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Review

Association between vaccine adjuvant effect and pre-seasonal immunity. Systematic review and meta-analysis of randomised immunogenicity trials comparing squalene-adjuvanted and aqueous inactivated influenza vaccines

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ABSTRACT

The immunogenicity benefit of inactivated influenza vaccine (IIV) adjuvanted by squalene over non-adjuvanted aqueous IIV was explored in a meta-analysis involving 49 randomised trials published between 1999 and 2017, and 22,470 eligible persons of all age classes. Most vaccines contained 15 μg viral haemagglutinin per strain. Adjuvanted IIV mostly contained 9.75 mg squalene per dose. Homologous pre- and post-vaccination geometric mean titres (GMTs) of haemagglutination-inhibition (HI) antibody were recorded for 290 single influenza (sub-)type arms. The adjuvant effect was expressed as the ratio of post-vaccination GMTs between squalene-IIV and aqueous IIV (GMTR, 145 estimates). GMTRs > 1.0 favoured squalene-IIV over aqueous IIV. For all influenza (sub-)types, the adjuvant effect proved negatively associated with pre-vaccination GMT and mean age. The adjuvant effect appeared most pronounced in young children (mean age < 2.5 years) showing an average GMTR of 3.7 (95% CI: 2.5 to 5.5). With increasing age, GMTR values gradually decreased towards 1.4 (95% CI: 1.0 to 1.9) in older adults. Heterologous antibody titrations simulating mismatch between vaccine and circulating virus (30 GMTR estimates) again showed a larger adjuvant effect at young age. GMT values and their variances were converted to antibody-predicted protection rates using an evidence-based clinical protection curve. The adjuvant effect was expressed as the protection rate differences, which showed similar age patterns as corresponding GMTR values. However for influenza B, the adjuvant effect lasted longer than for influenza A, possibly due to a generally later influenza B virus exposure. Collectively, this meta-analysis indicates the highest benefit of squalene-IIV over aqueous IIV in young children and decreasing benefit with progressing age. This trend is similar for seasonal influenza (sub-)types and the 2009 pandemic strain, by both homologous and heterologous titration. The impact of pre-seasonal immunity on vaccine effectiveness, and its implications for age-specific vaccination recommendations, are discussed.

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1. Introduction

Inactivated influenza vaccine (IIV) can be formulated as aqueous suspension or as emulsion. MF59® is an oil-in-water emulsion developed in the 1990ies [1]. Its oily constituent is squalene [2], a non-toxic and metabolisable lipid, naturally produced in liver cells as intermediate in cholesterol synthesis. Squalene in water, when processed under high pressure and stabilised by surfactants (polysorbate 80 and sorbitan trioleate), forms small oil droplets (~160 nm). The emulsion modulates cell- and cytokine-driven immune pathways at the injection site and local lymph nodes eventually leading to the induction of T-cell dependent, specific anti-HA antibodies against the vaccine antigens [3].

A meta-analysis that we published previously [4], described the immunogenicity and safety of IIV for intramuscular or subcutaneous application, as assessed in 33 eligible randomised controlled trials (RCTs) published between 1978 and 2009. Main immunogenic variables were the homologous post-vaccination geometric mean titre (post-GMT) for a vaccine arm, and the post-GMT ratio (GMTR) for a comparison of two vaccine arms within a randomised trial. It was found that the respective formulations of aqueous (not adjuvanted) IIV (whole virus, split, subunit and virosomal) were similar in mutual comparisons, and squalene-adjuvanted IIV induced, on average, modestly larger antibody titres than aqueous IIV (pooled GMTR $\leq$ 1.4-fold). The latter was based on a subset of 13 RCTs, all but one performed in middle-aged and older adults. The only RCT, which had been conducted in young children, provided larger GMTR values. Whether the latter observation was indicative of a generally larger adjuvant effect in children compared to adults, *i.e.*, of an association between adjuvant effect and pre-vaccination immunity, could not be decided on the basis of one RCT only.

In the meantime, more RCTs comparing squalene-IIV with aqueous IIV have been performed and published, in particular stimulated by the influenza A pandemic in 2009. The new clinical evidence warrants an update of the former investigation, and the larger number of trials in the respective age classes allows to explore the association between measures of pre-vaccination immunity (*i.e.*, mean age and pre-vaccination GMT) and the adjuvant effect of squalene.

Squalene adjuvancy was expressed as homologous GMTR, and, at least in a few trials, also as heterologous GMTR. Heterologous titrations (with a virus strain deviant from the vaccine strain) may predict how a vaccine would perform in the field in case of mismatch between vaccine strain and circulating strain. Moreover, since the work of Coudeville *et al.* [9] on the association between antibody titre and the chance of clinical protection against infection, antibody data can be transformed to antibody-predicted clinical protection rate (PR_{ab}) estimates, which may provide a more relevant indication for real vaccine performance in the field than antibody levels.

Safety data (local and systemic vaccine reactions and serious adverse events) in the 2011 meta-analysis revealed a slightly larger chance of local vaccine reactions for squalene-IIV, compared with aqueous IIV, in accordance with Pellegrini *et al.* 2009 [5] and Montana *et al.* 2010 [6], This is a finding of only marginal clinical importance in the light of the favourable safety profile of current inactivated influenza vaccines in general. This study does not consider safety data.

2. Materials and methods

2.1. Literature search and sources

Aim of the literature search was to identify all published articles describing randomised comparative immunogenicity trials between squalene-adjuvanted subunit IIV and aqueous IIV, and meeting the inclusion requirements as given in Table 1.

The literature search for the meta-analysis by Beyer *et al.* 2011 [4] was repeated and extended until December 12, 2018, with an improved keyword algorithm: The previously used keyword 'MF59' was replaced by 'MF59*' as it had turned out that the previous search could not always capture composed terms like MF59™ or MF59® in title or abstract. Reviews and meta-analyses identified in the search were exerted as additional sources for candidate articles (umbrella literature search strategy).

2.2. Treatment of trial variables, and data arrangement

Trial variables related to pre-vaccination immunity: In a provisional analysis, mean age, pre-vaccination GMT (pre-GMT), history of previous vaccinations and health state showed a strongly

Table 1
Requirements for inclusion of studies, and data extraction.

Item	Requirement for inclusion	Extracted data
Place and time of trial	Absence of naturally circulating influenza	Country and year
Study participants	(no restriction)	Numbers, mean age, age range, health state, history of previous vaccinations
Vaccines	Squalene-adjuvanted and aqueous inactivated influenza vaccines for intramuscular or subcutaneous application, with similar vaccine strains	Vaccine type, formulation, route, amount of haemagglutinin and, where applicable, adjuvant. Taxonomic designation of vaccine strains
Study design	Randomised comparative trial	Description of strata
Laboratory assay and outcome	Haemagglutination inhibition test	Taxonomic designation of titration strains. Pre- and post-vaccination GMT values with dispersion, per stratum and strain

positive correlation - in general, the higher the mean age of a trial population, the larger also the pre-GMT, the rate of previous vaccinations and the fraction of chronically ill persons. From single linear regression models with ln GMTR as dependent variable and for any of the trial variables, the coefficient of determination (R^2) was estimated as a measure of predictability. R^2-values were largest (up to 73%) and most significant for mean age, which was therefore chosen to represent pre-vaccination immunity. Pre-GMT values arranged according to year of trial, strongly varied, which may reflect previous virus circulation within the respective trial populations. For seasonal strains, no long-term time trend was detected. For the pandemic A-H1N1 strain, pre-GMT values increased in the period 2009 to 2015 in all mean age classes.

Age-defined classification of trials. Trials varied in their age ranges and could be narrow (*e.g.*, 1.5 to 3 years) or broad (*e.g.*, 65 to 88 years). When arranged according to their mean age, trials formed four clusters (very young and young children, and non-elderly and elderly adults); and there was one single trial in adolescents (9 to 17 years). We captured this pattern by forming four mean age classes (1 to 2.5, 3 to 6, 30 to 55, and greater than 65 years) and leaving the single trial unclassified.

Aqueous IIV: Most frequently, the aqueous comparators were subunit and split vaccines, and in some cases also whole virus and liposomal vaccines. Based on our previous study [4], these vaccine types were regarded similarly immunogenic and treated as one entity. In six trials, two aqueous vaccines were included; these trial arms were combined

Haemagglutinin and squalene amounts. Most vaccines (73%) contained a total amount of 15 μg viral haemagglutinin (HA) per strain, given either at one occasion (typically for adults) or at two occasions with half the total amount (typically for children). Also smaller (7.5 μg) and larger (30 μg) HA amounts were tested, in three trial arms even 60 μg. In 90% of trial arms, squalene-IIV and aqueous IIV contained the same HA amount, in the other 10% the HA amount in squalene-IIV was smaller than that in corresponding aqueous IIV (dose sparing approach). The standard squalene amount in adjuvanted IIV, 9.75 mg per vaccine dose, was used in most trials (69%). For trials, which did not report the squalene amount but mentioned the use of a commercially available brand like Fluad™, the amount was imputed as being 9.75 mg per vaccine dose. Some trials presented the squalene amount as volume (or percentage of total vaccine volume), which was multiplied by the density of squalene (0.585 g/cm^3) to receive the mass amount per dose. In some trials with experimental pandemic vaccines, also smaller amounts than the standard were tested. In provisional meta-regression models, positive associations between squalene and HA amount on the one hand, and the endpoints (see below) on the other hand, could be detected after adjustment for mean age, but they were quantitatively negligible in this data collection. When formulations were classified as either standardised (commercial) or investigative (developmental, experimental), the distributions of GMTR estimates in the two formulation classes were similar. Thus, no adjustments for squalene or HA amounts were applied. When a trial had more than one squalene arm, or more than one HA arm, these trial arms were combined.

2.3. Endpoints representing adjuvant effect. Statistical methods.

Geometric mean titre ratio (GMTR). From a randomised, paired comparison between squalene-IIV and aqueous IIV, the ratio (quotient) between the post-GMT estimate of squalene-IIV and that of aqueous IIV (GMTR) was calculated, and a 95% confidence interval (CI) for the GMTR estimate was derived from the standard deviations of the two post-GMT estimates, as described in [4]. A GMTR value larger than 1 would favour squalene-IIV over aqueous IIV, and *vice versa*. In the context of non-inferiority and superiority testing, squalene-IIV was regarded non-inferior to aqueous IIV when the lower limit of the CI of their GMTR exceeded 0.67-fold, and superior when it exceeded 1.5-fold. Meta-analytical (pooled) GMTR values and their CIs were calculated using the inverse variance-weighted method according to Sutton *et al.* [7].

Protection rate difference (PRD). As described previously [8], post-GMT values and their variances were transformed to antibody-predicted protection rate (PR_{ab}) estimates using the clinical protection curve by Coudeville *et al.* [9]. Per comparison, the protection rate difference (PRD) was formed from the PR_{ab} estimates of squalene-IIV and aqueous IIV. A PRD value larger than 0 would favour squalene-IIV, and *vice versa*. Limits for a 95% confidence interval around a PRD value were obtained, by means of bootstrapping, as the 2.5th and 97.5th percentile of a set of 1,000,000 bootstrap estimates as described [10]. For non-inferiority and superiority testing, no accepted margins for PRD are available. Arbitrarily, the respective margins were set at −10% and 10%.

Other details on data extraction, statistical methods, in particular meta-regression, and software used, were described previously [4,8]. Significance level in all procedures was 0.05.

3. Results

3.1. Sources and properties of data collection

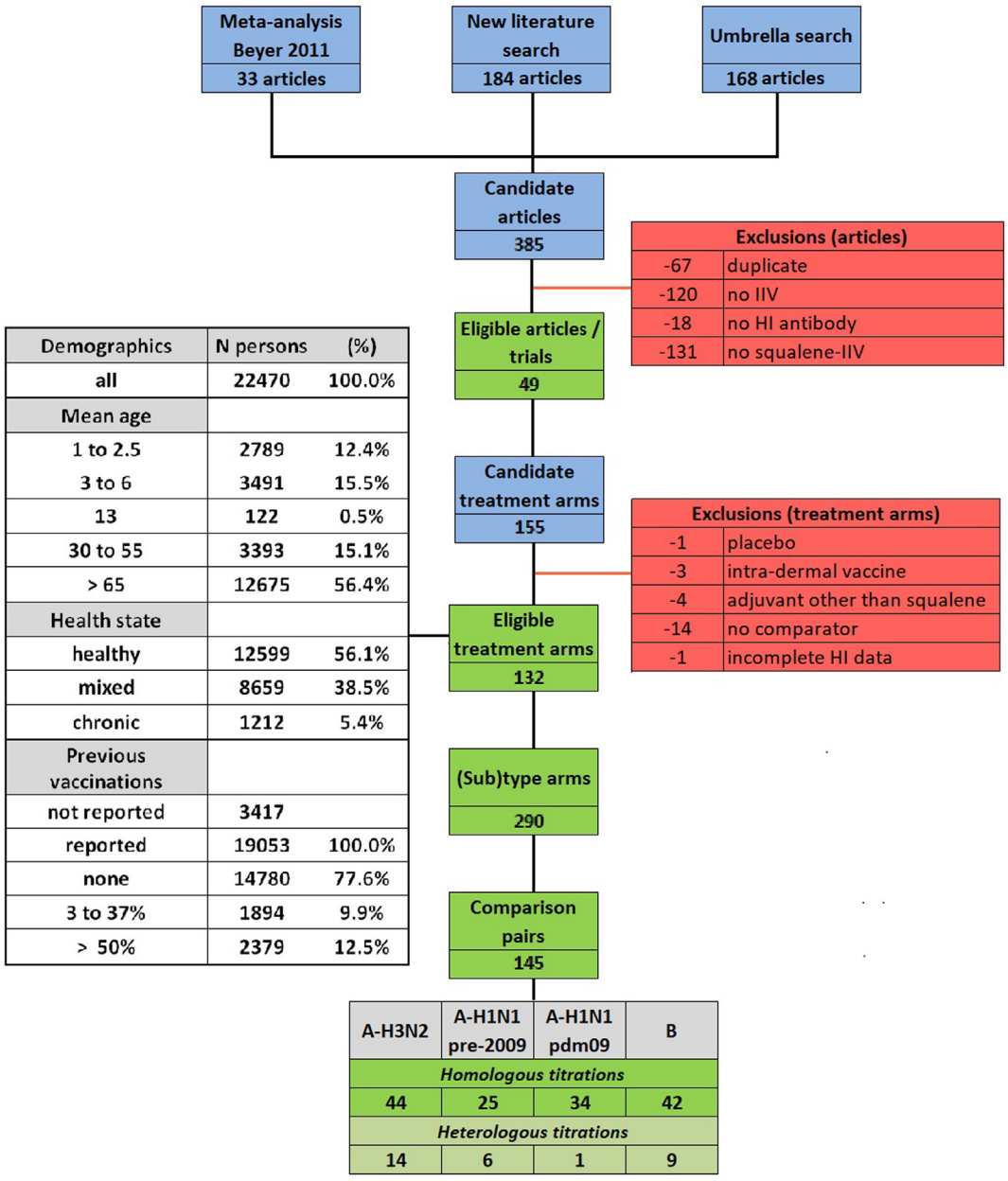
Our previous meta-analysis study had already identified 33 candidate articles. The renewed literature search identified titles and abstracts of 184 articles. From 25 meta-analyses and reviews (**Supplementary Material 1**), many of these published after 2009, another 168 references were contributed. In total, 385 candidate articles were considered and 336 excluded, for the reasons given in the flowchart (Fig. 1).

Thus, 49 articles, each describing one eligible trial, were identified (**Supplementary Material 2**). In total, 155 groups of persons had undergone any kind of intervention (treatment arms). Of them, 23 were excluded for reasons indicated in Fig. 1, and 132 treatment arms with either squalene-adjuvanted or aqueous IIV were extracted. When appropriate, treatment arms were combined with respect to HA or squalene amounts, and others divided with respect to mean age or health state. As vaccines contained one or more virus antigens, a treatment arm could include one or more sets of antibody data, resulting in 290 virus (sub-)type arms, thus 145 comparison pairs squalene-IIV *versus* aqueous IIV with homologous titration. Of these, 30 comparison pairs provided also antibody data with heterologous titration.

The entire collection of eligible trial arms comprised 22,470 persons of all ages, with 56.4% older people. More than half of the participants (56.1%) were explicitly described as healthy, and another large part (38.5%) included both healthy people and persons with age-related chronic conditions (“mixed” health state). Few trials were performed in people with defined diseases, *e.g.*, HIV infection. Not all articles reported the percentage of persons with influenza vaccination preceding the trial, but in those that did, the majority of persons (77.6%) had not received influenza vaccine previously.

3.2. Homologous titrations – overview

For an overview and regardless of virus (sub-)type, the 290 single post-GMT values were arranged according to mean age (Fig. 2**A**). Young children up to five years, and adults from 30 years onwards were well represented, but there was a data gap for adolescents and younger adults. At any mean age on the x-axis, the distance between best-fitting quadratic meta-regression lines for squalene-IIV (orange) and aqueous IIV (blue) expresses the average



Demographics	N persons	(%)
all	22470	100.0%
Mean age		
1 to 2.5	2789	12.4%
3 to 6	3491	15.5%
13	122	0.5%
30 to 55	3393	15.1%
> 65	12675	56.4%
Health state		
healthy	12599	56.1%
mixed	8659	38.5%
chronic	1212	5.4%
Previous vaccinations		
not reported	3417	
reported	19053	100.0%
none	14780	77.6%
3 to 37%	1894	9.9%
> 50%	2379	12.5%

Fig. 1. Flow of literature retrieval. Numbers of articles and trial arms. Numbers and characteristics of study participants.

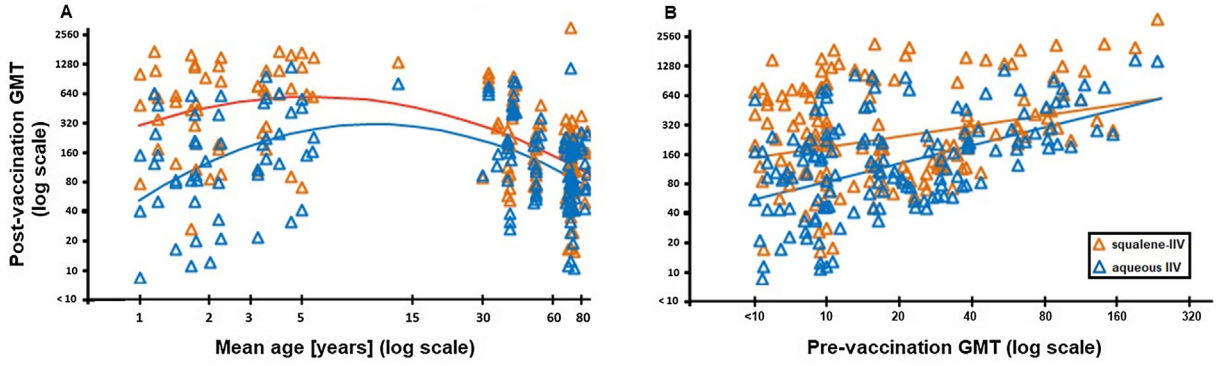


Fig. 2. Homologous post-vaccination antibody level according to (A) mean age, and (B) pre-vaccination antibody level. Symbols represent the post-vaccination GMT estimates (290 trial arms). General tendency for all virus (sub-)types combined, was visualised by meta-regression lines (A: quadratic regression, B: linear regression), separately for squalene-IIV and aqueous IIV.

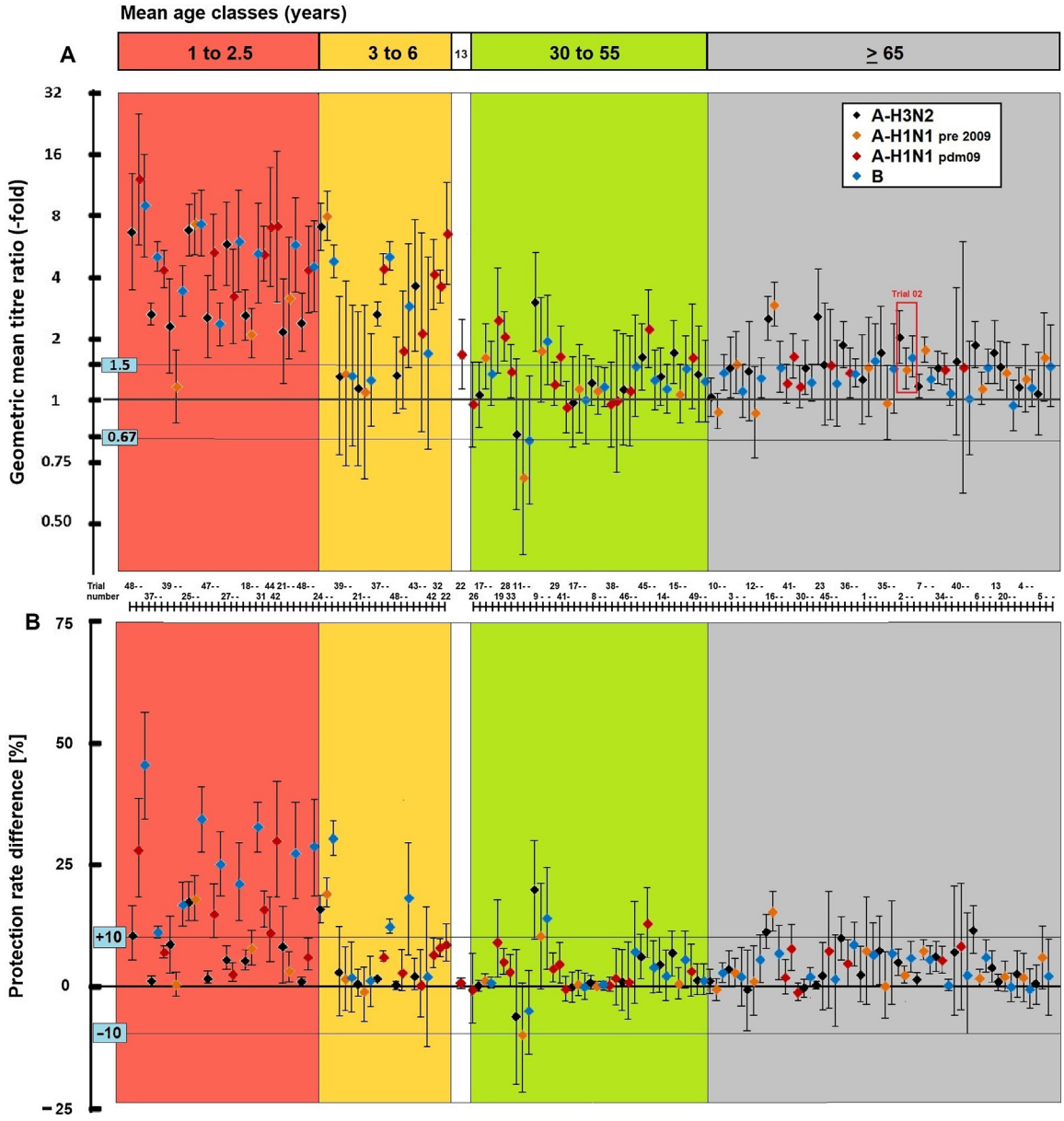


Fig. 3. Homologous adjuvant effect according to mean age. Symbols represent adjuvant effect estimates (145 comparisons squalene-IIV *versus* aqueous IIV) and their 95% CIs, sorted and classified by mean age, for antibody level (A) and antibody-predicted protection level (B). Trial numbers correspond to the original articles in Supplementary Material 2. When a trial number occurs more than one time, the respective trial provided data stratified for different age ranges. Red rectangle: trial number 02 [11] (see Discussion).

adjuvant effect. The distance was large at youngest age. With increasing mean age (up to ~6 years), post-GMT values of aqueous IIV tended to increase stronger than those of squalene-IIV suggesting a decreasing adjuvant effect. At high age, post-GMT values of the two vaccines converged.

When the same post-GMT values were arranged according to pre-GMT values (Fig. 2**B**), the distance between the meta-regression lines (best-fitting here: linear) was largest in seronegative persons (pre-GMT < 10), and shrank with increasing pre-GMT.

This overview suggests that the average adjuvant effect is not a constant, but is associated with measures of pre-seasonal immunity: generally higher in younger and lower in older persons. It appears that the fraction of primed and (frequently) exposed persons in a trial limits the trial outcome related to the adjuvant effect.

3.3. Homologous titrations – adjuvant effect according to virus (sub-)type and mean age

Fig. 3 presents the GMTR and PRD values of all 145 single comparisons between squalene-IIV and aqueous IIV, arranged according to increasing mean age and distinct for virus (sub-)types. Table 2 (left part) provides the meta-analysed means for the respective mean age classes.

On the antibody level (GMTR values, Fig. 3A), virtually all GMTR values in the youngest age class (≤2.5 years) were significantly elevated. With increasing mean age, GMTR values decreased. Children 3 to 6 years still showed elevated values, but 95% confidence intervals often included 1.0. In adults (30 to 55 years, and ≥65 years), most GMTR values were slightly larger than 1.0 with 95% CIs including 1.0. In older adults, only few trials (with tri-valent vaccines) showed a significant adjuvant effect for all three vaccine com-

Table 2
Meta-analysed GMTR and PRD for age classes and virus (sub-)types. [Two pages]

Influenza (sub-)type	Titration	Homologous					Heterologous			
	Mean age class (years)	1 to 2.5	3 to 6	13	30 to 55	≥65	1 to 2.5	3 to 6	30 to 55	≥65
A-H3N2	Number of comparisons	9	6		10	19	3	3	1	7
	Meta-analysed GMTR (95% CI)	3.2 (1.5 to 6.8)	2.0 (0.5 to 7.7)		1.3 (0.6 to 3.0)	1.5 (0.8 to 2.6)	2.7	1.7	1.6	1.6 (0.4 to 6.0)
	Meta-analysed PRD (%)	5.1 (3.3 to 7.1)	6.0 (4.7 to 7.5)		3.7 (−0.4 to 7.9)	1.7 (−0.9 to 4.3)	13.4	12.4	6.8	−5.0 (−12.2 to −2.2)
A-H1N1 pre-2009	Number of comparisons	4	3		6	12	2	2		2
	Meta-analysed GMTR (95% CI)	2.7 (0.4 to 19.0)	4.1		1.1 (0.3 to 4.1)	1.3 (0.6 to 2.7)	1.7	1.9		1.5
	Meta-analysed PRD (%)	9.4 (5.9 to 13.1)	17.0		1.0 (−1.9 to 4.1)	5.0 (1.8 to 8.2)	8.7	16.7		2.7
A-H1N1 pandemic 2009	Number of comparisons	8	6	1	12	7	0	1		0
	Meta-analysed GMTR (95% CI)	5.1 (1.8 to 14.4)	3.7 (*) (0.9 to 15.8)	1.7	1.3 (0.6 to 3.0)	1.4 (0.4 to 5.1)		1.7		
	Meta-analysed PRD (%)	11.9 (8.6 to 15.4)	6.8 (5.0 to 8.7)	0.8	3.4 (-0.3 to 7.3)	0.6 (-1.9 to 3.1)		12.0		
All A combined	Number of comparisons	21	15	1	28	38	5	6	1	9
	Meta-analysed GMTR (95% CI)	3.6 (2.2 to 5.9)	2.6 (1.2 to 5.5)	1.7	1.3 (0.8 to 2.0)	1.4 (1.0 to 2.1)	2.3 (0.4 to 12.0)	1.8 (0.4 to 8.7)	1.6	1.6 (0.6 to 4.3)
	Meta-analysed PRD (%)	8.6 (5.9 to 11.5)	7.8 (6.0 to 9.8)	0.8	3.1 (−0.6 to 6.9)	1.9 (−0.8 to 4.5)	11.2 (5.8 to 28.2)	13.0 (2.8 to 28.8)	6.8	−4.7 (−12.4 to −2.5)
B	Number of comparisons	9	6		10	17	3	3		3
	Meta-analysed GMTR (95% CI)	3.9 (1.9 to 8.1)	2.1 (0.6 to 7.7)		1.2 (0.6 to 2.6)	1.3 (0.8 to 2.2)	2.1	1.7		1.4
	Meta-analysed PRD (%)	20.0 (16.1 to 23.9)	17.3 (14.2 to 20.5)		2.9 (−1.3 to 7.1)	2.9 (0.2 to 5.7)	17.2	16.0		2.1
All (sub)types combined	Number of comparisons	30	21	1	38	55	8	9	1	12
	Meta-analysed GMTR (95% CI)	3.7 (2.5 to 5.5)	2.4 (1.3 to 4.4)	1.7	1.2 (0.8 to 1.8)	1.4 (1.0 to 1.9)	2.2 (1.0 to 4.9)	1.7 (0.6 to 5.1)	1.6	1.5 (0.7 to 3.6)
	GTMR non-inferiority met N trials (%) (**)	30 (100%)	15 (71%)	1	28 (74%)	51 (93%)	8 (100%)	6 (67%)	1	12 (100%)
	GTMR superiority met N trials (%) (***)	27 (90%)	10 (48%)	0	2 (5%)	5 (9%)	5 (63%)	5 (56%)	0	0 (0%)
	Meta-analysed PRD (%)	12.0 (9.0 to 15.2)	10.4 (8.2 to 12.7)	0.8	3.0 (−0.7 to 7.0)	2.2 (−0.5 to 4.9)	13.3 (7.8 to 18.8)	14.0 (11.0 to 16.9)	6.8	−2.4 (−5.6 to 0.3)
	PRD non-inferiority met N trials (%) (#)	30 (100%)	20 (95%)	1	35 (92%)	55 (100%)	8 (100%)	8 (89%)	1	12 (100%)
	PRD superiority met N trials (%) (##)	13 (43%)	4 (19%)	0	0 (0%)	1 (2%)	3 (38%)	3 (33%)	0	0 (0%)

Confidence intervals were not calculated for classes < 3.
(*) Estimate dependent on year of trial. In a linear meta-regression model weighed by inverse variance, GMTR was 4.2-fold in 2009, and 1.7-fold in 2015.
(**) Non-inferior when lower limit of 95% CI exceeds 0.67-fold.
(***) Superior when lower limit of 95% CI exceeds 1.5-fold.
(#) Non-inferior when lower limit of 95% CI exceeds −10%.
(##) Superior when lower limit of 95% CI exceeds 10%.

ponents (one of them, Trial 02 [11] marked in Fig. 3A, will be mentioned in the Discussion). The pattern of decreasing adjuvant effect estimates was similar for the four virus (sub-)types (Table 2). When data for all (sub-)types were combined to grand averages (lower part of Table 2), squalene-IIV induced 3.7-fold (95% CI: 2.5 to 5.5) larger GMT than aqueous IIV in the youngest age class, and 1.4-fold (1.0 to 1.9) in older adults. Non-inferiority of squalene-IIV *versus* aqueous IIV was demonstrated in most comparisons, regardless of age (100% in young children, 93% in older adults), but superiority was predominantly found in children (90% of comparisons in young children, 9% in older adults).

The pandemic A-H1N1 strain showed three particular features. First, most pre-GMT values were smaller than the HI detection threshold of 10 in 2009 and generally increased in trials performed in consecutive years, modestly for children < 2.5 years and adults (average pre-GMT in 2015: 10 to 22), but largely for the mean age class 3 to 6 years (average pre-GMT in 2015: 97). Second, the GMTR values in the younger age classes were large, but while those of children < 2.5 years remained large in the consecutive years, those in the 3 to 6 years class remarkably dropped from 4.2-fold to 1.7-fold. Third, in the two adult age classes, GMTR values were close to 1.0 already in 2009, and remained low during the consecutive years. The pandemic strain behaved like a seasonal one with respect to the adjuvant effect.

On the level of antibody-predicted protection (PRD values, Fig. 3B), most estimates for influenza A were smaller than 10%, even in children; the meta-analysed grand PRD for all influenza

A subtypes was 8.6% (95% CI: 5.9 to 11.5%) in the mean age class 1 to 2.5 years, and 7.8% (6.0 to 9.8%) in the mean age class 3 to 6 years. For influenza B in the same mean age classes, however, meta-analysed PRD values were clearly larger than for influenza A: 20.0% (16.1 to 23.9%) and 17.3% (14.2 to 20.5%), respectively. A possible explanation for this distinction between influenza types will be discussed. In the adult classes, individual and meta-analysed PRD values were mostly close to 0% for both influenza types, and no significant adjuvant effect was detected. The fraction of comparisons showing superiority of adjuvanted over non-adjuvanted IIV declined from 43% in the youngest mean age class to 2% in older persons.

3.4. Heterologous titrations

In twelve studies (**Supportive Material [B]**), also heterologous titrations were performed in order to predict the performance of the vaccine in case of a mismatch. After subdivision into treatment, mean age, and virus (sub-)type trial arms, 60 trial arms and 30 comparisons were available. Results should be interpreted cautiously, as often small subsets of available sera were titrated, which decreased statistical power. Moreover, the trials differed in the choice of titration viruses. Four classes of simulated antigenic mismatch could be formed:

1. Retrospective titration (eleven comparisons): Vaccine and titration viruses belonged to the same influenza A subtype or influenza B lineage, but the titration virus was 'older' than the vaccine virus. *E.g.*, in the study by Zedda *et al.* 2015 [12], sera of persons vaccinated with B/Brisbane/60/2008 (Victoria) were titrated against B/Malaysia/2506/2004 (Victoria), a strain isolated four years earlier than the vaccine virus. The relevance of these data to predict future mismatch may be limited.
2. Prospective titration - antigenic drift mismatch (thirteen comparisons). Here, the titration virus was 'younger' than the vaccine virus, still within the same influenza A subtype or influenza B lineage, *e.g.*, B/Yamagata/16/88 (vaccine strain) and B/Panama/45/90 (titration strain) in the study by Minutello *et al.* 1999 [13].
3. Prospective titration - influenza B lineage mismatch (five comparisons). *E.g.*, B/Florida/4/2006 (Yamagata) and B/Brisbane/60/2008 (Victoria) in the study by Vesikari *et al.* 2011 [14].
4. Prospective titration - antigenic shift mismatch (one comparison). This single study titrated sera raised against the last pre-pandemic A-H1N1 vaccine strain A/Brisbane/59/2007 with the pandemic strain A/California/7/2009 in older adults (Song *et al.* 2013 [15]).

The numbers of comparisons per titration class were too small to analyse the data separately for each mismatch class, which would have been desirable seen the biological differences between classes. Surprisingly yet, when the GMTR and PRD values from all comparisons together were arranged according to increasing mean age (Fig. 4), they appeared to mirror their homologous counterparts. As confirmed by meta-analysis (Table 2 right part), the average adjuvant effect was large in young children but small in older persons.

4. Discussion

Clinical evidence for the effect of squalene as an adjuvant to inactivated influenza vaccine was reviewed in a systematic collection of published immunogenicity trials. The extent of the adjuvant effect was found to be associated with pre-seasonal immunity (pre-vaccination HI antibody level and mean age): on average, it was larger in young children, decreasing in older children and young adults, and barely detectable in older adults. The finding applied for vaccines antigenically matched with circulating strains (homologous titration) and also for antigenically mismatched vaccines (heterologous titration, performed in fewer trials). It applied for seasonal A-H3N2, A-H1N1 and B strains, and the 2009 pandemic strain, but it is not necessarily predictive for future pandemic strains.

Pre-seasonal immunity against influenza viruses comprises all innate and adaptive immune functions, which contribute to the protection against upcoming influenza infection and disease (for review see [16]). Adaptive immunity is strongly age-related as exposure events accumulating in lifetime, modulate immune functions [17]. The very first influenza A exposure and infection (priming) usually occurs early in lifetime: more than 90% of seven-years-old children already carry anti-A antibodies, according to Bodewes *et al.* 2011 [18] and Sauerbrei *et al.* 2014 [19]. In general, the first influenza B infection occurs later in lifetime. At the age of seven years, Bodewes *et al.* found ~70%, and Sauerbrei *et al.* ~35% anti-B seropositivity. Interestingly, also our data show a virus type distinction at young age, related to the level of antibody-predicted protection: The meta-analysed protection rate differences of the two youngest age classes (≤six years) are larger for influenza B than for influenza A (Fig. 3B, Table 2). It seems that the fraction of naive children was larger for influenza B, and received a larger benefit from adjuvant, than for influenza A. Unfortunately, trials performed in older children and adolescents are not available to confirm this trend. At adult age, when virtually every person has experienced influenza A and B priming and accumulated exposure events during lifetime, the virus type distinction can no longer be demonstrated.

Not unexpectedly, the extent of the squalene adjuvant effect appears to be ruled by the individual history of influenza exposure events and the consecutive modulation of the immune system; most benefit is provided to the unprimed. Immunogenicity trials with vaccines containing influenza A-H5N1, an avian subtype not circulating in the human population, have found a strong squalene adjuvant effect in all ages. *E.g.*, in the meta-analysis by Feldstein *et al.* 2016 [20] including 22 A-H5N1 immunogenicity trials in healthy persons aged 18 to 99 years, the meta-analysed GMT of adjuvanted vaccines was found 3.5-fold larger than that of non-adjuvanted vaccines. Similarly, we found a grand average of 3.7-fold in the youngest mean age class (1 to 2.5 years, Table 2).

Support comes also from studies in naive and primed animals. *E.g.*, the experiments by Higgins *et al.* 1996 [21] in BALB/c mice aged eight weeks, vaccinated with aqueous or squalene-adjuvanted trivalent vaccines and assayed by a total Ig ELISA, demonstrated a strong adjuvant effect for all three vaccine components: 12-, 13- and 31-fold for A-H3N2, A-H1N1 and B, respectively. However, when eight weeks old mice were first infected by an A-H1N1 virus strain and vaccinated ten weeks thereafter, no adjuvant effect was found (1.0-, 1.3- and 1.1-fold), remarkably not only for A-H1N1, but also for A-H3N2 and B, suggesting that the priming event be a hetero(sub-)typic phenomenon in mice. When the same experiments were done in 18 months old mice, a moderate adjuvant effect did occur: 3.9-, 2.4- and 7.5-fold, respectively. The authors predicted that squalene may improve antibody induction of IIV in older humans to the same extent as in old mice; yet, they also note that the old mouse model may not well represent the human older population: the mice were infected only once, while older humans have experienced many influenza infections during lifetime.

A negative association between effect and pre-seasonal immunity had been found not only for squalene-adjuvanted IIV, but also for live attenuated influenza vaccine (*versus* IIV) [22] and quadrivalent IIV in case of influenza B lineage mismatch (*versus* trivalent IIV) [8]. It seems that some important vaccine innovations of the

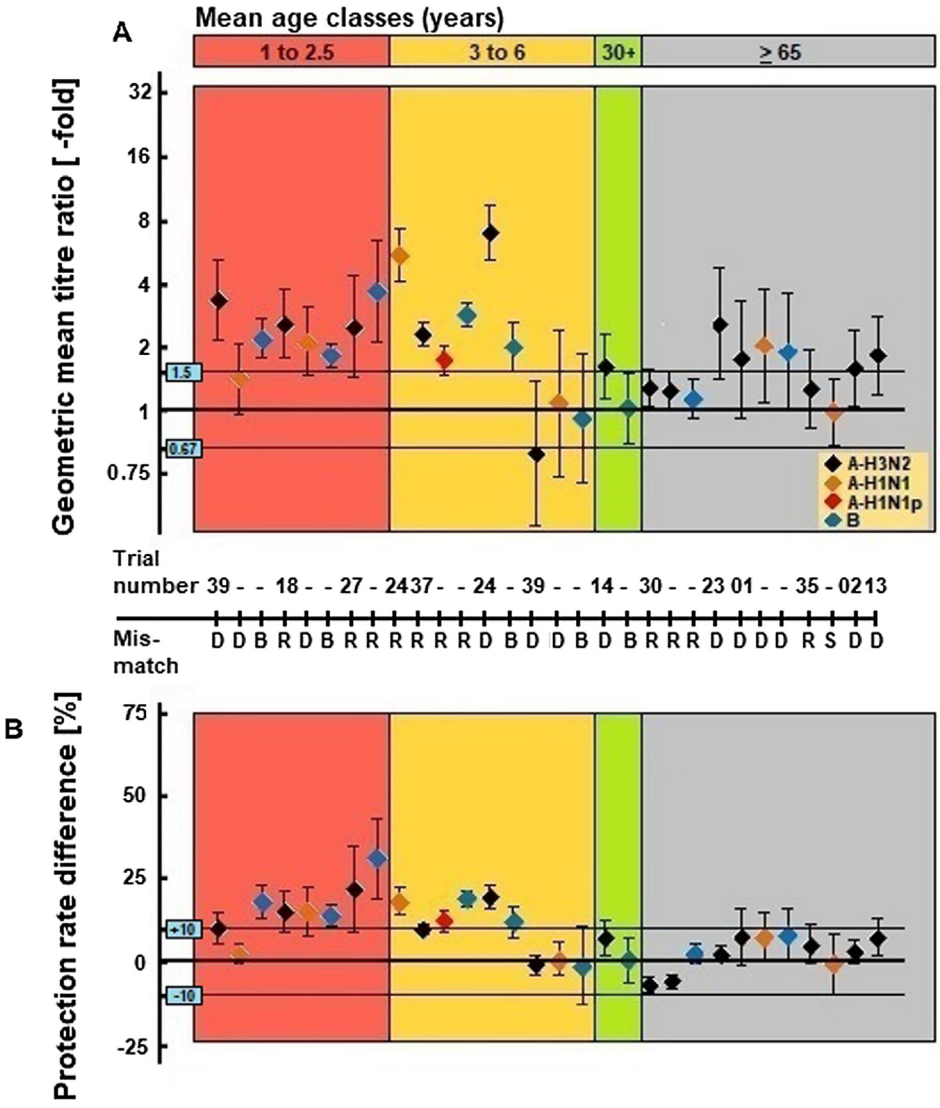


Fig. 4. Heterologous adjuvant effect according to mean age. Symbols represent adjuvant effect estimates (30 comparisons squalene-IIV *versus* aqueous IIV) and their 95% CIs, sorted and classified by mean age, for antibody level (A) and antibody-predicted protection level (B). Trial numbers correspond to the original publications in Supplementary Material 2. Mismatch: R, retrospective titration; D, drift mismatch; B, influenza B lineage mismatch; S, shift mismatch. Trial 23 included 14 clade- and drift-variants of the vaccine virus A/California/7/2004 (H3N2); here, the variant A/Genoa/47/2005 is presented.

last decades have been promising in naive animal models, beneficial for unprimed children, but of more limited value in older adults.

A strong feature of our investigation may be the sheer number of observations: 145 comparison pairs in 22,470 persons of all ages. Single trials are modulated by various geographic, seasonal, societal and individual factors unrelated to vaccination; the inter-trial variation of influenza studies is notorious. Only a sufficiently large number of trials identified by systematic search, may reveal a representative pattern. To generalise results from single trials to entire populations, may sometimes be inappropriate. *E.g.*, a review article on squalene as a vaccine adjuvant [1] presented just one comparative immunogenicity trial in older persons (of more than ten possible trials) and then seemed to claim superiority of squalene-adjuvanted IIV in older populations in general. The cited trial was one of the few, which did show a significant adjuvant benefit for all three vaccine components [11]. In most other elderly trials, at least one vaccine component induced just similar antibody levels in adjuvanted and aqueous trial arms. Inspection (Fig. 3) and meta-analysis (Table 2) of the entire data collection do not support the claim that squalene IIV is superior to aqueous IIV in older persons.

As a limitation, our findings are entirely based on trials measuring antibody, which do not necessarily directly assess real vaccine effectiveness in the field. But given the association between HI antibody and the chance of protection as established by Coudeville *et al.* [9], we explored protection rates predicted from antibody distributions and found a pattern similar to the antibody level. It would be desirable if these predictions could be confirmed by observations in the field. The classical study design to compare two vaccines is the randomised comparative trial (RCT) with specific (laboratory-confirmed) endpoints. However, a large number of volunteers would be required to detect a truly existing adjuvant benefit. The RCT by McElhany *et al.* [23] comparing another adjuvanted IIV ($AS03_B$ formulation containing 5.93 mg α-tocopherol and 5.93 mg squalene per vaccine dose) with aqueous IIV, included more than 40,000 vaccinees. An alternative study design requiring less participants, may be the test-negative case-control trial (tn-CCT) in care-seeking persons [24]. A current standardized protocol on brand-specific tn-CCT (DRIVE report 2018 [25]) recommends a

number of at least 1000 (cases and controls) when the combined true vaccine effectiveness is at least 50%, the attack rate during epidemic at least 5%, and the vaccine coverage at least 20%. Other observational study designs, like cohort and case-control, carry large risks of bias and confounding, and cannot be recommended to assess relative vaccine effectiveness.

To our knowledge, no RCT to compare the field effectiveness of squalene-IIV with that of aqueous IIV has ever been performed. Domnich *et al.* [26] identified five comparative field trials in older persons (two cohort trials, two case-control trials, and one tn-CCT), of which three used non-specific (clinically defined) endpoints and two laboratory-confirmed endpoints. Mostly due to low virus circulation, numbers of endpoint cases were small. *E.g.*, the tn-CCT by Van Buynder *et al.* [27] included 282 participants, of whom 84 were cases - numbers far below the DRIVE recommendations mentioned above. Three of the field trials detected an adjuvant benefit of borderline significance, the other two did not find a significant distinction between squalene-IIV and aqueous IIV. Thus, the available evidence from field trials can be regarded as limited. If anything, it would support our finding in antibody trials, namely that the additional benefit of squalene in older persons is small. Surprisingly, the review by Domnich *et al.* concludes that "*the available evidence suggests that MF59-TIV is ... superior to conventional non-adjuvanted vaccines*". But we do agree with their call for "*well-designed and sufficiently powered*" comparative field trials in older persons, with the restraint that trials of even the highest quality can barely detect an adjuvant effect.

In summary, our findings from comparative immunogenicity trials has found evidence for a negative association between adjuvancy and pre-seasonal immunity. Squalene-adjuvanted IIV may not significantly overcome the inferior vaccine effectiveness of aqueous IIV often found in older adults, but is more effective than aqueous IIV in unprimed persons.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.vaccine.2019.12.037.

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