Influenza immunology evaluation and correlates of protection: a focus on vaccines

(Article begins on next page)

11 July 2024

Influenza Immunology Evaluation and Correlates of Protection: a Focus on Vaccines

Claudia Maria Trombetta[1] & Emanuele Montomoli[1,2*]

1 Department of Molecular and Developmental Medicine, University of Siena, 53100 Siena, Italy.
trombetta@unisi.it; emanuele.montomoli@uni.it .
2 VisMederi srl, Enterprise of services in Life Sciences, Via Fiorentina 1, 53100 Siena, Italy.
*Author for correspondence: Tel: +39 0577234134; e-mail: emanuele.montomoli@uni.it .

Summary

Vaccination is the most effective method of controlling seasonal influenza infections and preventing possible pandemic events. Although influenza vaccines have been licensed and used for decades, the potential correlates of protection induced by these vaccines are still a matter of discussion. Currently, inactivated vaccines are the most common and the haemagglutination inhibition antibody titer is regarded as an immunological correlate of protection and the best available parameter for predicting protection from influenza infection. However, the assay shows some limitations, such as its low sensitivity to B and avian strains and inter-laboratory variability. Additional assays and next-generation vaccines have been evaluated to overcome the limitations of the traditional serological techniques and to elicit broad immune responses, underlining the need to revise the current correlates of protection. The aim of this review is to provide an overview of the current scenario regarding the immunological evaluation and correlates of protection of influenza vaccines.

Keywords: correlates of protection, influenza vaccines, immunological assays, guidelines on influenza vaccines, haemagglutination inhibition titer.

Vaccination is the most effective method of controlling seasonal influenza infections and the most important strategy for preventing possible pandemic events [1]. The degree of protection elicited by vaccination depends on a complex interplay between vaccine composition and circulating influenza viruses, the age of vaccine recipients and their previous exposure to influenza, and product-specific factors such as formulation and the use of adjuvants.
Currently, the most effective means of counteracting influenza infection are inactivated influenza vaccines [2].
Traditional seasonal vaccines are trivalent, containing three different influenza viruses, A H1N1, A H3N2 and B strains. More recently, quadrivalent vaccines have been marketed in order to cover both Victoria and Yamagata lineages of B strain [3, 4].

The composition of vaccines requires to be evaluated annually, in order to determine if any vaccine strain needs to be updated because of antigenic drift of the influenza viruses. In this case, even the immunogenicity of vaccines (their ability to induce an immune response) needs to be evaluated. Previous studies have shown that antibodies against viral haemagglutinin (HA) are an important correlate of protection, and vaccine composition is usually standardized according to the HA titer [3, 5]. In order to be licensed, every vaccine needs to fulfill the criteria issued by the Committee for Proprietary Medical Products (CPMP) [6]. Although influenza vaccines have been licensed and used for decades, the potential correlates of the protection induced by these vaccines are still a matter of discussion.

The aim of this review is to provide an overview of the current scenario regarding the immunological evaluation and correlates of protection of influenza vaccines.

CORRELATES OF PROTECTION

Although the concept of the "correlate of protection" is an important milestone in the process of developing and licensing influenza vaccines, it remains very confused. Different definitions have been suggested. According to Plotkin and Gilbert [7], "a correlate reflects a statistical relation between an immune marker and protection but does not necessarily imply causal agency of the marker", while Qin et al. [8] claim that correlates predict "protection for new settings and describe the data requirements for rigorous validation of an immunological measurement at each level". The concept of correlate of protection is based on the immunogenic capacity of the vaccine to induce an antibody and/or cell-mediated immune response in recipients [9]. Two types of correlates of protection can be distinguished, absolute and relative. Although the ideal scenario aspires to an absolute correlate of protection meaning that protection is almost guarantees by a definitive threshold of response (such as in the case of diphtheria, tetanus and rubella), many correlates of protection are relative [10].

CORRELATES OF PROTECTION FOR INFLUENZA VACCINE

For many years, the CPMP criteria fulfilled by vaccines licensed in Europe included exclusively Single Radial Haemolysis (SRH) and Haemagglutination Inhibition (HI) assays and two different target groups – healthy adults between 18-60 years old and those over 60 [6] (Table 1).

Currently, an HI titer ≥ 40 is considered an immunological correlate of protection and the best available parameter for predicting protection from influenza infection. The first evidence dates back to Hobson et al., who established that an HI titer of 18-36 was associated with 50% protection from infection. Their study consisted of challenge experiments in small groups of segregated volunteers and larger-scale field trials in industrial workers, with the infective challenge dose being administered by nasal spray or droplets [5].

Subsequent studies confirmed the validity of the HI titer, showing that a titer of 42 for H1N1 and 44 for H3N2 influenza viruses was 50% protective against the intranasal inoculation of attenuated viruses [11, 12].

A recent meta-analysis estimated the HI protection curve by considering both the data from Hobson and those from 15 studies reported in the literature. The results suggest that there is a significant relationship between the increase in protection and the increase in HI titer, regardless of strains and the vaccination status of the individuals,

and that the best representation is a curve rather than a threshold. A titer of 30 corresponds to 50% protection; however, clinical protection shows a progressive and significant increase at titres up to 100 (included the common titer of 40) while advantages become marginal beyond a titer of 150 [13, 14].

While these findings support the concept that an HI titer ≥ 40 is an immunological correlate of protection, they prompt two considerations. First, as mentioned above, the relationship between protection and HI titer is better depicted by a curve rather than a threshold, underlining the fact that the relationship is not correctly represented by a single threshold titer; second, an HI titer ≥ 40 indicates 50% protection, as a relative correlate of protection, which may not be deemed satisfactory [14, 15].

In a broader prospective, the crucial question is how reliable is a threshold defined a long time ago and based on challenge studies with potentially attenuated viruses. Indeed, in this regard, Hobson noted that ".... it is possible that the low PD50 merely reflects the essential requirement of the present trials that the challenge virus strains should be selected chick embryo-grown variants of somewhat attenuated virulence, likely to produce less persistent or clinically severe infections than parental wild-type strains. It is thus not possible to extrapolate from the present experimental procedures to forecast that similar low titres of serum HI antibody would protect against natural infection with highly virulent epidemic strains of virus" [5]. The challenge study performed by Hobson et al. did not consider the possibility that individuals may need a higher antibody level in order to be protected, especially in the event of later onset of influenza in the community [5, 16, 17]. Moreover, it is debatable whether an HI titer ≥ 40 can also provide protection in older adults, children and other groups at high risk. Indeed, Black et al. provided evidence that an HI titer ≥ 40 is not an appropriate correlate of protection in children under 6 years of age, as they need an HI titer of 110 to reach 50% protection and titres of 215, 330 and 629 to achieve protection levels of 70%, 80% and 90%, respectively [15, 18, 19].

Another question concerns the use of the HI assay, given its low sensitivity to B strains and avian viruses, in addition to other technical aspects, which could influence the assay [2, 20].

ASSAYS FOR INFLUENZA VACCINE EVALUATION

Traditional serological assays

Haemagglutination Inhibition assay

Officially recognized by the international regulatory authorities, HI is currently the assay most widely used to measure the immune response to influenza vaccines. The assay detects both IgM and IgG antibodies able to inhibit the interaction between red blood cells and the head domain of haemagglutinin (HA). This region displays high variability, mostly due to the mechanism of antigenic drift. The antibodies detected by the HI assay seem to be strain-specific, not cross-reactive and not protective against mismatching influenza strains [16, 21]. The assay has the advantage of being easy to perform, simple and cheap, but has some limitations, such as its low sensitivity to B and avian strains [22, 23]. With regard to avian strains, the use of horse red blood cells seems to significantly improve HI sensitivity, while in the case of B strains, ether antigen treatment can enhance HI performance, though it also reduces assay specificity and increases HI variability [19, 23, 24]. Collaborative studies have revealed

a high degree of variability among laboratories, owing to the lack of standardized assays, harmonized protocols and sources of red blood cells (Table 2) [25, 26].

The HI antibody titer is expressed as the reciprocal of the highest serum dilution showing complete inhibition on using 4 or /8 haemagglutination units of virus. As previously mentioned, an antibody titer of 40 is generally regarded as a protective threshold level, beyond which there is a 50% or greater reduction in the possibility of contracting influenza infection. An HI titer of 40 or higher is applied as an immunological correlate of protection and is regarded as the best currently available parameter for predicting protection from infection, according to international regulatory guidelines for influenza vaccines [5, 12, 27]. However, in the case of a population with a pre-vaccination titer due to previous exposure to the influenza virus or vaccination, the antibody response could be significantly overestimated, as the titer may represent the protection induced by antibodies in general, rather than being a real measure of the protection induced by the vaccine. Statistical analysis should take into account the pre-vaccination titer and other variables, such as previous vaccination one year earlier, that could affect the immunogenicity of the vaccine [24, 28, 29]. It should, however, be noted that two further parameters seroconversion and mean geometric increase, which have already been included in vaccine assessment, are able to distinguish high antibody titres prior to vaccination.

Single Radial Haemolysis assay

The other officially recognized technique is the SRH assay, which is based on the passive haemolysis of red blood cells; this haemolysis is mediated by complement and induced by the antibody-antigen complex. The result is an easily identifiable "area of haemolysis", which is proportional to the concentration of antibodies against influenza viruses present in the serum samples [30, 31]. The assay is rapid, simple, reliable and reproducible. The main advantage is its ability to analyze a large number of serum samples simultaneously and rapidly and to provide unbiased results. These features make the SRH assay particularly suitable for large-scale investigations, especially epidemiological studies. Other significant advantages are the ability to detect small differences in antibody levels and to distinguish between closely related influenza strains (Table 2) [32-37].

The SRH assay measures antibodies, mainly IgG, against surface glycoproteins and internal antigens [38]. The detection of IgG antibodies is an advantage in pediatric trials [16, 30, 38].

A haemolysis area of 25 mm^2 or greater is generally regarded as a protective threshold level, beyond which there is a 50% or greater reduction in the probability of contracting influenza infection [39].

There is a good correlation between SRH and HI assays. While both are equally sensitive to influenza A viruses, the SRH assay seems to be more sensitive to influenza B viruses and more reproducible [40]. Two other important advantages of the SRH assay are its adequate sensitivity and specificity in detecting antibodies against avian influenza viruses and its safety, since it can be performed with inactivated viruses [20, 38, 41].

Virus Neutralization assay

The Virus Neutralization (VN) assay, commonly used in a Micro Neutralization (MN) format, is not mandatory for the licensing of influenza vaccines. However, it is recommended for the quantification of neutralizing antibodies and for the confirmation of H5N1 infection [42]. The technique detects functional antibodies able to neutralize the ability of the virus to enter or replicate in mammalian cells; it therefore measures all antibodies involved in protection. In addition, the assay recognizes antibodies against the neuraminidase (NA) glycoprotein, which may participate in the neutralization of the virus, especially in the late stage of infection [16, 43].

Compared with HI, the VN assay seems to be more complete and better suited to evaluating the serological response to influenza vaccines [16, 43]. However, the detected antibodies are cross-reactive including those that recognize epitopes in the stem region of HA and are conserved in several influenza A subtype viruses. This feature could make the VN assay less specific than HI, especially in adult and older populations with previous exposure to influenza viruses. At the same time, the VN is more sensitive than HI, particularly in detecting low-titer seroconversions. The different properties of these assays suggest that they could be used together in order to ensure sensitivity and specificity in influenza vaccine evaluation (Table 2) [16, 44].

The VN titer is expressed as the reciprocal of the serum dilution showing at least 50% inhibition of cytopathic effect (CPE) in mammalian cell cultures, which means that tests are laborious and slow. The ELISA method of detecting virus-infected cells is less variable than the CPE method, and provides results within a few days [19, 45, 46].

The main drawback of the VN assay —arises when the live wild type viruses are required as-in the case of highly pathogenic influenza viruses where high-level bio-containment facilities and extensive training for laboratory personnel are needed. Moreover, issues have arisen with regard to standardizing cell preparations, virus inoculations and incubation times. In addition, the assay lacks common reference protocols and is hindered by discrepancies in the determination of assay endpoints and limited knowledge of correlates of protection. The complexity of the methodology, in comparison with the HI assay, makes the VN assay more susceptible to high inter-laboratory variability. However, this variability seems to be reduced by the inclusion of an appropriately calibrated antibody standard in order to normalize all titres [26, 43, 47].

To date, no correlates of protection have been established with regard to the VN assay, which makes it difficult to compare vaccine assessments based on VN. Owing to the variability of the assay, a VN titer equivalent to an HI titer of 40 is highly specific to each antigen-laboratory combination and therefore can not be generalized [48]. Some studies on H5N1 and other influenza infections have regarded a titer ≥ 80 as an efficacy endpoint for avian influenza vaccines, whereas others have considered a seroprotection cut-off of 20 to be suitable, on the basis of its correlation with an SRH area of 25 mm^2 [46, 49-52].

Regarding H1N1 pandemic virus, a titer of 64 has been suggested as a seropositive threshold, while other studies have concurred in considering a titer ≥ 10 as the minimum detection limit and a titer ≥ 40 as a significant response. A titer ≥ 10 could be regarded as an effective indicator of population exposure to the virus through either natural infection or vaccination [44, 53-56] . In accordance with previous studies in which an HI titer of 40 was considered equivalent to an VN titer of 160, recent study has shown that an HI titer of 40 corresponds to a VN of approximately 200 and 140 for H1N1 and H3N2 strains, respectively, in children and adolescent populations [57]. A

further study has demonstrated that a VN titer of 42 corresponds to an HI titer of 260 for subjects that naturally acquired the H3N2 influenza infection [58].

The use of influenza HA pseudotypes as surrogates for wild-type viruses is a safe and easy alternative to the VN assay. A pseudotype virus is composed of the "core" of one virus (e.g. a retrovirus) and the outer "envelope" of another (e.g. the HA/NA of influenza virus). This assay is more suited to high-throughput processing of large serum panels. Moreover, it is safe and economical, since antibody responses against two viruses are assessed on the same serum samples and the entire process can be carried out outside of high BSL facilities. Particular interest should also be addressed both to the potential of pseudotype neutralization assays in studying the antigenic evolution of influenza viruses and to the ability of the assay to provide an adequate evaluation of the immune response induced by current influenza vaccines [59]. Currently, the pseudo type assay is in a research stage, lacks of correlates of protection and needs to be validated, standardized and compared to the traditional serological assays [60].

Additional assays

Other assays should be considered in order to overcome the limitations of the traditional serological techniques and to increase the knowledge of immunological responses to influenza vaccines.

Neuraminidase assay

Neuraminidase is the second glycoprotein on the viral surface and is involved in viral release and spread from infected cells. Antibodies against NA are not associated with the prevention of infection, but are able to contribute significantly to immune protection by reducing the severity and duration of infection and by curbing viral shedding and transmission [61-65]. The great advantage of NA is its slower antigen evolution and the resulting capacity to induce longer-lasting immunity and cross-protection than that provided by HA or conventional vaccines [61, 66, 67]. Notably, when HA and NA are provided in equal amounts, and as purified proteins separated from the other viral proteins, they prove to be equivalent from an immunogenic viewpoint [67].

While recent studies have highlighted the role of NA-inhibiting antibodies as a predictor of immunity [68-70], no correlates of protection have yet been established for the NA antibody response, and only the HA antigen content is standardized and controlled in current vaccines.

However, it could be important to develop harmonized and validated assays in order to have a useful and common tool for the interpretation of results [69]. Currently, several assays have been developed, but most of these are still in an experimental stage in various laboratories. The most common and prevalent assay is the Enzyme-Linked Lectin assay (ELLA), which is based on the release of terminal sialic acid residues from fetuin, usually used as a substrate [71]. The advantages are that it evaluates specific NA antibodies, offers better safety and sensitivity and requires no hazardous reagents [72, 73]. In order to avoid the activity of anti-HA antibodies in the NA assay, the use of recombinant strains of influenza with appropriate NA and an HA from non-human subtypes, such as H6 or equine origin, has been suggested (Table 2) [24, 61].

ELISA assay

The ELISA assay is a useful technique for the detection of influenza antibodies. The great advantage is its ability to detect class-specific IgM, IgA and IgG antibodies in serum and nasal wash especially in nasal secretions in which the titres are low and difficult to detect by means of conventional assays [19, 24, 74]. This technique yields unbiased results in a few hours, enables the process of high-throughput testing to be completely automated, does not require the pretreatment of serum samples or the frequent titration and manipulation of antigens, and does not utilize red blood cells. It is therefore particularly suitable for large-scale serological investigations (Table 2) [19, 75, 76].

The drawback of the assay is the lack of specificity for influenza A and B subtypes, owing to the use of the whole virus, which contains both internal and surface viral antigens [75, 77]. The use of purified HA antigens or a recombinant fragment of the HA globular head may considerably improve the specificity of the assay. Currently, there is no standardized protocol or reagents for the ELISA assay. Before the assay is implemented as a research tool, validation of its specificity may be advantageous [19, 74, 76].

Assays for cellular immune response

The cell-mediated immunity (CMI) plays an important role in viral clearance, decreasing severity and complications following influenza infection. One of the main action is performed by the T cells that mediate the immune response and recognize internal viral proteins, such as the nucleoprotein (NP), conserved among influenza strains. This feature makes the T cell responses particularly suitable for providing long lasting cross-reactive immunity and being the potential basis for a universal influenza vaccine [61, 78-80].

Several powerful methods such as flow cytometry, ELISA/enzyme-linked immunospot (ELISpot) and cellular cytotoxicity have been developed for the CMI evaluation (Table 2) [81]. Nevertheless, the current correlates of protection based on antibodies induction are improper for the evaluation of T cell-inducing vaccines and need to be updated [80].

The ELISA and ELISpot assays aim to quantify the cytokine production in serum, alveolar lavage samples or culture supernatant. The first one measures the cytokines associated with specific T cell subsets while the ELISpot identifies the number of cells producing a particular cytokine [24, 82].

The flow cytometry is a powerful method for the study of T cells phenotypes and the use of fluorescent-labeled monoclonal antibodies allows the simultaneous measure of multiple markers. The technique is more complicated and expensive than ELISpot but its versatility, in addition to the ELISpot advantages as cheapness, high throughput processing, sensitivity and accuracy, makes both assays the best candidates for the evaluation of CMI and correlates of protection [24, 82].

INFLUENZA VACCINES

The inactivated influenza vaccines (TIV), which are the most common and widely used, consist of whole virus, split virus or subunits, and are administered by intramuscular or subcutaneous injection. Conventionally, subunit or split virion vaccines are the most widespread, owing to the fact that whole virus vaccines may elicit adverse events, especially in children. Initially developed in eggs, the production

of inactivated vaccines moves forward cell-based platform in order to overcome egg-based production shortfalls [3, 4, 61, 83].

TIV vaccines provide 70-90% efficacy in young adults when there is a good match between the vaccine and circulating influenza strains, but lower efficacy in elderly and pediatric populations [61, 84, 85]. Currently, as the correlates of protection are specific to egg-based TIV vaccines, there is some concern over the use of these correlates for the evaluation of cell-derived vaccines [6, 86]. TIV vaccines induce a predominant humoral response, increasing IgG serum levels against HA, and are recommended for persons aged 6 months and older [61]. Despite this recommendation, the correlates of protection only target healthy adults aged 18-60 years and the over 60s, thus excluding children and other subjects at high risk, such as immunocompromised patients. As already mentioned, the appropriateness of using correlates of protection that are not exactly defined for the target group is still under debate. The study by Black et al. [15] demonstrates that an HI titer ≥ 40 is not an appropriate correlate of protection for children under 6 years of age, since they need an HI titer of 110 in order to reach a 50% protection level and titres of 215, 330 and 629 to achieve protection levels of 70%, 80% and 90%, respectively. Disparate factors could lead to the need for a higher HI titer in children; the most significant is probably the condition of naïve population, which entails that children have not had previous experience of influenza vaccination or infection and need a higher immune response to reach an adequate protection level. Furthermore, the ability of the HI assay to detect both IgG and IgM antibodies could overestimate the immune response in the pediatric population, while the measure of IgG antibodies alone, as provided by SRH, could be more appropriate in this group [16, 18]. A further consideration concerns the need for distinct target groups to have different correlates of protection [59].

In order to increase the low efficacy of TIV vaccines in pediatric and elderly subjects, adjuvanted vaccines have been developed. Clinical studies in these populations have demonstrated the great advantage of adjuvanted vaccines that are able to induce an enhanced immune response in the elderly and children even against B strains and in a condition of low pre-immunization HI titres and mismatching viruses [84, 87-90].

Adjuvanted vaccines allow to reach an HI titer ≥ 40 with a single low dose in children compared to non adjuvanted vaccines that need higher doses and injections, particularly, in the younger subjects [91, 92]. The dose sparing strategy of adjuvanted formulation needs not to be underestimated in the event of pandemic, as this strategy would lead to a greater availability of vaccines and a higher coverage of vaccination. Further investigations show that a high proportion of children receiving the adjuvanted formulation achieves an HI titer ≥ 330. Non adjuvanted vaccines are not able to induce the same immune response and a booster of adjuvanted vaccine is needed to achieve an high proportion of subjects with an HI titer ≥ 330, underlining the convenience of using this formulation in the pediatric population [91, 93-95]. The other advantage is the capacity to induce a broad cross-reactive immunity, a long-term antibody persistence up to one year after the vaccination and a high CMI response [95-99]. The same benefits have been observed in the elderly population as the adjuvanted vaccines seem to be highly immunogenic and induce a persistent humoral and cell mediated immune response [100-102]. There is no safety concern regarding adjuvanted vaccines even though solicited local reactions seem to be more common than non adjuvanted vaccines [95].

The seasonal quadrivalent TIVs, with or without adjuvant, induce superior antibody response against the additional B strain and a comparable immunogenicity to the

traditional TIVs highlighting the great potential of this new formulation, particularly in children [103-107].

The current correlates of protection do not distinguish between adjuvanted and non adjuvanted vaccines nor whether there are slight differences among them [15]. Guidelines specific to the type of vaccine, with or without adjuvant, should be more specific and appropriate [16].

The other licensed vaccine is the live attenuated vaccine (LAIV), which is administered intranasally to persons aged 2-49 years in the US, Europe, India and Russia [3, 108]. Intranasal administration mimics the natural pathway of infection and induces a broader humoral and cellular response than TIV vaccines, as LAIV vaccines elicit local secretory IgA antibodies that primarily protect the mucosal surface, serum HI, IgG and NA-inhibiting antibodies, and the production of virus-specific cytotoxic T lymphocytes (CTLs), which play a significant role in cell-mediated immune protection. The LAIV vaccine provides protection against both well-matched and antigenically drifted strains [3, 24, 61, 109-111]. Currently, no correlates of protection are specific to LAIV vaccines; this is partly due to the difficulty of sampling and assaying mucosal antibodies [3] and also to the inadequacy of the HI assay, which could underestimate the broader protection induced by LAIV vaccines [61, 85, 112]. It is evident that these two types of vaccine induce different immunological responses to influenza viruses [111]. In adults, previous exposure and/or immunity to influenza viruses could inhibit viral replication, making the LAIV vaccine more effective than TIV in children without pre-existing immunity and TIV more effective in adults. Thus, TIV vaccine is more suitable for boosting previous immunity, while LAIV is better suited to priming [3, 108]. This evidence, in addition to the more attractive administration route of LAIV, highlights the need to develop correlates of protection and specific assays for LAIV vaccines, which seem to be a promising alternative or adjunct to TIV vaccines.

Currently, there is also a lack of correlates of protection specific to pandemic vaccines, the evaluation of which is based on the fulfillment of criteria for seasonal influenza vaccines [113]. This approach, however, raises a number of concerns. Indeed, it is questionable whether criteria based on seasonal vaccines should be extrapolated to the evaluation of pandemic vaccines and the role played by pre-existing immunity in reaching protection threshold levels and its absence or reduction in the event of a pandemic outbreak. Finally, the use of traditional immunological assays may be inappropriate. Thus, for the evaluation of pandemic vaccines, the VN assay has been added to SRH and HI, given the low sensitivity of this latter assay to pandemic strains such as H5 and H7. Moreover, the possibility to include alternative, justified threshold levels, instead of an HI titer of 40, has been granted to the companies testing candidate pandemic vaccines [113, 114]. The utility of introducing alternative assays to the traditional ones is being evaluated, with a view to providing assays that can adequately measure the immunogenicity of new-generation vaccines. It must be borne in mind that, in addition to HI antibodies, other immunological parameters, such as mucosal antibodies or cell-mediated immunity, also contribute to protection against influenza viruses. Therefore, it is quite unrealistic to image that one parameter may be appropriate to different target age-groups with different previous experience of influenza, different health status and different types and formulations of vaccines [3].

A FOCUS ON REVISED "GUIDELINE ON INFLUENZA VACCINES"

Since 2014, the Committee for Medicinal Products for Human Use (European Medicines Agency) has revised the guidelines on influenza vaccines in order to develop one single influenza guideline (draft) covering the regulatory, quality, non-clinical and clinical aspects [115].

The main change in the required immunological data is the exclusion of the SRH assay and the official inclusion of the VN assay. In recent years, the VN assay has proved to be useful in avian strains of influenza A, and influenza B viruses, as it identifies a wider range of neutralizing antibodies than the HI assay. While inclusion of the VN assay in the criteria is certainly a great advantage in the evaluation of influenza vaccines, a doubt may arise with regard to the exclusion of SRH. This is an established technique, particularly in the serological filed, is widely used to measure antibodies against influenza viruses, and is particularly useful in pediatric clinical trials. Its two main advantages lie in its safety, since it requires inactivated viruses, and the possibility to analyze a large number of serum samples simultaneously and rapidly. Thus, the question that arises is whether it is preferable to exclude the SRH assay or to use all three serological assays (HI, SRH and VN) in order to enable more complete influenza vaccine evaluation.

Although the conventional serological parameters, i.e. geometric mean titers, pre-/post-vaccination ratio and seroconversion rates, have been considered in the revised draft, careful attention should be paid to the pre-vaccination status of the population. This should be regarded as "an unpredictable population characteristic unrelated to the vaccine" that needs to be corrected by means of generally approved procedure [28].

The suggestion that studies for the evaluation of anti-NA antibody responses and antibody kinetics should be performed is very interesting. However, given the current scenario regarding these assays, an indication of which assay should preferably be used would be helpful, in addition to validation of the assay. The same applies to the VN and HI assays. Moreover, the guideline should require that the validation of these assays be recognized by the regulatory agency and conducted according to International Conference of Harmonisation (ICH) guidelines, in order to reduce inter-laboratory variations.

EXPERT COMMENTARY & FIVE-YEAR VIEW

Despite containing both HA and NA viral glycoproteins, the currently administered vaccines are standardized exclusively according to their HA content [116, 117]. Therefore, the evaluation of vaccine immunogenicity is mainly based on the HI assay, and an HI titer ≥ 40 is considered an immunological correlate of protection and the best available parameter for predicting protection from influenza infection. Despite its wide use, the assay shows some limitations, such as the strain-specific antibodies detected, its low sensitivity to B and avian influenza strains and the high degree of inter-laboratory variability. Moreover, the assay does not seem to be adequate for different age groups, such as the elderly and high-risk groups [24, 118]. A more functional technique than HI is the VN assay, which is able to detect the functional neutralizing antibodies that are really involved in protection against disease. The need to officially include the VN assay in the evaluation of influenza vaccines is increasingly clear. At the same time, however, it is necessary to overcome the current drawbacks, such as the lack of specific correlates of protection for the VN assay and the high inter-laboratory variability due to the scarcity of common validated standardized protocols

[26, 43]. The disadvantage of vaccine evaluation based exclusively on serological evidence is its potential overestimation of TIV vaccines to the detriment of other vaccine types, such as the LAIVs [112]. These latter mainly induce mucosal immunity, which contributes to protection against influenza infection but is not well defined by neither the current correlates of protection nor the HI assay [18, 62].

The main limitation of current vaccines is that they need to be evaluated annually, in order to match the circulating strains, owing to the antigenic changes manifested by the HA head. The next-generation vaccines are aimed at eliciting broad immune responses through the implementation of various approaches. One possibility is to promote antibody responses against the stalk region of the HA glycoprotein, which has proved to be highly conserved among influenza types. Neutralizing antibodies can prevent infection through the inhibition of cell membrane attachment or can impede the release of viral content into the cells. They are efficient in inhibiting infection in influenza A and B strains, but are not elicited in all individuals following influenza vaccination or infection [21, 119, 120]. Establishing new targets for antibodies would necessitate revising the current correlates of protection, types of vaccine and immunological assays for the evaluation of immunogenicity, as these antibodies act differently from those elicited by the traditional approach [18, 62].

The other promising target is the NA glycoprotein, which is subject to slower antigenic evolution than HA and is probably able to induce longer-lasting immunity than that provided by HA or conventional vaccines [66, 67]. The role of NA antibodies in the event of a pandemic could be very significant, considering that most of the population may be naïve to the HA glycoprotein but may present previous immunity to NA [73]. Studies have shown that prior infection with seasonal or pandemic influenza vaccines provides cross-protection against the H5N1 viruses, which is especially due to the antibodies against NA [69, 121, 122].

Among influenza A subtypes, matrix protein 2 (M2) has a highly conserved amino acid sequence and is involved in the early step of the replication cycle of the virus. This protein is particularly interesting for the development of a universal vaccine, even though it is included in the current vaccine formulation in very low quantity and is not able to induce an immune response in vaccinees [18, 21, 62, 123].

The NP protein also seems to have interesting features that make it a candidate for inclusion in a universal vaccine. It is a stable internal protein of A and B strains, and could be involved in the generation of CTLs as the protein is displayed on infected cells during viral replication [61] . Cell-mediated immune response is an attractive target, as it may elicit greater cross-protection against influenza viruses than the traditional humoral approach. It plays a significant role in viral clearance, thereby limiting influenza infection, and may also prevent complications associated with influenza disease [62, 78]. Studies have proved that pre-existing T cells to seasonal or pandemic influenza strains (H1N1 2009) provide cross-protection against pandemic strains (H5N1) and are correlated with less severe illness [124, 125].

The current scenario on next generation vaccines seems to be very promising, although there is an increasing demand to revise the current correlates of protection and traditional assays for the evaluation of influenza vaccines.

KEY ISSUES

- For many years, the CPMP criteria fulfilled by vaccines licensed in Europe included exclusively Single Radial Haemolysis (SRH) and Haemagglutination Inhibition (HI) assays.
- Currently, an HI titer ≥ 40 is considered an immunological correlate of protection and the best available parameter for predicting protection from influenza infection.
- The HI is currently the assay most widely used to measure the immune response to influenza vaccines. The assay has the advantage of being easy to perform, simple and cheap, but has some limitations.
- The Virus Neutralization (VN) assay is not mandatory for the licensing of influenza vaccines. However, compared with HI, the VN assay seems to be more complete and better suited to evaluating the serological response to influenza vaccines. To date, no correlates of protection have been established with regard to the VN assay
- Other assays should be considered in order to overcome the limitations of the traditional serological techniques and to increase the knowledge of immunological responses to influenza vaccines.
- Establishing new targets for antibodies would necessitate revising the current correlates of protection, types of vaccine and immunological assays for the evaluation of immunogenicity, as these antibodies act differently from those elicited by the traditional approach.

Declaration of Interests

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

References
Reference annotations

* Of interest
** Of considerable interest
1. Onions, D., W. Egan, R. Jarrett, D. Novicki, and J.P. Gregersen, *Validation of the safety of MDCK cells as a substrate for the production of a cell-derived influenza vaccine.* Biologicals, 2010. **38**(5): p. 544-51.
2. Montomoli, E., B. Capecchi, and K. Hoschler, *Correlates of Protection Against Influenza*, in *Influenza Vaccines for the Future*, S.B. AG, Editor. 2011, Springer.
3. Sridhar, S., K.A. Brokstad, and R.J. Cox, *Influenza Vaccination Strategies: Comparing Inactivated and Live Attenuated Influenza Vaccines.* Vaccines (Basel), 2015. **3**(2): p. 373-89.
4. Manini, I., A. Domnich, D. Amicizia, S. Rossi, T. Pozzi, R. Gasparini, D. Panatto, and E. Montomoli, *Flucelvax (Optaflu) for seasonal influenza.* Expert Rev Vaccines, 2015. **14**(6): p. 789-804.
5. Hobson, D., R.L. Curry, A.S. Beare, and A. Ward-Gardner, *The role of serum haemagglutination-inhibiting antibody in protection against challenge infection with influenza A2 and B viruses.* J Hyg (Lond), 1972. **70**(4): p. 767-77.

6.   CPMP, *Note for guidance on harmonisation of requirements for influenza vaccines.* 1997.

7.   Plotkin, S.A. and P.B. Gilbert, *Nomenclature for immune correlates of protection after vaccination.* Clin Infect Dis, 2012. **54**(11): p. 1615-7.

8.   Qin, L., P.B. Gilbert, L. Corey, M.J. McElrath, and S.G. Self, *A framework for assessing immunological correlates of protection in vaccine trials.* J Infect Dis, 2007. **196**(9): p. 1304-12.

9.   Hannoun, C., F. Megas, and J. Piercy, *Immunogenicity and protective efficacy of influenza vaccination.* Virus Res, 2004. **103**(1-2): p. 133-8.

10.   Plotkin, S.A., *Vaccines: correlates of vaccine-induced immunity.* Clin Infect Dis, 2008. **47**(3): p. 401-9.

11.   Al-Khayatt, R., R. Jennings, and C.W. Potter, *Interpretation of responses and protective levels of antibody against attenuated influenza A viruses using single radial haemolysis.* J Hyg (Lond), 1984. **93**(2): p. 301-12.

12.   de Jong, J.C., A.M. Palache, W.E. Beyer, G.F. Rimmelzwaan, A.C. Boon, and A.D. Osterhaus, *Haemagglutination-inhibiting antibody to influenza virus.* Dev Biol (Basel), 2003. **115**: p. 63-73.

13.   Coudeville, L., F. Bailleux, B. Riche, F. Megas, P. Andre, and R. Ecochard, *Relationship between haemagglutination-inhibiting antibody titres and clinical protection against influenza: development and application of a bayesian random-effects model.* BMC Med Res Methodol, 2010. **10**: p. 18.

14.   Coudeville, L., P. Andre, F. Bailleux, F. Weber, and S. Plotkin, *A new approach to estimate vaccine efficacy based on immunogenicity data applied to influenza vaccines administered by the intradermal or intramuscular routes.* Hum Vaccin, 2010. **6**(10): p. 841-8.

15.   *Black, S., U. Nicolay, T. Vesikari, M. Knuf, G. Del Giudice, G. Della Cioppa, T. Tsai, R. Clemens, and R. Rappuoli, *Hemagglutination inhibition antibody titers as a correlate of protection for inactivated influenza vaccines in children.* Pediatr Infect Dis J, 2011. **30**(12): p. 1081-5.
        Inadequacy of current correlates of protection in children

16.   Granstrom, M. and A.C. Voordouw, *Registration of influenza vaccines for children in Europe.* Vaccine, 2011. **29**(43): p. 7572-5.

17.   Ohmit, S.E., J.G. Petrie, R.T. Cross, E. Johnson, and A.S. Monto, *Influenza hemagglutination-inhibition antibody titer as a correlate of vaccine-induced protection.* J Infect Dis, 2011. **204**(12): p. 1879-85.

18.   van Els, C., S. Mjaaland, L. Naess, J. Sarkadi, E. Gonczol, K.S. Korsholm, J. Hansen, J. de Jonge, G. Kersten, J. Warner, A. Semper, C. Kruiswijk, and F. Oftung, *Fast vaccine design and development based on correlates of protection (COPs).* Hum Vaccin Immunother, 2014. **10**(7): p. 1935-48.

19.   Katz, J.M., K. Hancock, and X. Xu, *Serologic assays for influenza surveillance, diagnosis and vaccine evaluation.* Expert Rev Anti Infect Ther, 2011. **9**(6): p. 669-83.

20.   Wood, J.M., D. Melzack, R.W. Newman, D.L. Major, M. Zambon, K.G. Nicholson, and A. Podda, *A single radial haemolysis assay for antibody to H5 haemagglutinin.* International Congress Series, 2001. **1219**: p. 761-66.

21.   *Soema, P.C., R. Kompier, J.P. Amorij, and G.F. Kersten, *Current and next generation influenza vaccines: Formulation and production strategies.* Eur J Pharm Biopharm, 2015. **94**: p. 251-63.
        Interesting review on the current status and novel developments in influenza vaccine field from different viewpoints.

22.   Goodeve, A., C.W. Potter, A. Clark, R. Jennings, G.C. Schild, and R. Yetts, *A graded-dose study of inactivated, surface antigen influenza B vaccine in*

volunteers: reactogenicity, antibody response and protection to challenge virus infection. J Hyg (Lond), 1983. **90**(1): p. 107-15.

23. WHO, *Serological detection of avian influenza A(H7N9) virus infections by modified horde red blood cells haemagglutination-inhibition assay.* 2013. p. 1-11.

24. Reber, A. and J. Katz, *Immunological assessment of influenza vaccines and immune correlates of protection.* Expert Rev Vaccines, 2013. **12**(5): p. 519-36.

25. Wood, J.M., E. Montomoli, R.W. Newman, A. Daas, K.H. Buchheit, and E. Terao, *Collaborative study on influenza vaccine clinical trial serology - part 2: reproducibility study.* Pharmeur Bio Sci Notes, 2011. **2011**(1): p. 36-54.

26. Stephenson, I., A. Heath, D. Major, R.W. Newman, K. Hoschler, W. Junzi, J.M. Katz, J.P. Weir, M.C. Zambon, and J.M. Wood, *Reproducibility of serologic assays for influenza virus A (H5N1).* Emerg Infect Dis, 2009. **15**(8): p. 1252-9.

27. Noah, D.L., H. Hill, D. Hines, E.L. White, and M.C. Wolff, *Qualification of the hemagglutination inhibition assay in support of pandemic influenza vaccine licensure.* Clin Vaccine Immunol, 2009. **16**(4): p. 558-66.

28. Beyer, W.E., A.M. Palache, G. Luchters, J. Nauta, and A.D. Osterhaus, *Seroprotection rate, mean fold increase, seroconversion rate: which parameter adequately expresses seroresponse to influenza vaccination?* Virus Res, 2004. **103**(1-2): p. 125-32.

29. Beyer, W.E., A.M. Palache, M.J. Sprenger, E. Hendriksen, J.J. Tukker, R. Darioli, G.L. van der Water, N. Masurel, and A.D. Osterhaus, *Effects of repeated annual influenza vaccination on vaccine sero-response in young and elderly adults.* Vaccine, 1996. **14**(14): p. 1331-9.

30. Schild, G.C., M.S. Pereira, and P. Chakraverty, *Single-radial-hemolysis: a new method for the assay of antibody to influenza haemagglutinin. Applications for diagnosis and seroepidemiologic surveillance of influenza.* Bull World Health Organ, 1975. **52**(1): p. 43-50.

31. Morley, P.S., L.K. Hanson, J.R. Bogdan, H.G. Townsend, J.A. Appleton, and D.M. Haines, *The relationship between single radial hemolysis, hemagglutination inhibition, and virus neutralization assays used to detect antibodies specific for equine influenza viruses.* Vet Microbiol, 1995. **45**(1): p. 81-92.

32. Clarke, M., J. Boustred, V. Seagroatt, and G.C. Schild, *The use of single-radial-haemolysis for rubella antibody studies.* J Hyg (Lond), 1977. **79**(3): p. 355-64.

33. Callow, K.A. and A.S. Beare, *Measurement of antibody to influenza virus neuraminidase by single radial hemolysis in agarose gels.* Infect Immun, 1976. **13**(1): p. 1-8.

34. Trombetta, C.M., D. Perini, L. Vitale, R.J. Cox, V. Stanzani, S. Piccirella, and E. Montomoli, *Validation of Single Radial Haemolysis assay: A reliable method to measure antibodies against influenza viruses.* J Immunol Methods, 2015. **422**: p. 95-101.

35. Farrohi, K., F.K. Farrohi, G.R. Noble, H.S. Kaye, and A.P. Kendal, *Evaluation of the single radial hemolysis test for measuring hemagglutinin- and neuraminidase-specific antibodies to H3N2 influenza strains and antibodies to influenza B.* J Clin Microbiol, 1977. **5**(3): p. 353-60.

36. Fulton, R.E., V.L. DiNinno, R.I. Frank, J. Fildes, and I.J. Turner, *Single radial hemolysis test for quantitation of complement-fixing antibodies to non-hemagglutinating viruses.* J Clin Microbiol, 1984. **20**(2): p. 248-54.

37. Goodeve, A.C., R. Jennings, and C.W. Potter, *The use of the single radial haemolysis test for assessing antibody response and protective antibody levels in an influenza B vaccine study.* J Biol Stand, 1983. **11**(4): p. 289-96.

38. Rowe, T., R.A. Abernathy, J. Hu-Primmer, W.W. Thompson, X. Lu, W. Lim, K. Fukuda, N.J. Cox, and J.M. Katz, *Detection of antibody to avian influenza A (H5N1) virus in human serum by using a combination of serologic assays.* J Clin Microbiol, 1999. **37**(4): p. 937-43.

39. Delem, A., *Serum SRH antibody level as a measure of the immunity against natural and artificial A/Victoria/3/75 infections.* Dev Biol Stand, 1977. **39**: p. 391-6.

40. Wood, J.M., R.E. Gaines-Das, J. Taylor, and P. Chakraverty, *Comparison of influenza serological techniques by international collaborative study.* Vaccine, 1994. **12**(2): p. 167-74.

41. Kayali, G., S.F. Setterquist, A.W. Capuano, K.P. Myers, J.S. Gill, and G.C. Gray, *Testing human sera for antibodies against avian influenza viruses: horse RBC hemagglutination inhibition vs. microneutralization assays.* J Clin Virol, 2008. **43**(1): p. 73-8.

42. WHO, *Recommendations and laboratory procedures for detection of avian influenza A(H5N1) virus in specimens from suspected human cases.* 2007: p. 1-28.

43. Wagner, R., C. Gopfert, J. Hammann, B. Neumann, J. Wood, R. Newman, C. Wallis, N. Alex, and M. Pfleiderer, *Enhancing the reproducibility of serological methods used to evaluate immunogenicity of pandemic H1N1 influenza vaccines-an effective EU regulatory approach.* Vaccine, 2012. **30**(27): p. 4113-22.

44. Veguilla, V., K. Hancock, J. Schiffer, P. Gargiullo, X. Lu, D. Aranio, A. Branch, L. Dong, C. Holiday, F. Liu, E. Steward-Clark, H. Sun, B. Tsang, D. Wang, M. Whaley, Y. Bai, L. Cronin, P. Browning, H. Dababneh, H. Noland, L. Thomas, L. Foster, C.P. Quinn, S.D. Soroka, and J.M. Katz, *Sensitivity and specificity of serologic assays for detection of human infection with 2009 pandemic H1N1 virus in U.S. populations.* J Clin Microbiol, 2011. **49**(6): p. 2210-5.

45. WHO, *Manual for the laboratory diagnosis and virological surveillance of influenza.* 2011: p. 1-137.

46. van der Velden, M.V., G. Aichinger, E.M. Pollabauer, A. Low-Baselli, S. Fritsch, K. Benamara, O. Kistner, M. Muller, M. Zeitlinger, H. Kollaritsch, T. Vesikari, H.J. Ehrlich, and P.N. Barrett, *Cell culture (Vero cell) derived whole-virus non-adjuvanted H5N1 influenza vaccine induces long-lasting cross-reactive memory immune response: homologous or heterologous booster response following two dose or single dose priming.* Vaccine, 2012. **30**(43): p. 6127-35.

47. Ansaldi, F., S. Bacilieri, D. Amicizia, L. Valle, F. Banfi, P. Durando, L. Sticchi, R. Gasparini, G. Icardi, and P. Crovari, *Antigenic characterisation of influenza B virus with a new microneutralisation assay: comparison to haemagglutination and sequence analysis.* J Med Virol, 2004. **74**(1): p. 141-6.

48. Stephenson, I., R.G. Das, J.M. Wood, and J.M. Katz, *Comparison of neutralising antibody assays for detection of antibody to influenza A/H3N2 viruses: an international collaborative study.* Vaccine, 2007. **25**(20): p. 4056-63.

49. Howard, M.K., N. Sabarth, H. Savidis-Dacho, D. Portsmouth, O. Kistner, T.R. Kreil, H.J. Ehrlich, and P.N. Barrett, *H5N1 whole-virus vaccine induces neutralizing antibodies in humans which are protective in a mouse passive transfer model.* PLoS One, 2011. **6**(8): p. e23791.

50. Ehrlich, H.J., M. Muller, H.M. Oh, P.A. Tambyah, C. Joukhadar, E. Montomoli, D. Fisher, G. Berezuk, S. Fritsch, A. Low-Baselli, N. Vartian, R. Bobrovsky, B.G. Pavlova, E.M. Pollabauer, O. Kistner, P.N. Barrett, and H.N.P.I.V.C.S.T. Baxter, *A clinical trial of a whole-virus H5N1 vaccine derived from cell culture.* N Engl J Med, 2008. **358**(24): p. 2573-84.

51. Cox, R.J., G. Pedersen, A.S. Madhun, S. Svindland, M. Saevik, L. Breakwell, K. Hoschler, M. Willemsen, L. Campitelli, J.K. Nostbakken, G.J. Weverling, J. Klap, K.C. McCullough, M. Zambon, R. Kompier, and H. Sjursen, *Evaluation of a virosomal H5N1 vaccine formulated with Matrix M adjuvant in a phase I clinical trial.* Vaccine, 2011. **29**(45): p. 8049-59.

52. Galli, G., D. Medini, E. Borgogni, L. Zedda, M. Bardelli, C. Malzone, S. Nuti, S. Tavarini, C. Sammicheli, A.K. Hilbert, V. Brauer, A. Banzhoff, R. Rappuoli, G. Del Giudice, and F. Castellino, *Adjuvanted H5N1 vaccine induces early CD4+ T cell response that predicts long-term persistence of protective antibody levels.* Proc Natl Acad Sci U S A, 2009. **106**(10): p. 3877-82.

53. Allwinn, R., J. Geiler, A. Berger, J. Cinatl, and H.W. Doerr, *Determination of serum antibodies against swine-origin influenza A virus H1N1/09 by immunofluorescence, haemagglutination inhibition, and by neutralization tests: how is the prevalence rate of protecting antibodies in humans?* Med Microbiol Immunol, 2010. **199**(2): p. 117-21.

54. Miller, E., K. Hoschler, P. Hardelid, E. Stanford, N. Andrews, and M. Zambon, *Incidence of 2009 pandemic influenza A H1N1 infection in England: a cross-sectional serological study.* Lancet, 2010. **375**(9720): p. 1100-8.

55. Hakim, H., K.J. Allison, L.A. Van De Velde, Y. Li, P.M. Flynn, and J.A. McCullers, *Immunogenicity and safety of inactivated monovalent 2009 H1N1 influenza A vaccine in immunocompromised children and young adults.* Vaccine, 2012. **30**(5): p. 879-85.

56. Adamson, W.E., E.C. McGregor, K. Kavanagh, J. McMenamin, S. McDonagh, P.J. Molyneaux, K.E. Templeton, and W.F. Carman, *Population exposure to a novel influenza A virus over three waves of infection.* J Clin Virol, 2011. **52**(4): p. 300-3.

57. Verschoor, C.P., P. Singh, M.L. Russell, D.M. Bowdish, A. Brewer, L. Cyr, B.J. Ward, and M. Loeb, *Microneutralization assay titres correlate with protection against seasonal influenza H1N1 and H3N2 in children.* PLoS One, 2015. **10**(6): p. e0131531.

58. Tsang, T.K., S. Cauchemez, R.A. Perera, G. Freeman, V.J. Fang, D.K. Ip, G.M. Leung, J.S. Malik Peiris, and B.J. Cowling, *Association between antibody titers and protection against influenza virus infection within households.* J Infect Dis, 2014. **210**(5): p. 684-92.

59. Trombetta, C.M., D. Perini, S. Mather, N. Temperton, and E. Montomoli, *Overview of Serological Techniques for Influenza Vaccine Evaluation: Past, Present and Future.* Vaccines, 2014. **2**(4): p. 707-734.

60. Carnell, G.W., F. Ferrara, K. Grehan, C.P. Thompson, and N.J. Temperton, *Pseudotype-based neutralization assays for influenza: a systematic analysis.* Front Immunol, 2015. **6**: p. 161.

61. Zakay-Rones, Z., *Human influenza vaccines and assessment of immunogenicity.* Expert Rev Vaccines, 2010. **9**(12): p. 1423-39.

62. McCullers, J.A. and V.C. Huber, *Correlates of vaccine protection from influenza and its complications.* Hum Vaccin Immunother, 2012. **8**(1): p. 34-44.

63. Kilbourne, E.D., R.B. Couch, J.A. Kasel, W.A. Keitel, T.R. Cate, J.H. Quarles, B. Grajower, B.A. Pokorny, and B.E. Johansson, *Purified influenza A virus N2 neuraminidase vaccine is immunogenic and non-toxic in humans.* Vaccine, 1995. **13**(18): p. 1799-803.

64. Kilbourne, E.D., W.G. Laver, J.L. Schulman, and R.G. Webster, *Antiviral activity of antiserum specific for an influenza virus neuraminidase.* J Virol, 1968. **2**(4): p. 281-8.

65. Schulman, J.L., M. Khakpour, and E.D. Kilbourne, *Protective effects of specific immunity to viral neuraminidase on influenza virus infection of mice.* J Virol, 1968. **2**(8): p. 778-86.

66. Kilbourne, E.D., B.E. Johansson, and B. Grajower, *Independent and disparate evolution in nature of influenza A virus hemagglutinin and neuraminidase glycoproteins.* Proc Natl Acad Sci U S A, 1990. **87**(2): p. 786-90.

67. Johansson, B.E., D.J. Bucher, and E.D. Kilbourne, *Purified influenza virus hemagglutinin and neuraminidase are equivalent in stimulation of antibody response but induce contrasting types of immunity to infection.* J Virol, 1989. **63**(3): p. 1239-46.

68. Couch, R.B., R.L. Atmar, L.M. Franco, J.M. Quarles, J. Wells, N. Arden, D. Nino, and J.W. Belmont, *Antibody correlates and predictors of immunity to naturally occurring influenza in humans and the importance of antibody to the neuraminidase.* J Infect Dis, 2013. **207**(6): p. 974-81.

69. Rockman, S., L.E. Brown, I.G. Barr, B. Gilbertson, S. Lowther, A. Kachurin, O. Kachurina, J. Klippel, J. Bodle, M. Pearse, and D. Middleton, *Neuraminidase-inhibiting antibody is a correlate of cross-protection against lethal H5N1 influenza virus in ferrets immunized with seasonal influenza vaccine.* J Virol, 2013. **87**(6): p. 3053-61.

70. Monto, A.S., J.G. Petrie, R.T. Cross, E. Johnson, M. Liu, W. Zhong, M. Levine, J.M. Katz, and S.E. Ohmit, *Antibody to Influenza Virus Neuraminidase: An Independent Correlate of Protection.* J Infect Dis, 2015. **212**(8): p. 1191-9.

71. Lambre, C.R., H. Terzidis, A. Greffard, and R.G. Webster, *Measurement of anti-influenza neuraminidase antibody using a peroxidase-linked lectin and microtitre plates coated with natural substrates.* J Immunol Methods, 1990. **135**(1-2): p. 49-57.

72. EMA, *Report from scientific workshop on serology assays and correlates of protection for influenza vaccines* 2010: p. 1-32.

73. Fritz, R., N. Sabarth, S. Kiermayr, C. Hohenadl, M.K. Howard, R. Ilk, O. Kistner, H.J. Ehrlich, P.N. Barrett, and T.R. Kreil, *A vero cell-derived whole-virus H5N1 vaccine effectively induces neuraminidase-inhibiting antibodies.* J Infect Dis, 2012. **205**(1): p. 28-34.

74. Murphy, B.R., E.L. Tierney, B.A. Barbour, R.H. Yolken, D.W. Alling, H.P. Holley, Jr., R.E. Mayner, and R.M. Chanock, *Use of the enzyme-linked immunosorbent assay to detect serum antibody responses of volunteers who received attenuated influenza A virus vaccines.* Infect Immun, 1980. **29**(2): p. 342-7.

75. Turner, R., J.L. Lathey, L.P. Van Voris, and R.B. Belshe, *Serological diagnosis of influenza B virus infection: comparison of an enzyme-linked immunosorbent assay and the hemagglutination inhibition test.* J Clin Microbiol, 1982. **15**(5): p. 824-9.

76. Alvarez, M.M., F. Lopez-Pacheco, J.M. Aguilar-Yanez, R. Portillo-Lara, G.I. Mendoza-Ochoa, S. Garcia-Echauri, P. Freiden, S. Schultz-Cherry, M.I. Zertuche-Guerra, D. Bulnes-Abundis, J. Salgado-Gallegos, L. Elizondo-Montemayor, and M. Hernandez-Torre, *Specific recognition of influenza A/H1N1/2009 antibodies in human serum: a simple virus-free ELISA method.* PLoS One, 2010. **5**(4): p. e10176.

77. Jennings, R., T. Smith, and C.W. Potter, *Use of the enzyme-linked immunosorbent assay (ELISA) for the estimation of serum antibodies in an influenza virus vaccine study.* Med Microbiol Immunol, 1981. **169**(4): p. 247-58.

78. Cox, R.J., K.A. Brokstad, and P. Ogra, *Influenza virus: immunity and vaccination strategies. Comparison of the immune response to inactivated and live, attenuated influenza vaccines.* Scand J Immunol, 2004. **59**(1): p. 1-15.

79. Thomas, P.G., R. Keating, D.J. Hulse-Post, and P.C. Doherty, *Cell-mediated protection in influenza infection.* Emerg Infect Dis, 2006. **12**(1): p. 48-54.

80. Soema, P.C., E. van Riet, G. Kersten, and J.P. Amorij, *Development of cross-protective influenza a vaccines based on cellular responses.* Front Immunol, 2015. **6**: p. 237.

81. Svitek, N., E.L. Taracha, R. Saya, E. Awino, V. Nene, and L. Steinaa, *Analysis of the Cellular Immune Responses to Vaccines.* Methods Mol Biol, 2016. **1349**: p. 247-62.

82. *Coughlan, L. and T. Lambe, *Measuring Cellular Immunity to Influenza: Methods of Detection, Applications and Challenges.* Vaccines (Basel), 2015. **3**(2): p. 293-319.
    Useful summary of cellular immunity concept and related techniques

83. WHO, *Correlates of vaccine-induced protection: methods and implications.* 2013: p. 1-65.

84. Podda, A., *The adjuvanted influenza vaccines with novel adjuvants: experience with the MF59-adjuvanted vaccine.* Vaccine, 2001. **19**(17-19): p. 2673-80.

85. Beyer, W.E., A.M. Palache, J.C. de Jong, and A.D. Osterhaus, *Cold-adapted live influenza vaccine versus inactivated vaccine: systemic vaccine reactions, local and systemic antibody response, and vaccine efficacy. A meta-analysis.* Vaccine, 2002. **20**(9-10): p. 1340-53.

86. Barrett, P.N., G. Berezuk, S. Fritsch, G. Aichinger, M.K. Hart, W. El-Amin, O. Kistner, and H.J. Ehrlich, *Efficacy, safety, and immunogenicity of a Vero-cell-culture-derived trivalent influenza vaccine: a multicentre, double-blind, randomised, placebo-controlled trial.* Lancet, 2011. **377**(9767): p. 751-9.

87. De Donato, S., D. Granoff, M. Minutello, G. Lecchi, M. Faccini, M. Agnello, F. Senatore, P. Verweij, B. Fritzell, and A. Podda, *Safety and immunogenicity of MF59-adjuvanted influenza vaccine in the elderly.* Vaccine, 1999. **17**(23-24): p. 3094-101.

88. Vesikari, T., N. Groth, A. Karvonen, A. Borkowski, and M. Pellegrini, *MF59-adjuvanted influenza vaccine (FLUAD) in children: safety and immunogenicity following a second year seasonal vaccination.* Vaccine, 2009. **27**(45): p. 6291-5.

89. Gasparini, R., T. Pozzi, E. Montomoli, E. Fragapane, F. Senatore, M. Minutello, and A. Podda, *Increased immunogenicity of the MF59-adjuvanted influenza vaccine compared to a conventional subunit vaccine in elderly subjects.* Eur J Epidemiol, 2001. **17**(2): p. 135-40.

90. Pellegrini, M., U. Nicolay, K. Lindert, N. Groth, and G. Della Cioppa, *MF59-adjuvanted versus non-adjuvanted influenza vaccines: integrated analysis from a large safety database.* Vaccine, 2009. **27**(49): p. 6959-65.

91. Knuf, M., G. Leroux-Roels, H. Rumke, L. Rivera, P. Pedotti, A.K. Arora, M. Lattanzi, D. Kieninger, and G.D. Cioppa, *Immunogenicity and safety of cell-derived MF59(R)-adjuvanted A/H1N1 influenza vaccine for children.* Hum Vaccin Immunother, 2015. **11**(2): p. 358-76.

92. Oh, C.E., J. Lee, J.H. Kang, Y.J. Hong, Y.K. Kim, H.J. Cheong, Y.J. Ahn, S.H. Kim, and H.J. Lee, *Safety and immunogenicity of an inactivated split-virus influenza A/H1N1 vaccine in healthy children from 6 months to <18 years of age: a prospective, open-label, multi-center trial.* Vaccine, 2010. **28**(36): p. 5857-63.

93. Knuf, M., G. Leroux-Roels, H.C. Rumke, K. Abarca, L. Rivera, M. Lattanzi, P. Pedotti, A. Arora, D. Kieninger-Baum, and G. Della Cioppa, *Safety and immunogenicity of an MF59-adjuvanted A/H1N1 pandemic influenza vaccine in children from three to seventeen years of age.* Vaccine, 2015. **33**(1): p. 174-81.

94. Nolan, T., L. Bravo, A. Ceballos, E. Mitha, G. Gray, B. Quiambao, S.S. Patel, S. Bizjajeva, H. Bock, N. Nazaire-Bermal, E. Forleo-Neto, G.D. Cioppa, and V. Narasimhan, *Enhanced and persistent antibody response against homologous and heterologous strains elicited by a MF59-adjuvanted influenza vaccine in infants and young children.* Vaccine, 2014. **32**(46): p. 6146-56.

95. Knuf, M., G. Leroux-Roels, H.C. Rumke, K. Abarca, L. Rivera, M. Lattanzi, P. Pedotti, A. Arora, D. Kieninger-Baum, and G. Della Cioppa, *Immunogenicity and tolerability of an MF59-adjuvanted, egg-derived, A/H1N1 pandemic influenza vaccine in children 6-35 months of age.* Pediatr Infect Dis J, 2014. **33**(12): p. e320-9.

96. Nolan, T., P. Izurieta, B.W. Lee, P.C. Chan, H. Marshall, R. Booy, M. Drame, and D.W. Vaughn, *Heterologous prime-boost vaccination using an AS03B-adjuvanted influenza A(H5N1) vaccine in infants and children<3 years of age.* J Infect Dis, 2014. **210**(11): p. 1800-10.

97. Ortqvist, A., R. Bennet, J. Hamrin, M.R. Rinder, H. Lindblad, J.N. Ohd, and M. Eriksson, *Long term effectiveness of adjuvanted influenza A(H1N1)pdm09 vaccine in children.* Vaccine, 2015. **33**(22): p. 2558-61.

98. Izurieta, P., M.J. Uy-Aragon, M. Drame, and D.W. Vaughn, *Assessment of Prime-boost Vaccination Using an AS03B-adjuvanted Influenza A (H5N1) Vaccine: A Randomized Trial in Children of Three to Less Than Eighteen Years of Age.* Pediatr Infect Dis J, 2016. **35**(2): p. e35-47.

99. Garcia-Sicilia, J., J. Aristegui, F. Omenaca, A. Carmona, J.C. Tejedor, J.M. Merino, P. Garcia-Corbeira, K. Walravens, V. Bambure, P. Moris, A. Caplanusi, P. Gillard, and I. Dieussaert, *Safety and persistence of the humoral and cellular immune responses induced by 2 doses of an AS03-adjuvanted A(H1N1)pdm09 pandemic influenza vaccine administered to infants, children and adolescents: Two open, uncontrolled studies.* Hum Vaccin Immunother, 2015. **11**(10): p. 2359-69.

100. Gillard, P., D. Giet, S. Heijmans, M. Drame, K. Walravens, and F. Roman, *Long-term outcome of the humoral and cellular immune response of an H5N1 adjuvanted influenza vaccine in elderly persons: 2-year follow-up of a randomised open-label study.* Trials, 2014. **15**: p. 419.

101. Reisinger, K.S., S.J. Holmes, P. Pedotti, A.K. Arora, and M. Lattanzi, *A dose-ranging study of MF59((R))-adjuvanted and non-adjuvanted A/H1N1 pandemic influenza vaccine in young to middle-aged and older adult populations to assess safety, immunogenicity, and antibody persistence one year after vaccination.* Hum Vaccin Immunother, 2014. **10**(8): p. 2395-407.

102. Frey, S.E., M.R. Reyes, H. Reynales, N.N. Bermal, U. Nicolay, V. Narasimhan, E. Forleo-Neto, and A.K. Arora, *Comparison of the safety and immunogenicity of an MF59(R)-adjuvanted with a non-adjuvanted seasonal influenza vaccine in elderly subjects.* Vaccine, 2014. **32**(39): p. 5027-34.

103. Greenberg, D.P., C.A. Robertson, M.J. Noss, M.M. Blatter, R. Biedenbender, and M.D. Decker, *Safety and immunogenicity of a quadrivalent inactivated influenza vaccine compared to licensed trivalent inactivated influenza vaccines in adults.* Vaccine, 2013. **31**(5): p. 770-6.

104. Beran, J., M. Peeters, W. Dewe, J. Raupachova, L. Hobzova, and J.M. Devaster, *Immunogenicity and safety of quadrivalent versus trivalent inactivated influenza vaccine: a randomized, controlled trial in adults.* BMC Infect Dis, 2013. **13**: p. 224.

105. Wang, L., V. Chandrasekaran, J.B. Domachowske, P. Li, B.L. Innis, and V.K. Jain, *Immunogenicity and Safety of an Inactivated Quadrivalent Influenza Vaccine in US Children 6-35 Months of Age During 2013-2014: Results From A Phase II Randomized Trial.* J Pediatric Infect Dis Soc, 2015.

106. Langley, J.M., L. Wang, N. Aggarwal, A. Bueso, V. Chandrasekaran, L. Cousin, S.A. Halperin, P. Li, A. Liu, S. McNeil, L.P. Mendez, L. Rivera, B.L. Innis, and V.K. Jain, *Immunogenicity and Reactogenicity of an Inactivated Quadrivalent Influenza Vaccine Administered Intramuscularly to Children 6 to 35 Months of Age in 2012-2013: A Randomized, Double-Blind, Controlled, Multicenter, Multicountry, Clinical Trial.* J Pediatric Infect Dis Soc, 2015. **4**(3): p. 242-51.

107. Tinoco, J.C., N. Pavia-Ruz, A. Cruz-Valdez, C. Aranza Doniz, V. Chandrasekaran, W. Dewe, A. Liu, B.L. Innis, and V.K. Jain, *Immunogenicity, reactogenicity, and safety of inactivated quadrivalent influenza vaccine candidate versus inactivated trivalent influenza vaccine in healthy adults aged >/=18 years: a phase III, randomized trial.* Vaccine, 2014. **32**(13): p. 1480-7.

108. Ambrose, C.S., X. Wu, and R.B. Belshe, *The efficacy of live attenuated and inactivated influenza vaccines in children as a function of time postvaccination.* Pediatr Infect Dis J, 2010. **29**(9): p. 806-11.

109. Belshe, R.B., K.M. Edwards, T. Vesikari, S.V. Black, R.E. Walker, M. Hultquist, G. Kemble, E.M. Connor, and C.-T.C.E.S. Group, *Live attenuated versus inactivated influenza vaccine in infants and young children.* N Engl J Med, 2007. **356**(7): p. 685-96.

110. Hoft, D.F., E. Babusis, S. Worku, C.T. Spencer, K. Lottenbach, S.M. Truscott, G. Abate, I.G. Sakala, K.M. Edwards, C.B. Creech, M.A. Gerber, D.I. Bernstein, F. Newman, I. Graham, E.L. Anderson, and R.B. Belshe, *Live and inactivated influenza vaccines induce similar humoral responses, but only live vaccines induce diverse T-cell responses in young children.* J Infect Dis, 2011. **204**(6): p. 845-53.

111. Clements, M.L., R.F. Betts, E.L. Tierney, and B.R. Murphy, *Serum and nasal wash antibodies associated with resistance to experimental challenge with influenza A wild-type virus.* J Clin Microbiol, 1986. **24**(1): p. 157-60.

112. Petrie, J.G., S.E. Ohmit, E. Johnson, R.T. Cross, and A.S. Monto, *Efficacy studies of influenza vaccines: effect of end points used and characteristics of vaccine failures.* J Infect Dis, 2011. **203**(9): p. 1309-15.

113. CHMP, *Guideline on influenza vaccines prepared from viruses with the potential to cause a pandemic and intended for use outside of the core dossier context.* 2006. p. 1-11.

114. Report. *FDA/NIH/WHO public workshop on immune correlates of protection against influenza A viruses in support of pandemic vaccines development, Bethesda, Maryland, US, December 10-11, 2007.* 2007. Vaccine.

115. EMA, *Guideline on influenza vaccines. Non-clinical and clinical module. Draft.* 2014.

116. Powers, D.C., E.D. Kilbourne, and B.E. Johansson, *Neuraminidase-specific antibody responses to inactivated influenza virus vaccine in young and elderly adults.* Clin Diagn Lab Immunol, 1996. **3**(5): p. 511-6.

117. Sandbulte, M.R., G.S. Jimenez, A.C. Boon, L.R. Smith, J.J. Treanor, and R.J. Webby, *Cross-reactive neuraminidase antibodies afford partial protection against H5N1 in mice and are present in unexposed humans.* PLoS Med, 2007. **4**(2): p. e59.

118. McElhaney, J.E., *Influenza vaccine responses in older adults.* Ageing Res Rev, 2011. **10**(3): p. 379-88.

119. **Impagliazzo, A., F. Milder, H. Kuipers, M.V. Wagner, X. Zhu, R.M. Hoffman, R. van Meersbergen, J. Huizingh, P. Wanningen, J. Verspuij, M. de Man, Z. Ding, A. Apetri, B. Kukrer, E. Sneekes-Vriese, D. Tomkiewicz, N.S. Laursen, P.S. Lee, A. Zakrzewska, L. Dekking, J. Tolboom, L. Tettero, S. van Meerten, W. Yu, W. Koudstaal, J. Goudsmit, A.B. Ward, W. Meijberg, I.A. Wilson, and K.

Radosevic, *A stable trimeric influenza hemagglutinin stem as a broadly protective immunogen.* Science, 2015. **349**(6254): p. 1301-6.
New prospective for the development of a universal vaccines.

120. **Yassine, H.M., J.C. Boyington, P.M. McTamney, C.J. Wei, M. Kanekiyo, W.P. Kong, J.R. Gallagher, L. Wang, Y. Zhang, M.G. Joyce, D. Lingwood, S.M. Moin, H. Andersen, Y. Okuno, S.S. Rao, A.K. Harris, P.D. Kwong, J.R. Mascola, G.J. Nabel, and B.S. Graham, *Hemagglutinin-stem nanoparticles generate heterosubtypic influenza protection.* Nat Med, 2015. **21**(9): p. 1065-70.
Important finding on the protective immunity induced by haemagglutinin stem nanoparticles in mice and ferrets.

121. Chen, Z., L. Kim, K. Subbarao, and H. Jin, *The 2009 pandemic H1N1 virus induces anti-neuraminidase (NA) antibodies that cross-react with the NA of H5N1 viruses in ferrets.* Vaccine, 2012. **30**(15): p. 2516-22.

122. Easterbrook, J.D., L.M. Schwartzman, J. Gao, J.C. Kash, D.M. Morens, L. Couzens, H. Wan, M.C. Eichelberger, and J.K. Taubenberger, *Protection against a lethal H5N1 influenza challenge by intranasal immunization with virus-like particles containing 2009 pandemic H1N1 neuraminidase in mice.* Virology, 2012. **432**(1): p. 39-44.

123. Osterhaus, A., R. Fouchier, and G. Rimmelzwaan, *Towards universal influenza vaccines?* Philos Trans R Soc Lond B Biol Sci, 2011. **366**(1579): p. 2766-73.

124. Kreijtz, J.H., G. de Mutsert, C.A. van Baalen, R.A. Fouchier, A.D. Osterhaus, and G.F. Rimmelzwaan, *Cross-recognition of avian H5N1 influenza virus by human cytotoxic T-lymphocyte populations directed to human influenza A virus.*

CPMP/BWP/214/96 criteria				
	18-60 years	**over 60**	**Assay**	**Assay**
Seroconversion or significant increase	> 40%	> 30%		
Mean Geometric Increase	> 2.5	> 2.0		
Seroprotection	> 70%	>60%	HI titre ≥ 40	SRH titre ≥ 25mm2

J Virol, 2008. **82**(11): p. 5161-6.

125. Sridhar, S., S. Begom, A. Bermingham, K. Hoschler, W. Adamson, W. Carman, T. Bean, W. Barclay, J.J. Deeks, and A. Lalvani, *Cellular immune correlates of protection against symptomatic pandemic influenza.* Nat Med, 2013. **19**(10): p. 1305-12.

Table 1. Committee for Proprietary Medicinal Products (CPMP) criteria. Seroconversion (HI): if pre-vaccination serum is negative, post-vaccination serum must have a titer ≥40; if pre-vaccination serum is positive, at least a fourfold titer increase is required. Seroconversion (SRH): if pre-vaccination serum is negative, the post-vaccination serum haemolysis area must be ≥25 mm2; if pre-vaccination serum is positive, there must be at least a 50% increase in the hemolysis area. Seroprotection (HI): a serum sample is considered seroprotected when it shows an HI titer ≥40 or an SRH titer >25mm2. Seroconversion rate: proportion of subjects showing seroconversion. Seroprotection rate: proportion of subjects showing seroprotection (6).

Assays for influenza vaccine evaluation	
Traditional assays	
Haemagglutination Inhibition assay (HI)	▫ IgM, IgG antibodies;
	▫ antibodies against head domain of heamagglutinin;
	▫ titre ≥ 40 as an immunological correlate of protection;
	▫ advantage: simple, cheap
	▫ disadvantage: low sensitivity to B and avian strains, variability among laboratories;
Single Radial Haemolysis assay (SRH)	▫ antibodies, mainly IgG;
	▫ antibodies against surface glycoprotein and internal antigens;
	▫ haemolysis area ≥ 25mm2 as correlated of protection
	▫ advantage: rapid, simple, reliable, reproducible, large number of analyzed serum samples, unbiased data;
Virus Neutralization assay (VN)	▫ functional neutralizing antibodies;
	▫ no established correlates of protection;
	▫ less specific tha HI assay but more sensitive particulary for detecting low-titer seroconversions;
	▫ disadvantage: wild-type viruses, costs of high-level biocontainment facilities, high intra laboratories variability;
Additional assays	
Enzyme-Linked Lectin assay (ELLA)	▫ antibodies against neuraminidase;
	▫ no correlates of protection and harmonized/validated assays;
	▫ advantage: specific NA antibodies, safety and sensitivity;
ELISA assay	▫ serum and nasal wash IgM, IgA and IgG antibodies;
	▫ no correlates of protection and standardized protocols or reagents;
	▫ advantage: unbiased results, automated process for high-throughput testing, no pre treatment of serum samples and no red blood cells;
	▫ disadvantage: lack of specificity for influenza A and B subtypes;
Assays for cellular immune response	
ELISA/ELISpot	▫ quantify cytokin production in serum, alveolar lavage samples or culture supernatant;
	▫ ELISpot advantage: cheap, high throughput processing, sensitivity and accuracy;
Flow cytometry	▫ T cells phenotypes;
	▫ advantage: versatility.

Table 2. Traditional and additional assays for the evaluation of influenza vaccine immunogenicity.