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Report of the Workshop on correlates for protection and serological assays for influenza vaccines

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This Report has been reviewed by Vaccine and Biologics Working Parties members (VWP and BWP)

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1. Executive Summary

The aim of the workshop was to discuss research updates on some aspects of influenza vaccination and serology, including epidemiology, immunity to the infection (humoral, cellular), correlates of protection, scientific progress in the field of the serological assays and standardisation.

The following is a summary of the main topics discussed at the meeting:

- How to improve the current understanding of the predictive value of the immunogenicity data for vaccine efficacy taking into account i) the type of vaccine under study; ii) humoral and cellular immune response; iii) protection from infection or from disease.
- Expectations for the serological evaluation of immunogenicity. For example: the concept of natural immunity and priming by vaccination and their characterisation by serology, exploration of pre-vaccination serostatus (residual immunity) and its effects on post-vaccination immune responses.
- Considerations on predicting efficacy for live attenuated influenza virus (LAIV) vaccines, including the role of non-clinical models and the most appropriate types of studies to provide an assessment of the immunogenicity and likely protective efficacy of a vaccine construct; the use of LAIV during pandemics.
- Update on currently circulating strains of influenza A with pandemic potential and implications on pre-pandemic vaccination.
- Use of animal models for predicting the efficacy of pandemic vaccines in humans.
- Issues with clinical efficacy studies and the role of effectiveness studies in seasonal and pandemic vaccination and their design.
- The development of novel influenza vaccines: a research update on universal influenza vaccines and alternative adjuvanting systems.
- The selection of antibody assays currently available and evaluation of their performance, in particular to address the standardisation of functional assays such as Haemagglutinin Inhibition (HI), SRH and Virus Neutralisation¹ (VN) and also to consider the possible role of novel assays (e.g. determination of antibodies against neuraminidase (NA), cell-mediated immunity (CMI) assays, alternative haemagglutinin (HA) assays).

In summary the need to delineate and agree on what appears to be quite an extensive research agenda for influenza vaccines is one of the preliminary objectives to achieve. Some of the questions posed to the experts at the workshop remained unanswered but key issues were identified, starting with the standardisation of HI and VN assay formats, as well as the investigation of new antigenic targets. The other most important question to address in the future is how to improve and clinically validate correlates of protection. Surely further investigation, also by means of public-private partnerships, and discussion on influenza serology and correlates of protection are urgently required.

¹ The Micro-Neutralisation (MN) assay is a VN assay done in a micro-format but the two assays are equivalent. The VN term will be used throughout the document as it is more accurate, although the MN is the format usually performed.

Wednesday 30 May

2. Predicting clinical efficacy for seasonal TIV vaccines

2.1. Sero-epidemiology of influenza

Katja Hoschler, HPA, London UK

The essential features of the immune response to influenza virus infection were described. Following primary infection there is immediate activation of CD8+ T cells; antibody response (both serum and mucosal) gradually increases afterwards and becomes more pronounced following reinfection. Viral shedding occurs during the first 10 days after infection; symptoms are dependent on priming history, health status, and virus strain. Antibodies remain detectable for 2-3 weeks. It was highlighted that antibody production is not linked to the presence of clinical symptoms; therefore serological surveillance can also detect asymptomatic cases.

Three sampling strategies for seroepidemiological studies were discussed: cross-sectional approach, the cohort / longitudinal approach and transmission studies. These studies can provide insight in age-specific levels of pre-pandemic cross reactive antibodies and the incidence of infection during a pandemic.

The usefulness of seroepidemiology for public health planning purposes is limited by some critical factors related to timing and variability of the data. Concerning the timing, critical factors include mismatch between information needs and provision at the start of the pandemic, delays caused by assay development, standardisation and up-scaling in the laboratories, obtaining rapid ethical approvals for clinical studies, sampling at the most appropriate time and publication of serological studies. Concerning the variability of the data, this can be attributed to differences in sampling approach linked to the samples characteristics and to the serological assays used or to assay performance. In recognition of the huge potential as well as the existing gaps in influenza sero-epidemiology a Global influenza sero-epidemiology expert working group was established.

The HPA established a seroepidemiology programme, with an archive of sera collected since 1986. This programme has proven crucial for surveillance for measles, mumps and rubella, but it is also important to monitor other infectious diseases, such as hepatitis C, helicobacter, parvovirus B-19, HPV, CMV, etc. This programme allowed useful information and data to be obtained during the last H1N1 pandemic in the UK.

The seroepidemiology studies conducted during seasons 2009-10-11 permitted to identify an unexpectedly intense A(H1N1)pdm09 activity during the 2010-2011 season for example, especially in older individuals, which could not be predicted based on the low levels of susceptibility to the virus after the second wave. Furthermore, a shift in age range of death and hospitalizations was detected, which could be explained by the depletion of susceptible pool in younger groups, although other factors could play a role (representativeness). Children were identified to have played a key role for the spread of A(H1N1)pdm09 in the early epidemic.

The predictive value of a seroepidemiology study is potentially high in a pandemic situation, whereas more difficulties are envisaged in a seasonal context where several strains would be circulating.

The difficulties of interpreting the seropositivity implications were highlighted; for example seropositivity does not necessarily predict protection against circulating strains or responsiveness to vaccination, because it cannot differentiate between vaccination and infection. Likewise, the absence of antibodies not always indicates susceptibility (not all individuals seroconvert), since such individuals

could be protected anyway. An increase in antibody levels could indicate a booster response, not necessarily infection. Immune correlates and booster effect can predict protection depending on the age group, i.e. there can be a good correlation in children but not in elderly. Serological data in children are indeed more easily interpreted than data from at risk groups or older people, as the interpretation of antibody levels becomes more difficult with increasing age. Younger children have no antibodies at baseline, as their lifetime exposure to influenza is limited – although there might be age-related differences.

Overall the immune response to the vaccine cannot be fully predicted based on the seroepidemiology data alone, other types of surveillance may be required or retrospective analysis. Modelling seroincidence might be a way forward as this may help with the interpretation of the data.

Seroepidemiology studies are a useful tool for modelling a pandemic and thus can be critically important but a cautious interpretation of the data is required.

2.2. State of the science: correlates of protection for influenza vaccines

Katja Hoschler, HPA, London UK

Some aspects of the correlation between levels of virus replication and clinical responses were discussed: i) illness correlates with peak virus titres, with titres of 10^1 - 10^3 being associated with asymptomatic or upper respiratory infection, and titres of 10^6 - 10^7 with 104-105 °F fever; ii) peak titres are achieved early in infection. As influenza illness seems to correlate with peak viral titre, the job of the immune system is to keep the viral load $<10^3$. This can be achieved with an adaptive immune response.

Immunity is due especially to anti-HA antibodies (IgG in higher amount vs. IgA/IgM), together with a smaller amount of anti-NA antibodies (IgG and IgA/IgM). In addition all other antibodies play a role, e.g. anti-M2 antibodies, although the latter cannot clear the virus by themselves. Because immunity is ultimately the result of the concerted action of all the above mentioned immune mediators, no single correlate or surrogate of immunity can be uniquely defined.

Improved knowledge on the immune repertoire through characterization of signature epitopes (e.g. from H5N1s survivors²) could inform the development of new vaccines. For example it was shown that some antibodies are specific to these survivors and lack in the controls, like antibodies against a peptide at the end of HA2, against the catalytic site of NA (not in H1N1 controls) and against the M2e and the PB1-F2 proteins.

A Pandemic Influenza Vaccine Evaluation Consortium (PIVEC) was established among the Veterinary Laboratory Agency (VLA), The National Institute for Biological Standards and Control (NIBSC) and the Health Protection Agency (HPA), and many different work packages were identified, including the development of a ferret lethal challenge model for the assessment of vaccine efficacy, the comparison of antibody response among animals and humans in order to identify correlates of protection, and the comparison of CD4 and CD8 responses between human vaccine recipients and survivors of avian flu infection. Some preclinical data from vaccinated ferret models were presented which showed differences between vaccines related to viral replication and antibody titres, although by day 3 post-challenge all vaccines tested induced a significant reduction in virus shedding and viral load vs. controls. For example, adjuvanted vaccines were found to dampen virus replication in tissues of vaccinated animals by day 5 following viral challenge, whereas whole virion vaccines did not. It should

² Golding & Khurana, 3rd ESWI Portugal Sept 2008

be considered that endpoints in ferret models must be virological, not clinical, due to the mildness of H1N1 disease; therefore, although animal models mirror human clinical studies, the clinical relevance of the animal findings may be difficult to extrapolate.

The consortium was also involved in head to head vaccine-comparison trials in adults and children. Two head-to-head clinic trials (AS03-adjuvanted vs. whole-virion (Celvapan)) were performed in children 6mo-12 years (n=1000) and adults (n=347) including elderly. Immunogenicity showed clear differences between vaccines, especially in the children, with superior response in the Pandemrix vs. Celvapan group. An age effect was also obvious, with both vaccines, with lower immune responses in the elderly. The vaccine effectiveness was satisfactory in particular in the younger age groups. These differences in vaccine immunogenicity are puzzling and may require a more sophisticated analysis of the antibody repertoire.

A follow on study was performed looking at the response to H3N2 and B components of TIV during the subsequent influenza season and some interesting results were obtained. For example, a high prevalence of H3N2 antibodies pre-vaccination was detected, likely induced by exposure to previous H3N2 strains. These baseline antibodies were similar across different vaccine's groups, which indicated that they are not resulting from prior H1N1 vaccination. However the H3N2 response to TIV in the trial differed by vaccine group, i.e. a booster effect was seen only when the adjuvanted H1N1 vaccine was previously given, vs. the non-adjuvanted whole virion vaccine. There was a clear difference in long-lasting and cross-protective (cross-reactive) immunity, with possibly an effect of priming long-term. The youngest age groups showed the most evident benefit of an adjuvanted vaccine.

These observations however require further research to be conducted. More work is planned on the ferret immunogenicity study, the immunogenicity follow-up children study and on the epitope mapping work concerning the quality of antibodies' response.

2.3. Basic principles to establish correlates for protection

Sara Thomas, London School of Hygiene & Tropical Medicine, UK

To evaluate surrogate endpoints three questions need to be answered: i) how to identify an immune marker (IM) as a potential surrogate endpoint for clinical protection, ii) how to assess whether an identified IM is a valid surrogate endpoint for clinical protection, and iii) what is the relationship between the distribution of vaccine-induced titres of the IM in populations and clinical protection. Both experimental and observational studies can be used to evaluate potential substitute endpoints, by assessing associations between the vaccine and the IM and between the IM and occurrence of the clinical endpoint, as well as overall clinical vaccine efficacy (VE).

Two sets of criteria to validate vaccine-induced substitute endpoints were described:

- The Prentice criteria (1989) adopt a hypothesis-testing approach and require four conditions to be met: the vaccine has a significant effect on the clinical endpoint; the vaccine is significantly related to the IM; the IM is significantly related to the clinical endpoint; and the full effect of the vaccine on the clinical endpoint is explained by the IM. Criticisms of the Prentice criteria include the restrictive requirement that the IM lies on a sole causal pathway between the vaccine and occurrence of the clinical endpoint, and that the approach is susceptible to post-randomisation selection bias.
- The Qin et al hierarchical framework (2007) distinguishes three levels of association: a correlate of risk (CoR), where the IM correlates with protection against a clinical endpoint; a level 1 surrogate, which is a CoR that predicts VE in a specific setting (subdivided into a

statistical surrogate, which satisfies the Prentice criteria, and a principal surrogate, which uses a framework of causal inference); and a level 2 surrogate, which predicts vaccine efficacy across different settings (for example, using a meta-analytic approach).

Different methods to relate IM titres to clinical protection were presented.

Threshold methods assume an “all or none” model, i.e. titres above a specific threshold result in 100% protection, titres below the threshold result in 0% protection; the IM threshold which corresponds to clinical protection can be estimated from observed clinical vaccine efficacy and the proportion of vaccinated and unvaccinated individuals below specified thresholds.

Continuous methods relate IM titres to VE without assuming a threshold titre (i.e. a “partial” or “leaky” model) by fitting regression models (logistic regression, scaled logit, etc.) to IM titres in vaccinees and non-vaccinees.

Other factors may affect the relationship between IM titres and the degree of clinical protection. Heterogeneity in exposure intensity and/or the challenge dose needs consideration; a specific IM titre may confer less protection in settings with higher levels/more frequent exposure to pathogens. Host factors that could modify the relationship between IM titre and vaccine efficacy include age and socio-economic status. Different clinical endpoints (e.g. protection against infection or against disease) may require different IM titres and/or different markers, and immunological factors are also important (measurement of functional antibody vs. total antibody levels, timing of measurement in relation to the kinetics of the immune response, etc.).

2.4. Research update on parameters to predict clinical efficacy: Industry views

Andrew Dunning and Melanie Saville, Sanofi Pasteur for EVM

A recent definition of a correlate of protection resolves some earlier inconsistencies in terminology. Plotkin and Gilbert (2012) propose:

“A correlate of protection is a marker of immune function that statistically correlates with protection after vaccination and that can be either a mechanistic cause of protection or a non-mechanistic correlate – i.e. which does not cause protection directly but nevertheless predicts protection through its partial correlation with another immune marker that mechanistically protects.”

However, a number of open questions remain to be resolved prior to consideration of statistical models:

- Is a correlate of protection the titer at which 100% individuals are protected, or 90% are protected, or a ‘population average’ (50%) is protected?
- Is a CoP a titer to be achieved post-vaccination (when immune response is at its highest, possibly more relevant in vaccines research) or pre-exposure (possibly more relevant in scientific terms)?
- Is the protective titer the same for infants, children, adults and elderly?
- Is the individual protected only at the time the assay sample is taken or for a number of years or for life?

- Is the protective titer the same for different strains of pathogens and does it depend on how protection was induced (natural infection vs. inactivated or live vaccine or asymptomatic carriage)?
- Is there a 'one size fits all'?
- How may these questions be answered in a rigorous, quantitative manner?

Efficacy trials provide an opportunity to conduct sub-studies for investigating correlates of protection. However several aspects require careful consideration, e.g. aspects of the study design (who to bleed and type of assay) and which statistical methods are most appropriate. Special statistical methods are needed because protection is not directly observable, but must be inferred from occurrence and non-occurrence of disease. If occurrence of disease implies absence of protection, non-occurrence of disease can imply either protection or absence of exposure.

Two statistical methods have been broadly used for estimating protection from disease-occurrence data: Method of Chang and Kohberger, and Scaled logit model.

The Chang and Kohberger method is based on the principle that "the titer differentiating 'protected' from 'susceptible' individuals is the titer at which the relative risk of being below the titer equals a relative risk of disease" and assumes the same threshold for vaccinees and non-vaccinees. It's particularly efficient in immunogenicity subsets type of design and may be interpreted as a population average measure predictive of vaccine efficacy; however it does not provide estimates of levels of protection and covariates are not readily included. It has been used in the 7-valent pneumococcal conjugate vaccine efficacy trial³ and to estimate a serological correlate of protection for meningococcal C conjugate vaccine⁴.

The Scaled logit model explicitly models both protection and exposure and estimates a protection curve and titers for specific levels of protection (50%, 80% etc.). Protection is modeled as increasing in a continuous sigmoid curve from 0% in individuals with assay value of zero towards 100% in individuals with high assay values. This model may be used with 'all subjects' designs and is readily adaptable to case-cohort design; it loses power and precision in immunogenicity subset design but allows covariates to be included in the analysis.

Other statistical models are currently being developed⁵.

Sample size, precision and power of a typical efficacy trial were further discussed, and importantly it was noted that knowing the precision (i.e. the width of the confidence interval: $\pm 10\%$, $\pm 15\%$) of an estimated protective titer is critical for the interpretation of the results; indeed point estimates without confidence intervals can be misleading. A typical placebo-controlled efficacy trial, designed to show with 80% power that the lower limit of a 95% CI for VE exceeds 35% with an assumed VE of 70%, requires a number of cases equals to 67. It is not possible to make a sufficiently precise estimate of correlate of protection in a single efficacy trial as $>3,000$ cases would be needed for a CI of $(-10\%, +10\%)$. The conclusion was that a vaccine efficacy trial is typically underpowered for identifying correlate of protection endpoints.

Moreover, the generalizability of an individual trial is questionable, that is, in the absence of a clear demonstration of consistency, results of an individual study should be considered specific to the circumstances of the study e.g. study population, viral type/subtype and strain, case definition, assay

³ Black, 2000

⁴ Andrews 2003

⁵ The a:b model, suggested in Siber 2007 and developed by Chen et al. –to be published-, and a method based on diagnostic testing - Barrett et al., 2011

standardization, duration of surveillance etc. Precision and generalizability of data should always be rigorously considered. Performing meta-analysis can overcome the limitations of single studies by pooling data together; e.g. it may also be possible to apply the Chang-Kohberger method to a number of previously published efficacy trials. The use of a variety of statistical methods would also be useful by allowing synthesizing conclusions from different methods. Seroepidemiological studies may serve the same scope.

However key issues remain to be answered: i) protection is mediated through multiple immunological mechanisms of action, but assays only measure a single characteristic; and ii) are the immunological characteristics measured by the assay causally related to protection or is the assay merely differentiating individuals with strong or weak immune systems⁶.

In conclusion the identification of CoP for influenza is a complex matter with no single method available and which needs to take into account the different mechanisms of action of each influenza vaccine. In addition, it is still not clear whether or not one single correlate of protection is possible regardless of age, virus strain, vaccine type, assay type and immunological responses. Defining a CoP is overall a huge undertaking requiring prioritization and collaborative effort and prior to which harmonization is mandatory (assay standardization, case definition, trial design/statistical methods).

EVM recommendation would be to initiate with EMA' support defining a Public Private Partnership and in this context feasibility of the above undertaking should be assessed. Prioritization of age group/assay/vaccine type and timelines should also be defined.

2.5. Research update on parameters to predict clinical efficacy: antibodies against HA and NA, and protection

Walter Beyer, Dept. of Virology, Erasmus MC, Rotterdam NL

It seems generally accepted that anti-haemagglutinin antibodies are a strong predictor of protection. The publication by Ohmit et al., *J Infect Dis* 2011 states that: "While HAI antibody is the major correlate of protection, post-vaccination titres alone should not be used as a surrogate for vaccine efficacy. Vaccine failures from clinical trials need to be examined to determine why seemingly protective HAI titres may not protect." However, an absolute protective titre threshold does not exist. Instead, the relationship between the amount of antibody produced and the actual clinical protection should be described by an S-shaped protection curve.

The study by Coudeville et al., *Hum Vaccin* 2010 was discussed as it provides a 'state-of-the-art', evidence-based protection curve. A similar model was presented by Nauta et al., *Biologicals* 2009, whereby protection is predicted from several serological parameters, including the CHMP criteria. Based on this model, geometric mean titres (GMTs) plus standard deviation (SD) should be the optimal parameter to predict protection, GMT alone is a good/moderate predictor, seroprotection rate is moderate/poor and seroconversion is a poor or no predictor at all. Thus, seroprotection rate (based on a HI titre threshold of 40) is disappointing; its underlying protection curve is Z-shaped, which deviates from the real (S-shaped) protection curve. In conclusion, post-vaccination HI antibodies can still be considered excellent correlates of protection if used correctly (i.e. where mean and dispersion are considered). For a meaningful interpretation, a protection curve is needed, as exemplified and provided by Coudeville et al. The CHMP criteria ignore the underlying biology and suffer from mathematical flaws; hence it is not surprising that they poorly correlate with protection. Biology and mathematics are a better guide to CoP than artificial cut-off points.

⁶ see Follman, 2006 and Gilbert 2008 et seq

2.6. Research update on parameters to predict clinical efficacy. Assays suitability: sero-neutralisation (SNA) and haemagglutinin-inhibition (HI) assays

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The HI assay measures a type-specific response to the HA antigen of influenza virus. This assay was historically developed in the 1960s and 70s and the cut off defined for diagnosis was 1:1280 in adults (for reinfections) and 1:320 in children (primary infection). The major limitation of this assay though is that it measures both IgG and IgM, which some scientists believe that, although potentially irrelevant in adults and elderly, it may lead to overestimation of vaccine efficacy in children.

The trigger for the use of HA antibodies as correlates of protection for influenza lies in the human challenge study by Hobson et al. (1972). This study was however conducted with non-pathogenic strains; therefore, as human challenge studies should reproduce human disease (and not merely infection) in order to identify protective antibodies, the relevance of the 1:40 threshold identified by Hobson could be questionable.

Neutralising antibodies are those which correlate best to protection for all viral infections. Also measuring cell mediated immunity should be more predictive of protection, but technical issues are yet to be solved. If human disease is to be reproduced using VN testing in a microplate (i.e. MN), permissive cells (possibly human or human-like) and an incubation period that reproduces human disease are essential (i.e. at least 5 days of incubation for influenza). The incubation period in the original VN test consisted of at least 5 days in the 1960-70s, but this has later been substantially shortened. Since flu infection is a sequential event, shortening the infection period may lead to only measuring anti-HA antibodies. The cells that were used for VN in the 1960-70s were primary monkey cells. The currently used cells, MDCK, require the addition of trypsin for flu virus isolation, which is not a natural model of human influenza. Naturally permissive cells should be used such as CaCo-2 cells. As shown by some authors⁷, MDCK cells undergo rapid apoptosis after influenza infection, while CaCo-2 cells show no apoptosis but display cytopathic effects with necrotic signs significantly later after infection, which correspond to the normal cytopathogenic effect (CPE) of influenza virus. The importance of the incubation time is also evident with non-human adapted strains such as H5N1, because with the short incubation only few subjects are found already sero-positive to H5N1 before vaccination whilst up to 80% are found positive with the long incubation. Therefore there is the risk to overestimate vaccine responses (i.e. booster vs. priming).

In conclusion: i) the HI assay is of questionable relevance for measuring protection against clinical disease; ii) the 1:40 HI protective threshold is unlikely to sufficiently correlate with protection; iii) the VN with a short incubation period does not measure the full antibody responses; iv) MDCK cells do not reproduce in a microplate the natural disease that occurs in humans.

2.7. Factors impacting on correlates for protection in young children

Susanna Esposito, University of Milan, Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico, Milan Italy

Correlates for protection specific for children have not yet been established. The CoPs defined for adults may not be generalized to children because younger patients have a reduced capability for

⁷ Zhirnov et al., *Virology* 2003

cellular immunity and may lack prior experience with influenza virus infection. Many historical papers that support a correlation between antibody titres and clinical protection in children were mentioned⁸.

The data by Black et al., *Pediatr Infect Dis J* 2011 was also discussed in support of the conclusion that following two doses of TIV the 1:40 HI titre identified for adults is only associated with 22% clinical protection in children, whilst to achieve a 80% or 90% protection, HI antibodies titres must be respectively 1:330 and 1:629. A cut-off of 1:110 may be used to predict a 50% clinical protection rate.

The question remains whether CoPs in children should be defined against clinical disease or infection; moreover post-vaccination titres alone should not be used as a surrogate for vaccine efficacy.

Challenges for the future include: i) identification of CoPs in young children for live or adjuvanted influenza vaccines; ii) ascertain whether various age strata may pose differences in terms of CoPs; iii) investigate the role of secretory IgAs and CMI in the first years of life; iv) investigate the reasons why seemingly protective Hi titres are associated with influenza infection, i.e. are not protective.

2.8. Factors impacting on correlates in the elderly (>60 years of age): Vaccine preventable disability in older adults: Influenza as a model

Janet E. McElhaney, Dept. of Medicine, University of British Columbia (moved to Advanced Medical Research Institute of Canada)

Influenza is the single most vaccine-preventable disease and vaccination programs are cost-saving in older people but influenza is still a serious illness in this age group. New influenza vaccines are being developed but the reliance on antibody responses as a measure of vaccine efficacy seems to limit our ability to test these vaccines.

The effects of immunosenescence must be taken into account, as they involve the increase in incidence of serious outcomes of influenza (e.g. 90% of influenza deaths occur in older people and for every influenza death there are 3–4 influenza hospitalizations) and the decrease of response to vaccination. Efficacy with current influenza vaccines is 70–90% in preventing respiratory illness in healthy adults and only 30–40% in older people. Nevertheless vaccination is cost-saving and this indicates a clear margin for improvement.

Inactivated influenza vaccines trigger antibody production and stimulate T helper cells (Th2/Th3 response with cytokines IL-4 and IL-10). Instead new improved vaccines should aim at targeting both Th and Cytotoxic T lymphocytes (CTL) responses and should also shift the Th response to a Th1 response, with stimulation of IFN- γ and suppression of IL-10. The normal decrease in immune functions observed with increasing age may have a greater impact on cellular responses and therefore these become more important. The mucosal barrier is important as a first line of defence but especially in frail elderly may be impaired resulting in a permissive state for infection, therefore improving cell-mediated immunity (CMI) could improve the success rate of vaccination. In fact studies in elderly showed that humoral responses were comparable between individuals with influenza disease and individuals with no signs of disease⁹. PCR testing showed that individuals unable to seroconvert following infection suffer from the most serious disease¹⁰.

By means of in vitro cell mediated immune assays that were validated, it was found that granzyme B correlates with protection in the elderly⁹. The reported data showed that individuals who did not develop influenza up to 10 weeks post-vaccination possessed higher concentrations of granzyme B

⁸ Potter et al., *Brit Med Bull* 1979; Davies et al., *Epidem Inf* 1989; Wesseliuss-De Casparis et al., *Bull World Health Org* 1972; Fox JP et al., *Am J Epidemiol* 1982.

⁹ McElhaney et al., *J. Immunol.* 2006; McElhaney et al., *Vaccine* 2009.

¹⁰ Shahid et al., *Vaccine* 2010

than those who became infected; granzyme B levels were especially higher in those who suffered influenza in a previous year⁹, suggesting that T cell memory could effectively be stimulated by live virus (i.e. infection) and re-stimulated with a subsequent vaccination. In the absence of a recent influenza infection, inactivated influenza vaccines instead did not stimulate granzyme B production, indicating that current vaccines may only weakly stimulate cellular immunity in the elderly population. Additional reported data showed correlation between protection and INF- γ /IL-10 ratio⁹, with an increase in INF- γ and IL-10 production following vaccination but without change in ratio. Generally the elderly show a decline in the INF- γ /IL-10 ratio because of a higher IL-10 production compared to younger individuals¹¹.

Weaknesses of experimental evidence with CMI CoPs include the small number of individuals studied vs. immunogenicity studies, due to the requirement for in vitro stimulation with live virus. In contrast, the sensitivity of CMI has been shown to be much greater than immunogenicity assays¹². However, due to the fact that current split virus influenza vaccines are poor stimulators of CMI, a CMI response to the vaccine is more difficult to detect relative to a “protective” antibody response to a vaccine.

In conclusion: 1. Current split-virus influenza vaccines provide a weak stimulus to the CTL response, thus influenza remains a serious illness in older adults. 2. New influenza vaccines are being developed and it should be underlined that correlates of CTL-mediated protection are needed to screen for enhanced efficacy. 3. Aging affects cellular immune mechanisms, thus vaccine development needs to target re-stimulation of memory T cells. This does not imply a fully new development for improved cellular immune response, but targeting virus internal proteins and adding adjuvants would already be helpful, or increasing the amount of antigen included in current formulations. 4. In elderly, vaccine preventable disability could be used as a model for improved vaccine efficacy.

2.9. Factors impacting on correlates of protection in young children and the elderly, industry views: evaluation of the influenza priming status

Martine Denis, Sanofi Pasteur for EVM

Immunological priming is defined as the exposure to an antigen leading to an adaptive immune response and development of immunological memory. The definition of immunological priming against influenza is affected by a number of factors: i) the identity of the target-antigen, i.e. conserved or strain specific antigens; ii) the timing of priming and identity of influenza strain(s) involved, which are most often unknown; iii) only persisting immune responses can be detected, i.e. a subject with no detectable immunity against influenza at a given point in time is not necessarily immunologically naïve. The main goal is not just to detect signs of immunological priming, but also to identify the type of priming that can predict an optimal response following a single vaccine injection, for which more research is actually needed.

Manufacturers can contribute by generating various types of relevant data in the course of developing seasonal and pandemic vaccines, including:

1. baseline immunogenicity data documenting past exposure to influenza (e.g. Tsai et al., *Euro Surveill.* 2010, concerning humoral baseline data on 7,962 subjects sampled in 2009; Galli et al., *PNAS* 2009, on the association between H5-specific CD4 T cell expansion during primary vaccination and neutralizing Ab response after booster injection);

¹¹ Skowronski et al., *J Infect Dis* 2011

¹² Gijzen et al., *Vaccine* 2010

2. comparison of the immune response to one vs. 2 vaccine doses (e.g. NCT00954798 and NCT00956202 on H1N1 pandemic vaccines-induced GMTs after 1 vs. 2 injections in different age groups: the increase in antibody response following the second injection is inversely correlated to the % of subjects with detectable HI Ab at baseline, which in turn increases with age from 0.25% among individuals aged up to 35 months to 46% among individuals aged 60 years and older);
3. immune response to an additional exposure to influenza antigen (booster); numerous booster studies were conducted with H5N1 vaccines e.g. Schwarz et al., *Vaccine* 2009 on the magnitude of the booster response following one- or 2-dose priming with adjuvanted vaccine;
4. homologous and heterologous immune response.

Manufacturers can also investigate the following topics:

1. Detailed characterization of baseline immunity in various populations of subjects (quantitative and qualitative; antigen- and epitope-specificity of the response; large sample size);
2. Prospective randomized trials assessing the impact of specific levels of baseline immunity on post vaccination responses;
3. Comparison of different types of priming. An example of comparing different types of priming assessed by additional vaccine dose (booster) is the study by Johnson et al., *J Infect Dis* 1986, demonstrating the priming effect of a single injection of a H3N2 live attenuated or inactivated vaccine in children upon challenge with a homologous live attenuated virus. Different vaccines induced different levels and type of antibodies (IgG, IgA and IgM) both in serum and in the nasal mucosal vs. H3N2 natural infection or vs. no previously reported exposure.

In conclusion, manufacturers contribute to the generation of relevant clinical data as discussed above. Further research should address the following points: i) definition of priming and ii) characterisation of the immune response after natural infection or vaccination. This research would benefit from collaboration with Academia or from the creation of a public private partnership in order to facilitate recommendation and implementation of influenza vaccination throughout Europe.

However it was remarked that screening of individual subjects prior to influenza vaccination to identify baseline immunity levels is not feasible from the manufacturers' perspective, hence vaccination recommendation can only be made at population level; likewise vaccination schedule cannot be defined at the individual level.

2.10. Discussion slot: how to improve prediction of clinical efficacy for seasonal vaccines (adj/unadj) in all age groups

1. Protection from infection (sterilizing immunity) or from disease?

Preventing infection would be desirable as it can reduce viral load, which consequently would limit both risk of transmission and severity of clinical symptoms. Viral load and shedding are important factors especially in the paediatric population as they affect the risk of transmission to the adults and elderly population. Children are regarded as a virus reservoir; hence protection from infection would be desirable in this age group.

Protection from infection is a clear cut parameter vs. protection from disease for which clinically validated endpoints are required. This is a key issue, as from a conceptual point of view prevention of infection is an objective outcome that can be measured. All other outcomes related to morbidity are more difficult to determine in an objective way. Thus from a vaccine developmental perspective,

protection from infection could be a clearer target, whereas from a public health perspective it may not be as relevant as protection from disease.

Concerning the latter, some critical elements must be defined, including the meaning of 'disease', which endpoints should be used, the sample size, issues linked with a low attack rate. The definition of 'clinical disease' may also have an impact on the sample size, hence on the study feasibility, therefore these interlinks should be taken into account. According to EVM efficacy trials with clinical endpoints are expected to be very complex, to require quite large sample size and additionally to be extremely costly.

In conclusion the opinion of the experts is split on this topic, with those in favour of protection from infection arguing that if infection is prevented, disease will be prevented too. However protection from disease is the aim of vaccination in the first place, i.e. to prevent complications from infection, and vaccines should be a primary target.

2. Immunogenicity endpoints:

a) Is HI still a valid serological test for predicting protection?

Although it is controversial to what extent the HI assay is fully capturing all facets of immunity, HI is still considered a valid serological test because of the well-established correlation between antibodies to HA and protection. Nevertheless the HI assay can and should be improved, for example regarding variability, which needs to be reduced. Moreover the absolute titre provided by this test should be flanked by other measurements of the immune responses, which can contribute to a more comprehensive characterisation of the immunity induced and hence may allow a better prediction of vaccine efficacy.

b) Would the virus neutralization assay be preferable and, if so, which are the best assay conditions for measuring the whole spectrum of neutralizing antibodies induced by vaccination?

The VN assay represent technically an approach closer to the biology of immunity than HI, hence it could provide more valid measurements. However it did not appear as a straightforward choice whether VN should support HI assay or should replace it altogether or whether other assays should be considered, e.g. SRH. In any case more research is needed on the VN assay, common protocols should be set and standardisation achieved. For example it was noted that the length of incubation could play an important role in defining the breadth of immune responses to infection/vaccination.

c) What is the validity of the CHMP criteria based on current knowledge?

The validity of the immunological criteria ('CHMP criteria') currently used as correlates of protection for influenza is generally under scrutiny and there is consensus over the fact that they don't represent validated correlates of protection. The use of an absolute antibody titre (1:40) as a threshold for acceptable protection is questioned due to the recognised limitations of the Hobson study, which represents the scientific basis. There are also recent publications of statistical models that using a meta-analytical approach propose a more robust quantification of the association between HI titre and protective efficacy¹³.

Improved correlates or definition of new ones and their clinical validation should thus be investigated. Antigens other than HA should be considered, e.g. NA antigens and/or antigens of internal viral proteins. However this implies an enormous investigative effort from the manufacturers view point. In their view, such effort should be laid out as a collaborative effort with Governmental Institutions and

¹³ Coudeville et al., *Human Vaccines* 2010 and *BMC Medical Research Method* 2010

Academia, in order to share costs/responsibilities and obtain results and protocols more efficiently. A broad database on correlates of protection should be created, which would allow the gathering of large amounts of information.

At the present stage, HA antibody titres are still viewed by the majority as useful immune markers of protection, provided GMTs plus SD are used. Coupling the HI titre with VN data should further contribute to a better estimate of efficacy.

Before concluding on new CoPs, the issue of assay variability between different laboratories should be addressed; an increased stringency would also be desirable. An external reference laboratory was deemed useful in addition to reference sera and assay standardization.

d) What is the role of anti-NA antibodies?

It was noted that NA is a more conserved protein because it is less prone to antigenic drift than HA. Consequently NA antibodies may appear to be a relevant antigen to immunise against, but more data are needed to draw definitive conclusions. It was noted that a number of published studies implicate a correlation between NA and protection and/or a reduced severity of clinical outcome. In other cases, only a weak correlation between NA antibodies and vaccine efficacy could be proven. However it could not be ruled out that the low NA content in vaccines is the main cause for such variability.

In fact the exact correlation between anti-NA antibodies and mitigation of disease is currently unknown, and this should be established in order to allow recommending specific amounts of NA in vaccines. On the other hand it was noted that the scientific grounds in support of vaccines' HA content could also be questioned.

As already mentioned, there is also the possibility to identify co-correlates with HI titres in conjunction with NI titres, which should be more predictive than HI titres alone. Such CoP should be established in a collaborative effort including optimisation and standardization of NA assays.

e) Should CMI be used as correlate of protection, e.g. for the elderly?

CMI measurements as endpoints were extensively discussed and it was concluded that they are extremely important, especially in certain age groups. However there are a number of uncertainties, including i) assays for CMI are even less standardized than assays to detect humoral antibodies; ii) CMI endpoints would be unpractical in children because large blood samples are currently necessary, and iii) overall experience and data are too limited to substantiate their use as CoPs. Some experts are of the opinion that memory B cells are as important as T cells in evaluating CMI responses and may require smaller samples.

From the perspective of manufacturers that are currently investigating universal vaccines, it is important that assays availability are addressed first, and that a general consensus should be achieved on the definitions before regulatory requirements are set for assays and endpoints. More research may be needed to define the exact relevance of CMI during clinical development for regulatory purposes, especially for novel vaccines and vaccines including adjuvants. Experts in this field pointed out that granzyme assays and other CMI assays have been standardized and validated at least for the elderly, but from the industry view these assays may entail feasibility issues, e.g. in large trials.

Mechanistically the question remains what is CMI correlating with. Also it should be taken into account that no influenza vaccine induces antibodies independent of T-cells. The cellular parameter that is found to correlate with clinical disease is CD4 cells and this would be the best parameter to look for, especially in the elderly.

It was acknowledged that CMI correlates may have less importance for HA-based vaccines (especially unadjuvanted). In contrast for vaccines containing internal viral proteins but even for some HA-based vaccines such as split vaccines, CMI CoPs would play a bigger role, especially for specific age groups like the elderly.

3. Do we need different levels of antibodies response for different age groups?

The data by Coudeville et al. suggest a relatively stable pattern of antibodies production across age groups from young adults up to 60 years old, whilst it is well known that humoral responses to vaccination are lower in elderly people. These data however is gathered from different types of studies, where different age groups account for small numbers of participants. It is believed that interaction between virus and antibody is not age-related, although evidence in support of this is limited.

Children should not be viewed as one population. The first year of life is completely different to subsequent years and children above the age of 5 are considered most similar to adults.

Additionally, it is expected that in the very young (<1 year) protection is solely mediated by vaccination, whereas in older children and adults immune memory plays a more important role. This is not different for influenza than for other infections/vaccines. In children parameters should be identified that can distinguish between priming and boosting effects of vaccination.

4. From a methodological point of view what is the best approach to establish correlate of protection: challenge studies or large clinical data sets from efficacy studies?

Challenge studies in young healthy individuals (18-45 years of age) would allow a detailed characterization of immunity in response to viral challenge and may help further to understand cross-protection across several strains. Because of the stringent controlled settings and types of testing, challenge studies may better support the understanding and identification of correlates of protection. Moreover challenge studies could also provide initial early data and design insights before embarking into large costly field efficacy trials, which for example could be useful for new vaccines.

However challenge studies do not represent a replacement for clinical efficacy studies.

Also, challenge studies are very expensive and difficult to perform and are limited to healthy young adults who additionally are very carefully screened for safety reasons. Furthermore, the obvious difficulties and safety risks inherent to the challenge with highly pathogenic viruses (e.g. H5N1) may limit the usefulness of these studies in identifying CoPs that are clinically relevant. In addition a small amount of antibody can be protective against a small viral challenge, but when confronted with high quantities of virus the same antibody titre may result in vaccine failure.

3. Predicting clinical efficacy for pandemic vaccines

3.1. Use of animal models for predicting the efficacy of pandemic vaccines in humans

Kanta Subbarao, Laboratory of Infectious Diseases NIAID, NIH, USA

The starting assumption for the use of animal models is that, if immunogenicity follows similar patterns in animal models and humans, it may be reasonable to extrapolate efficacy as well. The animal models used to study influenza include ferrets (most commonly) and mice, much less frequently guinea-pigs and non-human primates. The assessment of vaccine efficacy in ferrets includes challenge with high doses (10^5 ID₅₀ or a lethal dose) of homologous and heterologous pandemic viruses followed by measurements of clinical signs and viral replication (serial nasal washes). To provide data on viral

replication (peak, kinetics of viral replication and clearance) in the upper and lower respiratory tract, animals need to be sacrificed at serial time points (at least two time points are recommended).

The existing consistencies and inconsistencies between the animal models and humans were summarised as following.

Consistent findings from animal and human studies include: i) inactivated vaccines that elicit robust HI and VN responses also protect animals from experimental challenge; ii) adjuvanted vaccines are more immunogenic than unadjuvanted vaccines allowing antigen sparing; iii) unadjuvanted split vaccines are less immunogenic than adjuvanted split vaccines, whilst the latter and whole virion vaccines show similar immunogenicity. For example unadjuvanted whole virion vaccines were immunogenic at low doses and protected against live virus challenge in mice¹⁴; both immunogenicity and protection in this model were comparable to that of a whole virus H5N1 vaccine, which had previously been demonstrated to induce high titres of antibodies in clinical studies. A 2 dose-regimen of the split virion pH1N1-AS03 vaccine (3.75 or 1.9 µg) was required to protect naïve ferrets against homologous challenge whereas higher doses of unadjuvanted split vaccine (15 µg) or one dose of adjuvanted vaccine conferred only 17-33% survival in ferrets after challenge¹⁵.

Inconsistencies between animal and human studies include: a) a single dose of 15µg unadjuvanted split virion pH1N1 vaccine was immunogenic in older children and adults; b) a single dose of 3.75 µg of AS03- adjuvanted split virion pH1N1 vaccine was immunogenic in older children and adults; c) unadjuvanted and adjuvanted split virion pH1N1 vaccines were effective in humans in protecting against MAARI (Medically Attended Acute Respiratory Illness); d) immunogenicity of LAIV in animal models exceeds that seen in clinical studies; and e) alum enhances vaccine immunogenicity significantly in ferrets, but the benefit in humans is equivocal.

Examples of the above include an immunogenicity and efficacy study of pH1N1 LAIV (p-LAIV) in mice and ferrets¹⁶ that showed that the p-LAIV was immunogenic in both species and conferred complete protection against challenge with pH1N1, in contrast to seasonal (s-)TIV that did not confer protection in either animal model and to s-LAIV that did not confer protection in ferrets (in mice, 2 doses of s-LAIV led to complete protection in upper respiratory tract and partial protection in the lungs). In another ferret study¹⁷ (n=118) it was found that alum improved the efficacy of an H5N1 (A/Vietnam/1203/ 2004) split virion vaccine as measured by immunogenicity (HI, VN), mortality, morbidity and brain invasion, whereas alum is not an effective adjuvant for H5N1 vaccines in humans. In contrast Cox et al. reported that alum enhanced immunogenicity and/or efficacy of a H7N1 split virion cell-grown vaccine in mice and ferrets (*IRV* 2009) as well as immunogenicity in humans (*Vaccine*, 2009), whilst as expected the non-adjuvanted formulation was poorly immunogenic if at all.

Concerning the importance of priming in animal models, Dr Subbarao described five preclinical studies to show that in animal models prior experience with influenza infection or vaccination played an important role in the response to pH1N1 vaccines. For example some authors¹⁸ found that prior infection with seasonal H1N1 or s-LAIV (but not s-TIV) primed for a robust response to a single dose of p-LAIV and protection from challenge in mice. Similarly two different ferret studies¹⁹ showed that prior infection with seasonal H1N1 (but not vaccination with s-TIV) primed for a more robust response to split virion pH1N1 vaccine and altered morbidity to a pH1N1 challenge, despite the detection of only

¹⁴ Kistner et al., *PLoS One* 2010

¹⁵ Baras et al., *Vaccine* 2011

¹⁶ Chen et al., *J Inf Dis* 2011

¹⁷ Layton et al., *PLoS One* 2011

¹⁸ Chen et al., *PNAS* 2011

¹⁹ Ellebedy et al., *CIV* 2010 and *Vaccine* 2011

minimal levels of cross-reactive antibodies. Suguitan et al., *PLoS One* 2011, evaluated different prime-boost regimens in ferrets and found that priming with DNA vaccine boosts immunogenicity of H5N1 LAIV in those animals. In a study in mice on the impact of prior vaccination against s-TIV on antibody responses against pH1N1 vaccine, vaccination with one/two doses of pH1N1 unadjuvanted split virion vaccine induced consistently higher antibody response in s-TIV primed animal vs. naïve animals, whereas one low dose of an AF03-adjuvanted pH1N1 was able to induce higher titres vs. the unadjuvanted vaccine regardless of the priming status of the animals.

In summary, extrapolations from preclinical studies need to be carefully balanced over a number of aspects, most importantly that animals are immunologically naïve, whereas most humans have prior experiences with influenza, and this is difficult to model in animals. In addition viral challenge is quite stringent (i.e. high virus dose) and the intra-tracheal route of inoculation is not physiological; sometimes the vaccine preparations used in animals differ from the vaccine in clinical use and specifics of dose sparing cannot be extrapolated to humans. Overall the assessment in animal models is most useful for predicting efficacy in humans if correlates of protection are known and can be measured. A challenge for the future is to identify immune correlates of protection for LAIV and newer vaccine platforms that can be measured in animal models. Other challenges include developing models that reflect the immunological priming that occurs in humans, including responses to T and B cell epitopes, conserved internal proteins and HA stem. However it should be noted that there is no standardization of assays in animals to detect different immune responses and that there are a number of open questions, including how to properly prime and whether it's possible to cross-prime.

3.2. Immunogenicity endpoints in a pandemic context and impact of residual immunity on post-vaccination responses

Ernst Soethout, National Institute of Public Health and Environment (RIVM), NL

An overview of the human antibody response against the influenza A antigens HA, NA, M2e, M1 and NP was presented. Antibodies against HA can be strain-specific (target the globular head on 5 antigenic sites and are responsible for the antigenic drift) or heterosubtypic. The latter are divided into non-neutralising (against the unfolded protein) and neutralising antibodies (against the globular head or the stem). Stem-specific antibodies target the helix A or the fusion peptide and are not detected by HI assay. The M2e protein is expressed at high levels on infected cells and has a protective role via antibody-dependent cell-mediated cytotoxicity (ADCC). Antibodies against NA restrict viral spread by inhibiting viral budding from infected cells. As of today, current immunological endpoints used to evaluate pandemic vaccines are set by the influenza seasonal vaccines guideline EMEA/CHMP/VEG/4717/2003 (so called 'CHMP criteria') and are based on HI and SRH assays. These assays only reveal a part of the immune responses generated, thus a recent concept paper on the revision of the guideline for influenza (EMA/CHMP/VWP/734330/2011) proposes complementary methods e.g. VN, as well as other types of assays (CMI) and exploration of pre-vaccination status. Two studies were presented, which showed a high correlation between VN (MN with the "CDC protocol") and HI assays responses against pH1N1 2009²⁰. However this correlation should be considered carefully as HI and VN assays measure different antibodies and in addition antibodies differ also based on vaccine type and virus strain. The VN assay seemed more sensitive than the HI assay particularly for detecting low-titer sero-conversions among vaccinees, but also yielded higher titers in general. The well-known variability issue of the HI and VN assays could be reduced by using standards²¹. Meta-analyses of pandemic H1N1-2009 vaccines efficacy (both adjuvanted and

²⁰ Hancock et al., *NEJM* 2009 and Veguilla et al., *J Clin Microbiol* 2011

²¹ Wagner et al., *Vaccine* 2012 and Wood, *Vaccine* 2012

unadjuvanted) confirmed that one single dose appears generally to be sero-protective in healthy subjects aged ≥ 36 months, whereas very young children (6-35 months) may need to receive two doses of non-adjuvanted vaccine or one dose of adjuvanted product to achieve seroprotection²².

The baseline sero-status has important consequences on the efficacy of vaccination in both seasonal and pandemic scenarios, which are also interlinked. In a paper by Luytjes et al., *Vaccine* 2012, undetectable pre-vaccination HI titers are associated with a higher chance of getting infected by seasonal influenza. The paper also shows that previous TIV administration to adults 18-60 years of age enables the mounting of a cross-reactive immune response against drifted seasonal influenza strains or pH1N1 virus. During the pandemic 2009 the elderly showed the highest pre-vaccination HI titers and protection against pH1N1 infection, implying a positive correlation between pre-vaccination HI titers, increasing age and protection²³. Strengell et al., *PLoS One* 2011, studied the long-term duration of immune response by HI following vaccination with AS03-adjuvanted pH1N1 vaccine, showing that HI titers against homologous pandemic strains may persist for one year post-vaccination. This response was however specifically directed against the California strain, whilst isolates with one or two aminoacid changes in the major antigenic sites of the HA (N215D and/or N156K) had the ability to avoid vaccine-induced immunity. Most importantly, it was found that HI responses after vaccination with an adjuvanted vaccine against pH1N1 can be boosted by seasonal vaccination one year later²⁴.

In conclusion: i) antibodies detected by VN assay may correlate with antibodies detected by HI assay and may be considered as an endpoint for pandemic vaccines; ii) low pre-vaccination titers may negatively affect both seasonal and pandemic vaccine efficacy.

3.3. Parameters to predict clinical efficacy in a pandemic vs. seasonal context

Dominique Descamps, GSK for EVM

Vaccine manufacturers are not entirely convinced by the future directions expressed in the concept paper for the revision of the influenza guideline, especially concerning the so-called holistic approach of “bridging from surface composition and seroprevalence to optimal vaccination concepts”. A number of concerns and challenges were presented:

- i) there are practical challenges in ensuring the timely availability of a sufficient number of pandemic vaccines doses due to the unknown virus pathogenicity at the early stages of a pandemic. In addition manufacturers must select only one formulation as early as possible. Multiple presentations/dosages/formulations will impact on the timely vaccine availability. Basically industrial feasibility and timelines of availability of products are key elements.
- ii) Seasonal and pandemic approaches are fundamentally different and cannot be translated one into the other, e.g. extrapolation from H1N1/H5N1 to TIV is scientifically not valid.
- iii) Moreover, the lessons learnt with the H1N1 pandemic may not apply to future pandemic strains (e.g. H5N1-like).

In conclusion, the proposed flexible approach for extrapolation of data between seasonal and pandemic contexts is attractive but faces many challenges, mostly due to the necessity of an early decision on the formulation to be developed. It was also stressed that it is of critical importance to distinguish between regulatory requirements and medical research proposals/needs. Industry confirmed their

²² Manzoli et al., *PLoS One* 2011 and Yin et al., *Influenza Other Resp Viruses* 2011

²³ Ikonen et al, *Eurosurveillance* 02-2011

²⁴ Strengell M., *Influenza and other resp. viruses* 2012

willingness to engage in public-private partnerships (PPPs, including PPPs for assay standardisation), whereby budget allocation can be prioritised based on public health needs. Finally it was proposed to re-discuss gaps and necessities and to reassess entirely the strategy around influenza vaccination.

3.4. Discussion slot: how to improve prediction of clinical efficacy in all age groups; factors impacting extrapolation from seasonal to pandemic

1. How to define prior to vaccination the immunological naivety of an individual in a way that is clinically relevant (in epidemic or pandemic settings).

Immunological naivety occurs both in adults and children when a totally new virus strikes, i.e. very limited to null cross-protection from experience with seasonal influenza (infection or vaccination) would be expected. Historical data from vaccine use in previous pandemics were discussed and in general a 2-dose regimen vaccination has always been required in serologically naive subjects. But whether or not two doses are able to prime naïve individuals, it is not known.

Nevertheless, in adults it may be possible to detect some cross-reactivity against conserved regions common to all flu strains²⁵. It may also happen that a seasonal vaccine could boost a B-cell response against heterologous strains, such as H5 or H7. There is however limited knowledge on these aspects.

The very young children population are expected to be fully naïve also to seasonal strains, thus mimicking the immunological situation of a pandemic. On this regard, some experts consider it important to investigate efficacy of seasonal flu vaccines in naive children because these data can be informative for a real pandemic by a completely new virus subtype, e.g. H9 or H16. However, it was recognised that the predictive value of such data/experience is unknown.

Overall this question remains unanswered.

2. Should immunogenicity endpoints be the same in seasonal and pandemic context?

In pandemic settings it was agreed that a vaccine should protect against disease. Indeed the vaccine is intended to ameliorate severity of disease, hence reducing the overall impact of the pandemic.

However there was a split view on the use of the same set of immunogenicity endpoints in seasonal and pandemic situations. There was consensus that the current immunogenicity criteria should be modified and immunological endpoints should be correlated to an adequate level of protection. In addition it should be taken into account that serological assays like HI lack sensitivity with H5 and more in general with all avian strains.

3. In case a pandemic strikes for which the population is fully naive, to what extent can the efficacy of a pandemic vaccine construct in such a population and across all ages be predicted by its efficacy established in naïve children with seasonal strains?

The extrapolation of naivety between seasonal and pandemic situations remains an open question.

The problem is that there is no agreement on the definition of naivety in terms of immunological measurement. A pragmatical approach would be to request data in seronegative individuals based on an agreed definition of seronegativity. However, subjects during the 2009 pandemic were defined as seronegative by means of an HI titre <10, which turned out to be unsatisfactory as this threshold varied extensively among companies depending on the assay used.

²⁵ JM Garcia et al., *PLoS One* 2009

Further investigation is needed to define the concept of naivety, for example by exploring boosterability of immunity. It was remarked that cross-reacting memory T and B-cells should be investigated in seronegative individuals. Both antibody titres and B cells from the age of 6 months up to adolescence should be assessed to define the naivety status of an individual, keeping in mind that after the age of 4-5 years it can be expected that most subjects become immunologically primed.

Seroepidemiological studies are important tools to investigate naivety, but would be influenced by the assay used to assess antibody prevalence in different age groups. Indeed HI assay may not detect the totality of individuals that are actually primed. In fact we would see a booster response although “seronegative” at baseline. The VN would be the most relevant assay and should be used more widely, once common protocols are optimised and agreed which would include solving the issue around the use of a cell line permissive to human-adapted flu strains.

4. Strains with pandemic potential and pre-pandemic vaccination

4.1. Circulating strains with future pandemic potential

Derek Smith, Cambridge University, UK

A H5 influenza map based on data from CDC, St Jude, Hong Kong University and Erasmus University was described. Several different clades have evolved during the years (Clades 0, 1, 2.1- 2.5, 3, 7 and 9). In the map, the HI titres distances to seropoints were disclosed, creating like a street map distance. The relative distance corresponds to antigenicity, i.e. the closer the distance the more antigenic similarities. The map also shows the size of the antigenic cluster. It is based on ferret antisera evaluated by HI assay and checked with duck and rabbit hyper immune sera. A future plan is to establish antigenic differences based on human sera and to update the H5 map based on this data.

It is unknown why such variability of H5 strains exists and if it is vaccine-induced or selected by immune pressure or due by other factors.

H5 clades 3, 7 and 9 are considered vaccine candidates by the WHO. The vaccines so far developed are covering all known clades and new candidates are developed when new important strains are detected. If an adjuvanted vaccine would be developed with increased antigenicity, it may potentially cover alone the majority of clades.

The immune responses studied are directed against the HA global head. If the response against the HA stem or a T-cells response would be used, it would create a different map.

Avian H9N2 (G1 sg1, G1 sg2 and G1 sg3) have also been studied and strains recommended as vaccine candidates. Avian H7 have been mapped by virus, including European LPAI duck strain, prototype strain and HPAI human strain. This subtype has been relatively antigenically stable, in contrast to H5.

4.2. Circulating strains with future pandemic potential and pre-pandemic vaccination

Jacqueline Katz, Influenza Division, CDC, USA

Data on the H3N2 variant viruses that circulated during years 2009-2011 in the US were presented. Since 2009 there have been 19 human infections, of which 13 were detected since July 2011. The H3N2v possesses genes from avian, swine and human viruses but since 2011 they have acquired the M gene from the A(H1N1)pdm09 virus. The majority of infections have been detected in children <10

years old (with little or no immunity) and were mostly linked to direct/indirect pig contact with potential human-to-human transmission limited to few cases. However the virus was efficiently transmitted by droplets in ferrets. Early risk assessment prompted development of candidate vaccine virus and subsequent clinical trials.

This is one example of the importance to have in place an influenza risk assessment tool, but the risk of influenza pandemics is on a continuous basis: H5N1 is enzootic in at least 6 countries with a substantial genetic and antigenic diversity; H9N2 and H7 avian influenza subtypes have infected mammals; H1N1pdm09 is now enzootic in swine globally and genetic reassortment with other endemic swine viruses is occurring.

The influenza risk assessment tool developed by CDC enables the systematic evaluation of novel circulating influenza A viruses and includes:

- an approach for rapid risk assessment and management for emerging viruses
- the logical, transparent prioritization of counter-measures and judicious use of resources
- a framework developed by influenza experts for use by the influenza community; semi-quantitative approach
- a documented process to communicate to policy makers.

Other features are:

- it highlights information gaps and drives research to fill gaps
- it motivate sharing of information across human and animal health
- it can be easily and rapidly updated with new information
- it enables to choose viruses to prioritize ongoing pandemic preparedness work

Elements that are included in the risk assessment algorithm are i) properties of the virus (e.g. receptor binding or transmission in the lab), ii) attributes of the population (e.g. existing immunity or disease severity) and iii) ecology and epidemiology (e.g. global distribution in animals, animal species and humans infected). The different risk elements are scored as low risk (score 1-3), moderate risk (score 4-7) or high risk (score 8-10) and are weighted for each of 2 situations identified as Emergence (the risk that a novel virus possess human transmission potential) and Public health impact (in case of human transmission, what is the impact on the population based on current knowledge).

This tool can be applied to any potential pandemic virus in order to develop a rational decision approach, to prioritize the viruses at risk and if high scores are detected for one of them to develop a candidate vaccine virus.

Concerning pre-pandemic vaccination, over 85 clinical trials have been conducted with H5N1 traditional inactivated vaccines and novel recombinant strategies. In the absence of adjuvant use, H5N1 vaccines are poorly immunogenic and very high doses are needed to elicit immune responses. Importantly, H5N1 vaccines representing one clade are able to prime for immune responses elicited by subsequent boosting with vaccine of another clade. Durability of antibody response and cross-reactivity with newly diverging H5N1 viruses need however to be monitored.

With H9N2 only seven trials in adults have been conducted (WHO trials). Early studies with a whole virion G1 vaccine suggested that adults born before 1969 were primed by H2N2 experience. This was not confirmed in more recent trials with an Y280 G9 subvirion vaccine. Apart from the poor cross-reactivity between G1 and Y280 G9 lineages (regardless of the use of adjuvants), H9N2 vaccines are

more immunogenic than other avian vaccines. The reasons behind pre-existing cross-reacting anti-HA antibodies detected in unexposed population remains to be elucidated as well as the consequences of prior human influenza virus exposure on responses to H9N2 vaccines (e.g. side-by-side comparison of different products/H9 lineages).

With the H7 subtype, only two studies have been conducting demonstrating poor immunogenicity of H7 vaccines (Eurasian lineage) even at high doses and when adjuvanted with alum. Additional studies with other adjuvants are needed as well as more sensitive assays for detection of immune responses and thorough preclinical evaluation prior to clinical development.

The challenges with pre-pandemic vaccines against avian viruses include:

- Immune correlates of protection developed for human influenza viruses have been applied to avian virus vaccines but may not be appropriate;
- Appropriate animal models need to be used as a bridge to efficacy (methods to assess more complete set of immune parameters including anti-NA antibody and CMI should be developed);
- Poor immunogenicity of some avian viruses needs to be further investigated from the molecular and biological points of view;
- Assay standardization is needed to compare different vaccine strategies (there are no antibody standards for H5N1 clade 2, H7 or H9 viruses).

Thursday 31 May

5. Live attenuated vaccines / Efficacy and effectiveness studies

5.1. Live attenuated influenza vaccines: can correlates of protection be identified?

Raburn Mallory, MedImmune for EVM

The currently licensed live attenuated influenza vaccine (LAIV) is a trivalent intranasal spray (0.1 ml per nostril) that contains no preservatives and in the EU is authorised for use in children 2-<18 years of age. 50 million doses since the USA approval in 2003 have been distributed worldwide. Due to the lack of validated correlate of protection for this type of vaccine, 9 paediatric efficacy trials have been conducted, 6 placebo-controlled and 3 TIV controlled. Immunological correlates have been nevertheless evaluated during development for both humoral and cell-mediated responses.

Numerous assays and parameters such as HI and VN assays, nasal and saliva IgA, IFN- γ ELISPOPT, serum IgG, mRNA transcription and others have been evaluated over the past 20 years in an attempt to find correlates of protection for LAIV. The absolute efficacy of LAIV compared to placebo based on culture confirmed influenza in children was calculated in the range of 62-100% based on data from 6 different studies. HI responses were evaluated in 4 paediatric efficacy trials and 1 adult challenge study and did not appear to be an absolute correlate of protection, as high levels of efficacy can be seen when seroconversion rates are modest to low²⁶. HI responses are a useful strain-specific marker

²⁶ Bandell et al., *Exp Rev Vaccine* 2011

of a functional immune response and have been used to bridge from frozen to refrigerated formulation for LAIV, evaluate consistency of manufacturing process and evaluate concomitant administration of LAIV with other vaccines.

Neuraminidase inhibiting antibodies and neutralising antibodies were not investigated in LAIV efficacy trials and therefore their potential role as correlates cannot be directly determined. NAI and VN responses were evaluated in a subset of 2-5 years old children enrolled in quadrivalent LAIV studies, which showed in general similar responses to HI in that they do not appear to correlate well with the known efficacy of LAIV.

IgA responses were evaluated one month after each vaccine dose in three paediatric placebo-controlled efficacy studies on subjects 6 to <36 months of age. The responses were compared in vaccine recipients vs. placebo recipients and in subjects with or without influenza. Ratios of strain-specific IgA to total IgA were consistently higher in vaccine recipients without influenza disease suggesting that IgA may contribute to efficacy of LAIV and can provide evidence of vaccine-induced immunity. However the heterogeneity in nasal antibody levels and variability in nasal specimen collection hinders any further evaluation of mucosal responses.

IFN- γ ELISPOT responses were evaluated in one paediatric placebo-controlled efficacy study (1836 subjects 6- <36 months of age) and showed that the majority of children with >100 spot-forming cells/ 10^6 peripheral blood mononuclear cells were protected against influenza, but, since only small percentage (<10%) of subjects reached 100 spot forming cells, other factors might contribute to the overall LAIV efficacy.

In summary, of all the immune responses evaluated, none could be definitely identified as CoP and it actually appears that different mechanisms contribute to LAIV efficacy. Immune responses evoked by LAIV are indeed likely to mirror the broad immune responses induced by a wild-type influenza infection.

5.2. Live attenuated influenza vaccines: potential for use during pandemics

Kanta Subbarao, NIAID, NIH, USA

There are several potential advantages to the use of LAIVs during a pandemic, including the mounting of an immune response following a single dose, the induction of an innate antiviral state shortly after vaccination as well as serum/mucosal and T cell responses, the induction of a broader cross-protection to drift variants vs. TIV, the possibility to produce a larger number of doses per egg and the administration in a rapid and needle-free fashion, which is well suited to mass vaccination in community settings. The generation of the 2009 H1N1 live attenuated influenza virus was at first unsuccessful, due to genetic sequence variations in the HA. Also, the yield in eggs of the rescued viruses was 10-fold lower compared to seasonal LAIV. Following the introduction of targeted mutations in the HA gene, a mutant virus with changes at residues 119E and 186D met the required replication, plaque size, antigenicity and immunogenicity criteria. This virus was thus selected to manufacture the monovalent 2009 H1N1 vaccine using the same process and technology as the licensed trivalent LAIV. Two safety and immunogenicity studies with a 2 dose vaccination schedule were performed in children (2-17 years of age) and adults (18-49 years) and showed side effects and immune responses similar to seasonal LAIVs. Serum immune responses measured by HI antibodies are modest compared to levels achieved by inactivated vaccines; however levels of these antibodies have not been shown to correlate well with protection against influenza for LAIVs. In addition antibody levels seen in these studies are consistent with those reported in other clinical studies of LAIV in which clinically significant protection

against influenza illness has been demonstrated²⁷. Two effectiveness studies were also conducted, the larger of which showed 81.9% (95% CI: 13.6, 96.2) adjusted vaccine effectiveness in children 2-9 years old and 26.4% (95% CI: -91.3, 71.7) in 10-49 years old at 7 days post-vaccination²⁸. The other study showed after a single dose an effectiveness of -3% (95% CI: <0, 75) in 7-35 months old, and 82% ((% CI: 0, 100) in 3-9 years old²⁹. In this study, following analysis by vaccine type, the inactivated vaccine showed an effectiveness of 66% (95% CI: <0, 99%) in 3-9 years old, whilst the LAIV was 100% (95% CI: <0, 100) effective in children 2-9 years old.

In summary, there are several potential advantages for the use of LAIV in a pandemic, including earlier availability vs. inactivated vaccines, good tolerability and safety/immunogenicity profile similar to seasonal LAIVs (although as already known, serum HI responses did not correlate well with protection). The 2009 pH1N1 LAIV was effective despite the difficult evaluation because of the timing of vaccination with respect to the 2nd wave of the pandemic.

5.3. Issues with clinical efficacy studies from recent experience: endpoints for efficacy, optimisation of study design, extrapolation of study results, e.g. among strains.

Michel Stoffel, GSK for EVM

Influenza efficacy trials are challenged by several uncertainties and issues, including:

1. Incidence of the disease (or attack rate): it can change according to age group being extremely low for certain populations (e.g. the elderly), it can vary from season to season and by geographic location within the same season. Because the flu exposure is so unpredictable, the accuracy of the sample size calculation is difficult to predict as well.
2. Match between vaccine and circulating strains: it changes from season to season and the degree of matching affects the estimates of vaccine efficacy. Therefore demonstration of efficacy against each strain contained in the vaccine is extremely challenging. The highest degree of mismatch is observed for the B strain.
3. Control group: studies in certain age groups, e.g. the elderly, require an active influenza vaccine comparator that results in large sample sizes, consequently affecting costs and feasibility. In addition without knowing the absolute level of efficacy of the control group (variable between strains and populations) it is impossible to assess the absolute level of efficacy of the candidate vaccine.
4. Endpoints and methodology for case ascertainment:
 - there is no clear/standardised clinical definition of influenza as the CDC-ILI definition currently used may be too insensitive for certain age groups (e.g. it may be relevant for 20-25 year-olds but not similarly for the elderly where the most relevant outcomes are complications from influenza infection, e.g. hospitalisation, mortality etc...);
 - viral culture confirmation has limited sensitivity and, although RT-PCR is a valuable alternative, validated methods are not widely available;

²⁷ Mallory et al., *PLoS One* 2010

²⁸ Griffin et al., *PLoS One* 2011

²⁹ Hadler et al., *J Inf Dis* 2012

- the uncertainty regarding the translation of relative vaccine efficacy to absolute vaccine efficacy confounds the assessment of meaningful benefit when the value of active control is poorly established;
 - the requirements of the regulatory agencies do not necessarily match the requirements from recommending/pricing bodies. Also, designing efficacy trials using 'public health' endpoints increases the complexity and affects feasibility.
5. The cost of the clinical development vs. the cost of the vaccine once approved for use: designing an efficacy trial with complications from influenza infection as primary endpoint requires around 100,000 subjects³⁰. Such a trial represents a significant undertaking and investment whilst the success of the study remains unpredictable due to extrinsic factors (attack rate or strain mismatch) and the sale price of influenza vaccines remains low. The cost and complexity of conducting influenza efficacy trials was illustrated by two trials, of which one was a GSK study in 44,000 elderly with an investigational vaccine vs. Fluarix as comparator, conducted in 15 countries and around 300 centres for a cost of 193 million Euros. A total cost to develop the investigational vaccine for the elderly and for children was estimated at 320 million Euros. The other example was an ongoing Sanofi Pasteur study of Fluzone-high-dose vs. Fluzone-regular-dose as comparator, involving 30,000 elderly subjects over 2 seasons, in around 150 study sites. The primary endpoint was prevention of laboratory influenza illness.

In conclusion careful consideration should be given to the expectations related to efficacy/effectiveness/safety requirements in different populations also in relation to operational feasibility. Moreover from the industry perspective a balance is needed among expected requirements, anticipated investments and public health needs. There is also an increasing need to have a more flexible approach including post-licensure effectiveness approach and flexible pricing (e.g. adaptive licensing). Joint discussions between regulators, recommending/pricing bodies and companies will be key prior to initiating any new developments.

5.4. Discussion slot on clinical efficacy studies

The following questions were posed to the experts during the discussion slot:

- 1. Considering the limited evidence of efficacy in elderly, can the demonstration of non-inferiority based upon immunogenicity endpoints be sufficient or should comparative efficacy trials be considered?**

Based on the increasing doubts on the estimated efficacy of current TIVs, it was considered that immunogenicity studies in the elderly population may present weaknesses. Placebo-controlled trials for the elderly are however not considered possible or ethical but such trials could be conducted in children in Europe where most countries do not have a general recommendation for vaccination. In spite of the difficulties, efficacy trials were considered anyway the best option available.

- 2. What are the views on the design of clinical efficacy studies in elderly having as primary endpoint a composite endpoint which consists of "Influenza related mortality, pneumonia, influenza related hospitalisation AND lab confirmed ILI"?**

Composite end-points were not considered better than well-defined separate end-points, e.g. hospitalisation for pneumonia. Rare endpoints that would require an unrealistic number of subjects

³⁰ Nichol KL et al., Influenza vaccination and reduction in hospitalizations for cardiac disease and stroke among the elderly, *N Engl J Med* 2003

to power the study should be analysed in the post-marketing studies or in a mixed approach. Effectiveness was advocated instead of efficacy.

3. Is demonstration of clinical efficacy needed for each of the strains included in a seasonal vaccine formulation?

The demonstration of clinical efficacy for each strain was not considered possible or necessary.

5.5. The role of effectiveness studies in seasonal and pandemic vaccination: an update on the I-MOVE experience

Bruno C. Ciancio, ECDC, Stockholm SE

The effectiveness of influenza vaccines (VE) cannot be assumed because the virus is constantly evolving due to antigenic drift but the relation between drift and VE is not completely defined, as high VE was observed in years where drift was reported and low VE was observed in years where supposedly good strain match was achieved. Moreover VE differs by age and study outcome, with the additional issue that optimal correlates of protection are lacking. Monitoring of influenza VE is a public health priority for many reasons, including i) maintaining public and provider confidence in vaccination programmes, ii) allowing for rapid detection of low VE which in turns allows to recommend alternative measures (e.g. antivirals) and to contribute to the vaccine strain selection process, iii) supporting post-marketing evaluation for regulatory purposes, iv) having a system ready to measure pandemic VE and v) triggering research on more effective and cost-effective vaccines.

Based on published meta-analyses there is not much data on VE from randomised controlled trials (RCT) and from good quality observational studies. Furthermore, high quality observational studies yielded very similar VE estimates than RCT, although VE in target age groups was not impressive³¹.

The I-MOVE consortium for influenza VE monitoring in Europe was planned and built up during the years 2007-8 and developed in phases afterwards with the current network encountering 17 member states and 26 institutes. During the planning phase, methods to measure VE were identified (e.g. based on literature review, VE survey among EU member states, experts workshops on VE studies) and recommendations on study design were defined, including which variables to control for positive and negative confounders and the preferential use of laboratory-confirmed outcome (influenza VE generic study protocols are published on the ECDC website). VE studies are performed in 15 sites within the network and follow predefined methods. Eight sites contribute to a multicenter case-control study, and the others sites perform cohorts studies based on primary care registers and other population-based datasets. In addition a number of sites perform the screening method. The I-MOVE cohort studies are based on large electronic GP databases in England, Scotland and Navarra; the outcomes are ILI, all respiratory illness, hospitalization and death, and the data are adjusted for confounding factors.

The analysis for the 2011/12 season from Navarra showed an adjusted VE of 38% in primary health care patients. Adjusted results from the multicentre case control sentinel surveillance system for 2 seasonal vaccinations (2008/09 and 2010/11) showed a 55-60% VE. In contrast, the season 2011/12 showed an adjusted VE of 10%, with 3% in 0-14 year-olds, 69% in 15-59 year-olds and 14% in >60 year-olds.

The advantages of the I-MOVE approach include cost-effectiveness, a robust methodology, excellent data quality, timely provisions of results, strong support from the member states network and independency. Current challenges faced by the I-Move consortium are i) the need to increase sample

³¹ Osterholm et al., *The Lancet* 2011

size (especially at the beginning of the season) in order to increase the timeliness of estimates and the ability to stratify results by age, vaccine type, etc., ii) how to formally contribute to the WHO strain selection process and to the regulatory process by providing product specific estimates, iii) to obtain VE data against severe influenza outcomes and iv) long term financial sustainability.

In conclusion:

- VE should be monitored routinely until more effective, universal vaccines will become available;
- monitoring systems should be simple, timely, robust and cost-effective;
- a strong EU network coordinated by the ECDC is in place but the sustainability of this activity is at risk due to financial constraints;
- joining efforts from public health and regulatory agencies to meet reciprocal needs would be a convenient option.

During the discussion that followed this presentation, the possible causes for the low protective effectiveness of the 2011/12 vaccine, especially among young and older age groups, were discussed. VE decreased over time and this was consistent with data from virological surveillance showing virus changes towards the end of the season. However this was also consistent with a rapid waning immunity. Based on the available data it was not possible to differentiate the contributions of antigenic drift, waning immunity or suboptimal strain selection as the root cause. It was pointed out that for any vaccine in general such low effectiveness would be considered problematic.

In conclusion the value of the I-MOVE consortium was recognized and furthermore, following the question raised by EMA whether effectiveness data could be generated to replace seasonal immunogenicity trials, it was stated that with additional funding the sample size of I-MOVE studies could be increased to generate product-specific data. EVM is supportive of such initiative too.

6. Development of novel influenza vaccines - a research update

6.1. Universal influenza vaccines: facts or fiction?

Albert Osterhaus, Erasmus University NL

Human influenza can be differentiated in three appearances, all of which have different aetiologies but are strongly interrelated: seasonal, avian and pandemic influenza. Looking at recent zoonotic transmissions, in the past 15 years 8 known events have been reported whereby different influenza A viruses jumped across species infecting humans (H7N7, H5N1, H9N2, H7N2 and H7N3). The last 4 pandemics have been the 1918 H1N1 'Spanish Flu', the 1957 H2N2 'Asian Flu', the 1968 H3N2 'Hong-Kong Flu' and the 2009 H1N1 'Mexican Flu', i.e. they were all caused by other strains with the Spanish Flu showing the highest mortality rate. Pandemic preparedness activities include enhanced human and animal surveillance, preventive and therapeutic antiviral therapy (but the development of resistance is a risk) and vaccination, the cornerstone of prevention. For example that one dose of vaccine comes from 1 egg based on current manufacturing processes shows how ample the room for improvement is, however only small changes are being proposed today as they are easy to incorporate into the existing systems. Therefore challenges for the future include the discovery of new antigenic targets for vaccines, the identification of true correlates of protection, improved vaccine efficacy, new adjuvants and delivery systems. Improvements in production systems can be made but larger changes are urged

and might need a long path for implementation, e.g. seasonal vaccination enhances pandemic preparedness but universal vaccines are regarded as a better countermeasure. Some authors³² describe an epitope shared by the haemagglutinins of H1, H2, H5, H6 and H9 subtypes of influenza A viruses. This epitope could be used to design universal vaccines, but such strategy should be further investigated. New classes of monoclonal antibodies that shows prophylactic and therapeutic efficacy in ferrets have been identified³³ and could be used in humans to protect against or treat severe influenza. Identification of new targets is underway but still concerning the use of M2 protein the data produced is conflicting and concerning NA this could be a very good candidate because drift occurs to a lesser extent and there are only 9 types of NA. The role of cellular immunity in protection is key but more investigation should be carried out towards the identification of targets (e.g. conserved internal proteins and epitopes) and modes of antigen delivery (e.g. vectors, LAIV, DNA vaccines etc.). How to obtain cross-protective immunity between virus subtypes and whether or not clinical protection is more desirable vs. sterile immunity are still open questions. Anamnestic cross-reactive CTL responses have been shown to correlate with protection against infection with homotypic and heterotypic A viruses in mice³⁴. Human CTL specific for seasonal flu viruses are highly cross-reactive with H5N1 viruses³⁵, which fact posing the basis for the use of induction of CTL as target in the development of universal vaccines. Concerning the role of adjuvants, the most important candidates for pandemic influenza vaccines are aluminium salts and oil-in-water emulsions (MF59, AS03 etc.) as well as novel approaches, like virosomes and ISCOMs. Some authors³⁶ showed a reduction in virus replication in monkeys following MVA-HA vaccination, which interestingly induced a CD8 rather than a CD4 response. Definitely the use of adjuvants allows for an important antigen sparing and for broader cross-protection (e.g. valuable against heterologous challenge), which may not be obtained with classical unadjuvanted vaccines. However adjuvants do not provide the whole answer to the needs for improvement. In conclusion:

- several novel generations of influenza vaccines are being developed (adjuvanted, DNA-based, LAIV, vectored, VLP-based, protein/peptides-based etc.);
- novel correlates of protection should be defined (broadly reactive antibodies and T cells);
- novel adjuvants have led to flu vaccine candidates against seasonal, avian and pandemic flu that are broadly reactive and antigen-sparing;
- vectored vaccines are a promising next generation influenza vaccine candidate (e.g. MVA or adenovirus-based);
- LAIV is used for seasonal vaccination but are a promising more universal vaccine candidate.

6.2. Alternative adjuvanting systems

Rebecca Jane Cox, University of Bergen, Bergen NO

Adjuvants enhance humoral and cell-mediated responses, induce broader and longer lasting immunity, allow for dose sparing and reduce the number of vaccine doses required. Novel adjuvanting systems include oil-in-water emulsions squalene-based (MF59, AF03 and Squalene) or squalene/tocopherol-based (AS03), polyoxidonium (poly-electrolyte), virosomal vaccines (virus like particle-liposomes), Toll-like receptor (TLR) agonists (TLR4-agonist like rHA Glucopyranosil Lipid A or GLA-SE and

³² Smirnov et al., *Acta Virol* 1999, and Friesen et al., *PLoS One* 2010

³³ Friesen et al., *PLoS One* 2010

³⁴ Kreijtz et al., *Vaccine* 2009

³⁵ Bodewes et al., *PLoS One* 2009 and *Lancet* 2010

³⁶ Kreijtz et al., *J. Inf. Dis.* 2008

Covaccine; TLR5-agonist like flagella HA fusion protein, and TLR9-agonist like M2e NP ImmunoStimulatory Sequence or ISS C295 and IC31) and immunostimulating complex (ISCOM-Matrix, Matrix M).

ISCOMs or Immune Stimulating Complexes consist of spherical open cage-like structures of 40 nm in diameter for antigen presentation that spontaneously form when mixing together cholesterol, phospholipids and *Quillaia* saponins, which are well known for their ability to activate the immune system. The 1st and 2nd generation of ISCOM adjuvants have been extensively tested in man and induce good humoral and T cell responses at low antigen doses. The 3rd generation (Matrix M) shows additionally an improved safety profile.

Matrix M adjuvanted virosomal H5N1 vaccine for intramuscular administration was tested in humans in a phase I clinical trial, which involved 60 healthy adults split into 4 groups with a 2 dose vaccination schedule of respectively 30, 7.5, 1.5µg HA adjuvanted with 50µg Matrix M and 30µg HA without adjuvant. The vaccine was able to induce homologous antibody against the RG14 strain; the 3 CHMP criteria were met only with the adjuvanted formulation and only after the second dose irrespective of the amount of HA included (although higher HI titres were achieved by the 30µg dose). In addition, the vaccine induced cross-clade antibodies against A/Indonesia/5/05 or A/turkey/Turkey/1/05 with none, one or two of the CHMP criteria met based on the amount of HA. The kinetics of immune response were thus influenced by the vaccine dose, with antibodies and CD4+ Th1-cell responses showing a dose-dependent induction. Interestingly Matrix M was also able to increase the frequency of CD4 cells as well as the magnitude of immune responses but did not change the quality of the CD4+ Th1 response. Only low CD4 responses were detected in the group vaccinated with the virosomal vaccine alone. It was additionally shown that a 4-fold increase in CD4+ Th1 cells could be used as early predictor of antibody responses (measured by HI/SRH or VN).

In conclusion:

- novel adjuvants should undergo a thorough evaluation of humoral and cellular responses, including kinetics of response, in all age groups, including paediatric and elderly populations;
- the immune response is multifaceted and thus potential correlates of protection may vary with vaccine formulation.

6.3. Alternative adjuvanting systems: Industry views

Giuseppe Del Giudice, Novartis for EVM

A vaccine is composed of flu antigens, which determine the specificity of the immune response, and adjuvants, which are designed to enhance and module the immune response. In a vaccine formulation the antigen need to be compatible with the adjuvant and the vaccine should be evaluated as a combination of the antigen and the adjuvant.

Desirable characteristics of adjuvanted influenza vaccines include the induction of a broad immune response/protection, an enhanced immune response in immunologically naïve (children) and immunologically frail individuals (elderly), a persistent immune response and an acceptable safety and reactogenicity profile, for what seasonal vaccines are concerned. For pandemic influenza vaccines, the most desirable features include high immunogenicity at low dose, immunogenicity regardless of priming status, possibility to cover a broad age range (from paediatrics to elderly) and high production capacity for rapid worldwide supply delivery.

Most vaccines are adjuvanted; some being self-adjuvanted (e.g. killed whole virion vaccine) whilst others (e.g. subunit and purified antigens) are aluminium adjuvanted or adjuvanted with “new

adjuvants". Most of the data available so far have been obtained with oil-in-water emulsions type of new adjuvants (MF59, AS03 and AF03), which are all squalene based. Squalene is naturally produced by the human body, it does not induce antibody responses after vaccination and it has no adjuvant activity by itself but adjuvant activity is provided by the emulsion. Enhanced immunogenicity results have been shown with oil-in water emulsion in young children³⁷.

The use of alternative adjuvants for influenza vaccines requires extensive work and large databases for registration. Therefore despite many adjuvants are tested in preclinical settings, very few reach the clinical stage and a handful of them have been licensed for human use, all of which belong to the family of oil-in-water emulsions.

TLR agonists are the most advanced today as potential alternative. All TLRs are targets for vaccines adjuvants in pre-clinical models. TLR4 agonist MPL is approved as AS04 in the EU (HBV and HPV vaccines) and USA. Others (GPA, IRID) are in phase I trial. TLR5 agonist Flagellin for flu and TLR9 agonists CpG and CpG-like oligonucleotides (IC31) for anthrax, melanoma and HBV vaccines are in clinical trials. However, results with TLR agonists in flu vaccines are limited. So far poor results have been obtained with CpG and seasonal vaccines³⁸. Good immunogenicity results have been shown with seasonal HA and m2e linked to bacterial Flagellin; however severe symptoms were reported with higher doses of M2e-based vaccine³⁹. No benefit could be demonstrated with emulsion combined with MPL in the context of seasonal vaccine. There are also many challenges associated with the use of TLR agonists for seasonal and pandemic flu, including the added complexity in combination with antigens which needs testing, the limited production capacity for naturally derived TLR4 agonist (stockpiling necessary) and need for new dose range finding for both antigen and adjuvant in a naïve/unprimed population.

In conclusion, adjuvanted vaccines remain an important asset in pandemic preparedness and the best results so far have been obtained with oil-in-water adjuvants. TLR agonists are the most advanced alternative to oil-in-water emulsions but are still in early development and present many challenges. Adjuvanted vaccines remain an important option also for seasonal vaccine improvement, mainly in immunologically naïve (children) and immunologically frail (elderly) subjects who need improved vaccines the most. Many novel adjuvants are being tested to improve seasonal vaccines, including TLR agonists. As compared to oil-in-water adjuvants, the clinical data available so far with novel adjuvants are however very limited both in terms of safety and of immunogenicity in various age groups.

7. Serological assays for measuring immunogenicity – conclusions based on current knowledge

7.1. Summary of the laboratory group conclusions (Seroepidemiology meeting, Stockholm, Dec 2011)

Othmar Engelhardt, NIBSC, UK

This presentation summarised the main conclusions on assay-specific issues as drawn during a recent sero-epidemiology meeting⁴⁰ held on 1-2 December 2011 at the European Centre for Disease Prevention and Control (ECDC) in Stockholm:

- Hemagglutination inhibition (HI) assay as primary serological assay for the time being;

³⁷ Vesikari et al., *Pediatr Infect Dis J* 2009

³⁸ Cooper et al., *Vaccine* 2004

³⁹ Turley et al., *Vaccine* 2011

⁴⁰ for further information follow this [link](#)

- Further evaluation of VN assay to be achieved by a global laboratory network. Comparison of different assay protocols, i.e. 2 day versus 3 day assay using different experimental read-out systems (ELISA versus hemagglutination titre);
- Examine assays for detection of neuraminidase (NA) specific antibodies;
- EU EDQM and NIBSC would be asked to provide support on the coordination of proficiency studies and the provision of serological standards, respectively.

The discussion centred on individual aspects of the VN methodology. Specifically questions on the length of the incubation period (up to seven days) and on the suitability of MDCK cells were raised. The majority of experts agreed that the envisaged incubation periods (18 hours and 3 days) are well-chosen to detect potential impacts of different assay protocols and that MDCK cells have a long-standing and proven susceptibility to efficient virus propagation that renders them highly applicable as VN substrate. Mimicry of the real influenza disease by using more authentic cell substrates (such as human airway epithelial cells) as proposed by one expert was not considered to be the primary goal (if at all possible) for such analytical laboratory assay format performed in a 96-well plate. Hence the use of MDCK as substrate was deemed acceptable for the given purpose.

7.2. Achieving the standardization of serological tests for influenza vaccines

1. EMA sera retesting exercise - Ralf Wagner, PEI, Langen DE

This presentation briefly summarised the results of the “EMA Re-testing exercise”⁴¹. During this study a large number of serum samples from A(H1N1)pdm09 clinical trials conducted by vaccine manufacturers was re-tested by two designated central laboratories, NIBSC (HI) and PEI (VN). Substantial differences were observed between HI and VN titres measured by manufacturers’ laboratories and those determined by the central laboratories. These differences could however be markedly reduced when the original titres were adjusted in relation to the International Standard preparation IS09/194. Inclusion of a suitable standard serum is therefore considered one practical option to considerably improve inter-laboratory reproducibility.

2. Technical proficiency monitoring, use of standardized protocols and reagents, use of antibody standards - Jim Robertson, NIBSC UK

This presentation focused on several important aspects of standardisation such as technical proficiency monitoring, standardised protocols, reagents and serum standards. A range of studies conducted in the last 20 years to evaluate HI, SRH and VN inter-laboratory variability was discussed; extensive inter-laboratory variability was evident in all of these studies, and it was highest for VN titres. The findings of collaborative studies undertaken for the assignment of consensus titres for international standard serum preparations were reported as well as the improvements achieved in assay reproducibility when applying these standards for the adjustment of original titres. Some limitations on the use of serum standards (such as clade specificity of human sera and the inappropriateness of certain animal sera) were also highlighted.

The presentation was followed by an extensive discussion on specific issues of assay standardisation. The point was raised whether it would be preferable to diligently identify the inherent sources of variability rather than to apply standards for adjustment of raw data. It appeared that it is virtually impossible to definitively and completely identify the actual sources of variability and the small differences between individual laboratories that can lead to the observed fluctuation in results. It was

⁴¹ Wagner et al., *Expert Rev Vaccines* 2012

noted that even experimental approaches that were designed to achieve maximal harmonisation of procedures and materials often failed to improve reproducibility (such as a recent survey conducted by EDQM for HI and SRH assay – study BSP063). Up to now only one important source of variability was identified, that is the origin and quality of red blood cells (RBC) used in the HI and SRH assays. Therefore it is not too surprising that both these assays showed roughly the same degree of variability in the EDQM study⁴².

EVM suggested that specialised laboratories would require a common leadership in order to efficiently implement measures for improvement of assay reproducibility, which for example could be taken over by EDQM or NIBSC. In this respect having in place SOPs and standardised protocols was considered insufficient to actively promote assay standardisation, but additional activities such as offering lab-training or providing key materials would also play a role. However, an EDQM representative reminded the expert group that the benefit gained by offering training and materials can be rather limited as observed during recent collaborative studies such as BSP063.

Another question was raised regarding the applicability of lentiviral pseudotype particles for use in VN. This was seen as a potential option to accomplish a higher degree of reproducibility, since these particles represent a uniform viral-like platform which would allow for a more standardised quantifiable experimental read-out. However, currently this technology was considered not sufficiently advanced to propose regulatory requirements and additional developmental work will need to be performed before official implementation can be considered.

It was also pointed out that – although no correlate of protection has been validated for VN – it would nevertheless be very important to determine the pre-vaccination immunity (baseline titres) in subjects as accurately as possible. If baseline immunity is not measured correctly for all age groups, vaccine efficacy may be overestimated. Likewise, consistent determination of VN titres could also improve comparability of immunogenicity across different vaccines and vaccination schemes.

In an attempt to consolidate the partially divergent views expressed in the discussion, it was suggested to consider the HI test as the primary assay for the time being, but that the VN assay is quite important and relevant in order to capture functionally active, infection-blocking antibodies. For this assay there is an urgent need to develop a common protocol to begin with. This proposed strategy was agreeable by most of the experts; hence it could be considered a pragmatic approach to proceed with activities on assay improvement in the near future.

3. Industry views - Martine Denis, Sanofi Pasteur for EVM

A comprehensive overview of the existing assays for the detection of anti-HA (HI, SRH, ELISA) and anti-NA antibodies (ELLA) or both (VN) was presented, including the standardisation status of these assays and requirements for the future (reference standard for each new strain, quality controls including different titres for assay control and trending, standard protocols and proven correlates of protection). The major standardisation challenges include the fact that reference standards need to be provided each season for each strain and that standard protocols need to be independent of strain change.

In conclusion, the VN assay appears to be a desired assay for future evaluations of influenza vaccines, particularly in younger populations. Correlation and concordance between HI and VN assays are needed to explore the potential additional value of VN over HI, which additionally would have to be conducted for different strains in different age groups. If the VN assay becomes the reference assay, CoPs will have to be defined with it. It was recommended to have a reference laboratory distributing

⁴² Wood JM et al., *Pharmeur Bio Sci Notes* 2011

'calibrated' sera (e.g. a panel of sera measured by the reference lab) with any manufacturer having to show that its assay falls under the defined specification of these sera.

Overall industry was in agreement with independent experts, in particular regarding the high relevance of VN assay for the comprehensive determination of immunogenicity and proposals/recommendations on provision of standard sera and involvement of specialised laboratories.

4. Use of a reference laboratory for serological issues - Othmar Engelhardt, NIBSC UK

This presentation concerned the potential implementation of a reference laboratory for serological issues. The aim of this presentation was not to propose concrete solutions but to describe different potential scenarios for discussion. In scenario 1 (a) central reference lab(s) would do the entire serology testing for all manufacturers so that manufacturers would not test sera themselves but submit their samples to the reference lab(s). This would clearly improve comparability of results but would pose considerable challenges regarding logistics, funding, assignment of central lab(s) and also legal issues (formal responsibility for test results). Scenario 2 resembles the approach taken during the EMA re-testing exercise (see above), whereby central lab(s) would conduct re-testing of a selected subset of samples from manufacturer's clinical trials whereas all of the remaining samples would still be analysed by the manufacturers themselves. This scenario leaves the responsibility of analysis to companies and would also limit the workload for the central lab(s).

A 3rd scenario that could be applied at least for an interim period of time until either scenario 1 or 2 would be implemented was suggested during the discussion. This would involve the testing of standard serum preparations in proficiency studies in order to examine assay performance in different laboratories. Further, the panel of sera tested in proficiency studies could also be used to calibrate tests in individual laboratories. With regard to the rapid generation of suitable standard sera, it was pointed out that all standard sera successfully applied to improve assay reproducibility had been of human origin. More investigation is urgently needed to determine whether sera from certain laboratory animal species would also be suitable for this purpose.

5. Discussion on standardization issues

The difficulty in implementing any of the proposed scenarios for the annual clinical trials was stressed; no clear pre-defined operational pathway is currently foreseen for seasonal influenza vaccines and specific, practically applicable activities would need to be developed. Some participants argued that given the presently existing limited laboratory resources the general implementation of a central lab re-testing strategy appeared unfeasible. This approach might only work under special conditions, for instance for novel vaccines, when triggered by special observations or in cases where specific additional information is required for defined purposes. This approach could be integrated with future activities in the context of clinical efficacy trials and the determination or re-assessment of serological correlates of protection. The idea of central reference laboratories was questioned by some due to the current debate on the optimal assay protocol for VN.

It was suggested that a (partial) solution to these discrepant positions could be the commitment of the international sero-epidemiology group (also known as CONSIDE – the Consortium for the Standardization of Influenza Seroepidemiology, see above) to address not only the standard but also other VN assay formats. Hence, more work is needed to investigate VN assay performance when conducted following different experimental protocols. No such investigations related to procedural aspects are required for the HI assay. For the latter, availability of suitable standard sera and certain reagents would at present be the prime practical option for improvement of reproducibility. The involvement of WHO in the implementation and efficient distribution of standard sera was suggested.

7.3. Alternative anti-HA assays

Jackie Katz, Influenza Division, CDC, USA

This was an overview of progress towards alternative and new assays for the detection of anti-HA antibody responses. Preliminary data obtained at CDC using 'surrogate' red blood cells (RBCs) were shared. These artificial RBCs consist of beads coated with synthetic sialyl-glycans; they can be used in HA and HI assays. However, the data are preliminary and more work is required before these surrogate RBCs could be used more widely. Other platforms that can detect and quantify antibodies directed against the HA, such as the Fortebio platform, Luminex Multiple Analyte Profiling (xMAP) and the Chembio Dual Path platform, were presented. All of these make use of recombinant HA or HA1 domain. In addition, the pseudotype virus neutralisation assay that employs a replication-deficient retroviral vector system expressing influenza virus HA was also described.

During the discussion, specificity and general applicability across influenza virus subtypes of the synthetic HI assay were raised as important questions. It was confirmed that the ideal synthetic HI assay would be a generic assay that would not need to be adapted to every new (drifted or pandemic) strain of influenza virus.

7.4. Detection of NA-specific antibodies in sera from vaccinated humans and animals

Ralf Wagner (PEI) and Mark Galinski (Medimmune for EVM)

The results obtained at PEI with various assays for anti-NA antibodies were presented, including a ferret immunisation study that involved the use of viruses containing various combinations of HA and NA subtypes in order to detect the contribution of anti-NA antibodies to the immune response. It was concluded that the microneutralisation assay did not efficiently detect anti-NA antibodies. In contrast, an assay using fetuin as substrate and detecting soluble sialic acid released by the neuraminidase activity (following a protocol described by WHO) detected the presence of anti-NA antibodies in sera of ferrets from the same study, as well as in human sera. Western Blot assays were also described as means of detecting antibodies against NA.

EMV presented additional data on the detection of anti-NA antibodies. Formats of available assays were described and the implementation of an ELLA (enzyme-linked lectin-binding) assay for the testing of sera from subjects (2-5 years old children) vaccinated with live-attenuated influenza vaccine (LAIV) was reported. In this study, responses were generally similar to HI response and correlation between anti-NA antibody responses and vaccine efficacy was poor. Some of the problems and complexity associated with the use of anti-NA assays in post-vaccination serological analysis were described, such as the variability in NA protein content between vaccines, the lack of standardised methods for detecting anti-NA immune responses and the lack of knowledge about correlation between NI assays and HI or VN assays.

7.5. Assays to measure CMI

Stefan Kostense, Crucell for EVM

This was an overview of assay methodologies to measure cell mediated immunity (CMI) to influenza virus. The unresolved issues with regard to measuring CMI were listed: it is as yet unclear which assay(s), influenza virus antigens or cytokines should be used; other outstanding questions regard the quality control of CMI assays, in particular due to the frequent change of viruses. However, a proposal was made towards CMI assays implementation: a T cell assay using one selected antigen and detecting

a single cytokine would be defined and validated as a standard read-out of CMI, serving as a secondary endpoint. In parallel, various other assays, antigens and cytokines would be explored with regard to potential new correlates of protection (CoP) (exploratory endpoint); if and when a new CoP would be identified, the corresponding CMI assay would be incorporated into studies as the primary endpoint. Other points discussed in this presentation were sample integrity and validation strategies.

It was acknowledged that the topic of CMI requires more discussion and investigation.

8. Concluding remarks

The meeting concluded with the chairmen's remarks. It was stated that much work remains to be done in influenza serology, but that priorities should be set without delay. The first priority would be the standardisation of HI and neutralisation assay formats; the investigation of new targets should also be a priority. The most important question to be answered in the future is on improved and clinically validated correlates of protection. It was mentioned that existing influenza guidelines are under revision and that this exercise was delineating an extensive research agenda for influenza vaccines.

In conclusion, many questions remain unanswered and thus further investigation and discussion on influenza serology and correlates of protection are urgently required.