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NEISSERIA MENINGITIDIS SEROGROUP B CARRIAGE BY ADOLESCENTS AND YOUNG ADULTS LIVING IN MILAN, ITALY: GENETIC CHARACTERISTICS AND POTENTIAL CORRELATION WITH PRESENTLY AVAILABLE MENB VACCINE.

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1. Introduction to vaccinology.

1.1 Historical background.

The use of immunization to protect subjects from disease dates back hundreds of years.

Vaccination was tested at the end of the 18th century by Edward Jenner, considered the founder of vaccinology in the West in 1796. He demonstrated immunity to smallpox[1] by inoculation a 13 year-old-boy with vaccinia virus (cowpox). Subsequently, the smallpox vaccine was developed and a systematic vaccination campaign culminated in its global eradication in 1979 (Fig.1).

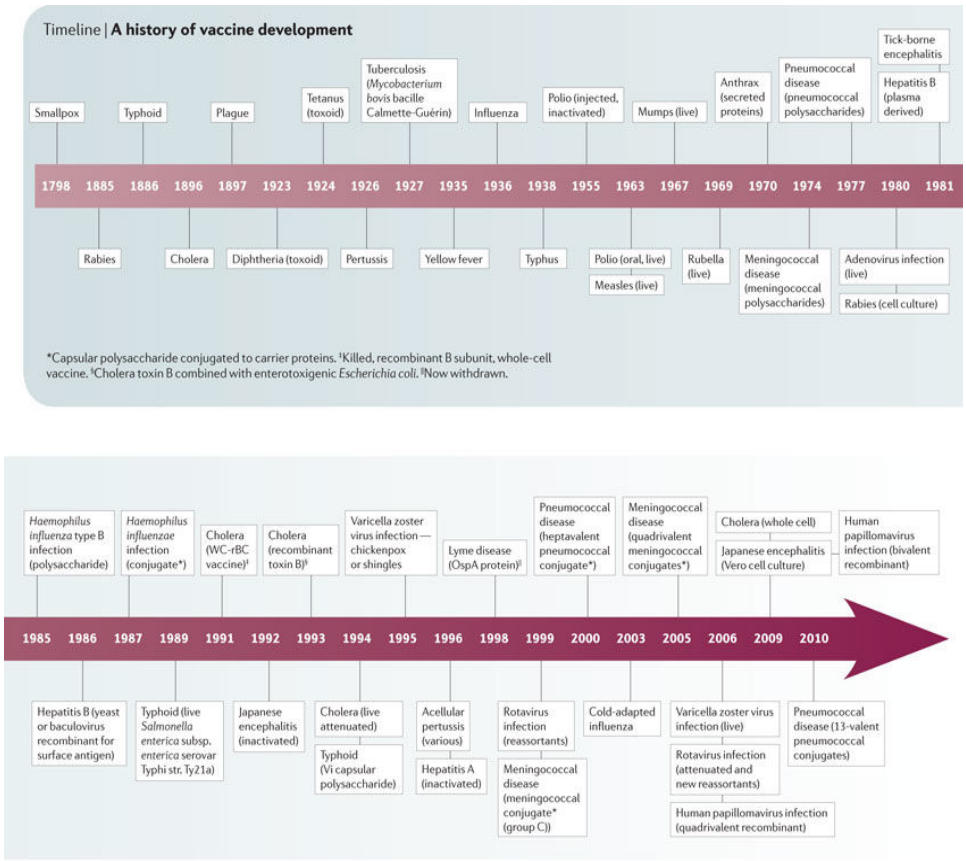

Fig.1: The timeline of vaccine development until 2010.

A century later, Louis Pasteur developed methods for attenuating bacteria, he produced live attenuated cholera vaccine[2] and inactivated

anthrax vaccine in humans. All these advances have opened the era of vaccinology. In the late 19th century bacterial vaccine development proliferated, plague vaccine and the bacillus-calmette-guerin (BCG) vaccine (which is still in use today) was created.

Salmon and Smith developed methods for inactivation of microorganisms[3]. The inactivation method with formaldehyde was used to develop a vaccine against diphtheria in 1926.

Viral tissue culture methods led to the advent of the Salk (inactivated) polio vaccine and the Sabin (live attenuated oral) polio vaccine[4]. Mass polio immunization has greatly reduced the disease caused by wild-type virus from thousands of cases per year to only twenty-two cases recorded during 2017[5].

Attenuated strains of mumps, measles, and rubella were developed for addition in vaccines. Measles is actually the next possible target for eradication via vaccination.

New technologies such as the conjugation of proteins to capsular polysaccharides and recombinant DNA methods have allowed the development of vaccines against bacterial pneumonia, meningitis, hepatitis B and recently the vaccine against human papillomavirus (HPV)[6-7].

Molecular genetics sets nowadays the condition for a bright future for vaccinology, including the development of new vaccine delivery systems (e.g. viral vectors, DNA vaccines and plant vaccines), new adjuvants, the development of more effective tuberculosis vaccines, and vaccines against cytomegalovirus (CMV), herpes simplex virus (HSV), respiratory syncytial virus (RSV), pandemic influenza, staphylococcal disease, streptococcal disease, Shigella, schistosomiasis and HIV among others. Therapeutic vaccines may

also soon be available for autoimmune diseases, allergies and addictions.

The list of illnesses that can be avoided through the administration of vaccines is far from being complete, but despite this, there is no doubt that vaccines play an important role in reducing mortality and morbidity of the most common infectious diseases among which meningitis, diphtheria, pertussis, tetanus, measles, mumps, rubella, hepatitis B and others[8].

1.2 The immune system and biological basics of vaccinations.

The immune system can be divided into two main subsystems, the innate system and the adaptive system (Fig. 2). Both interact with each other to provide an effective immune response.

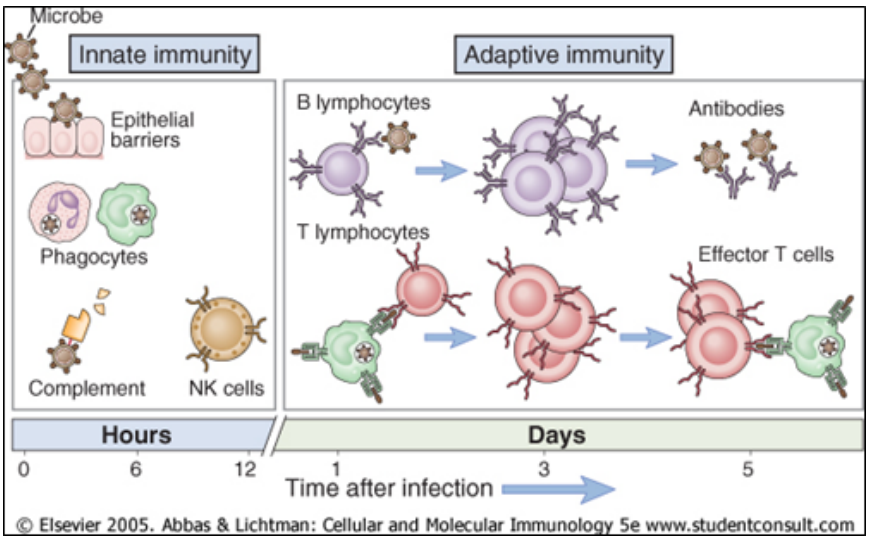

Fig. 2. Innate and adaptive immunity scheme.

The innate immune system is characterized by mechanisms which provide a first line of defense against pathogenic agents. These are not specific to a particular bacteria, the innate immune cells such as leukocyte, phagocytes (neutrophils and macrophages), natural killer (NK) are specific for conserved molecular patterns found on all microorganisms called PAMPs (Pathogen Associated Molecular

Patterns) recognized by Pattern recognition receptors (PRRs). This prevents the innate immune system from recognizing host cells and attacking them. However, the innate immune system responds identically to repeated infections from a pathogen, the system does not have memory[9].

The protective defenses of the innate immune system begin with the anatomic barriers such as skin and mucous membranes which prevent the entrance of many microorganisms. The skin has an acidic environment of pH 3-5 which retards the growth of microorganisms and a normal flora which compete with other microorganisms for nutrients and attachment sites. Further, the mucus and cilia on the mucous membranes aid in trapping microorganisms and propelling them out of the body[9]. The innate immune system include physiologic barriers as fever, gastric acidity, interferon, lysozyme and collectins. The complement pathways and the inflammatory response are another essential parts of the innate immune system.

The actions of the adaptive immune system, in contrast to the innate system, are specific to the particular pathogenic agent. This response will take longer to occur but the adaptive immune system has memory. This means that the adaptive immune system will respond more rapidly to that particular pathogen with each successive exposure acquiring a better defensive potential[9].

The adaptive immune system is composed of humoral and cell-mediated immunity, with the B–cells and the T-cells respectively.

The humoral immunity fight against extracellular pathogenic agents and toxins. B–cells can recognize antigens in their native form, these are called T-independent antigens because T-cell activation is not required to activate the B–cells. Examples of these antigens are

lipopolysaccharide, dextran, and bacterial polymeric flagellin. In contrast, activation of B–cells with T-helper cell activation results in a much better immune response and more effective memory. This long-term immune response is the type of reaction that is the objective of immunizations.

IgM is the first antibody produced and is a much larger antibody, IgG is a better neutralizing antibody, it binds more effectively to the antigen and aids in opsonization.

Other antibodies can be produced by plasma cells include IgD, IgA, and IgE. IgD is primarily found as a receptor bound to the surfaces of mature B–cells, IgA is found in secretions and IgE is involved in allergic reactions and parasitic infections.

On the other arm of adaptive immunity, cell-mediated immunity functions primarily against intracellular pathogens. T-cells develop in the thymus and are then, when mature, were released into the bloodstream. There are two main types of T-cells, CD4 cells and CD8 cells.

CD4 cells or T-helper cells recognize the major histocompatibility complex (MHC) II protein and are essential for antibody-mediated immunity and in helping B–cells control extracellular pathogens. There are two subsets of CD4 cells, Th1 and Th2: Th1 cells help promote cell-mediated immunity; Th2 cells help promote antibody-mediated immunity.

CD8 cells or T-cytotoxic cells recognize the major histocompatibility complex (MHC) I protein, are essential for cell-mediated immunity and in helping control of intracellular pathogens.

T-cells can recognize an antigen that has been processed and presented them by antigen-presenting cells. There are two types of antigen processing.

The first involves attaching intracellular antigens along with MHC I proteins to the surface of antigen-processing cells. This occurs with viral antigens and tumor cells.

The other type of antigen processing involves attaching extracellular antigens along with MHC II proteins to the surface of antigen-presenting cells. This occurs with bacterial and parasitic antigens.

1.3 Types of immunization.

Immunization can be derived from either passive or active means (Fig. 3). These means that can be from natural or artificial sources.

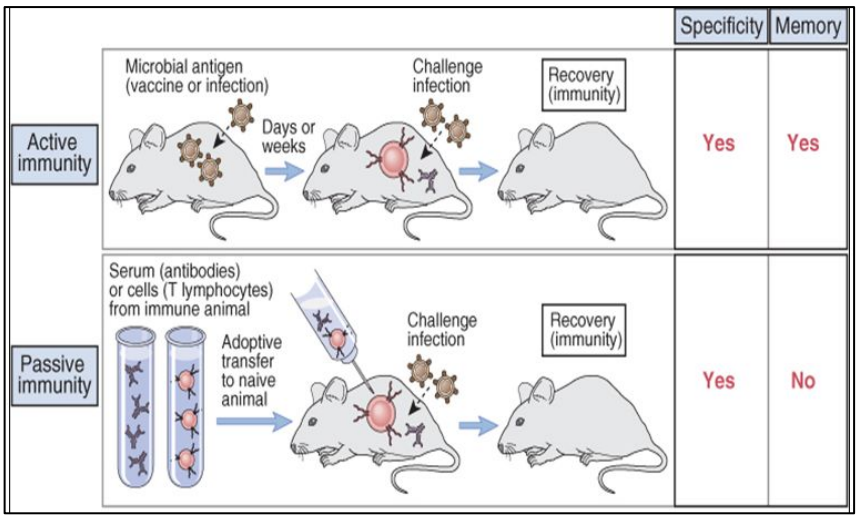

Fig. 3: Active and passive immunity.

Natural sources are due to exposure to the environment, humans, and animals followed by acquisition and natural eliminations of pathogens. In contrast, artificial sources are due to medical interventions such as vaccination or antibody inoculation. Vaccination is configured as active immunization and has the aim of artificially evoking, following the administration of antigens, an immune response against the components injected without the manifestation of the characteristic symptoms of the disease. This ensures durable protection but sometimes requires a latency time of around three

weeks before it becomes effective, for this reason, the vaccines are used for prophylactic purposes on healthy individuals.

Serotherapy occurs with the transfer to preformed antibodies to an unimmunized individual. This subjects so develop a temporary immunity to a particular organism or toxin.
Passive immunization can occur either naturally or artificially. Excellent examples of natural passive immunization are the passage of maternal antibodies through the placenta to the fetus and the passage of these maternal antibodies to the infant through the colostrum and milk.
Excellent examples of artificial passive immunization include the administration of pooled human immune gamma globulin and antivenin that provide temporary immunity.

Vaccinations have a dual objective, the defense of the subject and also the protection of the community. The herd immunity is reached when the percentage of vaccinated is sufficiently high to prevent the spread of the pathogen and to guarantee the protection also of the unvaccinated subjects.

1.4 Stimulation of immunity by vaccines.

The initial identification of pathogen typically is done by the innate immune system; although, B-cells may also perform this function. This detection starts when the immune system recognizes epitopes on antigens.
Many components of the innate immune system will then activate to respond to this challenge. These components will opsonize or bind to the agent and aid in its engulfment by antigen-presenting cells such as macrophages or monocytes. These antigen-presenting cell will then

process the antigens from this pathogenic agent and insert the processed antigen along with the MHC protein onto the surface on the antigen-presenting cell.

If it is a viral antigen, this will be bound with MHC I protein and presented by the antigen-presenting cell to a CD8 cell which will trigger cell-mediated immunity. If it is a bacterial or parasitic antigen, the antigen will be bound with MHC II protein and presented by the antigen-presenting cell to a CD4 cell which will trigger antibody-mediated immunity.

1.5 Types of vaccines.

Vaccines are made using several different processes and according to their constitution, are classified into categories. They may contain live viruses that have been attenuated, inactivated or killed organisms or viruses, inactivated toxins, or merely segments of the pathogen (this includes both subunit and conjugate vaccines).

a. Live attenuated vaccines.

The most common methods involve the culture of the virus through a series of cell cultures or animal embryos (typically chick embryos). With each passage, the virus loses partially its ability to replicate in human cells and acquire a better replicating capacity in chicken cells.
When the resulting vaccine is given to a human, it will be unable to replicate enough to cause illness, but will still provoke an immune response that can protect against future infection.
One concern that must be considered is the potential for the vaccine virus to revert to a form capable of causing disease, mutations that can occur when the vaccine virus replicates.

b. Killed or inactivated vaccines.

Vaccines of this type are created by inactivating a pathogen, using heat or chemicals such as formaldehyde or formalin. These destroy the pathogen's ability to replicate but keeps it "intact" so that the immune system can still recognize it.

Because killed or inactivated pathogens can't replicate, they can't revert into a virulent form capable of causing disease. However, they provide a shorter length of protection than live vaccines and are more likely to require boosters to create long-term immunity. Killed or inactivated vaccines include the inactivated polio vaccine and the seasonal influenza vaccine.

c. Toxoids.

Some bacterial diseases are not directly caused by bacterium itself, but by the toxin produced. One example is tetanus: symptoms are caused by the neurotoxin produced by *Clostridium tetani.* Immunizations can be made by inactivating the toxin that causes disease. As with organisms or viruses used in killed or inactivated vaccines, this can be done via treatment with a chemical such as formalin, or by using heat or other methods.

Immunizations created using inactivated toxins are called *toxoids*.

Toxoid immunizations include tetanus and diphtheria immunizations, which are available in a combined form.

d. Subunit and conjugate vaccines.

Subunit vaccines use a part of a target pathogen to provoke a response from the immune system. This may be done using a specific protein isolating from a pathogen and presenting it as an antigen. The acellular pertussis vaccine, the polysaccharide meningococcal A, C,

W135 and Y vaccine and the influenza vaccine are examples of subunit vaccines.

Subunit vaccine can be created using genetic engineering. A gene coding for a vaccine protein can be inserted into virus or producer cells in culture and then will be harvested and used in the vaccine.

A vaccine made using genetic engineering is the human papillomavirus (HPV) vaccine.

Conjugate vaccines are similar to recombinant vaccines: they are made using a combination of two different components. Conjugate vaccines are made using pieces from bacteria. These are chemically linked to a carrier protein, and the combination is used as a vaccine (Fig. 4).

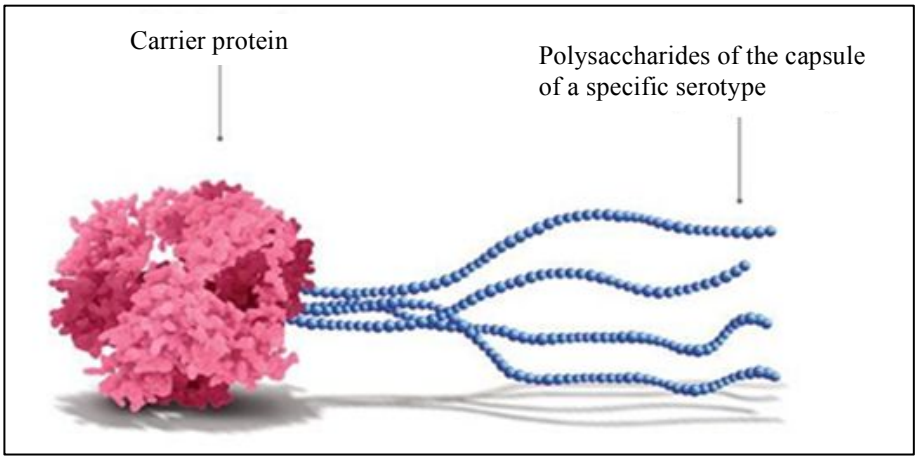

Fig. 4: Example of protein conjugation to a capsule polysaccharides.

Conjugate vaccines are used to induce in the subject a more powerful, combined immune response. The vaccines currently in use for children against *Streptococcus pneumoniae* infection are made using this method.

1.6 Italian national vaccine prevention plan 2017-2019.

Italian national vaccine prevention plan and the related vaccination calendar have the primary purpose of guaranteeing to the population,

regardless of their place of residence, income and socio-cultural level, the full benefits derived from vaccination, intended both as an instrument of individual protection and collective prevention. In addition, citizens who fall into target vaccination categories can use it for free.

The objectives identified by the national vaccination prevention plan 2017-2019 are:

1. Maintain polio-free status
2. Reach the free status for measles and rubella
3. Guarantee the active and free vaccination offer in the indicated age groups and in the population considered at risk
4. Increase the conscious adhesion to vaccination
5. Fight against inequalities, promoting vaccination intervention in marginalized groups of the population
6. Complete the computerization of the vaccine register at regional and national level
7. Improve the surveillance of preventable diseases by vaccination
8. Promote in population and in health professionals a vaccination culture consistent with the principles of the Italian national vaccine prevention plan
9. Support the sense of responsibility of health professionals and full adherence to the prevention plan providing adequate sanctions if a behavior of non-compliance is identified

In addition to vaccinations for which, for years, national immunization programs have been planned (diphtheria, tetanus, polio, hepatitis B, Haemophilus influenzae b, whooping cough, pneumococcus, measles, mumps, rubella, meningococcus C in newborns, HPV in 11-year old girls and flu in subjects aged ≥65 years) the national vaccination

prevention plan 2017-2019 (Fig. 5) includes in the vaccination calendar the meningococcal B, rotavirus and varicella vaccinations in newborns, HPV in 11-years old males, the tetravalent ACYW135 meningococcal vaccine and the polio booster with IPV in adolescents and pneumococcal (PCV13 conjugate or PPV23 polysaccharide) and herpes zoster vaccination in 65 years.

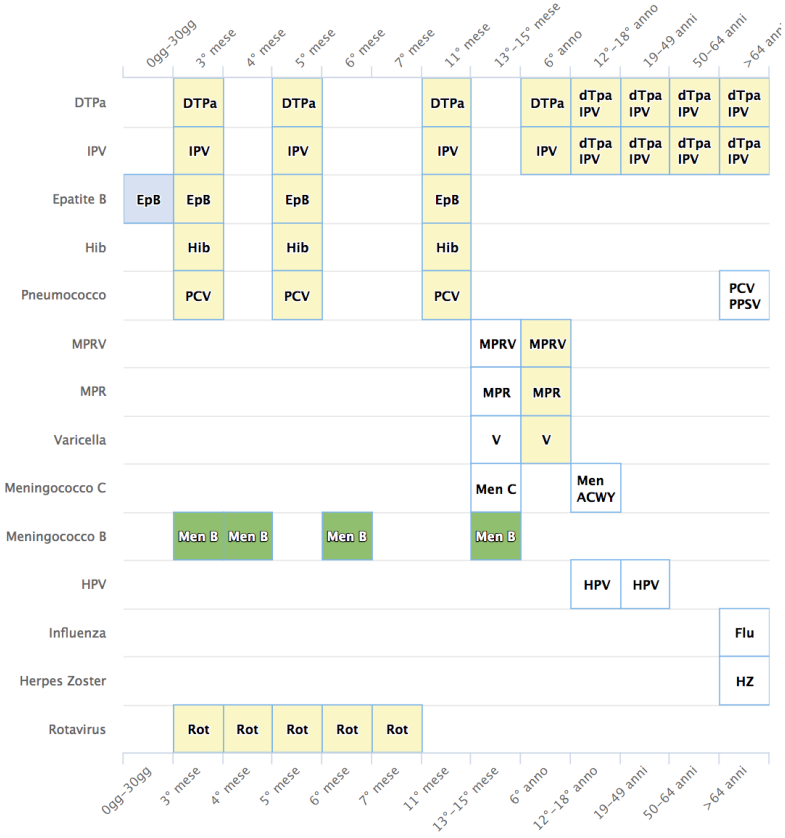

Fig. 5: Italian vaccination calendar plan 2017-2019.

2. The genus Neisseriae.

The genus Neisseriae includes many species, among these there are *Neisseria lactamica*, *Neisseria mucosa*, *Neisseria sicca*, *Neisseria flava*, *Neisseria subflava*, nonpathogenic commensals of the upper respiratory tract and two pathogenic species: *Neisseria gonorrhoeae* and *Neisseria meningitidis* identified respectively by Albert Neisser (1879) and Albert Weichselbaum (1887).

Neisseria gonorrhoeae is the etiologic agent of gonorrhea, sexually transmitted disease, this is one of the most common contagious bacterial illness in humans.

Neisseria meningitidis exclusively infects humans, is frequently found in the nasopharyngeal tract where causes asymptomatic infections similar to a colonization of non-pathogenic species of Neisseria (about twenty percent are healthy carriers)[10].

The levels of colonization are different, especially in relation to age, with a peak in adolescence, the incidence of the meningococcal disease depends on the geographical area, can reach up to 1000 cases per 100,000 per year[11].

In the establishment of the carrier state, the adhesion of the microorganism to the mucous membranes of the airways is essential; in particular conditions, especially susceptible individuals, the bacterium spreads in the organism causing serious diseases such as meningococcal meningitis, bacteremia and septic arthritis that leads to death in ten to fifteen percent of cases or cause permanent disability up to twenty percent of affected[12]. The causative agents responsible for the transition from the commensal to the pathogenic state are not yet completely clarified, it is thought that the expression of virulence factors is involved. In fact, some of these genes are not present, not expressed or will be expressed differently in strains that do not cause

pathology. The pathogenic potential can, therefore, be related to the presence of pathogen-specific sequences.

2.1 Neisseria meningitidis: characteristics and epidemiology.

Neisseria meningitidis is a gram-negative bacterium, asporogenous and provided with pili, often cells are found in pairs (diplococci) with a diameter of about 0.6-1 μm (Fig. 6).

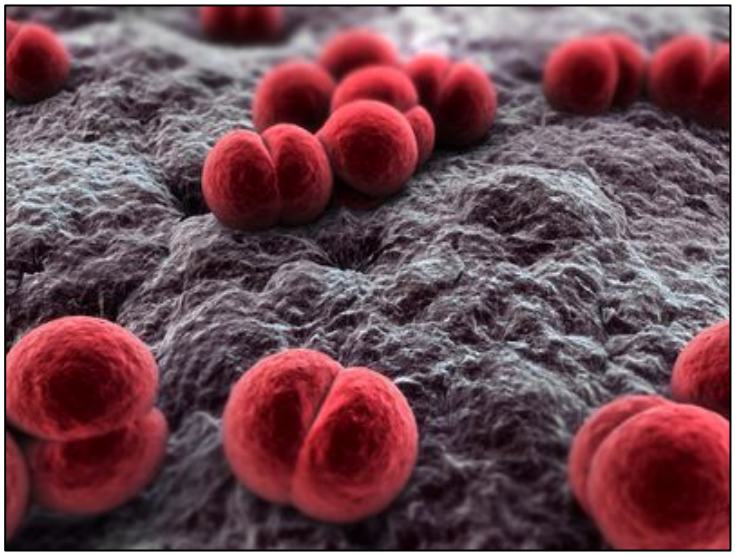

Fig. 6: *Neisseria meningitidis* scanning electron microscope.

Meningococcus grows in an aerobic environment at five to ten percent of CO2 and at temperatures between thirty-five and thirty-seven degrees centigrade, produces the cytochrome C which is responsible for the positivity to the oxidase test. The various *Neisseriae* strains diverge according to the different ability to use simple or a mixture of sugars as sources of energy: meningococci ferment both maltose and glucose while gonococci exclusively glucose. For cultural isolation, enriched soils such as agar-chocolate and GC agar (GonoCoccal Agar) are used.

Compared to all the Neisseriae strains, meningococci have a polysaccharide capsule and, according to the different antigens presented, are divided into thirteen serogroups (A, B, C, D, 29E, H, I, K, L, W135, X, Y, Z). These colonize the nasopharynx but most

invasive infections, up to ninety-five percent of the total, are caused by the strains belonging to serogroups A, B, C, X, Y and W-135[13].
Serogroups can be further subdivided into twenty serotypes (based on PorB protein antigens), in ten serum subtypes (determined by PorA antigens) and in immunotypes based on the immunological properties of the outer membrane proteins and of the lipo-oligosaccharide . The capsule, composed mainly of polysaccharides, is essential for the survival of the bacterium in the bloodstream allowing to resist the killing mediated by antibodies or the complement system, to evade phagocytosis and to mask the outer membrane proteins, a relevant target of the immune response

The transmission of bacterium occurs through the inhalation of infected oropharyngeal drops and the main reservoir is represented by carriers, the disease is rarely transmitted by a sick subject. Indeed, colonization, even chronic, rarely progresses until the disease, because specific antibodies against the colonizing strain are produced in the carrier. The disease, on the other hand, is manifested within a few days of acquisition of a new meningococcus strain, since immune system has not yet been able to produce a sufficient amount of neutralizing antibodies. The incidence of the disease is higher in children aged between six months and one year and in adolescents between fourteen and twenty years. Infections can occur both as isolated cases and in form of epidemic outbreaks that take place mainly in poor countries in which health facilities are not adequate to treat the disease.

The serogroup A meningococci are the main cause of the epidemics described in Africa, China, Nepal, where the disease is endemic; the B and C strains predominate in industrialized countries such as the United States, Europe and Australia (Fig. 7).

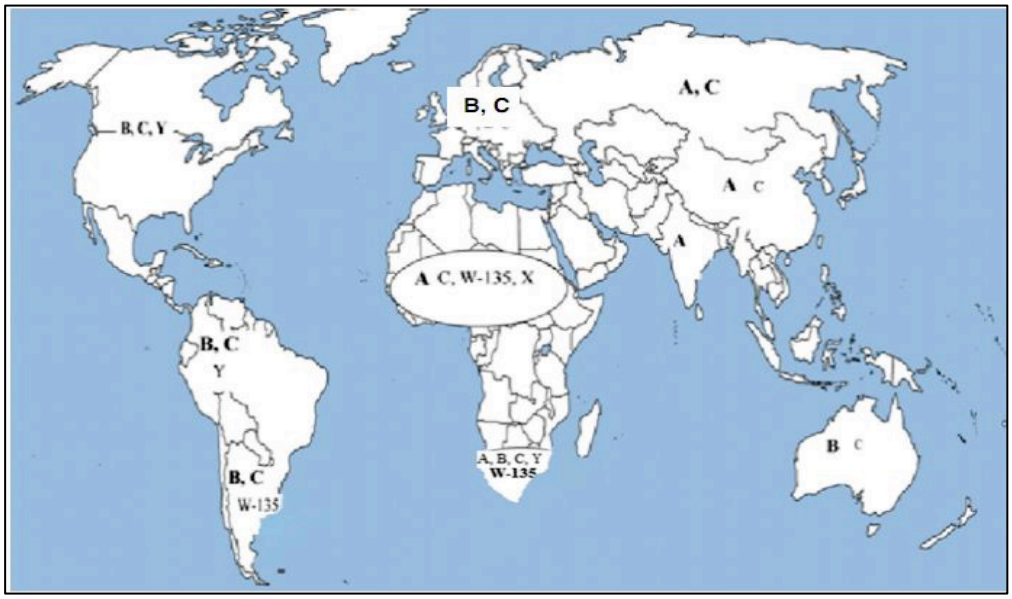

Fig. 7: Epidemiology of meningococcal serogroups in the world.

In Italy, we have an increased incidence of meningococcal disease, 232 cases of invasive meningococcal disease (IMD) were reported in 2016 with an incidence of 0.38 cases per 100,000 (0.23 in 2012, 0.29 in 2013, 0.27 in 2014 and 0.31 in 2015). An increase of meningococcus C infections was observed in Tuscany in 2015-2016, such that an extraordinary vaccination campaign was carried out. The incidence of the disease is highest in the age group from zero to four years with a peak in the first year of life (four cases per one hundred thousand) and remains high until age between fifteen and twenty-four years (0.3 per 100,000 in 2014 and 0.9 per 100,000 in 2016), decreases from the age of twenty-five. Considering the period from 2011 to 2016 about fifty-three percent of cases manifested as sepsis or meningitis/sepsis.

Examining the spread of serogroups, the B strain is the one most frequently identified until 2014 (65%, 51%, 48%, 48% for the years 2011, 2012, 2013 and 2014, respectively), until 2016 the predominant serogroup has become the C strain whereas the data for 2017 and 2018 show a greater number of cases from B strain.

The development of meningococcal disease depends on the host's immune status. Immunity is usually type-specific and develops within the first two decades of life. Meningococcal antibodies, therefore, are present in most cases and play an important role in the prevention of meningococcal meningitis whose invasive form occurs almost exclusively in subjects that do not possess protective levels of bactericidal antibodies against the invasive strain.

If the subject presents signs and symptoms of meningococcal disease, the immediate activation of the therapies is necessary; penicillin G, chloramphenicol and third-generation cephalosporins are among the most effective drugs used to fight the pathogen.

2.2 Features of the available meningococcal vaccines.

To date, several vaccines are available for the preventive treatment of meningococcal infection. These can be classified into following categories.

a. Polysaccharide-based vaccines.

Polysaccharide vaccines are composed of purified capsular polysaccharides obtained directly from the serogroups of the pathogens of interest. The currently administered meningococcal vaccine are quadrivalent, these contain capsular polysaccharide derived from serogroups A, C, W and Y. Monovalent (for figthing serogroups A and C) and trivalent (for serogroups A, C and W) vaccines are no longer used. Mencevax (GlaxoSmithKline) is licensed for use in Europe whereas Menomune (Sanofi Pasteur) is licensed for use in the USA and Canada. Conjugate vaccines elicit longer-lasting immune responses.

b. Glycoconjugate vaccines.

Carbohydrate-based glycoconjugate vaccines use bacterial capsular sugars linked to a carrier protein. Three types of carrier proteins have been used in vaccines against *Neisseria meningitidis*: diphtheria toxoid (DT), a non-toxic mutant of diphtheria toxoid with a 197 amino acid substitution (CRM197), and tetanus toxoid (TT). All of these carrier proteins are inactivated and are able to induce B cells and T cell-dependent immune responses leading to immune memory.

c. Monovalent conjugate vaccines.

There are three commercially available monovalent conjugate vaccines licensed for N. meningitidis serogroup C. Two vaccines, Meningtec from Pfizer and Menjugate from GlaxoSmithKline, use CRM197 as a carrier protein, while the other NeisVac-C by Pfizer uses TT. These three vaccines are effective in infants 2 months and younger.

d. Quadrivalent conjugate vaccines.

There are three licensed quadrivalent vaccines: Menveo (GlaxoSmithKline, Brentford, UK) contains CRM197 as a carrier protein, Menactra (Sanofi-Pasteur) is a conjugate vaccine of DT with and Nimenrix (Pfizer, New York, NY, USA) is a conjugate containing TT.

e. Outer membrane vesicles-based and protein-based vaccines.

Production of a vaccine against serogroup B has encountered some difficulty due to self-antigen concerns. Capsular polysaccharide from this serogroup is comprised of α 2-8 linked sialic acid, the same linkage of polysialic acid found on the mammalian neural cell adhesion molecule.

OMVs are naturally vesicles released by gram-negative bacteria.

They contain phospholipids, lipooligosaccharides, and membrane proteins. VA-MENGOC-BC is an OMV-based vaccine against *N. meningitidis* serogroup B and C and it was first licensed for use in Cuba in 1987.

The proteins that could be used as a targets for serogroup B vaccination were discovered using the reverse vaccinology technique. Reverse vaccinology essentially starts with a genomic search for potential antigens and the use of recombinant DNA technology to produce and test these antigens for suitability (Fig. 8).

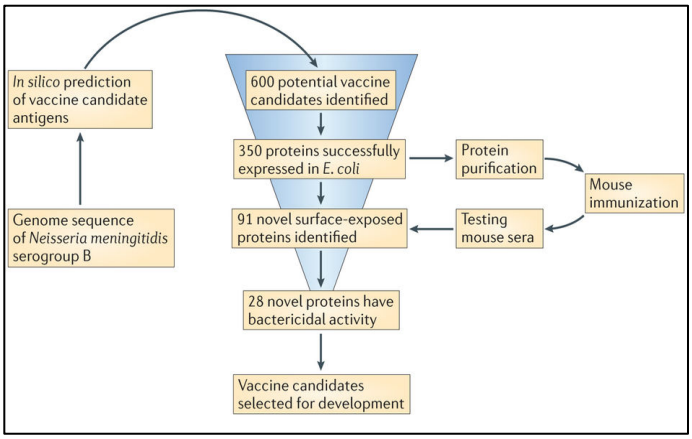

Fig. 8: Reverse vaccinology flowchart applied to the production of meningococcal B vaccine.

Vaccine development consists of two fundamental phases:

1. Characterization of the Neisseria meningitidis genome
2. Research for proteins that are common to all group B serotypes

The genomic sequence of the virulent strain *Neisseria meningitidis* MC58, determined by means of shotgun sequencing, revealed more than two thousand proteins produced by the bacterium. In the identification of potential vaccine antigens, proteins were analyzed using bioinformatics algorithms in order to predict their localization on the surface of the cell.

Once the proteins were selected, they were cloned and expressed in *E. coli*, purified and tested on mice to verify their potential ability to induce antibody formation. Finally, the serum bactericidal assay (SBA) was used to measure antibody activity in killing bacteria by activating the complement system.

Twenty-eight of the proteins tested were able to induce an antibody response, unfortunately, not all are present in different meningococcus strains. So, for the formulation of a vaccine, it was necessary to choose among the most widespread and which had more antigenic power. Among these, antigens selected for the vaccine allow broad-spectrum protection against different meningococcal strains. This ability was inferred via SBA or by the ability of antibodies to confer passive protection in mouse models.

This method has led to the licensing of Bexsero (GlaxoSmithKline) and Trumenba (Pfizer) vaccines. Bexsero vaccine contains OMV from NZ98/254 (an outbreak-specific strain), rNHBA (a recombinant Neisseria heparin binding antigen) fusion protein, rNadA (recombinant Neisseria adhesin A), rfHbp (a recombinant complement factor H binding protein). Instead, Trumenba is composed of two antigenic variants of rfHbp factors.
The reason for the choice of combining different antigens lies in the possibility of increasing the coverage spectrum, in order to minimize bacterial evasion and avoid the problem of possible selection of resistant mutants[16].

2.3 Bexsero and Trumenba components.

In detail, the final formulation of Bexsero®, launched on 14 January 2013, contains a mix of four main antigenic components:

- Factor H Binding Protein (fHBP) is also called GNA 1870, it binds the complement factor H increasing the ability of the microorganism to resist killing mediated by the complement system. The protein can be classified into three variants (fHBP-1, fHBP-2, fHBP-3) and further subdivided into sub-variants e.g. fHBP -1.x, fHBP -2.x and fHBP -3.x where x denotes the specific sub-variant. In a different nomenclature scheme, sub-variants are grouped into sub-families, A corresponds to variants 2 and 3 and B identifies variant 1. The degree of preservation within them ranges from ninety-two to one hundred percent, while between variants falls to sixty-three percent. This has a negative influence on the degree of cross-protection, a variant will induce a protective immune response against strains that have a similar variant while the response will be lower if the strain expresses different variants[14]. The sub-variant present in Bexsero® is fHBP 1.1 conjugated to the surface protein GNA2091.
- Neisseria Heparin-Binding Antigen (NHBA), also called GNA 2132, is a surface lipoprotein able to bind heparin in vitro (glycosaminoglycans such as heparan sulfate present in mucosal secretions in vivo) through a conserved region rich in arginine, this is ubiquitously expressed among meningococci. This capacity is related to the increased survival of non-capsulated strains in human serum and contributes to the interaction of meningococcus with the host cells. Much of the variability of protein is found in the amino-terminal region, whereas the carboxyl-terminal region is highly conserved. Bexsero® contains the variant NHBA peptide 2 (NHBA-2) conjugated to

the GNA1030 protein and evokes antibodies which are cross-reactive to the other sub-variants.

- Neisseria Adhesin A (NadA) is a protein involved in the adhesion of the bacterium and in the invasion through the epithelial cells of the mucosa, the gene is present in about thirty percent of the pathogenic strains and is absent in the strains carried by healthy subjects and in other neisseria species[15]. There are five variants of NadA (NadA-1,2,3,4,5), in the Bexsero® there is NadA-3 sub variant 8 that is able to induce cross reactive antibodies against NadA-1, NadA-2 and NadA-3.
- Outer membrane vesicles (OMVs) of the serogroup B strain NZ98 / 254, containing the outer membrane protein PorA P1.4. It plays a role in the evasion of the immune system and in the antibiotic resistance.

This vaccine is indicated for active immunization of subjects aged two months or more and contains: 50mg of recombinant NHBA protein, 50mg of recombinant NadA protein, 50mg of recombinant fHbp protein, 25mg of outer membrane vesicles strain NZ98/254 which contains PorA P1.4 adsorbed on 0,5mg of aluminum hydroxide (Fig. 9).

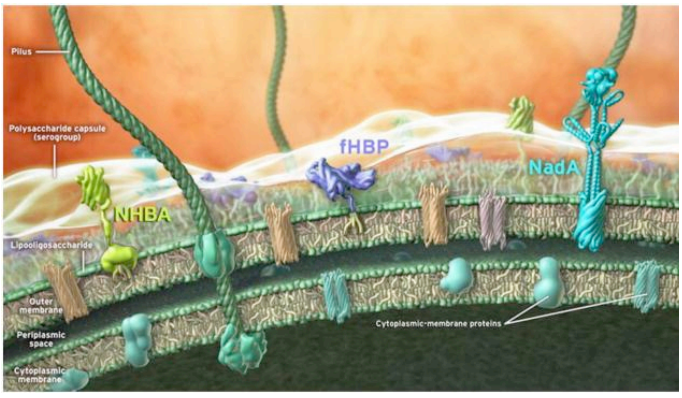

Fig. 9: Schematic representation of the Bexsero vaccine antigens.

Trumenba® contains two variants fHPB, one for each of the two sub-families A and B (A05 and B01 respectively). These proteins (also known as LP2086) are produced in *E. coli* and then isolated by purification. Each 0.5mL dose of vaccine contains 60µg of both fHBP (total 120µg), 0.018mg of polysorbate-80 and 0.25mg of Al3 + in pH6 buffer.

This vaccine is indicated for use on subjects aged ten to twenty-five years.

3. The aim of the study.

In this study we set different objectives, the first is to evaluate the degree of oropharyngeal colonization by meningococcus in adolescents in Milan, the second is to perform molecular typing to verify the presence of hypervirulent strains and the third is to analyze the proteins fHBP, NHBA, NadA and PorA present in strains of healthy carriers by comparing them to the sequences of the same protein contained in the two Bexsero® and Trumenba® vaccines in order to estimate the degree of identity.

For the study we assumed that children and adolescents are the most colonized: the first because have immunity defenses that are not yet fully developed, others because having repeated opportunities to live in close contact with their peers, increasing the risk of being infected.

Then, oropharyngeal swabs were collected from adolescents attending some schools in the Milan area, genomic DNA was extracted from them and analyzed by Real-Time PCR in order to identify *Neisseria meningitidis* and its serogroup.

Subsequently, swabs positive for meningococcus B were processed by Multilocus Sequence Typing (MLST) a system of genetic analysis based on the polymorphisms present in several constitutively expressed genes. Developed by Maiden in 1998 and today the gold standard of analysis[17] can also be used for the study of other bacteria. We proceed by amplifying the fragments of seven genes, obtaining a product of about 500 base pairs, these were sequenced on both strands to obtain the nucleotide sequence and were aligned with the reference by means of the online database (http://pubmlst.org/neisseria) allowing identification of the corresponding allele. The set of the seven alleles defines the allelic or sequence type (ST) profile of the

isolated strain. STs can then be further grouped into clonal complexes (CCs) that are defined as a group of STs that have at least four of seven loci in common with a central sequence type[18]. This method has shown that most of the isolates associated with disease have an exact ST and this has allowed to define those hypervirulent strains responsible for IMD.

Finally, the sequences encoding the proteins fHBP, NHBA, NadA and PorA were analyzed by amplification and sequencing and were compared to the sequences of the proteins contained in the two vaccines.

4. Materials and methods.

4.1 Collection of samples.

To perform the study, the posterior oropharynx secretions were collected from 2560 healthy adolescents aged between fourteen and twenty-one (907 males, 35.4%, median age 16.2 years) in the period January - March 2016 through the use of swabs Copan 482CE containing Amies liquid that maintains the vitality of microorganisms (Fig.10).

Fig. 10: Collection swabs with liquid transport.

Enrollment was voluntary, during the week preceding the swabbing students received a brochure providing information regarding meningococcal clinical relevance and purpose of the study. Moreover, during lesson time, science teachers reinforced the message.

Contemporaneously to the execution of throat swab in the medical room of the participating school, data regarding age and sex of participants were collected and in addition, each student was asked to fill a questionnaire to provide information on personal and family characteristics to be returned to teachers within a week.

This study was conducted in compliance with the ethical principles of the Helsinki Declaration within the guidelines of Good Clinical

Pratice. It was approved by the Ethical Committee of the Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico, Milan, Italy. Written informed consent was obtained from all subjects and from both parents of those under the age of eighteen.

4.2 Preparation and extraction of DNA from swabs.

In the laboratory, swabs were labeled with a unique code and subsequently were vortexed, centrifuged at 15,000g for 10 minutes to homogenate the solution and detach cells present in bristles of collection support. Then, 250 µL of amies liquid was incubated at 95 °C for 10 minutes to kill potentially pathogenic cells.

Finally, 200 µL of solution was used for extraction by means of High Pure PCR Template Preparation Kit (Roche, Cat. n°:11796828001) following the manufacturer's instructions. In detail:

1. Add 5µL of lysozyme to 200µL sample (10 mg/mL in 10nM Tris-HCl, pH 8) and incubate for 15 minute at 37 °C
2. Add 200µL of Binding Buffer and 40µL of proteinase K, shake and incubate for 10 minute at 70 °C
3. Add 100µL of isopropanol and mix
4. Transfer lysate into purification columns and centrifuge at 8,000g for 1 minute
5. Discard eluate and add 500µL of Inhibitor Removal Buffer to column, centrifuge at 8,000g for 1 minute
6. Remove eluate and add 500µL of Wash buffer to column, centrifuge at 8,000g for 1 minute
7. Remove eluate and add 500µL of Wash buffer to column, centrifuge at 8,000g for 1 minute
8. Centrifuge column at maximum speed for 10 seconds

9. Transfer column into a sterile 1.5mL tube and add 100µL of pre-heated Eluition Buffer at 70 °C, centrifuge at 8,000g for 1 minute to recover DNA

DNA was then stored at -20 ° C until use.

4.3 Bacterial identification and serogrouping by Real-Time PCR.

To carry out identification of meningococci and their serogroups, the Real-Time PCR technique was chosen because it is more sensitive than the cultural method to identifying a greater number of positive cases, probably thanks to the advantage of the molecular method in detecting even non vital meningococci unable to grow.

Real-Time PCR method work with a fluorescent dual-labeled probe (TaqMan probe) in addition to the other classic PCR reagents; this probe can be displaced and digested by the 5' exonuclease activity of the DNA polymerase upon primer extension. This cause the release of fluorophore that excited by laser emits a fluorescence that is measured during each PCR cycle; a digital camera collects fluorescence and data was so stored on computers (Fig. 11).

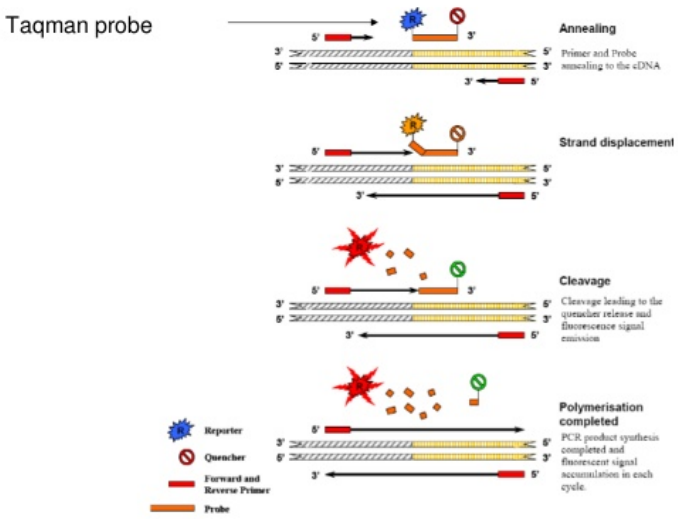

Fig. 11: Schematic representation of Real-Time PCR with TaqMan probe.

The selected primers and probe allow the detection of capsule transfer gene (ctrA gene), this protein is localized on the surface of meningococci and is common to the different serogroups of *Neisseria Meningitidis*[19] (Fig. 12, Tab. 1).

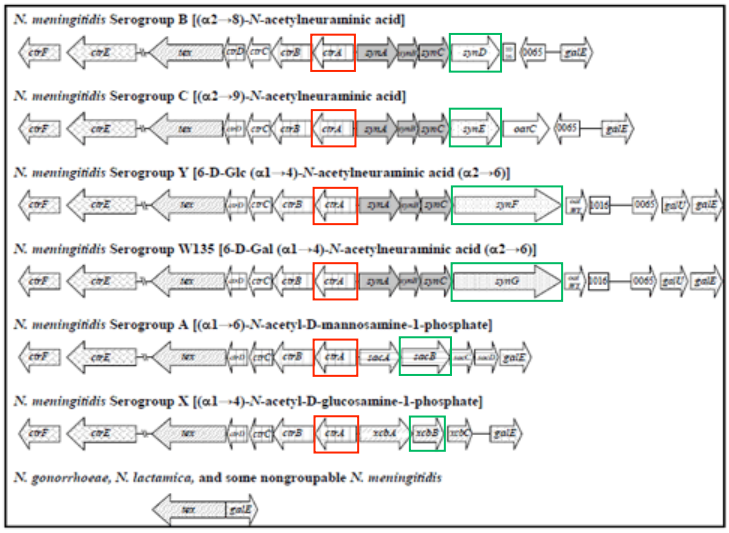

Fig. 12: Genetic map of the capsule gene complex (cps) of *Neisseria Meningitidis*. The capsule gene is marked in red and the gene for the serogroup in green.

ctrA gene oligos	**Sequence (5' – 3')**	**Label**
ctrA For	GCTGCGGTAGGTGGTTCAA	
ctrA Rev	TTGTCGCGGATTTGCAACTA	
ctrA Probe	CATTGCCACGTGTCAGCTGCACAT	FAM–BHQ1

Tab. 1: Primers used in bacterial identification.

Amplification was performed in a volume of 25µL containing: 2x TaqMan Universal Master Mix (Applied Biosystems), primer at a final concentration of 400nM, probe marked in FAM at a final concentration of 200nM and 5µL of extracted DNA. Each sample was analyzed in triplicate, including in each run a negative control (sterile water) and a positive control (DNA extracted from meningococcus).

Samples were amplified using the ABI 7900HT instrument (Applied Biosystems) with following parameters: 95 °C for 10 minutes for initial denaturation followed by 45 cycles of two steps denaturation and annealing/elongation at 95 °C for 15 seconds and 60 °C for 1 minute respectively. If no fluorescence signals are detected within the 40th cycle, the sample is considered negative. Furthermore, the specificity of primers was confirmed by specific Real-Time PCR using genomic extracts of *Neisseria gonorrhoeae*, *Neisseria lactamica*, *Neisseria sicca*, *Neisseria flavescens* and *Neisseria cinerea* obtained from National Collection of Type Cultures, Central Public Health Laboratory. The suitability of sample to molecular analysis has been assessed by amplification, using Real-Time PCR, on the human RNase P gene in order to understand if the possible failure of the bacterium's detection is due to its absence and not to presence of any inhibitors or to a total absence of DNA in extracts indicating a swabbing not effective.

For detection of serogroups, Real-Time PCR was used with primers and probes designed to detect specific genes for biosynthesis of the capsule of different meningococci, in detail the genes considered were: sacB, synD, synE, synG, xcbB and synF (https://www.cdc.gov/meningitis/lab-manual/chpt10-pcr.html), (Table 2).

Sero-group	**Capsule type**	**Gene target name**	**Alternative gene names**
A	(α1→6)-N-acetyl-D-mannosamine-1-phosphate	*sacB*	
B	(α2→8)- N-acetylneuraminic acid	*synD*	*siaD, siaD$_B$*

C	(α2→9)- N-acetylneuraminic acid	*synE*	$siaD_C$
W135	6-D-Gal(α1→4)-N-acetylneuraminic acid(α2→6)	*synG*	$siaD_W$
X	(α1→4)-*N*-acetyl-D-glucosamine-1-phosphate	*xcbB*	
Y	6-D-Glc(α1→4)-N-acetylneuraminic acid(α2→6)	*synF*	$siaD_Y$

Tab. 2: Genes used for detection of serogroups.

Amplification was performed in a volume of 25µL using 2x TaqMan Universal Master Mix, primers and specific probes (Tab. 3) and 5 µL of DNA extract.

Thermal cycles are common to all six reactions: 95 °C for 10 minute followed by 40 cycles of 95 °C for 1 minute and 60 °C for 1 minute[20-21].

Oligos	**Sequence (5' – 3')**	**Label**	**Final concentration**
Nem A For	AAAATTCAATGGGTATATCACGAAGA		300 nM
Nem A Rev	ATATGGTGCAAGCTGGTTTCAATAG		900 nM
Nem A Probe	CTAAAAG"T"AGGAAGGGCACTTTGTGGCATAAT	5' FAM – BHQ1 on "T"; 3' SpC6	100 nM
Nem B For	TGCATGTCCCCTTTCCTGA		300 nM
Nem B Rev	AATGGGGTAGCGTTG		300 nM

	ACTAACAA		
Nem B Probe	TGCTTATTCCTCCAGC ATGCGCAAA	5’ FAM – 3’ BHQ1	100 nM
Nem C For	CTTTCCCTGAGTATGC GAAAAAA		900 nM
Nem C Rev	TGCTAATCCCGCCTGA ATG		300 nM
Nem C Probe	TTTCAATGC”T”AATGA ATACCACCGTTTTTTT GC	5’ FAM – BHQ1 on “T”; 3' SpC6	100 nM
Nem W135 For	GTGAGGGATTTCCATA TATATTTA		900 nM
Nem W135 Rev	TTGCCATTCCAGAAAT ATCA		900 nM
Nem W135 Probe	TATGGAGCGAATGATT ACAGTAACTATAA	5’ FAM – 3’ BHQ1	100 nM
Nem Y For	GAGCAGGAAATTTAT GAGAATACAGA		300 nM
Nem Y Rev	CTAAAATCATTCGCTC CATAT		900 nM
Nem Y Probe	GTATGGTGTACGATAT CCCTATCCTTGCCTAT AAT	5’ FAM – 3’ BHQ1	100 nM
Nem X For	TGTCCCCAACCGTTTA TTGG		900 nM
Nem X Rev	TGCTGCTATCATAGCC GCC		900 nM
Nem X Probe	TGTTTGCCCACATGAA TGGCGG	5’ FAM – 3’ BHQ1	100 nM

Tab. 3. Sequences of primers and probes for detection of serogroups and concentrations of use.

4.4 Bacterial culture

N. meningitidis grows both on blood agar plate (BAP) and on chocolate agar plate (CAP) at 35-37°C with ~5% CO2. Colonies are grey and unpigmented on a BAP and appear round, smooth, moist, glistening, and convex, with a clearly defined edge, instead on a CAP appear large, colorless-to-grey and opaque (Fig. 13).

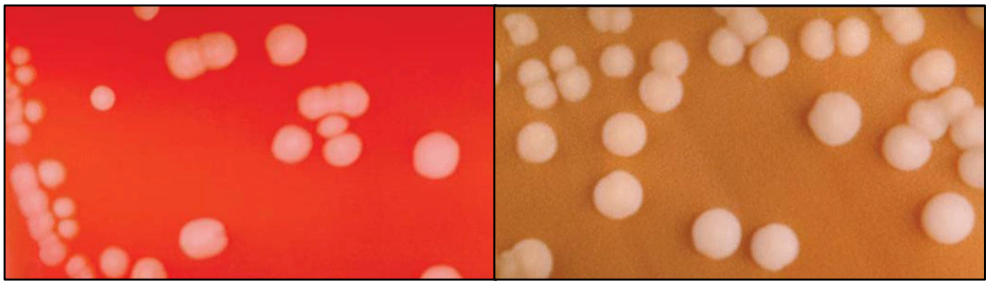

Fig. 13: N. meningitidis on a BAP plate at left and on a CAP plate at right.

Isolates should be inspected for the purity of growth and a single colony should be re-streaked, when necessary, to obtain a pure culture. For the following characterization procedures, testing should be performed on 18-24 hour growth from a BAP or CAP in a Biosafety Level 2 (BSL-2) environment.

4.5 Multilocus Sequence Typing, purification, quantification and sequencing.

Many methods of molecular typing are used in epidemiological studies for the characterization of pathogens, allowing to analyze the genetics of bacterial population and its evolution. These include ribotyping, random amplified polymorphic DNA (RAPD), restriction fragment length polymorphism (RFLP), multiple-locus variable-number tandem repeat analysis (MLVA), pulsed-field gel electrophoresis (PFGE) and multilocus sequence typing (MLST).

MLST[22] is a method based on PCR and sequencing of seven house-keeping genes and allows to define strain analyzed according to the allelic profile determined by the nucleotide sequence. For each isolate, seven alleles identify the allelic or sequence type (ST) profile of bacterium. This technique has numerous advantages, derived above all from the use of sequencing method, including:

1. Directly measure genetic variation
2. Is adaptable to the analysis of many samples simultaneously
3. Sequencing data are reproducible and comparable between different laboratories
4. Analysis of sequences can be done remotely and results can be viewed on the web
5. Sequence data can be found on an accessible online database (http://pubmlst.org/neisseria)
6. Information can be obtained by PCR on a clinical sample

The sequenced gene portions have a size of about 500 base pairs and those considered are: Putative ABC transporter (abcZ), Adenylate kinase (adk), Shikimate dehydrogenase (aroE), Fumarate dehydrogenase (fumC), Glucose-6-phosphate dehydrogenase (gdh), Pyruvate dehydrogenase subunit (pdhC) and Phosphoglucomutase (pgm). Several pairs of primers are used, one is useful for fragment amplification (Tab. 4) and the second pair is used in sequencing reaction (Tab. 5) (Fig. 14).

Gene name	**Primer's name**	**Sequence (5' – 3')**
abcZ	abcZ-P1C	TGTTCCGCTTCGACTGCCAAC
	abcZ-P2C	TCCCCGTCGTAAAAAACAATC
adk	adk-P1B	CCAAGCCGTGTAGAATCGTAAACC
	adk-P2B	TGCCCAATGCGCCCAATAC

aroE	aroE-P1B	TTTGAAACAGGCGGTTGCGG
	aroE-P2B	CAGCGGTAATCCAGTGCGAC
fumC	fumC-P1B	TCCCCGCCGTAAAAGCCCTG
	fumC-P2B	GCCCGTCAGCAAGCCCAAC
gdh	gdh-P1B	CTGCCCCCGGGGTTTTCATCT
	gdh-P2B	TGTTGCGCGTTATTTCAAAGAAGG
pdhC	pdhC-P1B	CCGGCCGTACGACGCTGAAC
	pdhC-P2B	GATGTCGGAATGGGGCAAACA
pgm	pgm-P1	CTTCAAAGCCTACGACATCCG
	pgm-P2	CGGATTGCTTTCGATGACGGC

Tab. 4: Amplification primers.

Gene name	**Primer's name**	**Sequence (5' – 3')**
abcZ	abcZ-S1A	AATCGTTTATGTACCGCAGR
	abcZ-S2	GAGAACGAGCCGGGATAGGA
adk	adk-S1A	AGGCWGGCACGCCCTTGG
	adk-S2	CAATACTTCGGCTTTCACGG
aroE	aroE-S1A	GCGGTCAAYACGCTGRTK
	aroE-S2	ATGATGTTGCCGTACACATA
fumC	fumC-S1	TCCGGCTTGCCGTTTGTCAG
	fumC-S2	TTGTAGGCGGTTTTGGCGAC
gdh	gdh-S3	CCTTGGCAAAGAAAGCCTGC
	gdh-S4C	RCGCACGGATTCATRYGG
pdhC	pdhC-S1	TCTACTACATCACCCTGATG
	pdhC-S2	ATCGGCTTTGATGCCGTATTT
pgm	pgm-S1	CGGCGATGCCGACCGCTTGG
	pgm-S2A	GGTGATGATTTCGGTYGCRCC

Tab. 5: Sequencing primers.

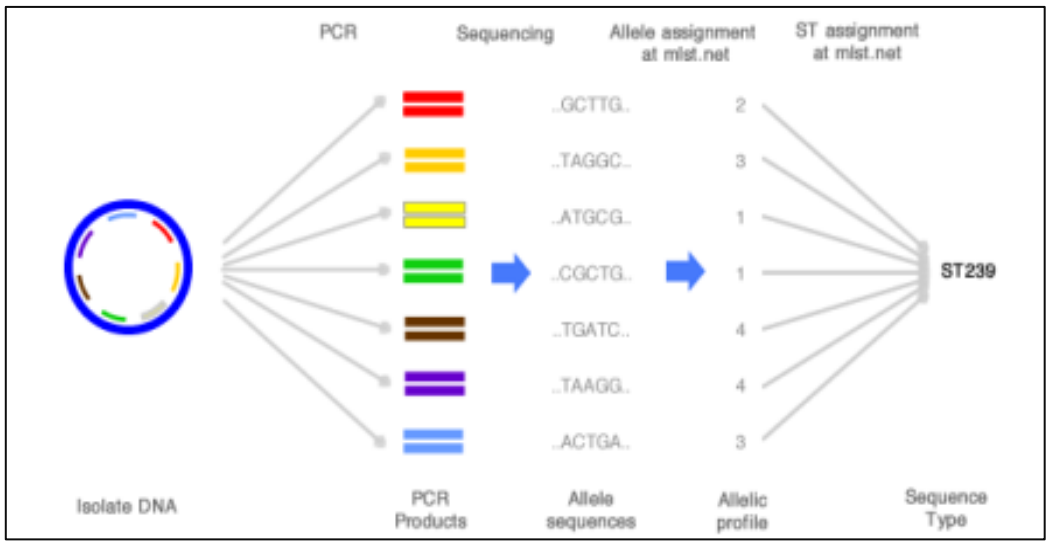

Fig. 14: Schematic MLST analysis.

Amplification was performed in a volume of 50 µL using 10x AmpliTaq Gold® 360 Buffer, 1 µL of both primers (initial concentration 10 µM), 6 µL of Magnesium MgCl2 (initial concentration 25mM), 1 µL of dNTPs (initial concentration 10mM), 2.5U of Taq Polymerase and 5 µL of extracted DNA. Thermocycler used is Applied Biosystems 2720 Thermal Cycler, cycles for amplification are common for all seven reactions and consist of: 94 °C for 5 minute, followed by 40 cycles of 94 °C for 1 minute, 55 °C for 1 minute and 72 °C for 1 minute, 72 °C for 5 minute and 4 °C at the end of the reaction. In each one a positive and a negative amplification control have been included.

The products are visualized by 1% agarose gel electrophoresis treated with Syber Safe (Thermo Fisher Scientific) intercalating the DNA and with the molecular weight marker 1kb Plus DNA Ladder (Thermo Fisher Scientific) that allows to discriminate amplicons ranging from 100 to 12.000 base pairs. Once the products of interest have been identified, these are recovered from gel by cutting with a sterile bistoury and placed in a 1,5mL tube for following purification phase (Fig.15).

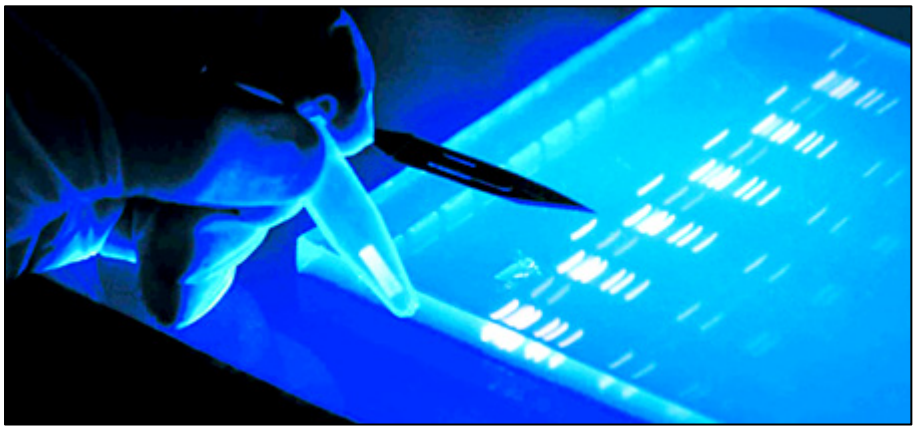

Fig. 15: Gel electrophoresis and cutting PCR products.

The fragment is purified using the PCR clean-up Gel extraction kit (Macherey-Nagel), in detail:

1. Determine the weight of gel's fragment
2. Add 200µL of NTI buffer per 100mg of gel
3. Incubate sample for 5-10 minute at 50 °C, shake every 3 minute
4. Transfer sample above the column and centrifuge at 11,000g for 30 seconds, discard the eluate
5. Add 700µL of NT3 buffer, to perform wash, and centrifuge at 11,000g for 30 seconds, then discard eluate
6. Add 700µL of NT3 buffer, to perform wash, and centrifuge at 11,000g for 30 seconds, then discard eluate
7. Centrifuge at 11,000g for 1 minute in order to eliminate washing residues from filter
8. Place column in a sterile 1.5 mL tube, add 30µL of NE buffer (elution) and incubate at room temperature for 1 minute
9. Centrifuge at 11,000g for 1 minute to recover DNA
10. Store DNA at -20 until use

Following the purification of fragments we proceeded with their quantification by means of the Quant-iT dsDNA Assay Kit, High Sensitivity (Thermo Fisher Scientific) and the Qubit 3 Fluorometer instrument, in detail:

1. Prepare working solution consisting of 199µL of HS Buffer and 1µL of dye for each sample to be tested (in duplicate) including two standard curve points
2. Two curve points consisting of 190µL of working solution and 10µL of DNA supplied with the kit (0-10ng/µL)
3. Prepare samples by adding 2µL of DNA into 198µL of working solution
4. Vortex and incubate in the dark for 2 minute
5. Calibrate instrument with two standard curve points and then read quantities, in nanograms per microliter, of DNA eluted from gel

After this quantification, samples are prepared for sending to GATC, a company based in Germany, which was responsible for sanger sequencing reactions. The samples are prepared in order to send:

- 5 µL of PCR product (20-80ng/µL)
- 5 µL of primer (concentrated 5µM)

Two sequence reactions, one with the forward primer and one with the reverse, were performed. Once sequences were obtained, they were assembled using the Sequencher 4.1.4 software and corrected, consensus sequence was submitted to the online database (http://pubmlst.org/neisseria) in order to obtain the number of the corresponding allele. Once obtained all numbers of alleles (seven

genes analyzed) these were submitted into the same database to obtain sequence type and clonal complex of strain examinated.

4.6 Analysis of vaccine proteins.

The analysis of nucleotide sequences and predicted amino acid sequences of proteins present in meningococcus B strains allows to estimate the degree of identity between protein encoded by the bacterium and the protein used in the formulation of the vaccine. So we proceeded with the analysis of gene sequences of fHBP, NHBA, NadA and PorA, by PCR amplification, gel electrophoresis, purification, quantification of DNA and sequencing following method published by Lucidarme[23]. The instrument used is Applied Biosystems 2720 Thermal Cycler, primers are shown in Tab. 6 and the amplification conditions in Tab. 7.

Target	**Primer use (direction)**	**Primer identifier**	**Sequence (5' – 3')**
fHBP	PCR/Seq (fwd)	gna1870F	TGACCTGCCTCATTGATGC
	PCR/Seq (rev)	gna1870R	CGGTAAATTATCGTGTTCG GACGGC
	PCR/Seq (rev)	gna1870v3 R	CGTGCCGTCGTGTCCTAG
	Seq (fwd)	gna1870S2	CAAATCGAAGTGGACGGG CAG
	Seq (fwd)	gna1870S3	TGTTCGATTTTGCCGTTTCC CTG
NHBA	PCR/Seq (fwd)	gna2132F	GGCGTTCAGACGGCATATT TTTACA
	PCR/Seq (rev)	gna2132R	GGTTTATCAACTGATGCGG ACTTGA
	Seq (fwd)	gna2132S2	GCGGACACGCTGTCAAAAC

			C
	Seq (fwd)	gna2132S4	GGCGTTCTGCACGGTCGAGG
	Seq (fwd)	gna2132S5	ATGGGTACGCAAAAATTCAA
	Seq (rev)	gna2132S7	AATGCAGTACTTCGCCGTTGT
	Seq (rev)	gna2132S8	CCTCGACCGTGCAGAACGCC
	Seq (rev)	gna2132S9	CCGCACCGCCATTGCCTGTA
nadA	PCR/Seq (fwd)	nadAF	GTGGACGTACTCGACTACGAAGG
	PCR/Seq (rev)	nadAR	CGAGGCGATTGTCAAACCGTTC
	PCR/Seq (fwd)	nadAintF	TATGTAAACAAACTTGGTGGGG
	PCR/Seq (rev)	nadAintR	GAAATAGAAAAGTTAACAACCAAGTT
	Seq (fwd)	nadAS1	TATGTAAACAAACTTGGTGGGG
	Seq (fwd)	nadAS2	GAAATAGAAAAGTTAACAACCAAGTT
	Seq (fwd)	nadAS3	GACATCAAAGCTGATATCGCTAC
	Seq (rev)	nadAS4	TTTCGAGGTGGCGCGTTCGGG
	Seq (rev)	nadAS5	GTAGCGATATCAGCTTTGATGTC
	Seq (rev)	nadAS6	CTTGGTTGTTAACTTTTCTATTTC
porA	PCR (fwd)	210	ATGCGAAAAAAACTTACCGCCCTC
	PCR (rev)	211	AATGAAGGCAAGCCGTCA

			AAAACA
porA (VR1)	Seq (fwd)	P3	CAAAGCCGGCGTGGAAG
	Seq (rev)	103L	AACGGATACGTCTTGCTC
porA (VR2)	Seq (fwd)	8U	TCCGTACGCTACGATTCTCC
	Seq (rev)	122L	GGCGAGATTCAAGCCGCC

Tab. 6: Primers used in amplification and sequencing reactions.

Target	**Denaturation**	**Annealing**	**Extension**	**N° of cycles**
fHBP	96°C, 30 sec	63°C, 30 sec	72°C, 1 min	30
NHBA	96°C, 30 sec	57°C, 30 sec	72°C, 1 min	30
nadA	96°C, 30 sec	56°C, 30 sec	68°C, 1min 20 sec	30
porA	94°C, 1 min	68°C, 1 min	72°C, 2 min	30

Tab. 7: PCR conditions.

Sequencing was performed at GATC and forward and reverse sequences were assembled with the software Sequencher 4.1.4, the obtained sequences were used to query the online database (http://pubmlst.org/neisseria) to have: the number of the nucleotide allele, the number of the corresponding peptide and the degree of identity towards the proteins contained in the two vaccines.

5. Results.

Real-Time PCR screening of 2560 subjects allowed to identify 135 meningococcal carriers (5.3%). Among these, serogroup B is the most frequently detected (n = 58, 43%) followed by serogroup Y (n = 32, 23.7%), by serogroup C (n = 7, 5.2%), by serogroup W135 (n = 6, 4.4%) and by serogroup X (n = 5, 3.7%), none belongs to serogroup A. In 27 cases none of the genes for capsule synthesis were detected (Fig. 16). No difference in Men carriage prevalence according to the type of school, age and gender were evidenced.

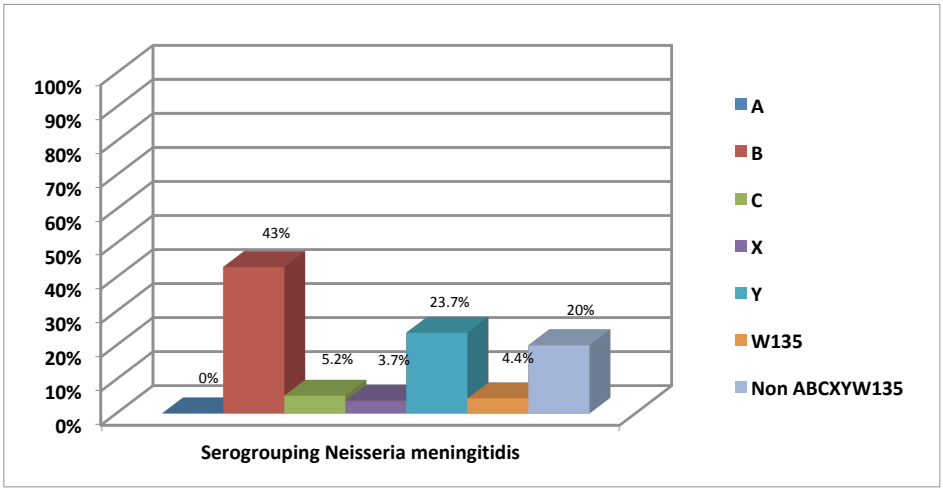

Fig. 16: Distribution of serogroups in the analyzed samples.

On samples positive for meningococcus B we proceeded with MLST analysis performed by PCR, gel electrophoresis and sequencing of products.

Amplification of abcZ gene provides a product that measures 897 base pairs and the next internal fragment to be sequenced measure 515 base pairs (Fig. 17, Fig. 18).

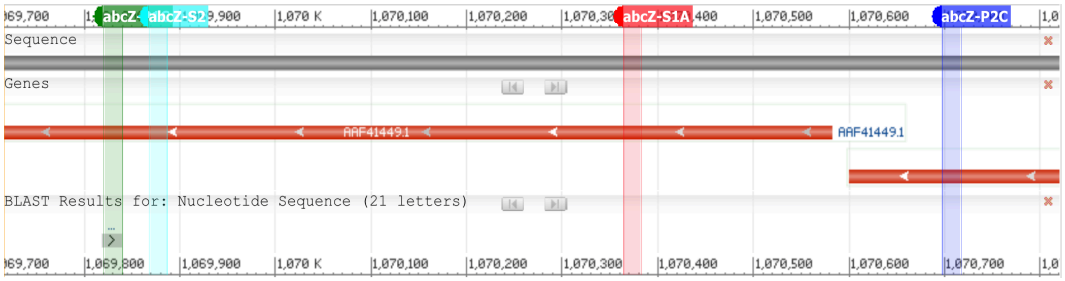

Fig. 17: Blast of abcZ primers on N. meningitidis MC58 genome.

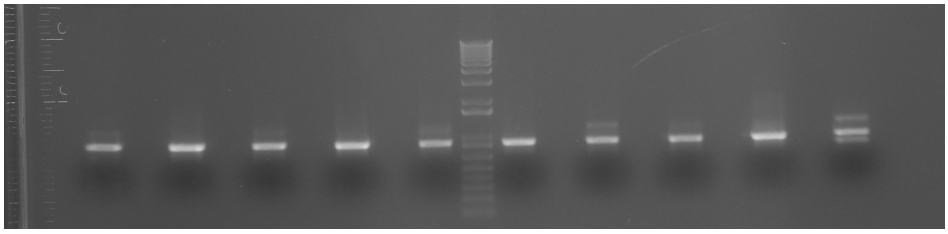

Fig. 18. Gel electrophoresis photo's of abcZ amplification.

The amplification of adk gene provides a product that measures 707 base pairs and the next internal fragment to be sequenced measure 511 base pairs (Fig. 19, Fig. 20).

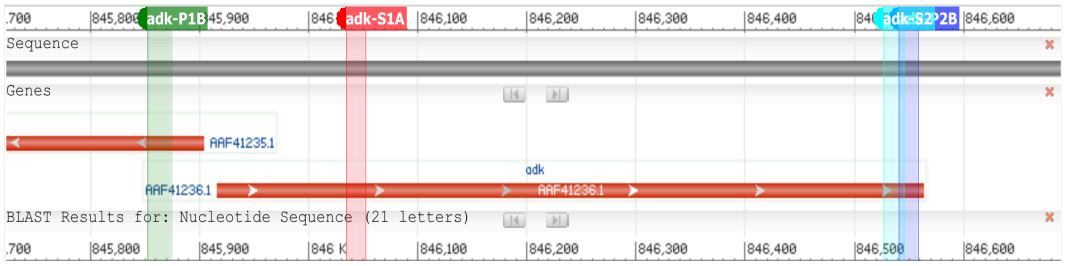

Fig. 19: Blast of adk primers on N. meningitidis MC58 genome.

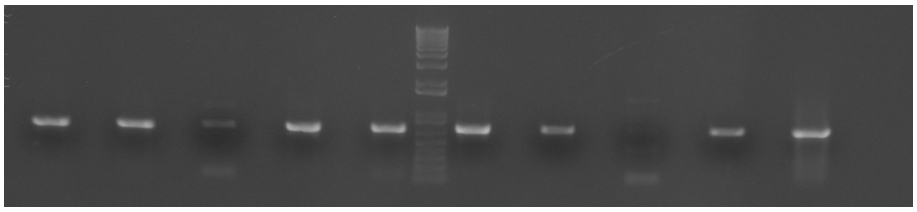

Fig. 20. Gel electrophoresis photo's of adk amplification.

The amplification of aroE gene provides a product that measures 834 base pairs and the next internal fragment to be sequenced measure 636 base pairs (Fig. 21, Fig. 22).

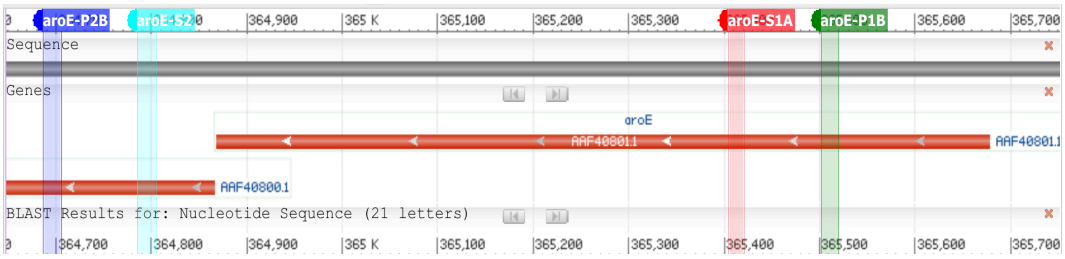

Fig. 21: Blast of aroE primers on N. meningitidis MC58 genome.

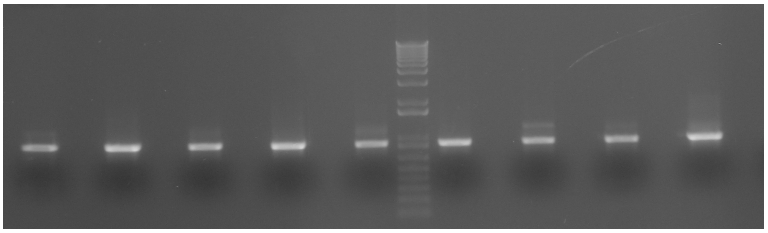

Fig. 22. Gel electrophoresis photo's of aroE amplification.

The amplification of fumC gene provides a product that measures 859 base pairs and the next internal fragment to be sequenced measure 529 base pairs (Fig. 23, Fig. 24).

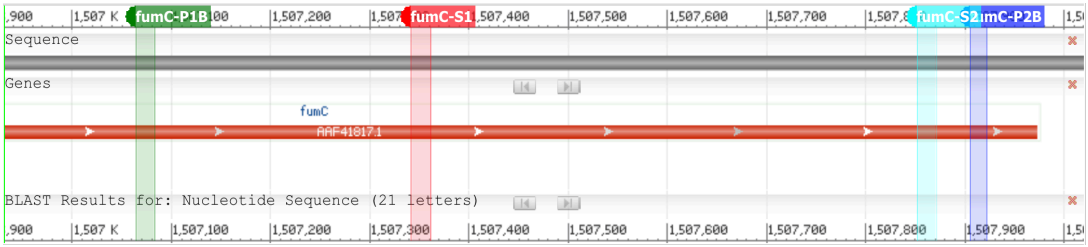

Fig. 23: Blast of fumC primers on N. meningitidis MC58 genome.

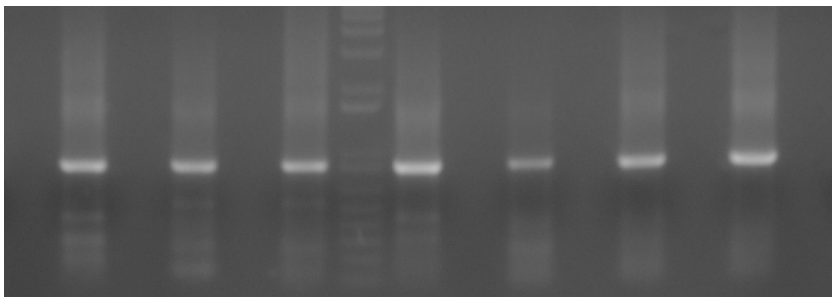

Fig. 24. Gel electrophoresis photo's of fumC amplification.

The amplification of gdh gene provides a product that measures 676 base pairs and the next internal fragment to be sequenced measure 597 base pairs (Fig. 25, Fig. 26).

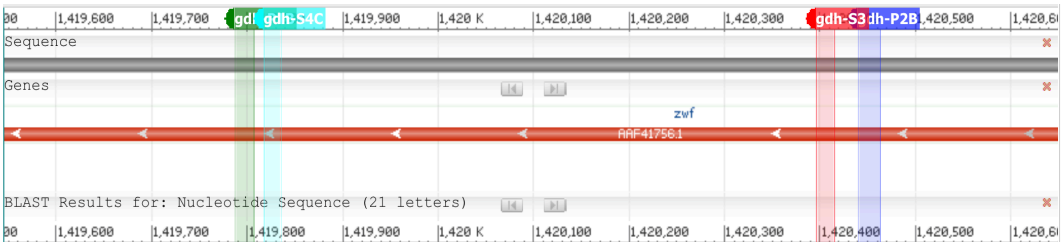

Fig. 25: Blast of gdh primers on N. meningitidis MC58 genome.

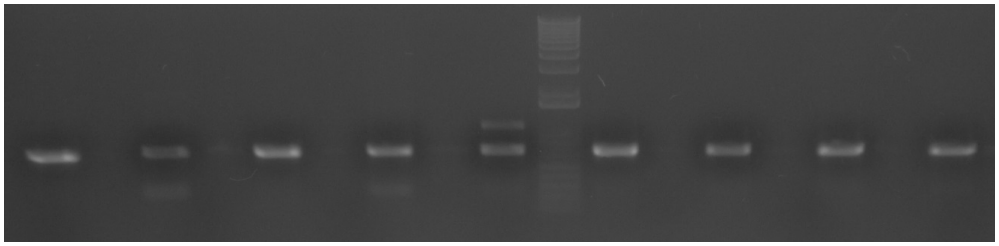

Fig. 26. Gel electrophoresis photo's of gdh amplification.

The amplification of pdhC gene provides a product that measures 817 base pairs and the next internal fragment to be sequenced measure 583 base pairs (Fig. 27, Fig. 28).

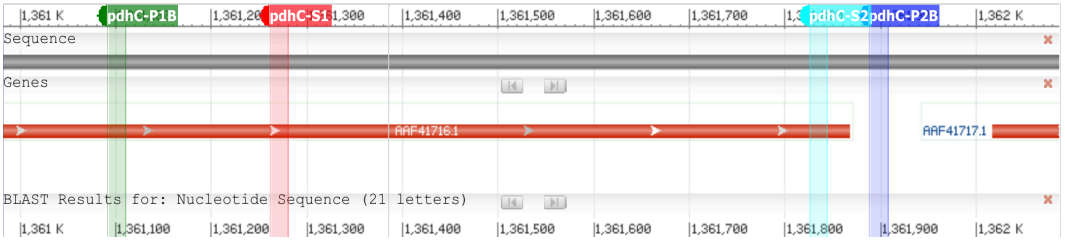

Fig. 27: Blast of pdhC primers on N. meningitidis MC58 genome.

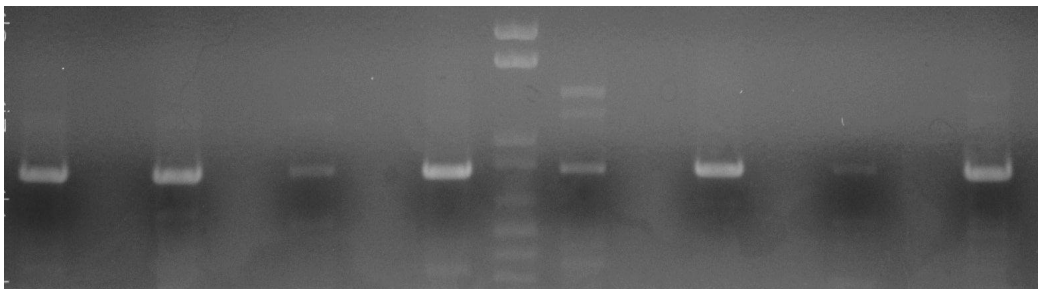

Fig. 28. Gel electrophoresis photo's of pdhC amplification.

The amplification of pgm gene provides a product that measures 1338 base pairs and the next internal fragment to be sequenced measure 492 base pairs (Fig. 29, Fig. 30).

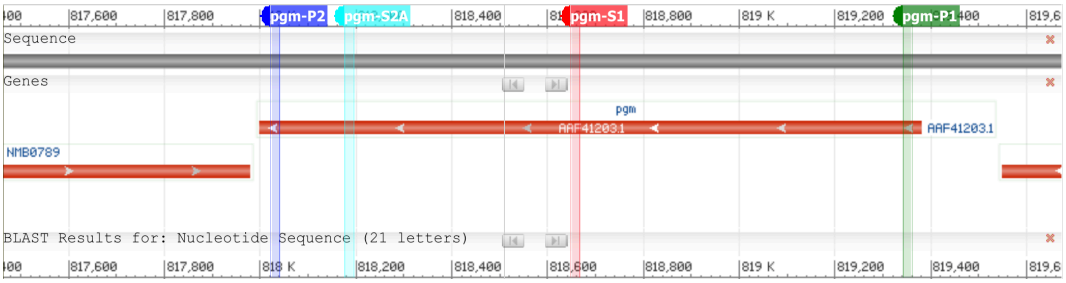

Fig. 29: Blast of pgm primers on N. meningitidis MC58 genome.

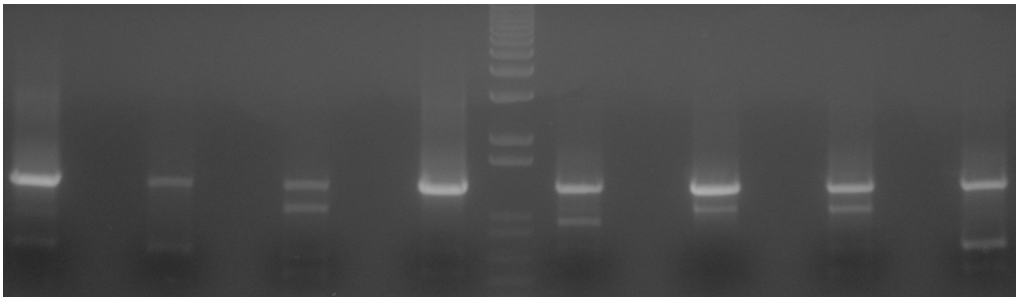

Fig. 30. Gel electrophoresis photo's of pgm amplification.

Data obtained from sequencing are used to query online MLST database (https://pubmlst.org/neisseria/) to obtain the number of the corresponding allele. Eleven clonal complexes were identified, four samples (6.9%) present a combination of alleles for which no clonal complex can be assigned from the online database (Tab. 8).

Clonal complex (CCs)	**N° of identification (%)**
ST-162	12 (20.7)
ST-865	12 (20.7)
ST-41/44/Lineage 3	11 (19)
ST-35	6 (10.3)
ST-32/ET-5	4 (6.9)
ST-269	3 (5.2)
ST-213	2 (3.4)
ST-198	1 (1.7)
ST-461	1 (1.7)
ST-549	1 (1.7)

ST-750	1 (1.7)
Unassigned CCs	4 (6.9)

Tab. 8: Distribution of clonal complexes in samples.

Generally, clonal complexes associated with hypervirulent strains have been found among all serogroups responsible for IMD, for serogroup B clonal complexes associated with higher virulence are: ST-41/44 / Lin3, ST-32 / ET-5, ST-11 / ET-37, ST-8 / Cluster A4[24] and ST-269 recently added[25]. In analyzed subjects, there are hypervirulent strains among which ST-41/44 / Lineage 3 is more represented (19%).

Data analysis of proteins included in two vaccines show that, regarding fHbp gene, all strains except 3 harbored fHbp alleles represented by a total of 31 sub-variants: 18 in variant 1 (v.1 n=25; 45.4%), 8 in variant 2 (v.2 n=25; 45.4%) and 5 in variant 3 (v.3, n=5; 9.1%). Variants 1 and 2 were mainly present in the ST-162, ST-41/44/Lin3 and ST-865 CCs instead variant 3 was primarily found in ST-269 CC (Fig. 31).

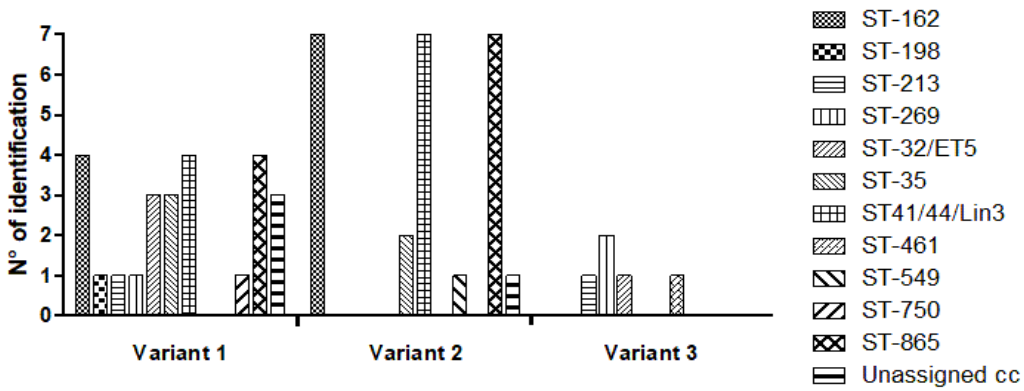

Fig. 31: Distribution of fHbp variants by the clonal complex.

Among the 18 sub-variants in variant 1: gene for fHbp sub-variant included in 4CMenB vaccine (fHbp 1.1) and the sub-variant included in MenB fHbp vaccine (fHbp 1.55) was not identified in any strains

whereas, regarding the 5 sub-variant in variant 3, the gene for fHbp sub-variant included in MenB.fHbp vaccine (fHbp 3.45) was detected in one strain. Data concerning percentage of amino acid identity shows that for detected fHbp sub-variants 1 identity towards 4CMenB sub-variant varies from 90.9% to 99.2% (median: 96.1%) instead that against MenB fHbp varies from 87.7% to 93.1% (median: 89.2%). The identity regarding detected sub-variant 3 towards the MenB fHbp varies from 93.7% to 100% (median: 97.6%) (Fig. 32-33).

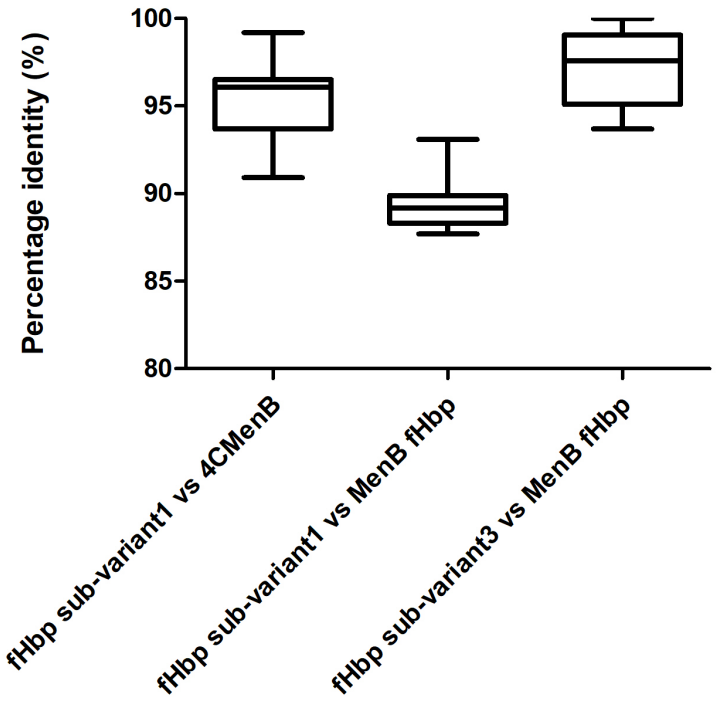

Fig. 32. Boxplot fHbp percentage identity.

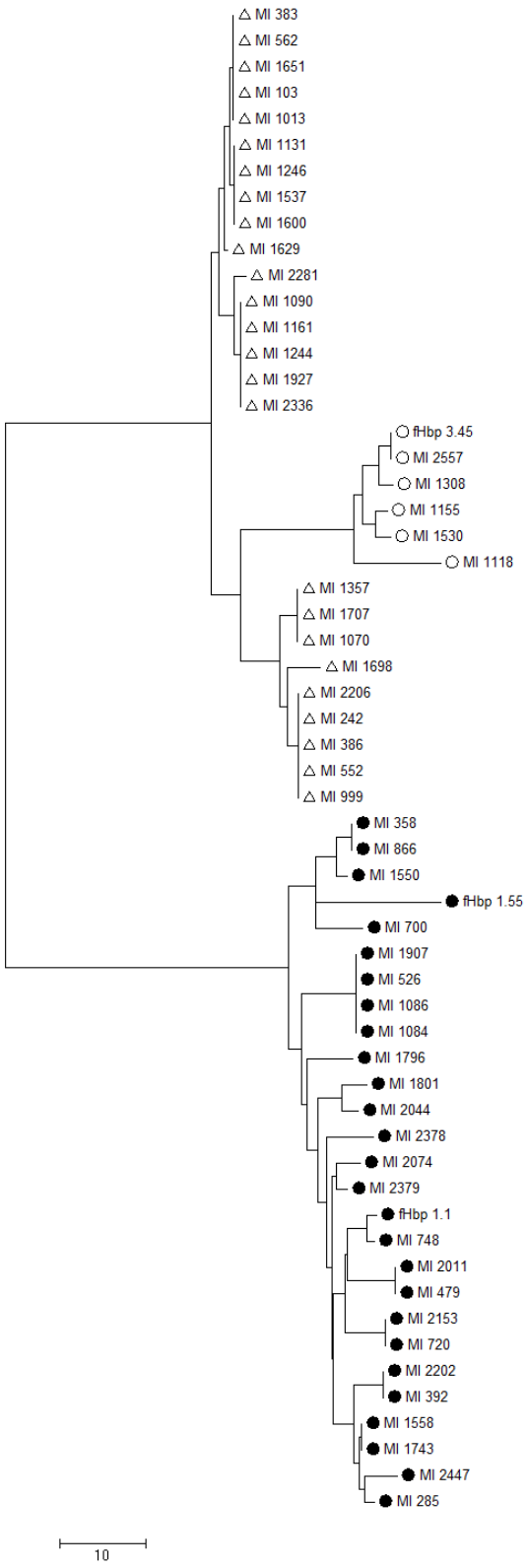

Fig. 33. Phylogenetic analysis of identified fHBP.

The gene for NHBA protein was found in all strains except for 6 (10.3%) and 21 sub-variants were identified, the gene for NHBA

protein included in the 4CMenB vaccine (sub-variant 2) was not found in any strains. The sequencing data regarding NHBA gene shows that the sub-variants most common were: 908 (n=10, 19.2%) in ST-865 (9 strain) and in ST-549; 879 (n=10, 19.2%) in ST-162 (9 strain) and ST-865; 21 (n=6, 11.5%) in ST-35 (4 strain) and in ST-269 (2 strain). The sub-variants had an amino acid identity towards protein included in the 4CMenB vaccine that varying from 71.3% to 99.8% (median: 76%) (Fig. 34-35). Among strains without NHBA gene, 5 had a gene for fHbp protein variant 1 and 1 a gene for fHbp protein variant 2.

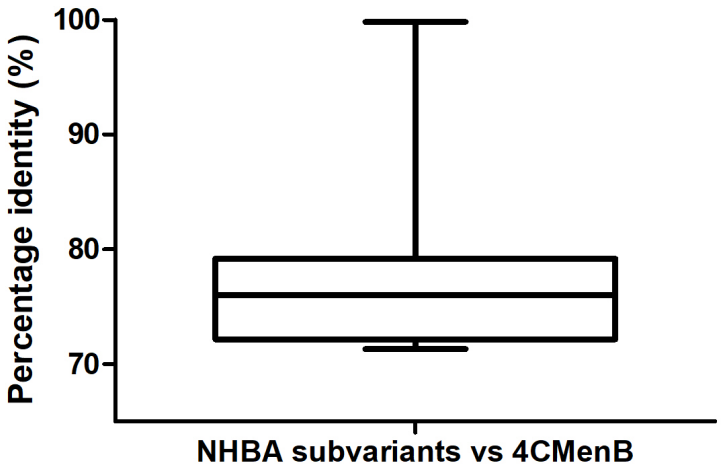

Fig. 34. Boxplot NHBA percentage identity.

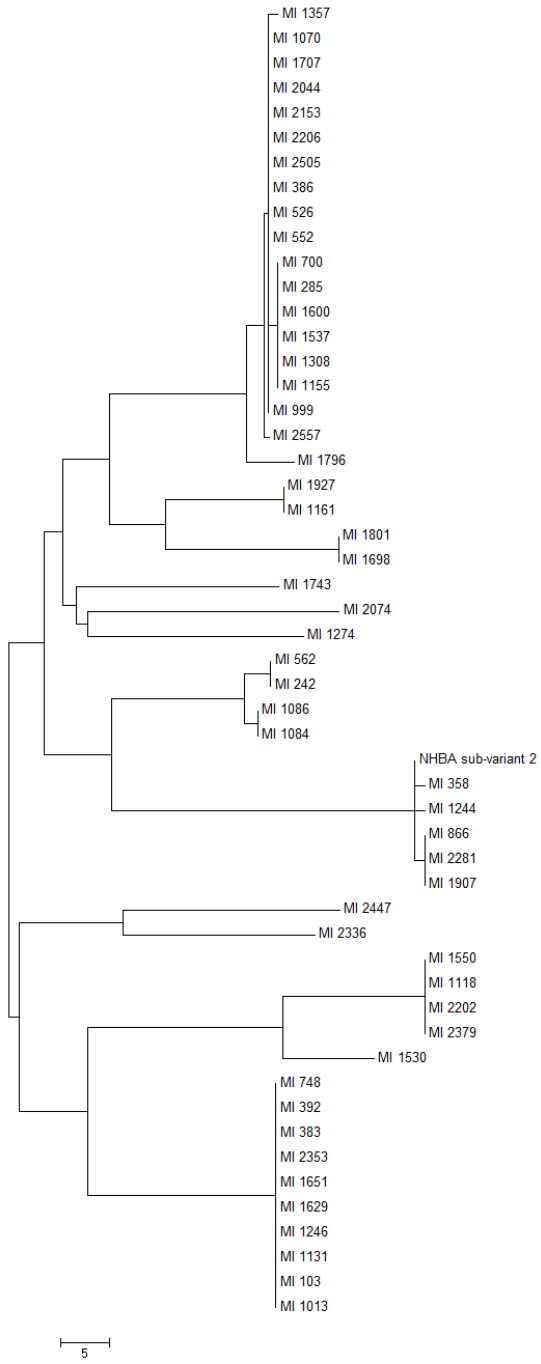

Fig. 35. Phylogenetic analysis of identified NHBA.

PorA proteins were identified in 53 out of 58 (91.4%) studied MenB strains and 22 porA sub-types were identified. The protein included in the 4CMenB vaccine (P1.4) was identified in 4 strains belonging to ST-162 (3 strain) and ST-41/44 Lin3 (1 strain). The amino acid identity between studied porA proteins and those were included in the

4CMenB vaccine was not less than 88.1% (median: 91.8%) (Fig. 36-37).

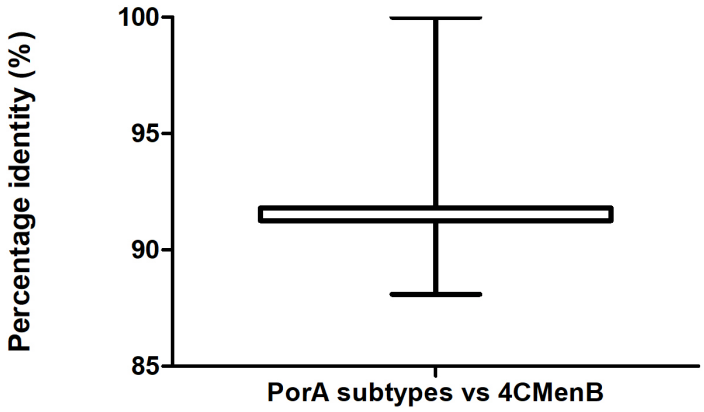

Fig. 36: Boxplot PorA percentage identity.

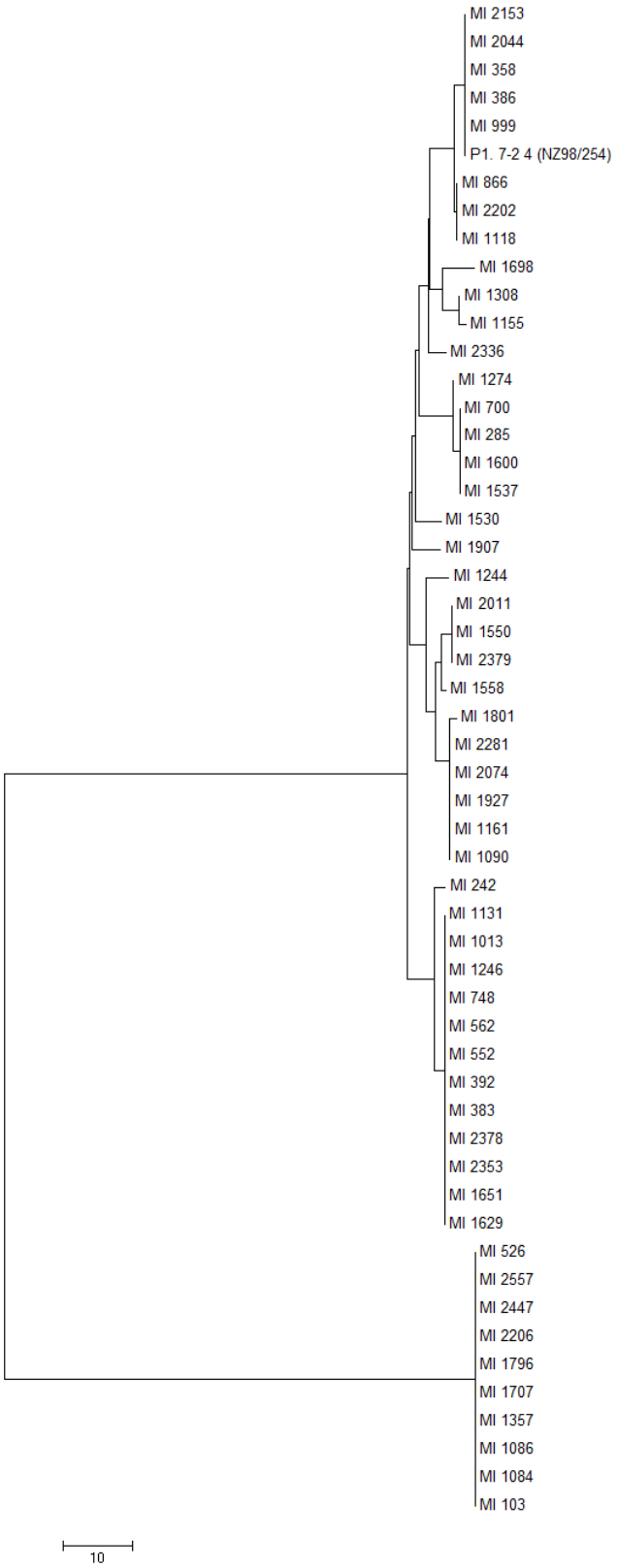

Fig. 37: Phylogenetic analysis of identified PorA.

The gene for NadA proteins was not detected in any of the 58 studied strains.

6. Conclusion

In this study was found that, in adolescent living in Milan, *Neisseria meningitidis* serogroup B was the most commonly carried followed by serogroup Y. This finding is in agreement with was reported in recent studies carried out in Europe where MenB is the most frequently identified meningococcus in the pharynx of both healthy individuals and patients with IMD[26-27-28]. This was expected because the introduction of MenC conjugate vaccine has significantly reduced MenC carriage and circulation evidencing the role of MenB in carriage and disease. In northern Italy, MenC vaccine coverage in children younger than 24 months is about 90%[29] and this could explain the low detection of MenC into studied population. On the contrary, in Italy, 4CMenB vaccine has been recently included in official vaccination schedule and recommended only for infants and toddlers, although a possible inclusion for the adolescent in next future was suggested[30]. This means that no effects for MenB vaccination may have occurred, on the other hand in Lombardy, the region of Italy where this study was performed, the incidence of MenB invasive disease has remained substantially unmodified in the last 10 years (i.e. around 4 cases out of 100,000)[31].

The genetic characteristics of MenB strains identified in this study are quite similar to those identified in a previous study performed several years earlier in a similar population living in the same geographic area[23]. This seems to confirm that, despite the high incidence of genetic exchange, meningococcal strains are relatively stable[13].

In analyzed strains was found that a great number could be included in some of the clonal complexes frequently associated with bacterial hypervirulence and development of IMD, such as ST-41/44 Lin3 and

ST-32, this suggests a potential virulence of meningococcal strains carried by the study population. The MenB vaccines presently licensed could have theoretically induced the production of antibodies effective against the greatest part of identified meningococci. Trumenba® contains two sub-variants of bacterial fHbp proteins and in studied strains 1 had the exactly sub-variant (fHbp 3.45) included in this vaccine. Bexsero® contains a variant of fHbp proteins (fHbp 1.1), a NadA-3 component, a variant of NHBA protein (sub-variant 2) and PorA P1.4 and in studied strain 4 had a protein that exactly matches with PorA included in the vaccine. In analyzed bacteria, the gene for NadA was never found as frequently occurs in carried stains[32]. These findings show that 1 subject is fully covered by Trumenba® vaccine and 4 subjects are fully covered by Bexsero® [33]. In all of the other cases, amino acid identity seem to suggest that this two vaccines could evoke antibodies able to eliminate carried strains. Indeed, for fHbp, each variant family (1, 2 or 3) induce a strong protective immune response against strains carrying homologous alleles but are ineffective against strains that express distantly related variant. Regarding NHBA, preclinical studies suggest that antibodies elicited against the vaccine peptide are cross-protective against strains expressing different NHBA peptides. Finally three main NadA variants (NadA-1, NadA-2, NadA-3) show highly conserved sequences and produce cross-bactericidal activity[18]. Therefore these vaccines could theoretically induce the production of antibodies effective against the greatest part of the identified MenB strains, although with slight differences between them .

Elimination of carriage seems essential to maximize the efficacy of a meningococcal vaccine. A relevant part of the positive effects obtained with the use of MenC vaccine was due to population

immunity induced by the reduction of carriage and pathogen transmission[34]. However, the potential positive effect of both MenB vaccines suggested should be considered with caution because the only presence of genes encoding proteins included in vaccines was studied. Gene expression was not evaluated and it has been demonstrated that MenB vaccines are theoretically effective when the invasive meningococcal strain is able to produce one or more of vaccine components at a concentration higher than the minimum required for the bactericidal protective activities of antibodies[35-36]. The meningococcal antigen typing system (MATS) is a suitable method to predict the susceptibility of individual MenB strains to be killed by bactericidal antibodies elicited by vaccination, this allows a qualitative and quantitative evaluation of the antigens expressed on the surface of meningococcus. This is a modified ELISA assay that quantifies expression and the level of matching with the corresponding antigen (fHbp, NHBA and NadA) included in the vaccine.

In another study authors reported that 3 months after dose two of 4CMenB vaccination, subjects had a 26.6% lower carriage of any meningococcal strain including capsular groups BCWY[29]. This findings is not surprising because proteins included in both vaccines are not exclusive of MenB but are present in meningococcal strain regardless of the serogroup.

Therefore, monitoring carriage remains essential to evaluate MenB circulation but a long-term studies will be necessary to evaluate the effects on the carriage and the final efficacy of both new MenB vaccines.

7. Papers published during PhD

During these years of PhD I participate in conducting several epidemiological studies focused in particular to identify, by molecular methods, different pathogens both bacteria or viruses cause of disease or carried by subject in order to understand the long term efficacy of vaccines or to advise, for particular categories of population, a booster vaccination.

These studies have generated some papers that I describe below:

1. **Interaction between *Streptococcus pneumoniae* and *Staphylococcus aureus* in paediatric patients suffering from an underlying cronic disease.**

   Esposito S, Marseglia GL, Colombo C, Iughetti L, **Terranova L**, Ierardi V, Gambino M, Principi N; Italian Pneumococcal Study Groups on Asthma, Cystic Fibrosis and Diabetes.

   Int J Immunopathol Pharmacol. 2015 Dec;28(4):497-507.

   doi: 10.1177/0394632015601486.

   Abstract:

   Little is known about the interaction between Streptococcus pneumoniae and Staphylococcus aureus in school-age children and adolescents suffering from an underlying chronic disease. To increase our knowledge in this regard, an oropharyngeal swab was obtained from school-age children and adolescents suffering from asthma (n = 423), cystic fibrosis (CF) (n = 212) and type 1 diabetes mellitus (DM1) (n = 296). S. pneumoniae detection and serotyping were performed using a real-time polymerase chain reaction, and S. aureus detection was performed using the RIDAGENE MRSA system. Among asthmatic, CF and DM1 patients, both pathogens were identified in 65/423 (15.4%), 21/212 (9.9%) and 62/296 (20.9%) children, respectively; S. pneumoniae alone was identified in 127/434

(30.0%), 21/212 (9.9%) and 86/296 (29.1%), respectively; S. aureus alone was identified in 58/434 (13.7%), 78/212 (36.8%) and 49/296 (16.6%), respectively. S. pneumoniae colonisation rates were higher in younger children and declined with age, whereas the frequency of S. aureus colonisation was quite similar in the different age groups. Among asthmatic and CF patients aged 6-9 years, S. aureus carriage was significantly higher in children who were positive for S. pneumoniae ($P < 0.05$). No significant association emerged between S. aureus carriage and carriage of S. pneumoniae serotypes included in the pneumococcal conjugate vaccines (PCVs). This study shows for the first time that school-age children and adolescents with asthma, CF and DM1 are frequently colonised by S. pneumoniae and S. aureus and that no negative relationship seems to exist between these pathogens. Moreover, the supposed protection offered by PCV administration against S. aureus colonisation was not demonstrated.

2. ***Streptococcus pneumoniae*** **pharyngeal colonization in school-age children and adolescents with cancer.**

Principi N, Preti V, Gaspari S, Colombini A, Zecca M, **Terranova L**, Cefalo MG, Ierardi V, Pelucchi C, Esposito S.

Hum Vaccin Immunother. 2016;12(2):301-7.
doi: 10.1080/21645515.2015.1090071.

Abstract:

Patients with cancer, particularly those with hematologic malignancies, are at an increased risk of invasive pneumococcal disease (IPD) and they are included in the list of subjects for whom pneumococcal vaccination is recommended. The main aim of this study was to evaluate Streptococcus pneumoniae colonization in school-aged children and adolescents with cancer to determine the potential protective efficacy of 13-valent pneumococcal conjugate

vaccine (PCV13). An oropharyngeal swab was obtained from 277 patients (age range 6-17 years) with cancer during routine clinical visits and analyzed for S. pneumoniae using real-time polymerase chain reaction. S. pneumoniae was identified in 52 patients (18.8%), including 47/235 (20.0%) with hematologic malignancies and 5/42 (11.9%) with solid tumors. Colonization declined significantly with an increase in age (odds ratio [OR] 0.34, 95% confidence interval [CI] 0.16-0.71, and OR 0.30, 95% CI 0.11-0.82 in children aged 10-14 and ≥15 years, respectively, as compared to those <10 years). Carriage was more common among patients with leukemia or lymphoma than in children with solid tumors. Co-trimoxazole prophylaxis was significantly associated with reduced pneumococcal carriage (OR 0.41, 95% CI 0.19-0.89). A total of 15/58 (25.9%) and 26/216 (12.0%) children were colonized by PCV13 serotypes among cancer patients previously vaccinated and not vaccinated with 7-valent pneumococcal conjugate vaccine (PCV7), respectively. In conclusion, this study indicates that children and adolescents with cancer are frequently colonized by S. pneumoniae. Because most of the carried serotypes are included in PCV13, this vaccine is presently the best solution to reduce the risk of IPD in these patients.

3. ***Streptococcus pneumoniae*** **colonization in children and adolescent with asthma: impact of the heptavalent pneumococcal conjugate vaccine and evaluation of potential effect of thirteen-valent pneumococcal conjugate vaccine.**

   Esposito S, **Terranova L**, Patria MF, Marseglia GL, Miraglia del Giudice M, Bodini A, Martelli A, Baraldi E, Mazzina O, Tagliabue C, Licari A, Ierardi V, Lelii M, Principi N.

   BMC Infect Dis. 2016 Jan 12;16:12. doi: 10.1186/s12879-016-1335-3.

Abstract:

Background:

The main aim of this study was to evaluate Streptococcus pneumoniae carriage in a group of school-aged children and adolescents with asthma because these results might indicate the theoretical risk of invasive pneumococcal disease (IPD) of such patients and the potential protective efficacy of the 13-valent pneumococcal conjugate vaccine (PCV13).

Methods:

Oropharyngeal samples were obtained from 423 children with documented asthma (300 males, 70.9%), and tested for the autolysin-A-encoding (lytA) and the wzg (cpsA) gene of S. pneumoniae by means of real-time polymerase chain reaction.

Results:

S. pneumoniae was identified in the swabs of 192 subjects (45.4%): 48.4% of whom were aged <10 years, 46.9% aged 10-14 years, and 4.7% aged ≥15 years ($p < 0.001$). Carriage was significantly less frequent among the children who had received recent antibiotic therapy (odds ratio [OR 0.41]; 95% confidence interval [95% CI] 0.22-0.76). Multivariate analyses showed no association between carriage and vaccination status, with ORs of 1.05 (95% CI 0.70-1.58) for carriers of any pneumococcal serotype, 1.08 (95% CI 0.72-1.62) for carriers of any of the serotypes included in 7-valent pneumococcal conjugate vaccine (PCV7), and 0.76 (95% CI 0.45-1.28) for carriers of any of the six additional serotypes of PCV13. Serotypes 19 F, 4 and 9 V were the most frequently identified serotypes in vaccinated subjects.

Conclusions:

These results showed that carriage of S. pneumoniae is relatively common in all school-aged children and adolescents with asthma,

regardless of the severity of disease and the administration of PCV7 in the first years of life. This highlights the problem of the duration of the protection against colonisation provided by pneumococcal conjugate vaccine, and the importance of re-colonization by the same pneumococcal serotypes included in the previously used vaccine.

4. Pneumococcal colonization in older adults.

Esposito S, Mari D, Bergamaschini L, Orenti A, Terranova L, Ruggiero L, Ierardi V, Gambino M, Croce FD, Principi N.

Immun Ageing. 2016 Jan 12;13:2. doi: 10.1186/s12979-016-0057-0.

Abstract:

Background:

Little is known about pneumococcal carrier states in older adults. The main aim of this study was to evaluate pneumococcal colonization patterns among older adults in two centres in Milan, Italy, before the widespread use of the 13-valent pneumococcal vaccine (PCV13) in this age group, to investigate demographic and clinical features that are associated with pneumococcal colonization and to estimate the potential coverage offered by PCV13.

Results:

Among 417 adults ≥65 years old (171, 41.1 %, ≥75 years), 41 (9.8 %) were pneumococcal carriers. Univariate and multivariate analyses revealed that pneumococcal colonization was significantly less common among individuals with underlying co-morbidities than among those without (odds ratio [OR] 0.453, 95 % confidence interval [CI] 0.235-0.875, p = 0.018; adjusted OR 0.503, 95 % CI 0.255-0.992, p = 0.047). Moreover, among these patients, those with cardiac disease had a significantly lower risk of colonization (OR 0.308, 95 % CI 0.119-0.795, p = 0.015; adjusted OR 0.341, 95 % CI 0.13-0.894, p = 0.029). Only one vaccinated subject who received 23-valent

polysaccharide pneumococcal vaccine (PPV23) was colonized. Twenty-five (89.3 %) of the subjects who were <75 years old and 9 (75.0 %) of those who were ≥75 years old were colonized by at least one of the serotypes that is included in PCV13, with serotype 19 F being the most common. Respiratory allergies as well as overall co-morbidities were more common in subjects who were positive for only non-PCV13 serotypes compared with negative subjects and those who were carriers of only PCV13 serotypes.

Conclusions:

Although this study included a relatively small number of subjects and has been performed in a limited geographic setting, results showed that pneumococcal colonization in older people is common, and the monitoring of carriers can offer useful information about the circulation of this pathogen among older people and the potential protective effect of pneumococcal vaccines. Because the colonization in most cases involves the strains that are included in PCV13, this vaccine could be useful in the prevention of pneumococcal infections in the overall population of older people. In subjects with respiratory allergies and in those with co-morbidities, the addition of the PPV23 to PCV13 should be recommended. Due to the low vaccination coverage, urgent educational programmes are required to inform older adults and their medical doctors of the risks of pneumococcal infection and the efficacy and safety of the available pneumococcal vaccines.

5. **Measurement of lipocalin-2 and syndecan-4 levels to differentiate bacterial from viral infection in children with community-acquired pneumonia.**

Esposito S, Bianchini S, Gambino M, Madini B, Di Pietro G, Umbrello G, Presicce ML, Ruggiero L, **Terranova L**, Principi N.

BMC Pulm Med. 2016 Jul 20;16(1):103. doi: 10.1186/s12890-016-0267-4.

Abstract:

Background:

In this study, we evaluated the lipocalin-2 (LIP2) and syndecan-4 (SYN4) levels in children who were hospitalized for radiologically confirmed CAP in order to differentiate bacterial from viral infection. The results regarding the LIP2 and SYN4 diagnostic outcomes were compared with the white blood cell (WBC) count and C reactive protein (CRP) levels.

Methods:

A total of 110 children <14 years old who were hospitalized for radiologically confirmed CAP were enrolled. Serum samples were obtained upon admission and on day 5 to measure the levels of LIP2, SYN4, and CRP as well as the WBC. Polymerase chain reaction of the respiratory secretions and tests on blood samples were performed to detect respiratory viruses, Streptococcus pneumoniae, and Mycoplasma pneumoniae.

Results:

CAP was considered to be due to a probable bacterial infection in 74 children (67.3 %) and due to a probable viral infection in 16 children (14.5 %). Overall, 84 children (76.4 %) were diagnosed with severe CAP. The mean values of the WBC count and the LIP2 and SYN4 levels did not differ among the probable bacterial, probable viral, and undetermined cases. However, the CRP serum concentrations were significantly higher in children with probable bacterial CAP than in those with probable viral disease (32.2 ± 55.5 mg/L vs 9.4 ± 17.0 mg/L, $p < 0.05$). The WBC count was the best predictor of severe CAP, but the differences among the studied variables were

marginal. The WBC count was significantly lower on day 5 in children with probable bacterial CAP ($p < 0.01$) and in those with an undetermined etiology ($p < 0.01$). The CRP and LIP2 levels were significantly lower 5 days after enrollment in all of the studied groups, independent of the supposed etiology of CAP ($p < 0.01$ for all comparisons). No statistically significant variation was observed for SYN4.

Conclusions:

Measuring the LIP2 and SYN4 levels does not appear to solve the problem of the poor reliability of routine laboratory tests in defining the etiology and severity of pediatric CAP. Currently, the CRP levels and WBC, when combined with evaluation of clinical data, can be used to limit the overuse of antibiotics as much as possible and to provide the best treatment to the patient.

6. **Serotypes not included in 13-valent pneumococcal vaccine as causes of acute otitis media with spontaneous tympanic membrane perforation in a geographic area with high vaccination coverage.**

Marchisio P, Esposito S, Picca M, Baggi E, **Terranova L**, Orenti A, Biganzoli E, Principi N; Milan AOM Study Group.

Pediatr Infect Dis J. 2017 May;36(5):521-523.
doi: 10.1097/INF.0000000000001485.

Abstract:

In 177 children living in an area with high 13-valent pneumococcal conjugate vaccine 13 coverage, who had acute otitis media complicated with spontaneous tympanic membrane perforation, Streptococcus pneumoniae was identified in the middle ear fluid of 48 (27.1%) subjects, with 37 (77.1%) cases caused by non-pneumococcal conjugate vaccine 13 serotypes.

7. **Acute flaccid myelitis associated with enterovirus-D68 infection in an otherwise healthy child.**

Esposito S, Chidini G, Cinnante C, Napolitano L, Giannini A, **Terranova L**, Niesters H, Principi N, Calderini E.

Virol J. 2017 Jan 11;14(1):4. doi: 10.1186/s12985-016-0678-0.

Abstract:

Background:

Reporting new cases of enterovirus (EV)-D68-associated acute flaccid myelitis (AFM) is essential to understand how the virus causes neurological damage and to characterize EV-D68 strains associated with AFM.

Case presentation:

A previously healthy 4-year-old boy presented with sudden weakness and limited mobility in his left arm. Two days earlier, he had an upper respiratory illness with mild fever. At admission, his physical examination showed that the child was febrile (38.5 °C) and alert but had a stiff neck and weakness in his left arm, which was hypotonic and areflexic. Cerebrospinal fluid (CSF) examination showed a mild increase in white blood cell count (80/mm3, 41% neutrophils) and a slightly elevated protein concentration (76 gm/dL). Bacterial culture and molecular biology tests for detecting viral infection in CSF were negative. The patient was then treated with intravenous ceftriaxone and acyclovir. Despite therapy, within 24 h, the muscle weakness extended to all four limbs, which exhibited greatly reduced mobility. Due to his worsening clinical prognosis, the child was transferred to our Pediatric Intensive Care Unit; at admission he was diagnosed with acute flaccid paralysis of all four limbs. Brain magnetic resonance imaging (MRI) was negative, except for a focal signal alteration in the

dorsal portion of the medulla oblongata, also involving the pontine tegmentum, whereas spine MRI showed an extensive signal alteration of the cervical and dorsal spinal cord reported as myelitis. Signal alteration was mainly localized in the central grey matter, most likely in the anterior horns. Molecular biology tests performed on nasopharyngeal aspirate and on bronchoalveolar lavage fluid were negative for bacteria but positive for EV-D68 clade B3. Plasmapheresis was performed and corticosteroids and intravenous immunoglobulins were administered. After 4 weeks of treatment, the signs and symptoms of AFM were significantly reduced, although some weakness and tingling remained in the patient's four limbs. MRI acquired after 3 weeks showed that the previously reported alterations were no longer present.

Conclusion:

This case suggests that EV-D68 is a neurotropic agent that can cause AFM and strains are circulating in Europe. EV-D68 disease surveillance is required to better understand EV-D68 pathology and to compare various strains that cause AFM.

8. **Prospective evaluation of the aetiology of acute otitis media with spontaneous tympanic membrane perforation.**

Marchisio P, Esposito S, Picca M, Baggi E, **Terranova L**, Orenti A, Biganzoli E, Principi N; Milan AOM Study Group.

Clin Microbiol Infect. 2017 Jul;23(7):486.e1-486.e6.
doi: 10.1016/j.cmi.2017.01.010.

Abstract:

Objectives:

To evaluate the aetiological role of the main bacterial pathogens associated with acute otitis media (AOM) in children with AOM and spontaneous tympanic membrane perforation (STMP).

Methods:

Between 1 May 2015 and 30 April 2016, 177 children, aged 6 months to 7 years, with AOM complicated by STMP within 12 h were prospectively enrolled. Middle ear fluid (MEF) was tested by real-time PCR for Streptococcus pneumoniae, non-typeable Haemophilus influenzae, Streptococcus pyogenes, Moraxella catarrhalis and Staphylococcus aureus.

Results:

Among the 177 children with AOM and STMP, 92/100 (92.0%) of those with recurrent AOM and 13/77 (16.9%) without recurrent AOM had recurrent STMP ($p < 0.001$). A single pathogen was identified in 70 (39.5%) MEF samples, whereas two, three and four bacteria were detected in 54 (30.5%), 20 (11.3%), and 7 (4.0%) cases, respectively. Non-typeable H. influenzae was the most common and was identified in 90 children (50.8%), followed by M. catarrhalis (62 cases, 35.0%) and S. pneumoniae (48 cases, 27.1%). Non-typeable H. influenzae was the most frequent pathogen in children with co-infections. Children with co-infections, including non-typeable H. influenzae, had significantly more frequent recurrent AOM (adjusted OR 6.609, 95% CI 1.243-39.096, p 0.029).

Conclusions:

Recurrent AOM episodes appear to be associated with an increased risk of AOM with STMP. In AOM with STMP, non-typeable H. influenzae is detected at a high frequency, especially in children with recurrent STMP and often in association with other pathogens.

9. **Severe pneumonia caused by Influenza A (H1N1) virus successfully managed with extracorporeal life support in a comorbid former preterm infant.**

Raffaeli G, Cavallaro G, Pugni L, Leva E, Artoni A, Neri S, Baracetti C, Cotza M, Gentilino V, **Terranova L**, Esposito S, Mosca F.
Int J Environ Res Public Health. 2017 Mar 31;14(4). pii: E360.
doi: 10.3390/ijerph14040360.

Abstract:

Influenza A (H1N1) virus infection is a global health burden, leading to significant pediatric morbidity and mortality. Prematurity, young age and comorbidities are important risk factors for unfavorable outcomes. Preventive strategies, such as healthcare workers and household contacts vaccination as well as the implementation of infection control practices during the epidemic season, are crucial to protect the most vulnerable populations. Early diagnosis, timely administration of antiviral drugs and supportive therapy are crucial to lead to a complete recovery. When conventional treatment fails, extracorporeal life support (ECLS) may be employed. In neonates and young infants, this high-tech support is burdened by specific technical complexity. Despite the potential risks related to this aggressive approach, ECLS is a life-saving procedure in 65% of pediatric viral pneumonia and in 73% of sepsis cases. Here, we report the successful outcome of a 51-day formerly preterm infant, suffering from a surgical necrotizing enterocolitis (NEC), complicated with hospital-acquired pneumonia due to influenza A (H1N1) virus. She developed a severe respiratory failure, unresponsive to conventional therapy, and successfully treated with ECLS. To our knowledge, this is the first report on the use of ECLS in a formerly preterm infant, suffering from NEC complicated by influenza A (H1N1) virus infection.

10. **Pertussis-associated persistent cough in previously vaccinated children.**

Principi N, Litt D, **Terranova L**, Picca M, Malvaso C, Vitale C, Fry NK, Esposito S; The Italian Pertussis Group For Persistent Cough In Children.

J Med Microbiol. 2017 Nov;66(11):1699-1702.

doi: 10.1099/jmm.0.000607.

Abstract:

To evaluate the role of Bordetella pertussis infection, 96 otherwise healthy 7- to 17-year-old subjects who were suffering from a cough lasting from 2 to 8 weeks were prospectively recruited. At enrolment, a nasopharyngeal swab and an oral fluid sample were obtained to search for pertussis infection by the detection of B. pertussis DNA and/or an elevated titre of anti-pertussis toxin IgG. Evidence of pertussis infection was found in 18 (18.7%; 95% confidence interval, 11.5-28.0) cases. In 15 cases, the disease occurred despite booster administration. In two cases, pertussis was diagnosed less than 2 years after the booster injection, whereas in the other cases it was diagnosed between 2 and 9 years after the booster dose. This study used non-invasive testing to show that pertussis is one of the most important causes of long-lasting cough in school-age subjects. Moreover, the protection offered by acellular pertussis vaccines currently wanes more rapidly than previously thought.

11. Neutrophil elastase in bronchiectasis.

Gramegna A, Amati F, **Terranova L**, Sotgiu G, Tarsia P, Miglietta D, Calderazzo MA, Aliberti S, Blasi F.

Respir Res. 2017 Dec 19;18(1):211. doi: 10.1186/s12931-017-0691-x. Review.

Abstract:

The role of neutrophil elastase (NE) is poorly understood in bronchiectasis because of the lack of preclinical data and so most of

the assumptions made about NE inhibitor potential benefit is based on data from CF. In this context, NE seems to be a predictor of long-term clinical outcomes and a possible target of treatment. In order to better evaluate the role of NE in bronchiectasis, a systematic search of scientific evidence was performed.Two investigators independently performed the search on PubMed and included studies published up to May 15, 2017 according to predefined criteria. A final pool of 31 studies was included in the systematic review, with a total of 2679 patients. For each paper data of interest were extracted and reported in table.In this review sputum NE has proved useful as an inflammatory marker both in stable state bronchiectasis and during exacerbations and local or systemic antibiotic treatment. NE has also been associated with risk of exacerbation, time to next exacerbation and all-cause mortality. This study reviews also the role of NE as a specific target of treatment in bronchiectasis. Inhibition of NE is at a very early stage and future interventional studies should evaluate safety and efficacy for new molecules and formulations.

12. **Neisseria meningitidis serogroup B carriage by adolescent and young adults living in Milan, Italy: prevalence of strains potentially covered by the presently available meningococcal B vaccines.**

Terranova L, Principi N, Bianchini S, Di Pietro G, Umbrello G, Madini B, Esposito S.

Hum Vaccin Immunother. 2018 Mar 27:1-16. doi: 10.1080/21645515.2018.1450121.

Abstract:

Recently, two vaccines against meningococcal serogroup B (MenB) have been developed. They are prepared according to the reverse

vaccinology approach and contain 4 (4CMenB) and 2 (MenB-FHbp) cross-reactive surface proteins. In Italy 4CMenB vaccine has been included in the official vaccination schedule only recently and recommended only for infants and toddlers, whereas MenB-FHbp is not licensed. In order to collect information about the present carriage of Neisseria meningitidis serogroup B (MenB) in Italian adolescents and to evaluate the potential protection offered by the presently available MenB vaccines, 2,560 otherwise healthy, high school students aged 14-21 years (907 males, 35.4%, median age 16.2 years) were enrolled in Milan, Italy. A swab to collect posterior pharynx secretions was collected from each subject and meningococcal identification, serogrouping, multilocus sequence typing analysis, sequence alignments and phylogenetic analysis were performed. A total of 135 (5.3%) adolescents were meningococcal carriers. Strains belonging to serogroup B were the most common (n = 58; 2.3%), followed by MenY (n = 32; 1.2%), MenC (n = 7; 0.3%), MenW (n = 6; 0.3%) and MenX (n = 5; 0.2%). The remaining bacteria were not capsulated. The identified MenB strains belonged to eleven clonal complexes (CCs): ST-162 CC (n = 12; 20.7%), ST-865 CC (n = 12; 20.7%), ST-41/44/Lin.3 CC (n = 11; 19.0%), ST-35 CC (n = 6; 10.3%), ST-32/ET-5 CC (n = 4; 6.9%), ST-269 CC (n = 3; 5.2%), ST-213 CC (n = 2; 3.4%), ST-198 CC (n = 1; 1.7%), ST-461 CC (n = 1; 1.7%), ST-549 CC (n = 1; 1.7%), and ST-750 CC (n = 1; 1.7%). This study showed that MenB was the most commonly carried meningococcal serogroup found in adolescents living in Milan, Italy. The MenB vaccines presently licensed could have theoretically induced the production of antibodies effective against the greatest part of the identified MenB strains (100% in the case of 4CMenB and 95% in case of MenB-FHbp) Monitoring carriage remains essential to

evaluate MenB circulation, but further studies are necessary to evaluate the effect on carriage and the final efficacy of both new MenB vaccines.

13. **Staphylococcus aureus colonization and risk of surgical site infection in children undergoing clean elective surgery: A cohort study.**

Esposito S, **Terranova L**, Macchini F, Bianchini S, Biffi G, Viganò M, Pelucchi C, Leva E, Principi N.

Medicine (Baltimore). 2018 Jul;97(27):e11097. doi: 10.1097/MD.0000000000011097.

Abstract:

Staphylococcus aureus persistently colonizes the skin and nasopharynx of approximately 20% to 30% of individuals, with the highest rates in younger children. To avoid clinical problems for carriers and the spread of S aureus to other hospitalized patients, screening and decolonization of carriers undergoing surgery has been recommended. However, the best approach to patients undergoing clean surgery is not precisely defined. To evaluate whether children carrying S aureus admitted to the hospital for clean elective surgery have an increased risk of postoperative surgical infections, 393 infants and children (77.1% males; mean age±standard deviation, 7.6±4.5 years) who were scheduled for clean elective surgery procedures were evaluated for S aureus carriage on the day of intervention and 5 days after it. Both anterior nares and pharyngeal swabs were collected. S aureus was identified using the RIDAGENE methicillin-resistant S aureus (MRSA) system (R-Biopharm AG, Darmstadt, Germany), according to the manufacturer's instructions. At admission, 138 (35.1%) children screened positive for S aureus. MRSA was identified in 40 (29.0% of S aureus positive subjects) cases. The carriage rates of

S aureus and MRSA varied considerably with age, and in children <2 years old the rate was significantly lower than in any other age group (P<.05). Surgical site infection was demonstrated in 4 out of 109 (3.7%) children who were initially colonized by S aureus and in 5 out of 201 (2.5%) children with a negative screening, without any statistically significant difference between groups (P=.72). None of these children had MRSA. These results seem to suggest that children undergoing clean elective surgery do not need to be screened for S aureus colonization because, although positive, they have no increased risk of surgical site infection. Following this statement, preoperative procedures should be simplified with relevant advantages from a clinical, social, and economic point of view.

14. **When and out rouling cystic fibrosis in adults patients with bronchiectasis.**

Gramegna A, Aliberti S, Seia M, Porcaro L, Bianchi V, Castellani C, Melotti P, Sorio C, Consalvo E, Franceschi E, Amati F, Contarini M, Gaffuri M, Roncoroni L, Vigone B, Bellofiore A, Del Monaco C, Oriano M, **Terranova L**, Patria MF, Marchisio P, Assael BM, Blasi F.

Abstract:

Bronchiectasis is the final result of different processes and most of the guidelines advocate for a careful evaluation of those etiologies which might be treated or might change patients' management, including cystic fibrosis (CF). CFTR mutations have been reported with higher frequency in bronchiectasis population. Although ruling out CF is considered as a main step for etiological screening in bronchiectasis, CF testing lacks of a standardized approach both from a research and clinical point of view. In this review a list of most widely used tests in CF is provided. Exclusion of CF is imperative for patients with

bronchiectasis and CFTR testing should be implemented in usual screening for investigating bronchiectasis etiology. Physicians taking care of bronchiectasis patients should be aware of CFTR testing and its limitations in the adult population. Further studies on CFTR expression in human lung and translational research might elucidate the possible role of CFTR in the pathogenesis of bronchiectasis.

15. **The Italian registry of pulmonary non-tubercolus mycobacteria - IRENE: the study protocol.**

Aliberti S, Codecasa LR, Gori A, Sotgiu G, Spotti M, Di Biagio A, Calcagno A, Nardini S, Assael BM, Tortoli E, Besozzi G, Ferrarese M, Matteelli A, Girardi E, De Lorenzo S, Seia M, Gramegna A, Del Prato B, **Terranova L**, Oriano M, Sverzellati N, Mirsaeidi M, Chalmers JD, Haworth CS, Loebinger MR, Aksamit T, Winthrop K, Ringshausen FC, Previdi G, Blasi F; IRENE Network.

Abstract:

Background:

A substantial increase in pulmonary and extra-pulmonary diseases due to non-tuberculous mycobacteria (NTM) has been documented worldwide, especially among subjects suffering from chronic respiratory diseases and immunocompromised patients. Many questions remain regarding the epidemiology of pulmonary disease due to NTM (NTM-PD) mainly because reporting of NTM-PD to health authorities is not mandated in several countries, including Italy. This manuscript describes the protocol of the first Italian registry of adult patients with respiratory infections caused by NTM (IRENE).

Methods:

IRENE is an observational, multicenter, prospective, cohort study enrolling consecutive adult patients with either a NTM respiratory isolate or those with NTM-PD. A total of 41 centers, including mainly

pulmonary and infectious disease departments, joined the registry so far. Adult patients with all of the following are included in the registry: 1) at least one positive culture for any NTM species from any respiratory sample; 2) at least one positive culture for NTM isolated in the year prior the enrolment and/or prescribed NTM treatment in the year prior the enrolment; 3) given consent to inclusion in the study. No exclusion criteria are applied to the study. Patients are managed according to standard operating procedures implemented in each IRENE clinical center. An online case report form has been developed to collect patients' demographics, comorbidities, microbiological, laboratory, functional, radiological, clinical, treatment and outcome data at baseline and on an annual basis. An IRENE biobank has also been developed within the network and linked to the clinical data of the registry.

Conclusions:

IRENE has been developed to inform the clinical and scientific community on the current management of adult patients with NTM respiratory infections in Italy and acts as a national network to increase the disease's awareness.

16. How to Process Sputum Samples and Extract Bacterial DNA for Microbiota Analysis.

Terranova L, Oriano M, Teri A, Ruggiero L, Tafuro C, Marchisio P, Gramegna A, Contarini M, Franceschi E, Sottotetti S, Cariani L, Bevinino A, Chalmers JD, Aliberti S, Blasi F.

Abstract:

Different steps and conditions for DNA extraction for microbiota analysis in sputum have been reported in the literature. We aimed at testing both dithiothreitol (DTT) and enzymatic treatments of sputum

samples and identifying the most suitable DNA extraction technique for the microbiota analysis of sputum. Sputum treatments with and without DTT were compared in terms of their median levels and the coefficient of variation between replicates of both DNA extraction yield and real-time PCR for the 16S rRNA gene. Treatments with and without lysozyme and lysostaphin were compared in terms of their median levels of real-time PCR for S. aureus. Two enzyme-based and three beads-based techniques for DNA extraction were compared in terms of their DNA extraction yield, real-time PCR for the 16S rRNA gene and microbiota analysis. DTT treatment decreased the coefficient of variation between replicates of both DNA extraction yield and real-time PCR. Lysostaphin (either 0.18 or 0.36 mg/mL) and lysozyme treatments increased S. aureusdetection. One enzyme-based kit offered the highest DNA yield and 16S rRNA gene real-time PCR with no significant differences in terms of alpha-diversity indexes. A condition using both DTT and lysostaphin/lysozyme treatments along with an enzymatic kit seems to be preferred for the microbiota analysis of sputum samples.

8. PhD courses

1. Introduzione alla statistica per l'epidemiologia e la sanità pubblica.
   Prof. Bossi, 11, 14 Apr 2016
2. Le strategie di sanità pubblica in ambito vaccinale.
   Prof. Romanò, Castaldi, Mapelli 10, 17, 24 Feb, 4, 31 May 2016
3. Misure di associazione in epidemiologia.
   Prof. Decarli 24 May, 27 Jun 2016
4. Open access – open data e il mondo delle pubblicazioni.
   Competenze trasversali 17 Jun 2016
5. Disegno e analisi di studi caso-controllo.
   Prof. La Vecchia 10-11 May 2016
6. Elementi di valutazione del rischio chimico.
   Prof. Moretto 23, 24, 30 Jun, 1 Jul 2016
7. Come scrivere un progetto di ricerca-parte 1. Le 100 cose che avrei voluto sapere quando ero un dottorando.
   Competenze trasversali 22 Feb 2017
8. General linear model and experimental design using "R"
   Prof. Micciolo 27, 28, 29, 30, 31 Mar 2017
9. Come scrivere un progetto di ricerca-parte 2. Le 100 cose che avrei voluto sapere quando ero un dottorando.
   Competenze trasversali 16 Jun 2017
10. Analisi della sopravvivenza.
    Prof. Ambrogi, Boracchi, Biganzoli 19, 20, 21, 22, 23 Feb 2018
11. Tutelare e valorizzare sul mercato i risultati della ricerca in UniMi.
    Competenze trasversali 8 Mar 2018
12. Valorizzare creando impresa: fare spin off in UniMi (prima parte).
    Competenze trasversali 20 Apr 2018
13. Revisioni sistematiche con meta-analisi: metodi statistici ed interpretazione dei risultati.

Prof. Casazza 22-23 May 2018

14. Valorizzare creando impresa: fare spin off in UniMi (seconda parte).
    Competenze trasversali 25 May 2018
15. Costruire un profilo professionale attraverso le competenze trasferibili. Quali possibilità per i dottori di ricerca?
    Competenze trasversali 20 Jun 2018

9. Bibiography

1) Jenner E. The Origin of the Vaccines Inoculation. Shury; London, UK: 1801

2) Pasteur L. De l'attenuation du virus du Choléra des poules. CR Acad. Sci. Paris. 1880; 91:673

3) Salmon D, Smith T. On a new method of producing immunity from contagious diseases. American Vet Review. 1886; 10:63.

4) Enders JF, Weller TH, Robbins FC. Cultivation of the Lansing Strain of Poliomyelitis Virus in Cultures of Various Human Embryonic Tissues. Science. 1949; 109:85.

5)http://www.polioeradication.org/Dataandmonitoring/Poliothisweek.aspx

6) Black S, Shinefield H, Fireman B, Lewis E, Ray P, Hansen JR, Elvin L, Ensor KM, Hackell J, Siber G, Malinoski F, Madore D, Chang I, Kohberger R, Watson W, Austrian R, Edwards K. Efficacy, safety and immunogenicity of heptavalent pneumococcal conjugate vaccine in children. Pediatr Infect Dis J. 2000 Mar;19(3):187-95.

7) Schneerson R, Barrera O, Sutton A, Robbins JB. Preparation, characterization, and immunogenicity of Haemophilus influenzae type b polysaccharide-protein conjugates. J Exp Med. 1980; 152:361.

8) Roush SW, Murphy TV. Historical comparisons of morbidity and mortality for vaccine-preventable diseases in the United States. JAMA 2007;298(18):2155–63.

9) Goldsby, R., Kindt, T.J., Osborne, B.A. and Kuby, J. (2003) Chapter 2: Cells and Organs of the Immune System. In: Immunology, 5th Edition, W. H. Freeman and Company, New York, 24-56.

10) Virji M. Meningococcal disease: epidemiology and pathogenesis. Trends Microbiol. 1996 Dec;4(12):466-9; discussion 469-70.

11) Caugant DA, Maiden MCJ. Meningococcal carriage and disease--population biology and evolution. Vaccine. 2009 Jun 24;27 Suppl 2:B64-70.

12) Rosenstein NE, Perkins BA, Stephens DS, Popovic T, Hughes JM. Meningococcal disease. N Engl J Med. 2001 May 3;344(18): 1378-88.

13) Rouphael NG, Stephens DS. Neisseria meningitidis: Biology, Microbiology, and Epidemiology. Methods Mol Biol. 2012; 799: 1–20.

14) Masignani V, Comanducci M, Giuliani MM, Bambini S, Adu-Bobie J, Aricò B, Brunelli B, Pieri A, Santini L, Savino S, Serruto D, Litt D, Kroll S, Welsch JA, Granoff DM, Rappuoli R, Pizza M. Vaccination against Neisseria meningitidis using three variants of the lipoprotein GNA1870. J Exp Med. 2003 Mar 17;197(6):789-99.

15) Comanducci M, Bambini S, Brunelli B, Adu-Bobie J, Aricò B, Capecchi B, Giuliani MM, Masignani V, Santini L, Savino S, Granoff DM, Caugant DA, Pizza M, Rappuoli R, Mora M. NadA, a novel vaccine candidate of Neisseria meningitidis. J Exp Med. 2002 Jun 3;195(11):1445-54.

16) Serruto D, Bottomley M, Ram S, Giuliani MM, Rappuoli R. The new multicomponent vaccine against meningococcal serogroup B, 4CMenB: immunological, functional and structural characterization of the antigens. Vaccine. 2012 May 30;30 Suppl 2:B87-97.

17) Maiden MC, Bygraves JA, Feil E, Morelli G, Russel JE, Urwin R, Zhang Q, Zhou J, Zurth K, Caugant DA, Feavers IM, Achtman M, Spratt BG. Multilocus sequence typing: A portable approach to the identification of clones within populations of pathogenic microorganisms. Proc Natl Acad Sci USA. 1998 Mar 17;95(6):3140-5.

18) Urwin R, Maiden MCJ. Multi-locus sequence typing: a tool for global epidemiology. Trends Microbiol. 2003 Oct;11(10):479-87.

19) Azzari C, Canessa C, Lippi F, Moriondo M, Indolfi G, Nieddu F, Martini M, De Martino M, Castiglia P, Baldo V, Resti M. Distribution of invasive meningococcal B disease in Italian pediatric population: implication for vaccination timing. Vaccine 2014 Feb 26:32(10):1187-91.

20) Mothershed EA, Sacchi CT, Whitney AM, Barnett GA, Ajello GW, Schmink S, Mayer LW, Phelan M, Taylor TH, Bernhardt SA, Rosenstein NE, Popovic T. Use of Real-Time PCR to resolve slide agglutination discrepancies in serogroup identification of Neisseria meningitides. J Clin Microbiol. 2004 Jan;42(1):320-8.

21) Esposito S, Zampiero A, Terranova L, Montinaro V, Scala A, Ansuini V, Principi N. Genetic characteristics of Neisseria Meningitidis serogroup B strains carried by adolescents living in Milan, Italy. Hum Vaccin Immunother. 2013 Nov;9(11):2296-303.

22) Jolley K, Maiden MC. BIGSdb: Scalable analysis of bacterial genome variation at the population level. BMC Bioinformatics 201011:595

23) Lucidarme J, Comanducci M, Findlow J, Gray SJ, Kaczmarski EB, Guiver M, Vallely PJ, Oster P, Pizza M, Bambini S, Muzzi A, Borrow R. Characterization of fHbp, nhba (gna2132), nadA, porA, and sequence type in group B meningococcal case isolates collected in

England and Wales during January 2008 and potential coverage of an investigational group B meningococcal vaccine. Clin Vaccine Immunol. 2010 Jun;17(6):919-29.

24) Racloz VN, Luiz SJ. The elusive meningococcal meningitides serogroup: a systematic review of serogroup B epidemiology. BMC Infect Dis. 2010 Jun 17;10:175.

25) Law DK, Lorange M, Ringuette L, Dion R, Giguère M, Henderson AM, Stoltz J, Zollinger WD, De Wals P, Tsang RS. Invasive Meningococcal Disease in Québec, Canada, Due to an Emerging Clone of ST-269 Serogroup B Meningococci with Serotype Antigen 17 and Serosubtype Antigen P1.19 (B:17:P1.19). J Clin Microbiol. 2006 Aug; 44(8): 2743–2749.

26) Jeppesen CA, Snape MD, Robinson H, Gossger N, John TM, Voysey M, Ladhani S, Okike IO, Oeser C, Kent A, Oliver J, Taylor P, Morales-Aza B, Clarke SC, Casey M, Martins F, Kitchin NR, Anderson AS, Jones H, Jansen KU, Eiden J, Pedneault L, Heath PT, Finn A, Faust SN, Pollard AJ. Meningococcal carriage in adolescents in the United Kingdom to inform timing of an adolescent vaccination strategy. J Infect. 2015;71:43-52.

27) Read RC, Baxter D, Chadwick DR, Faust SN, Finn A, Gordon SB, Heath PT, Lewis DJ, Pollard AJ, Turner DP, Bazaz R, Ganguli A, Havelock T, Neal KR, Okike IO, Morales-Aza B, Patel K, Snape MD, Williams J, Gilchrist S, Gray SJ, Maiden MC, Toneatto D, Wang H, McCarthy M, Dull PM, Borrow R. Effect of a quadrivalent meningococcal ACWY glycoconjugate or a serogroup B meningococcal vaccine on meningococcal carriage: an observer-blind, phase 3 randomised clinical trial. Lancet. 2014;384:2123-2131.

28) European Centre for Disease Prevention and Control. Invasive meningococcal disease - Annual Epidemiological Report 2016 [2014 data]. Available at: https://ecdc.europa.eu/en/publications-data/invasive-meningococcal-disease-annual-epidemiological-report-2016-2014-data Accessed on: October 9,2017.

29) Ministry of Health. Istituto Superiore di Sanità. Vaccination coverage in Italy. Meningococcus C. Available at: http://www.epicentro.iss.it/temi/vaccinazioni/dati_Ita.asp Accessed on: December 11,2017.

30) Ministry of Health. National vaccination prevention plan 2017 - 2019. Available at: http://www.salute.gov.it/imgs/C_17_pubblicazioni_2571_allegato.pdf Accessed on: December 11,2017.

31) Istituto Superiore di Sanità. Surveillance data for invasive bacterial diseases updated to 16 November 2016. http://www.iss.it/binary/mabi/cont/Report_MBI_20161116_v11.pdf Accessed on: December 14,2017.

32) Comanducci M, Bambini S, Caugant DA, Mora M, Brunelli B, Capecchi B, Ciucchi L, Rappuoli R, Pizza M. NadA diversity and carriage in Neisseria meningitidis. Infect Immun. 2004;72:4217-4223.

33) O'Connor L, Ward M, Bennett D, Mulhall R, O'Lorcain P, Cunney R, McDermott R, Neville E, Heslin J, Frizgerald R, Meyler K, Conlon M, Clarke A, Corcoran B, Fitzpatrick, O'Connor B, Flanagan P, O'Flanagan, Cotter S. A prolonged outbreak of invasive meningococcal disease in an extended Irish Traveller family across three Health Service Executive (HSE) areas in Ireland, 2010 to 2013. Euro Surveill. 2015 May 28;20(21).

34) Maiden MC, Ibarz-Pavón AB, Urwin R, Gray SJ, Andrews NJ, Clarke SC, Walker AM, Evans MR, Kroll JS, Neal KR, Ala'aldeen

DA, Crook DW, Cann K, Harrison S, Cunningham R, Baxter D, Kaczmarski E, Maclennan J, Cameron JC, Stuart JM. Impact of meningococcal serogroup C conjugate vaccines on carriage and herd immunity. J Infect Dis. 2008;197:737-743.

35) European Medicines Agency. Bexero. Available at: http://www.ema.europa.eu/ema/index.jsp?curl=pages/medicines/human/medicines/002333/human_med_001614.jsp&mid=WC0b01ac058001d124. Accessed on: October 9,2017).

36) Patton ME, Stephens D, Moore K, MacNeil JR. Updated Recommendations for Use of MenB-FHbp Serogroup B Meningococcal Vaccine - Advisory Committee on Immunization Practices, 2016.MMWR Morb Mortal Wkly Rep. 2017;66:509-513.