman's test for correlation between MATS and Luminex for Nad A (top) and NHBA (bottom). X and y-axis report Luminex and MATS RPs for each antigen, respectively (log-scale). Dot-dashed lines are the LLOQ of both assays. Black dots represent the RPs of strains killed in hSBA. On the contrary, red dots are the RPs of strains not killed
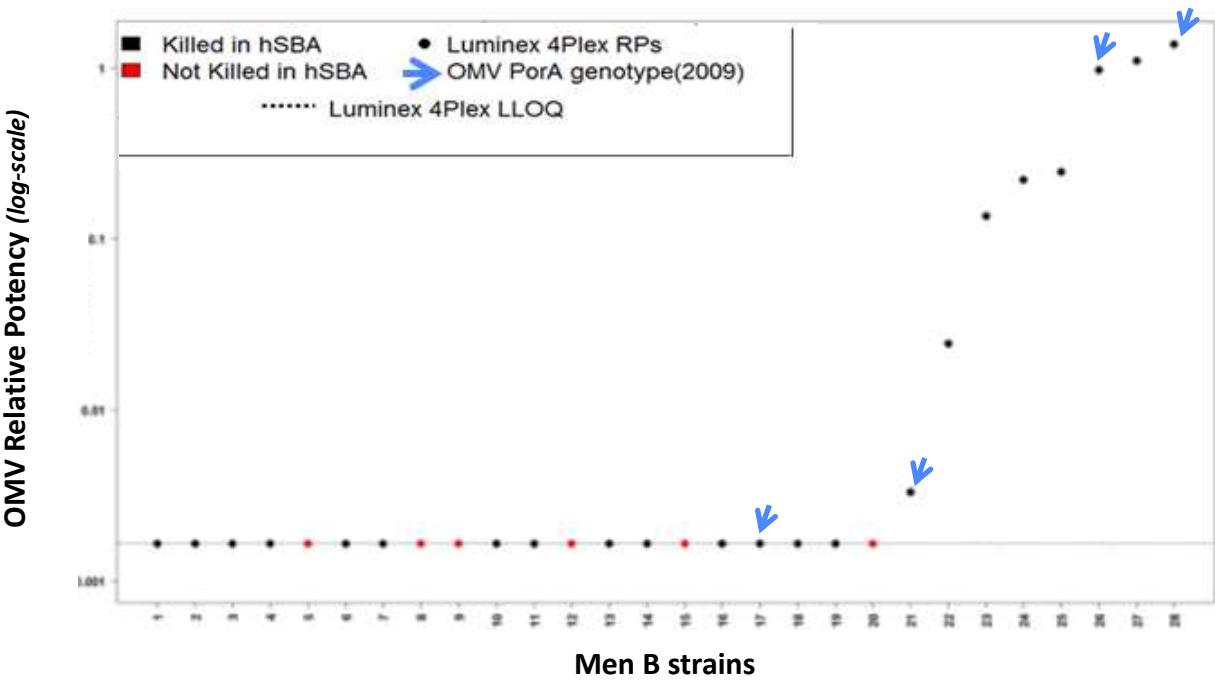
79

fHbp-assays correlation

		Not Measurable	Measurable
Luminex 4-plex assay		12	16
MATS-ELISA (2014)	13	15	

fHbp-assays correlation

	Not Measurable	Measurable
Luminex 4-plex assay	12	16
MATS-ELISA (2009)	13	15

Panel 15. Spearman's test for correlation between Luminex 4-plex assay and both MATS-2014 (top, data under publication) and MATS 2009 (bottom) for fHbp. X and y-axis report Luminex and MATS RPs for each antigen, respectively (log-scale). Dot-dashed lines are the LLOQ of both assays. Black and red dots represent the RPs of strains killed not killed in hSBA, respectively

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OMV-PorA assays correlation

Panel 16. Correlation between PorA genotype and Luminex RPs for OMVs. Light blue arrows represent the PorA P1.4 homologous strains and the dotted line is the Luminex OMVs LLOQ. Strains tested (from 1 to 28) and Luminex OMVs RPs are reported on x and y-axis, respectively. Black and red dots represent the RPs of strains killed not killed in hSBA, respectively

|	Not Measurable	Measurable
Luminex 4-plex assay	18	10
MATS-ELISA (PorA genotype, 2009)	22	4

NadA-RPs ranks comparison
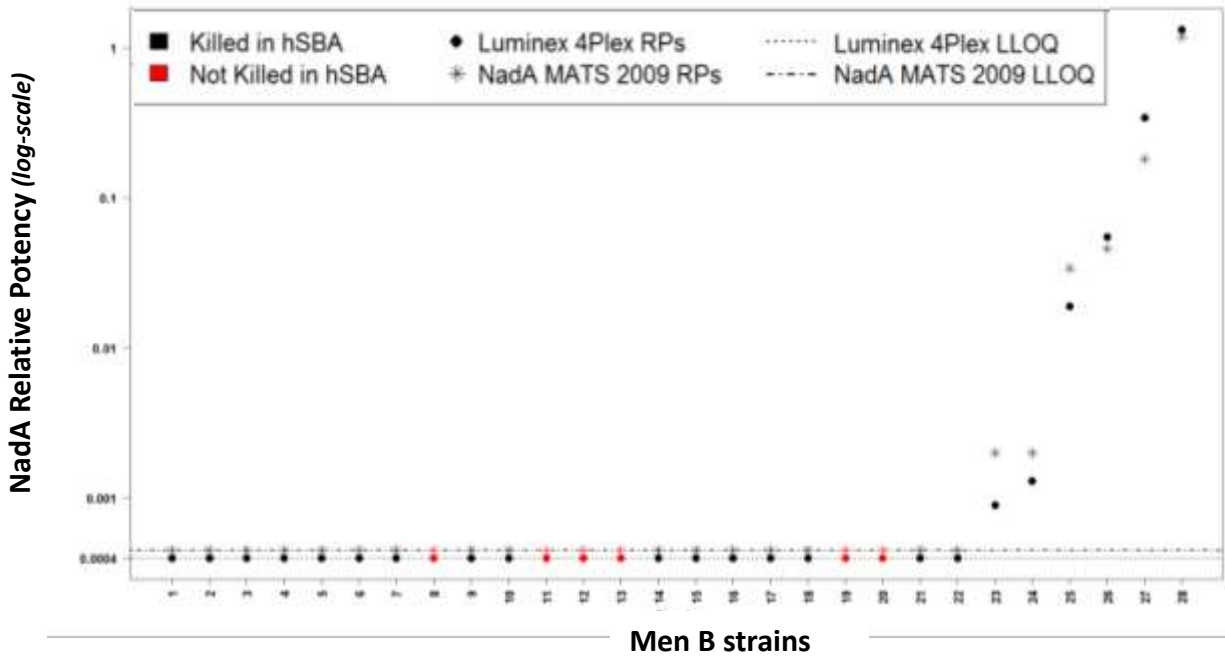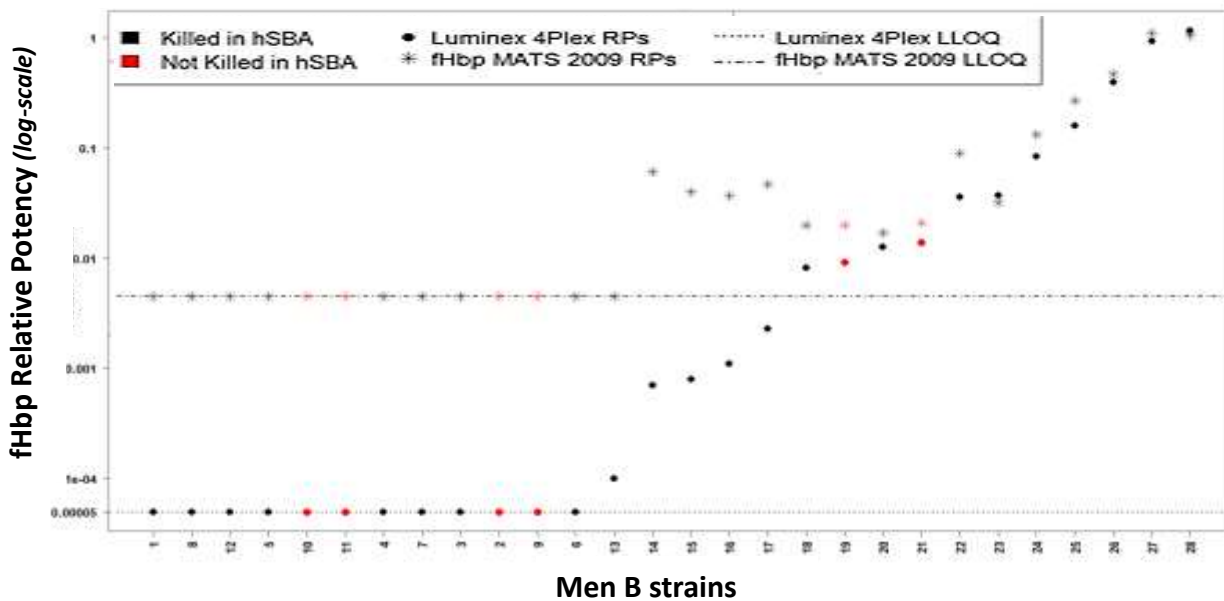

NHBA-RPs ranks comparison
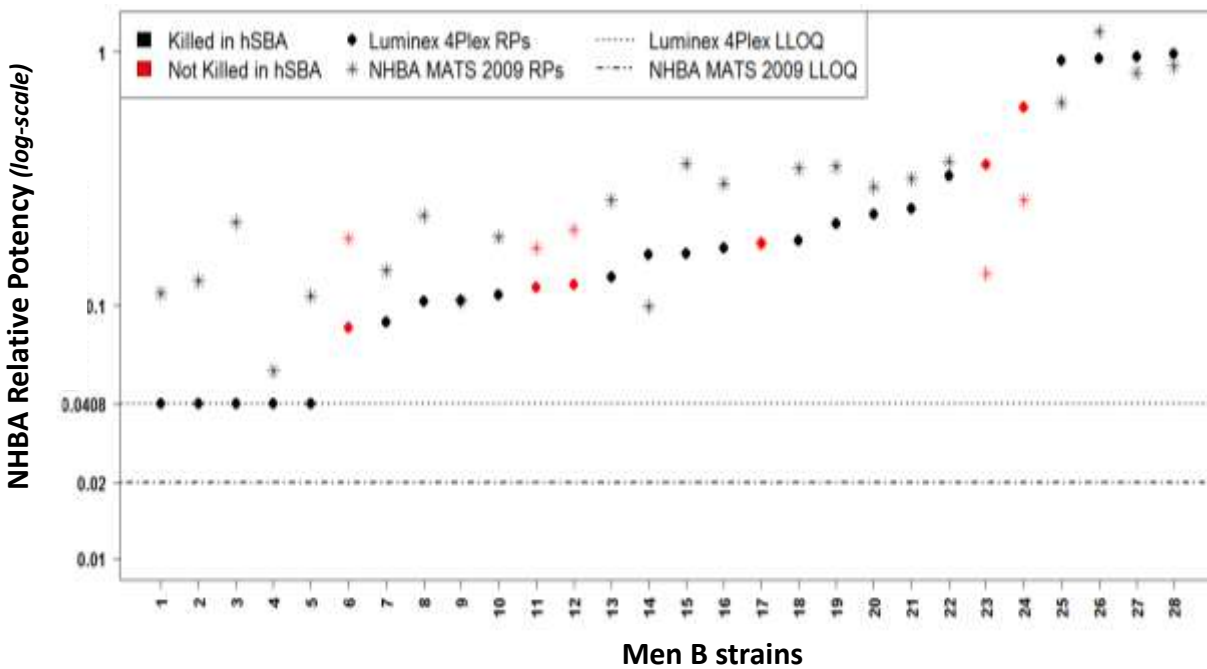
Panel 17. RPs strain rank comparison between the two assays for NadA and NHBA. Strains tested (from 1 to 28) and Luminex RPs are reported on x and y-axis, respectively. Black and red dots represent the RPs of strains killed not killed in hSBA, respectively. Stars are MATS RPs and dots represent Luminex RPs. Dotted and dot-dashed lines are the Luminex and MATS LLOQ, respectively

fHbp-RPs rank comparison (MATS-ELISA 2014)
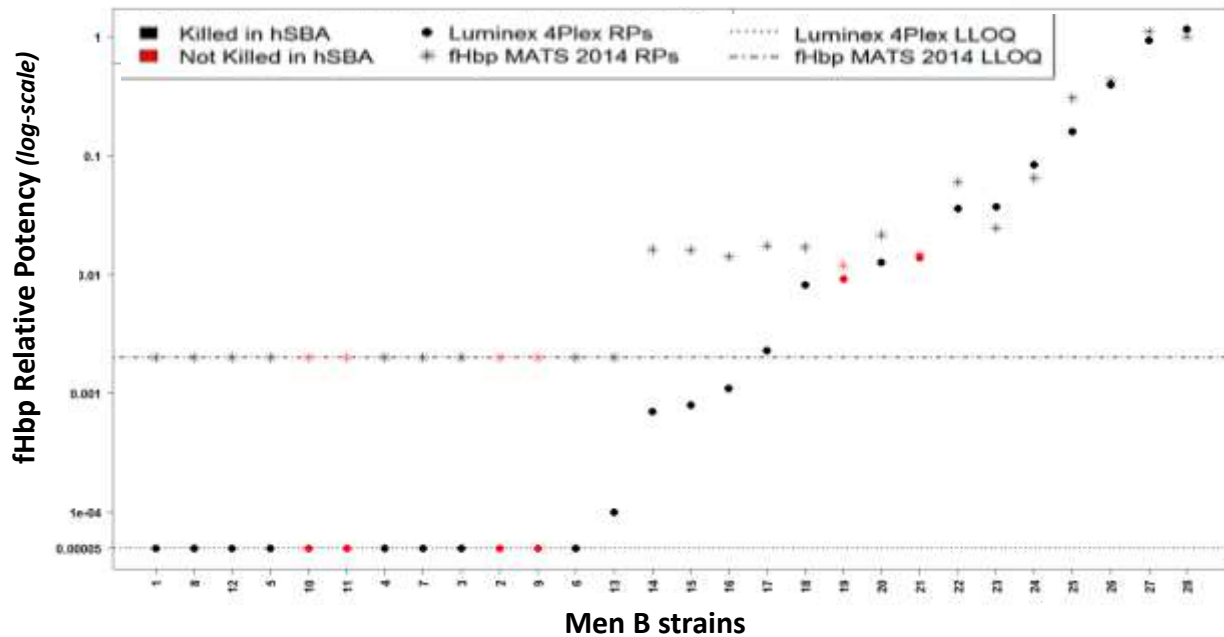
fHbp-RPs rank comparison (MATS-ELISA 2009)

Panel 18. RPs strain rank comparison between the two assays for fHbp MATS RPs calculated in 2009 and 2014 (data under publication). Strains tested (from 1 to 28) and Luminex RPs are reported on x and y-axis, respectively. In each graph, black and red dots represent the RPs of strains killed not killed in hSBA, respectively. Stars are MATS RPs and dots represent Luminex RPs. Dotted and dot-dashed lines are the Luminex and MATS LLOQ, respectively

7. DISCUSSION AND FUTURE PERSPECTIVES

Invasive meningococcal disease (IMD) is caused by invasion of the bacterium *Neisseria meningitidis* into the blood stream and subsequent development of septic shock and purpura fulminans in a subset of patients. Meningitis is the consequence of bacterium access to the central nervous system. IMD is characterized by a death-rate of 5-15 % in developed countries, also in treated cases (Stephen and Pelton, 2016).

The disease incidence is highest in infants and young children, with a second smaller peak in adolescents and young adults and varies by geographic region ranging from <.5 to .9 cases per 100,000 population in North America and Europe to 10 to 1,000 cases per 100,000 population in the African meningitis belt (Stephen and Pelton, 2016).

Bexsero[TM] vaccine against serogroup B meningococci (4CMenB) was registered first in Europe in January 2013; its composition is based on three Neisseria recombinant antigens, namely factor H binding protein (fHbp), Neisseria Heparin Binding Antigen (NHBA) and Neisseria adhesin A (NadA), combined with Outer Membrane Vesicles (OMVs) from MenB strain NZ98/254 containing porin A (PorA) serosubtype P1.4. 4CMenB vaccine components are present across most strains and are able to elicit bactericidal antibodies conferring protective immune responses (Pizza, et. al. 2000; Holst, et al. 2005, Wedege, et al. 2007).

In contrast to homogeneous and highly conserved polysaccharides, surface proteins are heterogeneous and it complicates the development of protein-based meningococcal vaccines (Giuliani, et al. 2010). Effectiveness measurement of these vaccines is assessed by the Serum Bactericidal Assay (SBA), the only accepted correlate of protection against meningococcal disease, against many different strains for each geographic region. Actually, this approach is impractical, especially for infants, whose serum volumes are very limited.

For this purpose, Meningococcal Antigen Typing System (MATS) was developed in 2010 (Donnelly et al., 2010). MATS is a combination of three modified sandwich Enzyme-Linked Immunosorbent Assays (ELISA) that quantify on bacterial lysates both protein content and level of matching with the corresponding antigen present in the vaccine (fHbp, NHBA, and NadA). In addition, PorA serosubtype is identified by PCR genotyping by amplification of variable region 2.

In 2013, it has been shown that MATS is a conservative predictor of Bexsero[TM] strain coverage in infants and adolescents since, vaccine coverage results underestimated as per comparison of MATS prediction with hSBA titers (Frosi, et al. 2013). In addition, conventional ELISA makes immunogenicity evaluation of a multi-component vaccine laborious, time-consuming and expensive, since only one immunogen per assay run can be tested.

In the last 20 years, microspheres-based multiplex immunoassays have been developed to identify infectious microorganisms and they represent a polyvalent approach which is able to unravel the complexity of bio-molecular interactions.

The simultaneous measurement of multiple analytes (multi analyte profiling, xMAP) is attractive for many reasons. The possibility to test multiple analytes starting from the same sample leads to a considerable material saving, especially given that samples (e.g. sera) are often available in limited quantity. As consequence, samples volume, experimental times and workforce could be significantly decreased. Moreover, multi-analyte profiles can be obtained by combining relevant targets that are usually evaluated by separate methodologies (e.g. ELISA).

Currently, multiplex assays based on microspheres are better applicable to traditional arrays (from 2 to 100 analytes) performed in liquid conditions, since they provide the best kinetic characteristics compared to ELISA planar arrays or glass chip. Multiplex liquid assays allow extremely fast interactions in high binding affinity reactions, and they can be easily modified by adding or removing microspheres population (one analyte=one microspheres set). In addition, laser detection is precise and consequently, Luminex Technology allows a good signal to noise ratio due to a minimum contribution of suspension buffer or sample matrix.

For these reasons and, taking into account a possible modification of the current Bexsero[TM] formulation according to epidemiological needs, this work was focused on the possibility to switch from ELISA to the xMAP Luminex Technology in order to simultaneously quantify each vaccine antigen) and eliminate PorA sequencing adding information about whole (not only PorA-related) OMVs-contribute to Bexsero[TM]-induced immune response.

The first attempt was based on transposition of the MATS Format, characterized by the same Ag-specific polyclonal rabbit Abs for both detection and capture steps, into the Luminex setting. Presumably, due to the liquid kinetics that amplifies the competition between the two Abs, no optimal signal, in terms of linearity and sensitivity, was observed.

Therefore, a different format (NEW Format), showing a better assay performance and based on usage of different species pAbs for capture and detection steps, was selected.

A 4-plex sandwich immunoassay was set up, optimized and qualified by using the New Format, in order to predict the coverage of Bexsero[TM] vaccine. The assay consists in a single multiplex sandwich test which simultaneously measures the four vaccine Ag content (fHbp, NadA, NHBA and OMVs) in Men B isolates. Vaccine Ags are captured from the bacterial lysates by mouse pAb-coupled beads and, the resulting complex, reacts with an Ag-specific rabbit pAb detected by a PE-labeled anti-rabbit Ab.

For this purpose, 28 serogroup B strains were selected out of the 57-strains panel chosen by Donnelly et al. in 2010 to evaluate the performance of MATS (in turn a subset of a 124-strains panel tested by hSBA using pooled sera from 141 infants who had received either three Bexsero[TM] immunizations or three immunizations plus one boost dose). This panel covers a wide range of responses for each of the three antigens and was tested at least twice, on different days, to evaluate assay performance (LLOQ, specificity, sensitivity, intra/inter-assay repeatability).

For each vaccine antigen statistical analysis on Luminex and MATS RPs dataset was performed by evaluation of the Spearman's correlation coefficient, demonstrating a statistically significant correlation between the Luminex 4-plex assay and MATS-ELISA relative potencies (NadA: Spearman's coefficient=0.99, p-value=0.0003; NHBA: Spearman's coefficient=0.7, p-value=0.0002; fHbp (2014): Spearman's coefficient=0.85, p-value<0.0001; fHbp (2009): Spearman's coefficient=0.62, p-value<0.05, see section 6.9 in the 'Results' chapter). As consequence, it is possible to speculate that Luminex 4-plex assay has a close correlation with the bactericidal titers as well.

A critical factor that endorses a high added value to Luminex 4-plex assay compared to MATS, is the quantification, for the first time, of overall OMVs component (not only PorA-correlated) contribution to Bexsero[TM]-induced immune response. This demonstrates a major finding that may reduce the MATS underestimation.

As far as the NHBA antigen is concerned, assay sensitivity resulted in underestimation since the LLOQ calculated for Luminex was quite conservative, probably due to the composition of the selected panel. In fact, the original Donnelly's 57-strains panel, from which our 28 strains were extracted, did not include a sufficient number of strains with low NHBA expression.

Before validation of Luminex 4-plex assay following the European immunoassay method validation's guidelines (European Medicine Agency, 2011), optimization of sensitivity assessment for each antigen, by testing a statistically significant number of strains expressing different levels of vaccine Ags would be needed.

Expanding the panel to be tested, it will be possible to evaluate the relationship between Luminex 4-plex assay and Bexsero[TM] coverage by calculating the Positive Bactericidal Threshold (PBT: minimum amount of antigen needed for bacterial killing) for this new assay.

A validated Luminex 4-plex assay would allow a fast and easy tracking of spatial and temporal changes in MenB epidemiology and their implications on 4CMenB coverage. Moreover, implementation of Bexsero[TM] could potentially modify the population structure of *N. meningitidis*, increasing the proportion of strains not covered by the vaccine probably, thus requiring additional surveillance efforts.

A possible implementation of the Luminex 4-plex assay would be testing whole inactivated bacterial samples, thus closer mimicking real conditions occurring in nature.

Last, the Luminex 4-plex assay is highly flexible and easy to be modified for application to different targets, such as a second generation MenB vaccine or other *N. meningitidis* serogroups. In fact, since the genetic diversity of other serogroups is substantially lower compared to B one and, Bexsero[TM] coverage on other serogroups carrying the vaccine antigens was demonstrated (Masignani, 2014), the establishment of a PBTs for each of them should be easily calculable. The analysis of non B serogroup meningococci by Luminex 4-plex assay will provide additional information on Bexsero[TM] potentialities and possible resulting effects on *N. meningitidis* epidemiology.

Moreover, reverse vaccinology has introduced the use of high-throughput technologies into vaccine research, affecting and improving vaccine antigen identification efficiency, development process, sample screening and vaccine coverage estimation (Donati and Rappuoli, 2013). Therefore, vaccine development based on reverse vaccinology could lead to the production of multi-component vaccines with broad protection and, immunoassays based on Luminex technology can be easily adapted in order to estimate the coverage of complex multi-component vaccines.

8. REFERENCES

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